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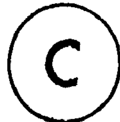
THE FIDELITY OF IN VITRO PROTEIN SYNTHESIS AND ITS IMPLICATIONS FOR THE AGING OF HUMAN CELLS

by

 Roman Ivan Wojtyk

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ABSTRACT

To test the error catastrophe theory of cell senescence, we have developed a cell-free protein synthetic system from human diploid fibroblasts capable of translating both the synthetic mRNA, poly(U), and endogenous cellular mRNA in vitro. We have measured the fidelity of poly(U)-directed protein synthesis in extracts from a variety of cells. The results contradict the error catastrophe in several ways:

- cells from subjects suffering maladies of premature aging, progeria and Werner Syndrome, exhibited error frequencies within the normal range.
- cells from an old donor did not have elevated error frequency of protein synthesis.
- early-passage (young) normal cells had error frequencies higher than those of late-passage (old) cells.

The error frequency in one normal strain of fibroblasts declined throughout its tissue culture lifespan. Cells maintained in the post-mitotic (terminal) state, in which there was no cell selection, exhibited a constant error frequency over 16 weeks. In a series of experiments using clonal populations of cells, it became evident that the decline in error frequency as a function of passage was dependent on the clonal heterogeneity of the mass culture. In no experiment, however, could any correlation between the error frequency of protein synthesis and cellular growth parameters be made.

Overall, the senescence characteristic of human diploid fibroblasts was independent of the measured fidelity of protein synthesis *in vitro*.

This thesis I dedicate to my wife

dawn marie

whose love and understanding over the
course of many years has enabled me
to undertake this effort. This work is
as much a product of her commitments
as it is of mine.

And the Lord said, My spirit shall not always strive with man... yet his days shall be an hundred and twenty years.

– Genesis 6:3.

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List of Abbreviations

A	adenosine
A_{260}	absorbance at 260 nm wavelength
aa-tRNA	aminoacyl transfer RNA
ADP	adenosine diphosphate
AIB	α-aminoisobutyric acid
A-site	ribosomal site which accepts incoming aa-tRNA
ATP	adenosine triphosphate
b	regression coefficient; slope
C	cytosine
^{o}C	degrees centrigrade
Ca^{+2}	calcium ion
CHO	Chinese Hamster Ovary
Ci	curie
CP	creatine phosphate
CPK	creatine phosphokinase
cpm	counts per minute
d	days
2-D	two dimensional
DB	dialysis buffer
df	degrees of freedom
DNA	deoxyribonucleic acid
DOC	deoxycholate
DTT	dithiothreitol
E. coli	Escherichia coli bacterium
EF	elongation factor

E.F.	error frequency
EGTA	ethylene guanidine tetraacetic acid
eIF	eukaryotic initiation factor
EM	energy mix
EtOH	ethanol
F	F-statistic
FBS	fetal bovine serum
FCS	fetal calf serum
fmet-$tRNA_f$	prokaryotic initiator formylated methionyl tRNA
fmoles	femtomoles
F-phe	para-fluorophenylalanine
GDP	guanosine diphosphate
GFC	glass fiber circles
g/l	grams per litre
glu	glutamic acid
gly	glycine
g_{max}	maximum centrifugal force (gravities)
G6PD	glucose 6-phosphate dehydrogenase
GTP	guanosine triphosphate
^3H	tritium; tritiated
H1	histone 1
HB	homogenization buffer
HCl	hydrochloric acid
HLA	histocompatibility locus antigen
HOAc	acetic acid
I	inosine

IF	prokaryotic initiation factor
K^+	potassium ion
kcal	kilocalorie
KCl	potassium chloride
L7/12	protein 7/12 of the large ribosomal subunit
leu	leucine
LF	limit flocculating unit
lys	lysine
M	molarity; molar
MEM	Eagle's minimum essential medium
met	methionine
met-$tRNA_i$	eukaryotic initiator methionyl-tRNA
mg	milligram
Mg^{+2}	magnesium ion
$Mg(OAc)_2$	magnesium acetate
ml	millilitre
mM, μM	millimolar, micromolar
mRNA	messenger RNA
mRNP	messenger RNA particle
MS2-RNA	RNA from bacteriophage MS2
N-Ac-phe-tRNA	N-acetylated phenylalanyl tRNA
NAD^+	nicotinamide adenine dinucleotide
NaOH	sodium hydroxide
nm	nanometer
OAB	Optimum Adjusting Buffer
p	probability

P	progeric cell strain
PBS	phosphate-buffered saline
PCS	phase combining solution
6PGD	6-phosphogluconate dehydrogenase
phe	phenylalanine
pI	isoelectric point
P_i	inorganic phosphate
P-site	ribosomal site containing nascent chain peptidyl-tRNA
poly(phe)	polyphenylalanine
poly(U)	polyuridylate
poly(U,C)	heteropolymer of uridine and cytosine
poly(U,G)	heteropolymer of uridine and guanosine
P(U)	poly(U)
Q_{10}	temperature coefficient
r	correlation coefficient
RF	release factor
RGM	regular growth medium
RNA	ribonucleic acid
RNase	ribonuclease
rRNA	ribosomal RNA
^{35}S	sulphur-35
S1	protein 1 of small ribosomal subunit
S-30	supernatant from 30,000 x g_{max} centrifugation
Sm^D	streptomycin-dependence
SV	simian virus

TdR	thymidine
TPCK	p-tosylphenylchloroketone
tRNA	transfer RNA
$tRNA_3^{Leu}$	third isoaccepting species of leucine tRNA
$tRNA_{phe}$	deacylated tRNA for phenylalanine
U	uridine
UV	ultraviolet
wks	weeks
μ	micro, 10^{-6}
λ	lambda, microlitres
χ^2	chi-square statistic

CHAPTER ONE

INTRODUCTION

To understand aging has always been a great dilemma for man. Just as the old find a prophecy in death, so does senescence and old age forbode the future for the young. Although aging has been an important item in the thoughts of men throughout the ages and has been the subject of legends and myths of most societies (Gruman, 1977), it has become of central importance to modern man. The modern era has exhibited a precipitous decline of faith in supernatural salvation for man, replaced by industry, technology and science. Cures from infirmities of old age will be achieved in what may be deemed as part of a secular salvation for man.

Following the understanding of germ theory of infectious disease by Pasteur and Lister scarcely a century ago, the meliorist efforts of public health officials and medical scientists have resulted in greatly increased life expectancy of man, yet the biologically determined maximum lifespan has remained unimproved. A new problem has arisen, that of an aging population. Today's society is finding itself burdened with unprecedented numbers of old people. Thus the dilemma with aging at its focus remains, and now the community is diverting large sums to research into the nature of degenerative diseases of old age and to aging itself.

The study of old age, gerontology, is a multifaceted science drawing from many of the more fundamental disciplines: psychology, sociology, biology, etc. Until recently aging studies were mentioned only in passing within the broader context of these general sciences. However, the last two decades has seen a tremendous upsurge of interest in gerontology as evidenced by the appearance of numerous publications dealing specifically with gerontology in all its facets. The biology of aging has been the subject of many recent monographs, as well (i.e. Cutler, 1976; Finch and Hayflick, 1977; Strehler, 1977; Behnke et al., 1978; Bergsma and Harrison, 1978; Schneider, 1978; Comfort, 1979; Dietz, 1980).

1.1 A Cellular Model for Aging

A differential approach to the problem of senescence has led to the study of aging as a cellular phenomenon. The central theorem of cell biology is that a phenomenon observed in an intact organism can also be studied in the organism's component parts (i.e. cells and their products). Cytogerontology is a relatively new discipline however. Early work by cell culturist Alexis Carrel (1914) apparently showed that cells derived from chick embryo connective tissue grew for exceedingly long periods of time and could replicate ad infinitum. This evidence had a negative effect on further cytogerontological research since the belief was taken that the fundamental mechanisms of aging resided not

at the cellular level but at higher levels of tissue organization. Eventually, there accrued enough evidence that the growth of normal human cells ceased in time that limited cellular lifespan could no longer be accepted as a tissue culture artifact (Swim and Parker, 1957).

1.1.1 The Hayflick Phenomenon

The now classic work by Hayflick and Moorehead (1961) confirmed that normal human diploid fibroblasts possessed a limited lifespan in tissue culture and thus rekindled research in cytogerontology. Their original work illustrated that cells from fetal tissue grew with vigor in tissue culture initially but after (an average of) 50 subcultivations (mean population doublings, MPD) the culture ceased to grow. This effect has been termed the "Hayflick phenomenon" and has been confirmed by dozens of laboratories across the world.

Hayflick described 3 phases of cellular lifespan in tissue culture (Figure 1-1). In Phase I, cells are extracted from the living tissue and those capable of growth under culture conditions, usually fibroblasts, are removed from the surrounding mesenchyme and the cell <u>strain</u> becomes established. With successive subcultivation (Phase II), normal cells grow to monolayer thickness; at confluence, further growth of the culture is contact-inhibited. Each subcultivation, also termed <u>passage</u>, represents a mean of one replicative event (i.e. cell cycle) per cell. Initially, while growth is

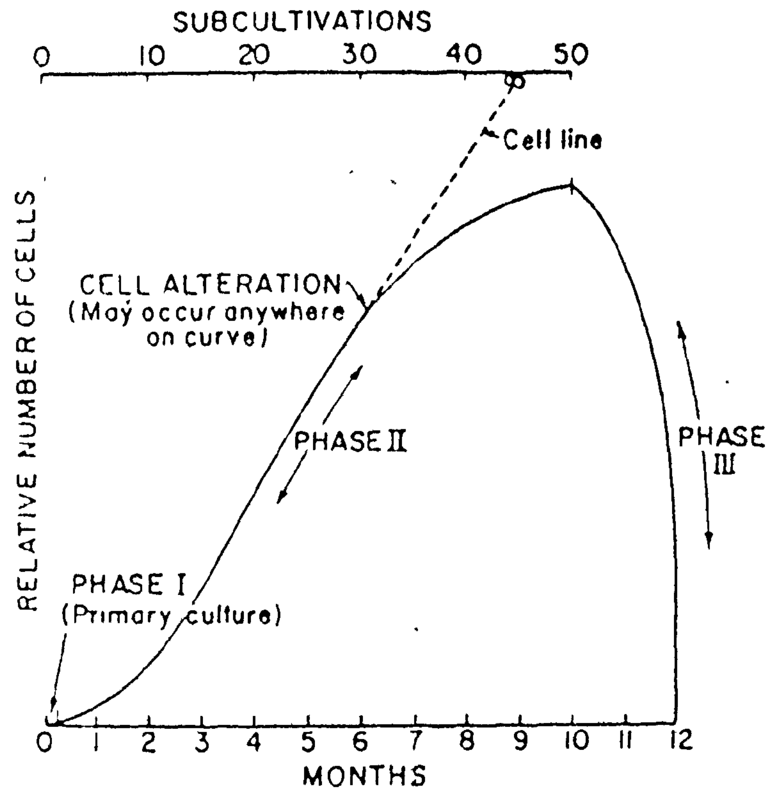


Figure 1-1 Tissue culture lifespan of human diploid fibroblasts. After tissue explantation, Phase I, the primary culture, terminates with the formation of the first confluent monolayer. Vigorous growth occurs throughout Phase II necessitating subcultivation. Human diploid fibroblasts are capable of a limited number of divisions prior to Phase III and cell death. - from Hayflick (1977).

still vigorous, cells are termed (in) early passage or young cells. As subcultivation continues and passage number accrues, the culture begins to decline in growth rate. When growth has slowed considerably, cells are labelled old or (in) late passage. A culture no longer capable of further subcultivation has entered Phase III or terminal passage. In early Phase III a few cells are still capable of replication. All cell replication ceases in late Phase III and the culture is termed post-mitotic. The number of passages through which the culture grew is the "Hayflick limit" of that particular cell strain. The Hayflick limit is a function of the number of replicative events (i.e. number of passages) the culture has undergone and not determined by the passage of "metabolic" time (i.e. the number of months in culture) (Dell'Orco et al., 1974; Harley and Goldstein, 1978). The position was taken by Hayflick that the limited replicative lifespan of normal human fibroblasts represents senescence at its most fundamental (i.e. cellular) level. A considerable body of data now supports the validity of this model for studies of biological aging (Cristofalo, 1972; Martin, 1977; Hayflick, 1980).

There have been some reports that normal cells possess unlimited growth potential. However, these cell lines have invariably been shown to have subtle chromosomal rearrangements (Martin, 1977a). Escape from in vitro

senescence has implications for cancer research since apparently normal cells which have become immortal result in neoplasms when injected into host animals (Deal et al., 1971). A possible exception to this rule has been illustrated in a culture of skeletal muscle "satellite" cells established from a regenerating lizard tail (Martin, 1977a). Normal human fibroblasts are under exquisite growth control and do not escape senescence in tissue culture, unlike, for example, cell strains of mouse fibroblasts, a considerable proportion of which undergo spontaneous transformation with associated chromosomal alterations resulting in permanent cell lines (Meek et al., 1977).

Considerable evidence has been gathered describing a functional relationship between cellular senescence in vitro and in vivo (Strehler, 1977). Limited replicative lifespan of normal cells has been demonstrated in vivo. Serial transplants of mammary tissue (Daniel et al., 1968; Daniel, 1977), skin (Krohn, 1962), and hematopoietic cells (Siminovitch et al., 1964; Hellman et al., 1978) into syngeneic hosts can outlive the original donor (Krohn, 1966) but do not survive indefinitely, whereas cancer cells do (Daniel et al., 1975). Although cells apparently age in vivo most cell types do not exhaust their ability to replicate in one lifetime and grafts from old donors can still proliferate rapidly in a suitable host (Young et al., 1971). In tissues which are constantly required to undergo self-

renewal, decreased proliferative capacity is observed in cells from aged animals (Post and Hoffman, 1964; Lesher and Sacher, 1968).

Hayflick (1965) reported a significant decrease in growth potential of cells derived from adult donors as compared to cells derived from fetal tissue. Working with skin fibroblasts, Goldstein et al., (1969) first showed an inverse relationship between the doubling potential of a cell strain and the age of the tissue donor, although the significance of the relationship is lessened when cells from diabetic and non-diabetic donors are considered separately (Goldstein, 1978). Decreasing growth potential as a function of the donor's age has been confirmed in cells from skin (Martin et al., 1970) and liver (LeGuilly et al., 1973). The size of colonies to which cells grow is another indication of remaining cellular doubling potential. Schneider and Mitsui (1976) found that fibroblasts from old donors yielded smaller colonies than cells from young donors. A parallel result has been observed in studies using keratinocytes, cells of ectodermal origin (Rheinwald and Green, 1975). Taken together, the findings listed above concur that the replicative ability of cells within the living organism decreases as the organism ages.

It can be extrapolated, therefore, that a relationship may exist between a species maximum lifespan and the maximum replicative potential of cultured cells. That such a relation-

ship exists has been claimed by some (Hayflick, 1973; 1977) and discounted by others (Stanley et al., 1975). It is difficult to interpret the existing data since an appropriately controlled study with cells from a variety of animal species using comparable methods, media, etc., has yet to be done.

Another finding which supports the fibroblast model of aging is found in work on genetic disorders featuring premature aging and demise.

1.1.2 Genetic Variants of Aging

Aging, like many life processes, is determined both by the organism's genetic endowment and its interaction with the environment (Schneider, 1978). The genetic component to the rate of aging is most evident when comparing interspecies lifespans (mouse 5 years, man 120 years), or even lifespans of inbred lines of animals of the same species. Twin studies (Bank and Jarvik, 1978) also show smaller variation in the lifespans of monozygotic twins compared to dizygotic twins and sibs. Although the conclusion that aging is in part genetically determined is unavoidable, the nature of the genetic processes in aging are very poorly understood. A classical approach to solving this difficult task is studying genetic variants of aging in which the typical aging process is accelerated or delayed.

Longevity syndromes have been reported. For

Table 1a

Table Selected Pathophysiological and Cellular Criteria of Aging[a]

1. Intrinsic mutagenesis hypothesis of aging
2. Increased frequency of nonconstitutional chromosomal aberrations
3. Increased susceptibility to one or more types of neoplasms relevant to aging
4. Defects in stem cell populations or in the kinetics of stem cell proliferation
5. Premature graying or loss of hair or both
6. Dementia or certain types of related degenerative neuropathology or both
7. Increased susceptibility to slow virus
8. Increased amyloid deposition
9. Increased deposition of lipofuscin pigments
10. Diabetes mellitus
11. Disordered lipid metabolism
12. Hypogonadism
13. Autoimmunity
14. Hypertension
15. Degenerative vascular disease
16. Osteoporosis
17. Cataracts
18. Abnormalities of mitochondria in one or more systems
19. Regional fibrosis
20. Abnormal amounts or distributions of adipose tissue
21. A miscellaneous group of syndromes with potential relevance to the pathobiology of aging

[a] From G. M. Martin (1977)

Table 1b

Table Rank Order of Premature Aging Syndromes Scored by Criteria in Table

Number of times scored	Syndrome
14	Down syndrome
12	Werner syndrome
12	Cockayne syndrome
9	Progeria (Hutchinson–Gilford syndrome)
8	Ataxia telangiectasia
8	Cervical lipodysplasia, familial
8	Seip syndrome generalized lipodystrophy hereditary type
8	Klinefelter syndrome
7	Turner syndrome
6	Myotonic dystrophy (Steinert disease)

– from Goldstein (1978).

example, individuals with familial hypobetalipoproteinemia and hyperalphalipoproteinemia (Glueck et al., 1976) possess lifespans significantly increased over the general population as a result of genetically-acquired protection from atherosclerosis.

Based on several selected pathological changes and cellular criteria that accompany normal aging (Table 1-1a) several human genetic disorders (Table 1-1b) were shown to exhibit accelerated aging and decreased lifespan as a component of their phenotype (Goldstein, 1978; Martin, 1978; Brown, 1979). It is readily apparent that few of the diseases are expressions of accelerated normal aging alone. Thus comparisons between the accelerated aging syndromes and normal aging must be made with prudence.

By far the most dramatic disorder featuring rapid aging is progeria or Hutchinson-Gilford Syndrome. It was described almost a century ago though few cases have been carefully studied. Although information on its mode of inheritance is tenuous, it is considered an autosomal recessive disorder with a frequency of one in five million births (DèBusk, 1972). Progeria presents as marked growth retardation in the first year of life. Loss of hair and development of a senile appearance occur in the first few years. Internally skeletal abnormalities are characteristic and the child usually suffers an atherothrombotic incident within the first decade of life. Sexual maturity is never

Table 1-2

Pathological similarities in normal aging and progeria

Disease Feature	Normal Aging	Progeria	Werner Syndrome
Vascular disease	common	very common	increased
Hypertension	common	increased	slight
Diabetes	some	rare	common
Neoplasia	common	rare	increased
Senile dementia	common	absent	rare
Cataracts	common	rare	severe
Skin atrophy	slight	severe	very severe
Gonadal atrophy	common	immature	severe
short stature	slight	dwarfism	slight

attained although some patients have lived into their third decade (mean lifespan 13.4 years) before succumbing usually to severe atherosclerosis. Endocrinological problems are found only in some cases. The disease exhibits some variability and partial expressions of the disease have been described (Goldstein, 1978).

Another disorder thought to involve accelerated aging is Werner Syndrome which usually expresses itself late in the second decade of life, with premature onset of senile characteristics of skin and hair being diagnostic. Unlike progerics, patients with Werner Syndrome are fertile, despite hypogonadism, and can procreate normally. These patients can survive into their sixties but the average lifespan is between 45 and 50 years, death being caused by malignant and/or cardiovascular disease. Some of the similarities and differences of progeria and Werner Syndrome are outlined in Table 1-2 and have been discussed in detail elsewhere (Goldstein, 1978; Tice and Schneider, 1976).

In vitro, skin fibroblast cultures have been established from both progeric and Werner patients and exhibit generally poor ability for growth and a significantly curtailed lifespan in tissue culture (Goldstein, 1978) (Figure 1-2). Thus, the etiology of these diseases may well have a cellular basis. Several studies have attempted to locate the primary defect in progeric and Werner cells. Fine ultrastructural similarities exist between late-passage

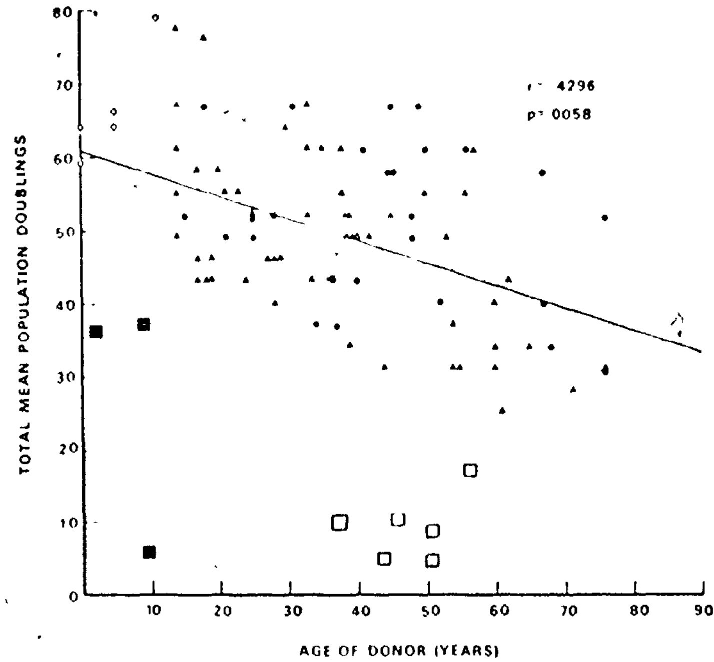


Figure 1-2 Relationship between age of donor and replicative life span of cultured skin fibroblasts. The majority of the symbols represent a single comparative study of three groups of subjects: (●) 25 normal controls; (▲) 30 genetically prediabetic subjects; (Δ) 25 overt diabetic subjects. Additional symbols represent other cell strains interpolated to show the effect of accelerated physiological aging on the cellular life span *in vitro*: (■) progeria; (□) the Werner syndrome; ( ) normal newborns and young controls.

– from Goldstein (1978).

and prematurely-aged cells (Basler et al., 1979), including heavy vacuolation, nuclear dysmorphology, increase in lysosome size and number and dilation of intracellular membranes (being evident features). Dysmorphology of mitochondrial networks has been shown to be characteristic of progeric cells as well (Goldstein and Moerman, unpublished results).

Fibroblasts from progeric and Werner patients have been shown to contain a substantial fraction of defective (heat-labile) cytoplasmic enzymes (Goldstein and Moerman, 1976). A smaller, but similar, increase in enzyme heat-lability has been observed in late-passage normal cells. Progeric and Werner fibroblasts also show reduction in histocompatibility locus antigen (HLA) markers (Singal and Goldstein, 1973; Goldstein et al., 1975) unlike normal fibroblasts which retain HLA antigenicity throughout their tissue culture lifespan (Brautbar et al., 1973).

It has been suggested that a primary impediment in the processing of molecular information may account for these findings. Errors in protein synthesis may result in increasing amounts of altered proteins containing amino acid substitutions. These may be enzymes or structural proteins. However, post-translational modification of proteins may also play a substantial role in altering proteins (Kahn et al., 1977). Recent work illustrates that progeric cells may be defective in controlling their pro-

teolytic systems and thus show abnormal protein turnover (Elliot, 1980).

Several aspects of DNA metabolism have been implicated as primary defects in premature aging disorders. Epstein (1973) suggested that deficiency in DNA repair may cause the shortened in vitro lifespans of progeric cultures. The deficiency in progeric cells to repair single strand breaks in DNA has been questioned by some (Regan and Setlow, 1974; Bradley et al., 1978) and supported by others (Brown et al., 1976; rainbow and Howes,1977).

Werner cells on the other hand exhibit normal DNA repair but markedly decreased rates of DNA synthesis (Fujiwara et al., 1977). The defect in DNA replication is overcome by fusion of Werner cells with normal fibroblasts (Tanaka et al., 1979) and there is evidence that both nuclear and cytoplasmic environments are involved in retarded DNA synthesis (Tanaka et al., 1980). Recently, Salk et al.(1980) have illustrated minor chromosomal rearrangements in Werner cells though not all the cell strains examined possessed them (G.M. Martin, personal communication).

Elucidating the primary biochemical defect in progeria and Werner syndrome requires further investigation and will certainly shed light on the nature of these premature aging syndromes.

1.1.3 Biochemical Studies into Cellular Aging

By meticulously investigating how any measurable biochemical process is altered in fibroblasts from progeric subjects or during in vitro senescence of normal fibroblasts, information pointing to the primary biochemical defect of aging may be revealed. A review of all the biochemical parameters checked for relevance to cellular aging is beyond the scope of this work and the reader is guided to a comprehensive, if not critical, literature review by Leonard Hayflick (1980a). Items from that review are outlined in Table 1-3. It is immediately clear that some disagreement has been raised. The observed biochemical changes occur at all levels of cellular function and point to a defect in one (or more) of the cell's primary mechanisms. Many of the functional alterations found in cultured cells occur well before cessation of replicative ability.

Efforts were made to determine whether cells cease replication at a particular point in the cell cycle (Figure 1-3). Considerable evidence (Yanishevsky et al.; 1974; Grove and Cristofalo, 1977) shows that the majority of senescent fibroblasts have stopped cycling in G_1 phase. Prolongation of cell cycle time in late-passage culture was also due, predominantly, to increase in G_1 phase (Grove et al., 1976). It is not clear, however, whether all replicative function was ceased in these cells, since some DNA synthesis was possible (Macieira-Coelho, 1974b).

Table 1-3

Biochemical changes in human diploid fibroblasts during in vitro aging

Increase in:	No change in:	Decrease in:
- DNA repair	- heat-lability of DNA, RNA	- DNA synthesis and repair
- RNA content and turnover	- DNA repair	- RNA synthetic rate
- lysosomes and lysosomal enzymes	- activity of numerous enzymes	- RNA template activity
- heat-lability of enzymes (G6PD, 6PGD)	- S phase of cell cycle	- collagen synthesis
- activity in RNAse, DNase and protease associated with chromatin	- mis-synthesized proteins	- proteolytic capacity
- protein content	- degradation of normal or analogue-containing protein	- pH 7.8 protease
- proportion of rapidly degraded protein	- number of mitochondria	- activity of numerous enzymes
- proteolysis of analogue-containing protein	- respiration	- protein synthetic rate
- misincorporation of Met into H1		- response to growth stimulus
- Met incorporation		
- cell size		
- cell cycle time		
- non-mitotic cells		
- residual bodies		
- dysmorphic mitochondria		
- membrane changes		

after Hayflick (1980a).

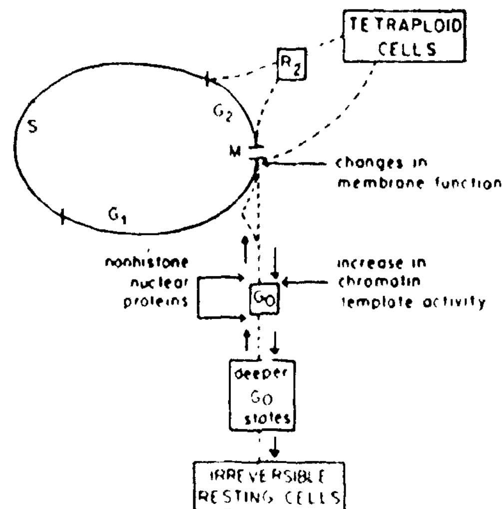


FIGURE 1.3 The perils of a cell. Continuously dividing cells (lining epithelium of the crypts of the small intestine, exponentially growing cells in culture) are cells that go around the cycle, from one mitosis (M) to the next through G_1, S and G_2. They can leave the cycle either from G_2 or immediately after mitosis. Cells leaving the cycle in G_2 can return to the cycle by reentering it before (epidermal cells of mouse skin) or after M (tetraploid hepatocytes). In the latter case the tetraploid cell goes once around the cycle and divides into two tetraploid cells. Cells leaving the cycle after M go into a brief G_1, then into G_0 (certain density inhibited cells, uterine cells in the castrated animal and so forth). The cell can return from G_0 to G_1 (a step characterized by a prompt increase in chromatin template activity) or it can go into a deeper G_0 state (lymphocytes, cells left confluent for long periods of time). Cells can return to the cycle even from this deeper G_0 state. However, in some instances, they go into an irreversible state of quiescence which they do not leave except to die (polymorphonuclear leukocytes, keratinizing cells of the epidermis.

1.1.4 Theoretical explanations of the Hayflick Phenomenon

The nature of cellular senescence has generated much speculation as to its primary cause. Although numerous theories have been advanced, no simple hypothesis has received universal acceptance. One prevalent criticism of cellular aging research is indeed the apparent excess of theories, many generated on a minimum of experimental evidence and also fail to make predictions which are testable experimentally. Although the knowledge of what parameters increase or decrease as cells age is important, much of the data collected has offered very little in the way of definitive evidence for or against any particular theory.

To further confound the issue, for each theory there exists an opposing one (Table 1-4). Although no definitive statements can be made, previous research has favoured some of the theories over others. The weight of evidence favours an "intrinsic" mechanism responsible for cellular aging, although Macieira-Coelho (1980) maintains that environmental factors synergistically influence intrinsic cellular properties which lead to aging.

A prevalent view has been that, whatever the aging properties of a cell may be, errors arise within enzymes responsible for information processing and other essential cell functions. The general error theory of aging has been widely debated although most of the tests of this theory

Table 1-4

Theories of Cellular Aging

theory	opposes	theory
A. Epiphenomenal - extrinsic factors - hormones[1] - mutagens[2]		Fundamental - intrinsic factors - waste products[3] - spontaneous mutation - genetic programs - error theories
B. Non-error - mutations (environmental and/or spontaneous) → aberrant molecules → aging.[4]		Error - inherent infidelity in information processing.[5] → errors → mutations → aging
C. Error Increase - increasing infidelity error catastrophe.[6]		Repair Decrease - decreasing ability to sequester errors, DNA repair[7], selective proteolysis.[8]
D. Stochastic - random loss of cells[4], genes[9], etc. - commitment.[10] - errors		Deterministic - genetic aging programs[11] - counting mechanisms[12]

1. Finch, 1976.
2. Lima et al., 1972
3. Lesher et al., 1957
4. Szilard, 1958; Maceira-Coehlo, 1980.
5. Medvedev, 1980; Fulder, 1979.
6. Orgel, 1963; 1970.
7. Moore & Schwartz, 1978.
8. Goldberg & St.John, 1976.
9. Strehler et al., 1979.
10. Kirkwood & Holliday, 1975.
11. Comfort, 1979.
12. Good & Smith, 1974.

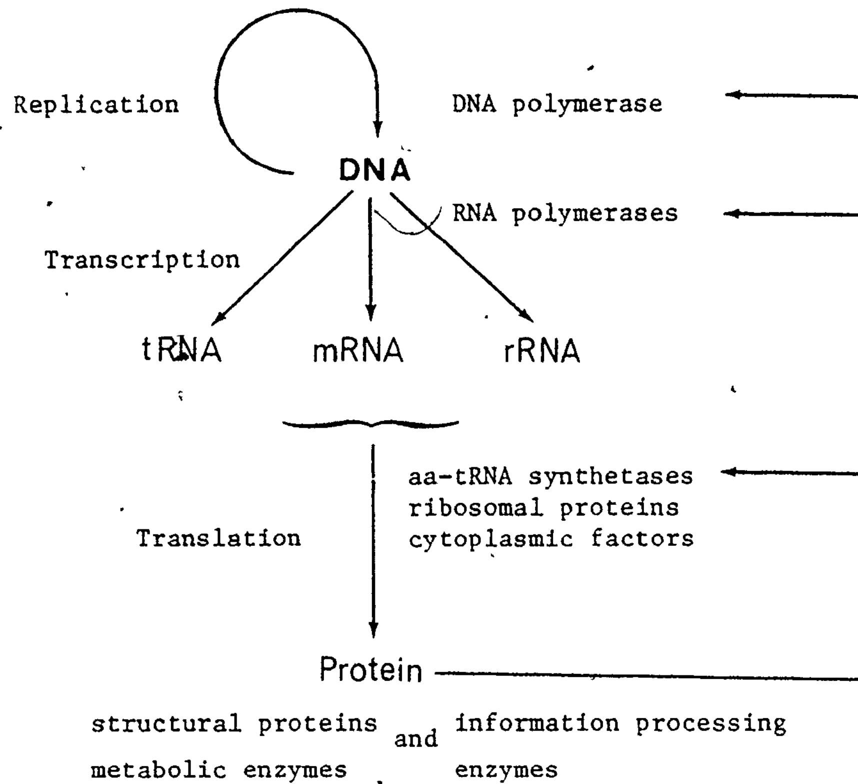


Figure 1-4 Cellular information flow and error feedback.
Since the processing of genetic information is accomplished by a variety of proteins, the translational apparatus resides at the focus of maintaining the fidelity of information flow.

have been relevant to Orgel's "Error Catastrophe Theory" (section 1.1.5). Changes at the RNA level (Figure 1-4) have been viewed as the most decisive in age-related impairment of cellular function. Hypotheses concerning errors at different levels of cellular activity also have been advanced. At the DNA level, somatic mutations (Hirsch, 1978), deficient repair mechanisms (Tice, 1978), or faulty replication (Linn et al., 1976; Good, 1977) may affect aging. Deterioration of nuclear template function may also be centrally implicated in aging (Whatley and Hill, 1980). After translation, the final protein products may be subject to aberrant modifications such as cross-linkage (Bjorksten, 1968) or deamidation (Robinson et al., 1976).

Due to its diffuse nature, no simple test of the general error theory seems feasible. The literature features both contradictory (Cutler, 1976; Morrow and Garner, 1979; Gupta, 1980; Whatley and Hill, 1980) and supportive (Fulder, 1979; Macieira-Coelho, 1980; Medvedev, 1980) evidence.

A current debate on the general nature of cellular aging centres around the senescence phenomenon (Phase III) as an expression of "terminal differentiation" (Hayflick, 1973; Martin et al., 1974) in which specific genetic systems which induce aging would be activated. It is unclear whether terminal differentiation is a stochastic event or whether it is predetermined by a specific genetic program or counting mechanism.

The "commitment" theory of cellular senescence holds that the cultured fibroblast was originally immortal and a stochastic event during subculture caused irreversible differentiation to a mortal cell. Kirkwood and Holliday (1975) proposed that the probability of differentiation was fixed throughout subculture and that the committed (differentiated) fibroblast was capable of numerous although limited replicative cycles. They concluded, therefore, that the Hayflick phenonemon was an artifact of culture conditions. Assertions made by this model (Holliday et al., 1977) have been criticized on several grounds including the inability to detect immortal fibroblasts in culture (Harley and Goldstein, 1980). An opposing model of cellular commitment (Shall and Stein, 1979) treats the committed cell as post-replicative, with the probability of commitment increasing with each successive generation. This model does not agree with experimental evidence (Smith et al., 1977) either, and a model compromising the former two has been derived (Prothero and Gallant, 1981).

Shmookler-Reis et al., (1981) have also observed that data concerning heterogeneity of proliferative potential of clonal populations of fibroblasts (Smith and Hayflick, 1974; Smith and Whitney, 1980; Smith and Lumpkin, 1981) cannot adequately distinguish a purely stochastic process from a deterministic mechanism modified by chance events. With each additional work the theories of cellular aging must be

reassessed and new models proposed.

1.1.5 The Orgel Hypothesis

One of the most generally tested theories of cellular senescence has been the error catastrophe theory. Proposed by Orgel (1963, 1970) the hypothesis concerns the self-propagation of errors in the translational (protein synthetic) machinery and is only part of the general error theory. The hypothesis has been popular due to its simplicity, its many specific predictions and has also been amenable to mathematical analysis (Orgel, 1970; Hoffmann, 1974; Goel and Ycas, 1975; Kirkwood, 1980; Gallant and Prothero, 1980).

Since the processing of genetic information (Figure 1-4) is conducted by many specific proteins (DNA and RNA polymerases, ribosomal proteins, aminoacyl-tRNA synthetases), Orgel (1963) postulated that if errors occurring in these molecules hampered the fidelity of their function, errors would be recycled during processing and the resulting propagation of errors would lead to accumulation of errors in proteins synthesized. This "positive error feedback" or "error propagation" would ultimately lead to an "error catastrophe", a point at which the error frequency of protein synthesis is so high that proteins necessary for cell function become too error prone and inefficient for further cell survival. Workers set out to test the theory by a) looking for altered proteins in aged cells b) testing the idea of error feedback c) monitoring the fidelity of protein synthesis as a function

of cellular age.

The theory was supported by early observations of apparent error catastrophes in mutants of Neurospora which synthesize increasingly abnormal proteins prior to cell death (Lewis and Holliday, 1970). Holliday and Tarrant (1972) also reported altered enzymes in late-passage human fibroblasts, however, other workers (Danot and Gershon, 1975; Pendergrass et al., 1976; Evans, 1977) have reported no increase in altered enzymes in senescent cells. The synthesis of viral particles also seems to proceed with comparable fidelity in cells infected at early or late passage (Holland et al., 1973; Danner et al., 1978). Goldstein and Moerman (1975, 1976) observed an increased proportion of heat-labile enzymes in cells from patients with premature aging diseases.

Many alterations in various enzyme activities in a variety of organisms have been reported (Rothstein, 1977). Not all enzyme activities measured decline with age (Gershon, 1979) and there is evidence that enzyme modification is post-translational (Kahn et al., 1974; Kahn et al., 1976). It is, as yet, difficult to implicate age-related enzyme alterations to the breakdown of translational fidelity.

There have been numerous attempts to induce error catastrophe mistranslation by forcing the translational machinery to synthesize error containing proteins. Using amino acid analogues to generate abnormal proteins, Harrison and Holliday (1967) could shorten the lifespan of Drosphila.

This was due, however, to inhibitory efforts on protein synthesis, not to error accumulation (Dingley and Maynard-Smith, 1969; Bozcuk, 1976). Holliday and his co-workers also showed that amino acid analogues were not as faithfully distinguished from their natural counterparts (Lewis and Tarrant, 1972) and could induce premature senescence in both cultured cells (Holliday, 1972) and mice (Holliday and Stevens, 1978). However, age-acceleration by amino acid analogues was not observed in either Drosophila (Shmookler-Reis, 1976) or cultured fibroblasts (Ryan et al., 1974; Duncan and Dell'Orco, 1979).

Drugs which reduced the fidelity of translation have also been used in tests of error feedback and attempts to induce error catastrophe. Streptomycin induces ribosomal misreading in E. coli (Davies et al., 1965; Old and Gorini, 1965) and upon treatment with the drug, the error frequency of protein synthesis is shown to increase (Branscomb and Galas, 1975; Edelmann and Gallant, 1977). With continued treatment, the error frequency increases for a few generations and then stabilizes at a higher level (Edelmann and Gallant, 1977; Gallant and Palmer, 1979; Gallant and Foley, 1980). The error frequency returns to normal upon removal of the drug (Branscomb and Galas, 1975). Gallant's conclusions that error catastrophe is not an inevitable consequence of error feedback have been criticized by Rosenberger et al., (1980) who observe different kinetics of error feedback but do not report

(stabilization of) error levels before the death of the bacteria.

Similar attempts to test error propagation in mammalian cells, using drugs known to increase ribosomal ambiguity in eukaryotes (such as paromomycin, Palmer and Wilhelm, 1978) have not yet been reported.

The fidelity of protein synthesis in aging systems is discussed in section 1.2.3.

1.2 Protein Biosynthesis

The synthesis of proteins is an extremely complex process becoming ever more irreducible. It requires ribosomes mRNA, a variety of tRNAs, aminoacyl-tRNA synthetases, a plethora of factors, ATP, GTP, etc. giving the appearance of an "incongruous assemblage of parts...where knowing ninety percent of the mechanism would not allow one to predict the remaining ten percent" (Woese, 1980). How more than 150 macromolecules unite to synthesize one molecule of protein has been the subject of intense research over the past two decades. Finally some of the molecular details are being understood. The first part of this section deals with some aspects of the mechanisms of protein synthesis and acts as a prelude to a more comprehensive look at the translation of poly(U). Finally the fidelity of protein synthesis with special emphasis on aging will be discussed.

1.2.1 Mechanisms of Translation

Much of the mechanics of protein synthesis has yet to be ascertained. For the purpose of this thesis, this discussion will consider only the basic steps of translation. Moreover, interactions between ribosomes and/or factors with regulatory compounds will not be considered.

Initiation of protein synthesis (reviewed recently by Grunberg-Manago and Gros, 1977; Hershey, 1980; Jagus et al., 1981) involves formation of an initiation complex, containing initiator tRNA, mRNA and the ribosome, which then enters into the elongation cycle. The general pathway of initiation is quite similar in both prokaryotic and eukaryotic cells.

Initiation in E. coli (Figure 1-5) has been extensively studied. The first step involves dissociation of the 70S ribosome. It is likely that IF-1 induces the dissociation while IF-3 binding to the 30S ribosomal subunit prevents reassociation. IF-2 then binds to the 30S subunit and a trifactor complex is formed. Once initiation factors are bound, binding of mRNA and fmet-$tRNA_f$ can occur. Though it is not certain which occurs first it is known that IF-3 mediates mRNA binding and initiator tRNA is bound as a fmet-$tRNA_f$·IF-2·GTP complex. Upon binding of mRNA, IF-3 is released and the 30S initiation complex is formed. It has been postulated that sequences to the 5'-side of the initiator

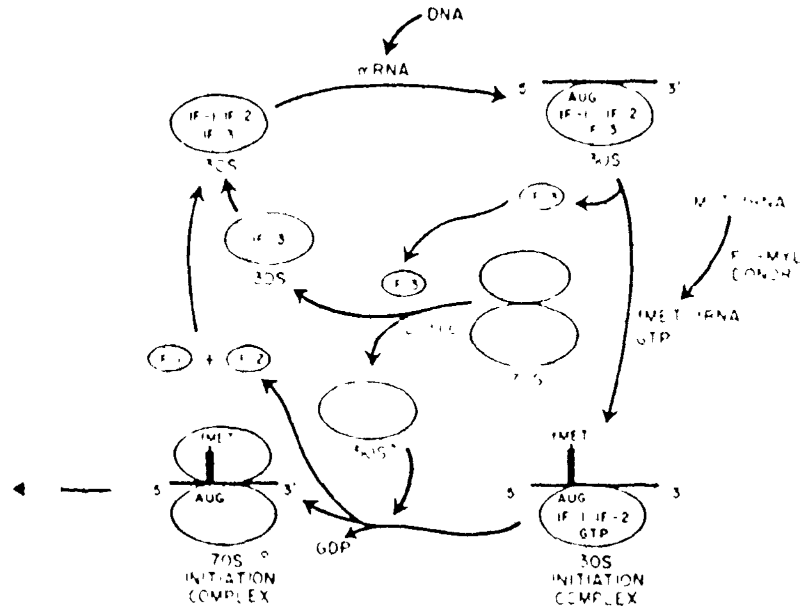


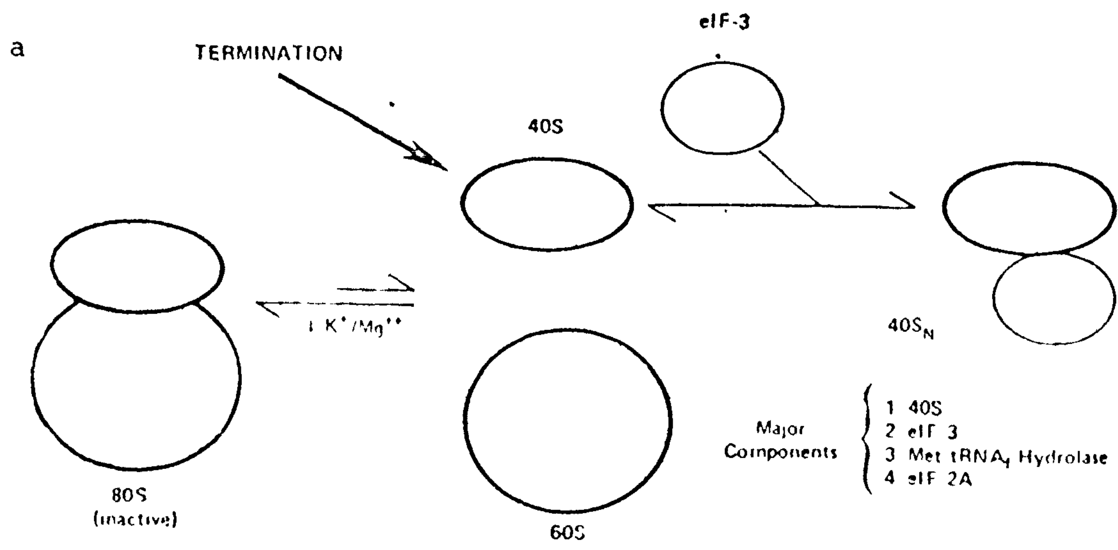
Figure 1-5 Initiation of protein synthesis in prokaryotes.
- from Weissbach (1980).

codon of mRNA are complementary to sequences on 16S ribosomal RNA and are necessary for mRNA-30S subunit binding (Grunberg-Manago and Gros, 1977). Finally, the 50S subunit is bound with concomitant hydrolysis of GTP and release of GDP with IF-1 and IF-2. After release of IF-2 the initiator fmet-$tRNA_f$ is found at the ribosomal P-site with the A-site ready to accept the second aa-tRNA and begin elongation.

The assembly of the eukaryotic 80S initiation complex (Figure 1-6) is more intricate involving many factors (Schreier et al., 1977) whose precise role is still unclear (Trachsel et al., 1977).

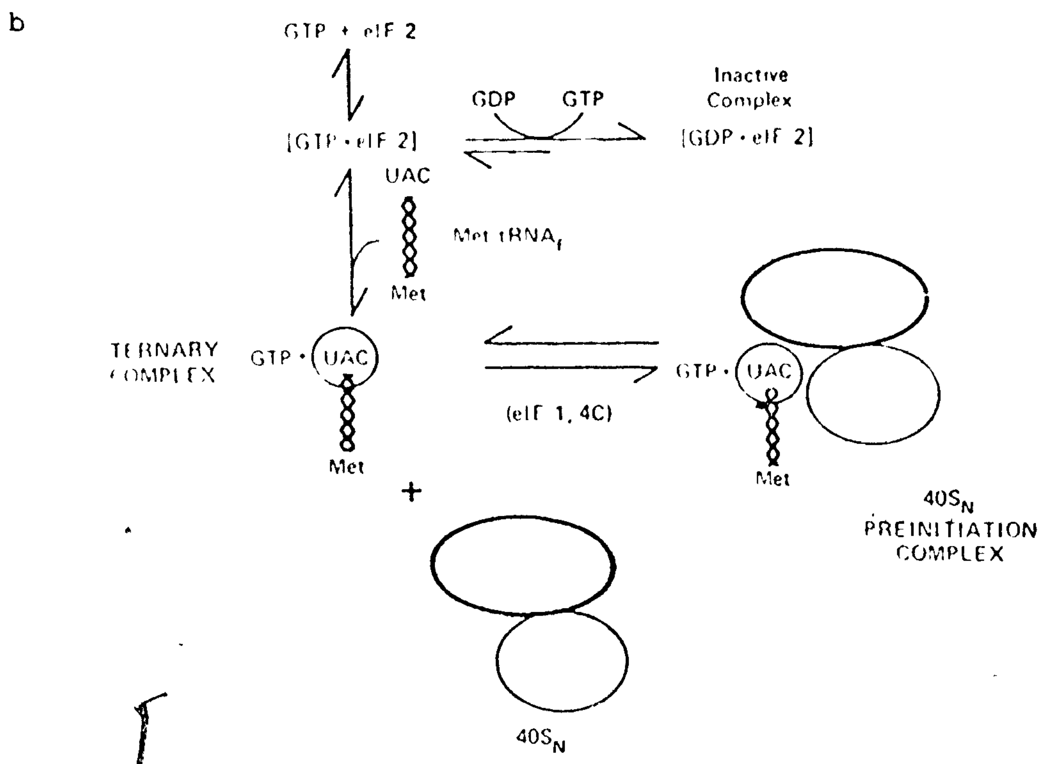
Nonfunctional 80S ribosomes dissociate into 40S and 60S subunits (aided by eIF-4C) (Jagus et al., 1981). The smaller subunit is bound by eIF-3 preventing reassociation (Figure 1-6a). Initiator tRNA is activated to a GTP·eIF-2·-met-$tRNA_i$ ternary complex which then binds the eIF-3·40S complex (Figure 1-6b). The 40S preinitiation complex is stabilized by factors eIF-1, and -4C. Only then does mRNA bind to the preinitiation complex (Figure 1-6c). The binding occurs at or near the 5'-terminus of the mRNA near 5'-m^7guanosine "cap" (Kozak, 1978,1979). The cap region is recognized by a polypeptide associated with the initiation factors (Sonenberg et al., 1978) and binding to the region is enhanced by eIF-3, -4A, -4B and marginally by eIF-1. Although ATP hydrolysis is essential for mRNA binding it is unclear which factors participate or what functional role

a



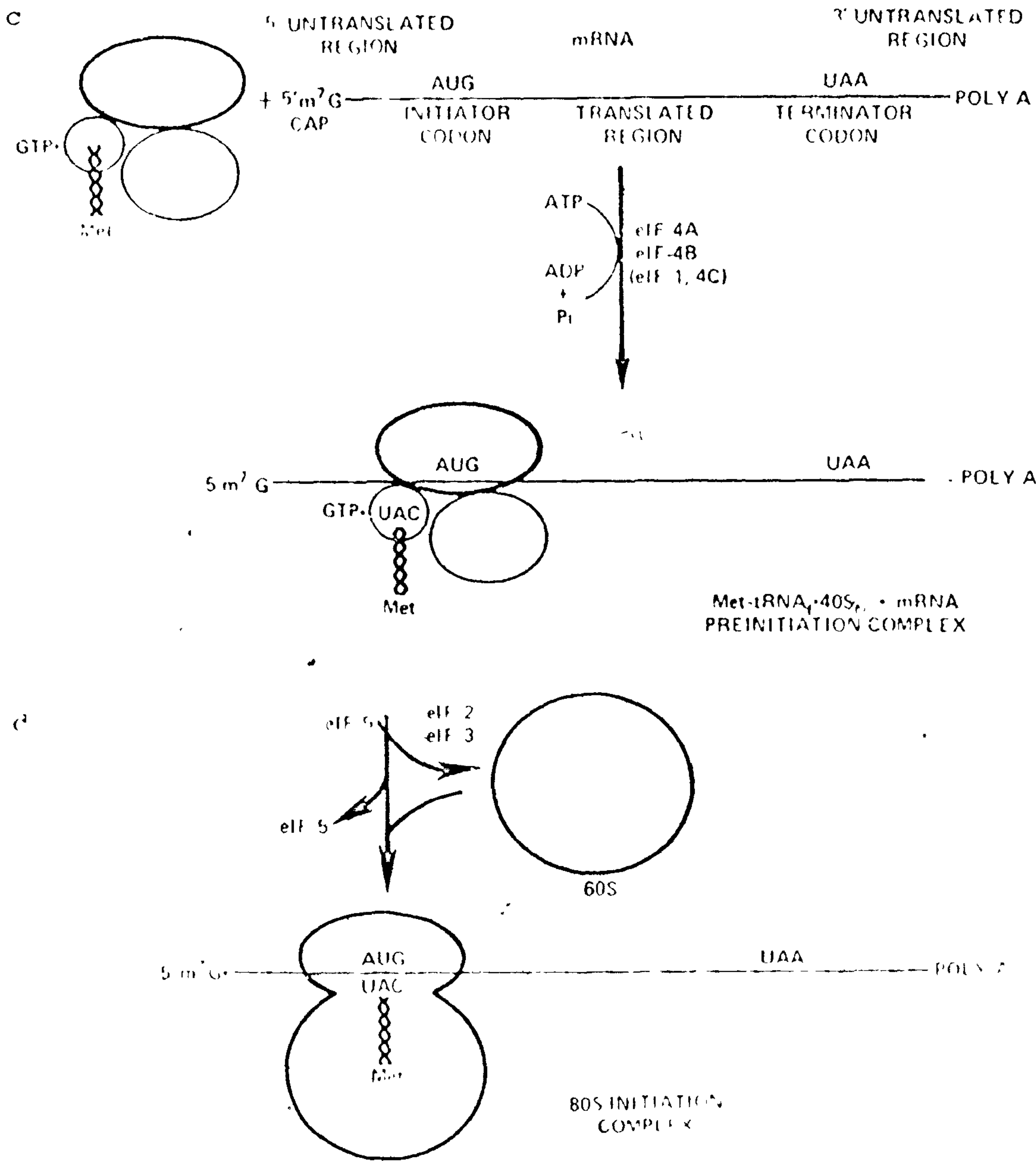
Formation of native 40S ribosomal subunits. The first step of 80S initiation complex formation is the binding of eIF 3 to the 40S ribosomal subunits which are released upon termination of polypeptide synthesis. No other initiation factors or energy source are required. Binding of eIF 3 to form $40S_N$ prevents the spontaneous reassociation of 40S and 60S ribosomal subunits that would occur under in vivo ionic conditions. In vitro, formation of inactive 80S ribosomal couples is promoted by decreased K^+/Mg^{++}. Other major components associated with $40S_N$ are Met tRNA$_f$ hydrolase and eIF 2A.

b



Met tRNA$_f$ binding to $40S_N$. The second step of 80S initiation complex formation is the binding of Met tRNA$_f$ to $40S_N$. This is accomplished through the formation of a ternary complex composed of Met tRNA$_f$, eIF 2, and GTP. eIF 1 and eIF-4C appear to stabilize the [40S eIF-3 eIF 2 Met-tRNA$_f$ GTP] preinitiation complex. Physiological regulation of this early step of protein synthesis may be mediated through the greater affinity of eIF 2 for GDP than GTP. This results in the formation of an inactive eIF 2 GDP binary complex when the energy charge of the cell is low. Binding of the ternary complex occurs independent of the initiation codon AUG. In contrast, mRNA [illegible] dependent on Met tRNA$_f$ binding to $40S_N$.

Figure 1-6 Initiation of translation in eukaryotes.



[illegible] mRNA to the [illegible] preinitiation complex. The third step of initiation [illegible] mRNA binding to the [illegible] Met-tRNA, GTP) complex. Three initiation factors are required for [illegible] of mRNA: eIF 3, eIF 4A and eIF 4B. It is not known at the present time whether the eIF 3 already bound to 40S [illegible] this function. Actively translated messenger RNA is associated with characteristic proteins which appear to be [illegible] and specifically bound to the mRNA. The relationship of these proteins to initiation factors is unknown, but no [illegible] evidence has been obtained for the general existence of message specificity factors. [illegible] Binding of the [illegible] ribonucleoprotein particles [illegible] ATP hydrolysis.

60S ribosomal subunit joining. Joining of the 60S ribosomal subunit [illegible] 40S, mRNA [illegible] eIF 2 and eIF 3. eIF 5 dep[illegible] GTP [illegible] activity appears to mediate factor release prior to 80S initiation complex formation. A stable [illegible] of eIF 5 with the Met-tRNA, 40S, mRNA complex has not been observed. Indirect evidence for an eIF 2 recycling [illegible] complex by release [illegible] obtained.

Figure 1-6 continued. - from Safer and Anderson (1978).

this serves. eIF-5 hydrolyzes GTP, and GDP, eIF-2, and eIF-3 are released leaving the mRNA·40S·met-$tRNA_i$ in a metastable state, which can bind available 60S ribosomal subunits. The resultant 80S complex contains met-$tRNA_i$ at the ribosomal P-site prepared for elongation.

The mechanisms of elongation have been the subject of current reviews (Bermek, 1978; Krayevsky and Kukhanova, 1979; Hershey, 1980). This phase of protein synthesis, when amino acids of the primary sequence of the protein are actually bonded together, is, by convenience, divided into 3 parts (Figure 1-7): binding of aa-tRNA to the ribosomal A-site, peptide bond formation and translocation of the nascent chain peptidyl-tRNA back to the P-site.

There are 2 elongation factors. In prokaryotes are factors EF-T and EF-G, with EF-T existing in a heat-labile (EF-Tu) and heat-stable (EF-Ts) form. EF-Tu complexes with GTP which in turn reacts with incoming aa-tRNA. This ternary complex binds to the ribosome only if the proper codon exists in the ribosomal A-site. Correct binding is followed by GTP hydrolysis by EF-Tu with liberation of P_i, GDP remaining bound to EF-Tu. (EF-Ts displaces GDP forming ER-Tu·Ts which binds GTP, releasing Ts and binding another aa-tRNA for yet another cycle of elongation). Upon GTP hydrolysis, transpeptidation occurs with the transfer of the nascent peptide of the peptide-tRNA at the P-site to the α-amino group of the aa-tRNA of the A-site (ester bond

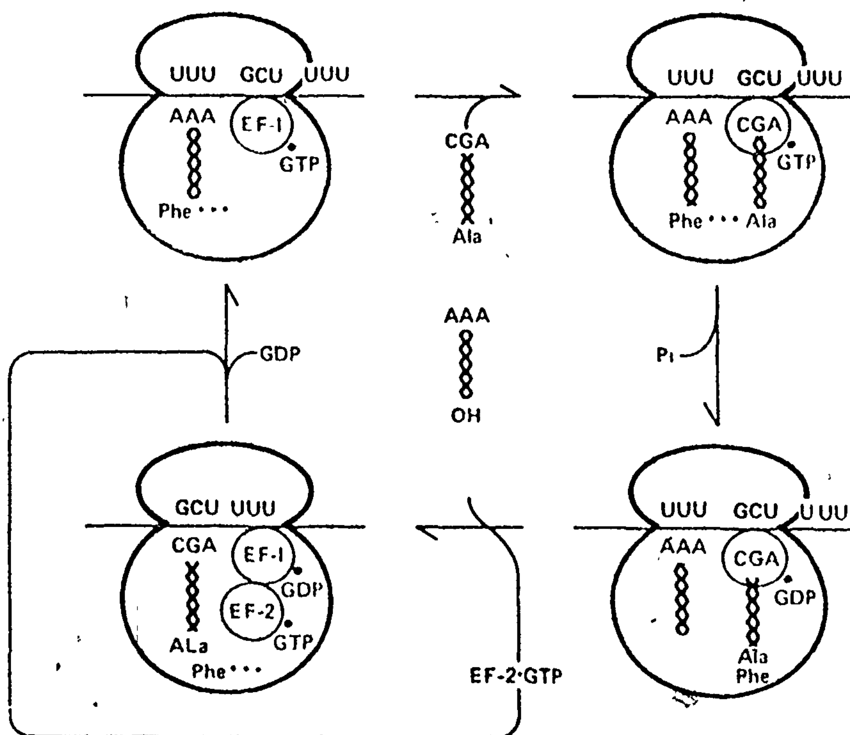


Figure 1-7 Polypeptide elongation. Following initiation factor release and 80S initiation complex formation, EF-1 binds to the 80S ribisomal couple and in the presence of GTP binds the aminoacyl-tRNA specified by the triplet-codon of mRNA. Upon hydrolysis of GTP, transpeptidation occurs, and the growing nascent chain is elongated by one amino acid. Binding of a stable EF-2 GTP complex mediates release of the uncharged tRNA from the P site, translocation of the peptidyl tRNA from the A site to the P site, and exchange of EF-1 bound GDP with GTP to complete the elongation cycle.

In this scheme, EF-1 remains associated with the 80S ribosomal couple until termination.[19] In an alternate mechanism, binding of an EF-1:aminoacyl-tRNA.GTP ternary complex, release, and reactivation analogous to the prokaryotic EF Tu-Ts cycle[19] is thought to occur

- from Safer and Anderson (1978).

broken, amide bond formed). The peptidyl transferase activity is a structural part of the larger ribosomal subunit. EF-G binds GTP, then complexes with the ribosome, only if stripped tRNA is present at the P-site, causing rapid translocation of the peptidyl-tRNA from the A-site to the P-site with concomitant movement of the mRNA by one codon position (Matzke et al., 1980). GTP hydrolysis occurs only for release and recycling of EF-G.

The elongation mechanism in eukaryotic cells is very similar to that in prokaryotes as evidenced by the interchangeability of eukaryotic and prokaryotic elongation factors (Grasmuk et al., 1977). Eukaryotic factors EF-1 and EF-2 behave like prokaryotic factors EF-T and EF-G, respectively. EF-1 exists as 2 complementary factors EF-1α and EF-1β. EF-1β may be analogous to EF-Ts being required for regeneration of EF-1α·GTP. EF-2, like EF-G, binds GTP and is also only transiently associated with the (80S) ribosome. Unlike EF-G, EF-2 exhibits a unique ADP-ribosylation reaction with diphtheria toxin. ADPR-EF-2 can still bind GTP but translocation activity is greatly inhibited.

Upon reaching the end of the coding mRNA with one of the three nonsense codons, UAA, UAG, or UGA in the ribosomal A-site, protein synthesis is terminated (Caskey, 1977). In prokaryotes, two releasing factors have been described. RF-1 recognizes UAA or UAG and RF-2 recognizes

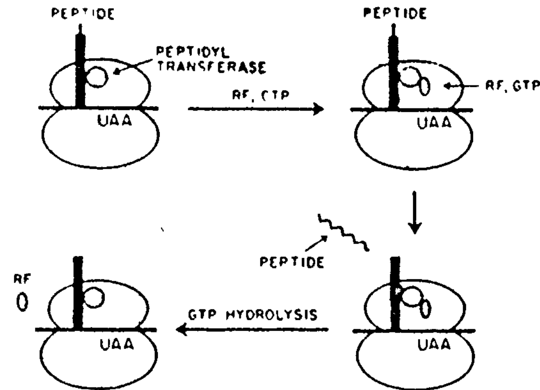


Figure 1-8 Proposed scheme for termination sequence in eukaryotes.
- from Weissbach (1980).

UAA or UGA. A third factor, RF-3, promotes RF-1 and RF-2 binding to the A-site, and possesses GTPase activity. There has been only one termination factor, RF, which recognizes all the stop codons, found in eukaryotic cells. RF requires GTP for binding (Figure 1-8) and in the presence of suppressor tRNA competes with the latter for the ribosomal A-site. The presence of RF at the A-site activates peptidyl transferase activity and the nascent peptide chain on the P-site is transferred to water (i.e. hydrolyzed). The various factors dissociate from the ribosome upon GTP hydrolysis. This allows the free ribosome to dissociate into subunits which once again partake in initiation.

The general mechanism of when and how the components of the translation apparatus interact is known, moreso for bacteria than eukaryotes. The similarities between the two systems are striking, however, showing considerable conservation throughout evolution.

1.2.2 Poly(U) Translation

An important question which must be addressed when studying protein synthesis directed by any synthetic messenger RNA, such as poly(U), is: How normal (or abnormal) is the translation of the synthetic template compared to translation of natural mRNA? Careful examination of the existing literature, which unfortunately is predominated by studies with the E. coli cell-free protein synthetic systems, does reveal, however, that during the various stages of translation: ribosome binding, initiation, elongation and termination, most events required by natural mRNA translation are also required by poly(U) though there are obviously some very important differences.

Initiation of protein synthesis is a complex process in which the ribosome interacts with mRNA and initiator tRNA to form the "initiation complex". The scheme of initiation complex was outlined in the preceding section (Figure 1-5, for prokaryotes; and Figure 1-6, for eukaryotes).

In prokaryotic cell-free systems, initiation of poly(phe) synthesis has been studied under two conditions: at high magnesium concentrations ($\sim$15 mM), and at low magnesium ($\sim$5 mM). At high magnesium concentrations, ribosomes are 100% associated and initiation on poly(U) requires only 70S ribosomes, and N-Ac-phe-tRNA, as the initiator tRNA. Initiation factors IF-1 and IF-2 are not involved in this process. Although there is no need for IF-3,

this factor stimulates poly(U) translation at 18 mM Mg^{+2} and constitutes a specific test for IF-3 anti-association activity (Grunberg-Manago and Gros, 1977). Initiation onto poly(U) at lower magnesium concentrations required both IF-1 and IF-3 (Revel et al., 1968). IF-1 is known to enhance IF-3 mediated ribosomal dissociation (Voorma et al., 1979). The 30S subunit in the presence of IF-3 binds poly(U) and then N-Ac-phe-tRNA to yield a poly(U)·30S·IF-3·N-Ac-phe-tRNA complex in a step that is analogous to the normal binding of fMet-tRNA to the 30S subunit. The latter process normally requires IF-2 which is not involved in poly(U)·30S·N-Ac-phe-tRNA formation. With natural mRNA, IF-3 is thought to be involved with 30S recognition of ribosomal binding sites on the mRNA, since partial disruption of the tertiary structure of the mRNA eliminates IF-3-dependant binding onto the 30S subunit. Aberrant initiation is the result (Vermeer et al., 1973).

Poly(U) binding of ribosomes was presumed non-specific and it has been shown that the basic proteins comprising the ribosome bind poly(U) spontaneously (Normura and Held, 1974). However, several lines of evidence suggest that poly(U) binding is specific. Poly(U) only binds to 30S and not to 50S subunits (Takanami and Okamoto, 1963). Ribosomal binding of both natural mRNA and poly(U) is inhibited by aurintricarboxylic acid (Grollman and Stewart, 1968) and poly(U) effectively competes with MS2-RNA for ribosome binding

(Szer and Leffler, 1974). These observations suggest that poly(U) and natural mRNA compete for an identical (or nearly identical) ribosomal binding site. The most compelling evidence for specific poly(U) binding comes from studies identifying the proteins in the 30S subunit that are involved in binding.

Several techniques have been employed to determine which proteins are essential for mRNA binding. Chemical inactivation of the 30S subunit by iodination (Shimizu and Craven, 1976) have implicated proteins S3, S14 and S19 as being involved with fMet-tRNA binding and S1, S2, S3, S14 and S19 involved in Phe-tRNA binding. Chemical modification of 30S subunit with maleic anhydride (Cantrell and Craven, 1977) inactivates both Phe-tRNA and poly(U) binding. Reconstitution with proteins S4, S11, S12, S13 and S18 restores poly(U) binding activity. These proteins coincide exactly with proteins modified by the attachment of brominated derivatives of the AUG codon to 70S and 30S ribosomes (Pongs et al., 1975). In this study, S21 was also shown to be involved in AUG binding. Fiser et al., (1975) also implicated S21 in the photoaffinity reaction of 70S ribosomes with poly(4-thiouridylic acid), a photoaffinity analog of poly(U). One difference in poly(U) and natural mRNA binding is exemplified in streptomycin-dependant mutants (Sm^D) of E. coli, in which protein S12 is modified (Wittmann and Wittmann-Liebold, 1974). In the absence of streptomycin,

Sm^D ribosomes translate poly(U) but not natural mRNA. It has been shown that IF-2 cannot be recycled on Sm^D ribosomes, thus fMet-tRNA is bound but cannot undergo peptide bond formation (Lazar and Gros, 1973).

Ribosomal protein S1 which is required for both poly(U)-dependent poly(phe) synthesis and MS2 RNA-dependent coat protein synthesis (Van Dieijen et al., 1975), has been purified from both 30S subunits and 70S ribosomes by its tight binding to poly(U), enabling 70S(-S1) ribosomes to be prepared (Khanh et al., 1979).

The RNA of the 30S subunit, 16S RNA, is also important in binding both fMet-tRNA and N-Ac-phe-tRNA. Treatment of E. coli with the bacteriocin Colicin E3 causes specific inhibition of protein synthesis by cleaving an oligonucleotide from the 3' end of 16S RNA. Ribosomes isolated from E3-treated cells are inactive in both poly(U)-dependent N-Ac-phe-tRNA binding and poly(AUG)-dependent fMet-tRNA binding (Normura et al., 1974).

Normally, upon formation of the 70S ribosome, the initiator fMet-tRNA is directed to the P-site by IF-2 occupying the A-site. Formation of poly(U)·70S·N-Ac-phe-tRNA is independent of IF-2 and GTP. Specific blockage of the A-site of 70S ribosomes with the antibiotic tetracycline does not inhibit poly(U)-dependent N-Ac-phe-tRNA binding in the absence of deacylated $tRNA_{phe}$ (Watanabe, 1972). However in the presence of uncharged tRNA which binds to the 70S

P-site, tetracycline inhibits N-Ac-phe-tRNA binding (Benne and Voorma, 1972) indicating that the initiator tRNA also occupies the P-site of the poly(U)·70S·N-Ac-phe-tRNA complex. Furthermore, with both natural and synthetic mRNA, the initiator tRNA binding to the P-site is codon specific (Grasmuk et al., 1975; Wurmbach and Nierhaus, 1979).

It is generally accepted that normal initiation of protein synthesis in E. coli occurs at the initiator codon AUG or GUG. In one case (Files et al., 1975), UUG acts as initiator codon on the mRNA of an amber mutant of lac repressor. Also, the triplets AUG, GUG and UUG are the most effective in stimulating fMet-tRNA binding to ribosomes in vitro (Ghosh et al., 1967). A recent report however describes poly(U)-dependent binding of fMet-tRNA to 30S ribosomal subunits (Van der Laken et al., 1979). This binding is as efficient as MS2 RNA-dependent binding and is stimulated by IF-1 and absolutely dependent on IF-2. IF-3, unlike the situation with MS2 RNA, however, slightly inhibits poly(U)-directed fMet-tRNA binding. Poly(A), poly(C) and poly(I) were not active in this regard, nor were U_3 or U_{14}. The fMet-tRNA bound to poly(U)·30S in this manner results in the synthesis of fMet-poly(phe) upon the addition of 50S subunits and cytoplasmic factors, and also formed fMet-puromycin when 50S subunits and puromycin were added. These results contradict the assumption that functional fMet-tRNA binding to ribosomes is absolutely dependent on the

presence of the AUG initiator codon.

Further evidence that initiation on a UUU codon is not as abnormal as previously supposed is the finding of two overlapping ribosome binding sites during translation of RNA4 of alfalfa mosaic virus by a heterologous (E. coli) cell-free system (Castel et al., 1977). On this mRNA, protein synthesis begins either with N-Ac-phe-tRNA at the UUU codon in the second triplet position or fMet-tRNA at the AUG codon in the 37th triplet position. The two initiator tRNAs do not compete with one another, indicating that the E. coli ribosome chooses one of the two initiation sites before binding of either initiator tRNA.

Initiation on the UUU triplet of alflafa mosaic virus RNA does not occur in a wheat-germ cell-free system and gives an indication that conditions for initiation on eukaryotic (80S) ribosomes are much more stringent than on 70S ribosomes. There is limited information in eukaryotic systems to allow extensive comparison between initiation on natural and synthetic mRNA. Besides lacking an AUG initiation codon, another major difference between natural templates and poly(U), is the presence of a 7-methylguanosine cap, at the 5'-terminus of most eukaryotic mRNA. The presence of the 5'-cap is required for proper translation, or more specifically, initiation (Muthukrishnan et al., 1975). It has been shown that initiation factor, eIF-2, has high affinity for the 5'-capped terminus (Kaempfer et al., 1978).

Poly(U) has profound inhibitory effects on the translation of eukaryotic mRNA being a potent inhibitor of AUG-directed Met-tRNA binding to 40S ribosomes (Chatterjee et al., 1979). This inhibitory capacity of poly(U) may have physiological importance. Translational control RNA (tcRNA), a low-molecular weight form of RNA usually found associated with cytoplasmic non-ribosomal mRNA particles (mRNPs) in several cell types, contain many oligo(U) sequences which may exert an effect on cellular protein synthesis (Heywood et al., 1979).

As in prokaryotic systems, initiation factors are not needed at high magnesium ion concentrations (Hardesty et al., 1969). At Mg^{+2} concentration less than 6 mM, however, several eukaryotic initiation factors including eIF-2A, 4C, 4D and 5 are required for initiation onto poly(U) (Merrick, 1979). Factor eIF-2A is distinguished from eIF-2 since the former promotes both Phe-tRNA binding onto poly(U)·40S complexes and Met-tRNA binding onto natural mRNA·40S complexes (Cimadevilla and Hardesty, 1974). Both processes involve GTP hydrolysis upon formation of the initiator tRNA·mRNA·80S complex. Note that Phe-tRNA (as well as N-Ac-phe-tRNA) can function as initiator tRNA in eukaryotic systems. Initiator tRNA (Met or Phe) occupies the P-site of the 80S ribosome; however, if de-acylated $tRNA_{phe}$ is present with poly(U) the initiator Phe-tRNA occupies the A-site (Pranger et al., 1974). In many studies, E. coli $tRNA_{phe}$ is preferably used since it

is equally active in poly(phe) synthesis as eukaryotic $tRNA_{phe}$ and exhibits a greater difference in Mg^{+2} optima between factor-dependent and -independent poly(U) translation (eg. Merrick, 1979; Iwasaki and Kuziro, 1979)

Although almost all work with synthetic polynucleotides is done in cell-free systems, poly(U) can be translated efficiently in lymphocytes rendered permeable by toluene treatment (Burrone, 1978). Such a system can be considered intermediate between classic cell-free systems and the intact cell.

Since most of the biochemical events of elongation (section 1.2.1) have been ascertained using poly(U)-directed translation in both prokaryotic and eukaryotic systems rendered non-dependent on initiation factors i.e. high Mg^{+2} concentration, it is difficult to assess differences between elongation on poly(U) and on natural mRNA.

A phenomenon observed and intensely studied with poly(U) is the so-called "factor-free" translation by E. coli ribosomes (Spirin, 1978). In the absence of EF-Tu, and EF-G and GTP, translation of poly(U) occurs at a slow but appreciable rate. Phe-tRNA is bound to 70S ribosomes non-enzymatically (i.e. without EF-Tu and GTP) but in a codon-dependent manner at Mg^{+2} concentrations above 10 mM (Wagner and Sprinzl, 1979) and the amino acid is incorporated into peptide linkage as shown in Figure 1-9. Other homo- and heteropolymers such as poly(A) and poly(U,C) can also act

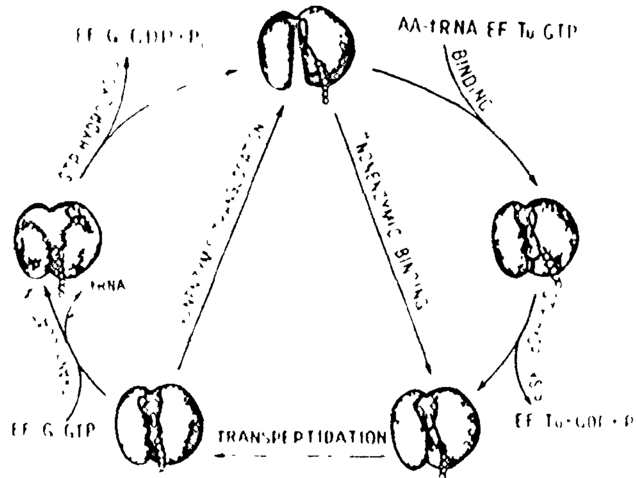


Figure 1-9 Elongation cycle of a prokaryotic ribosome during factor-promoted and factor-free translation. - from Spirin (1978).

as substrates. With poly(U), factor-free translation is slow compared to a factor-promoted system although comparable rates of poly(phe) synthesis are attained if the S12 protein of 70S ribosomes is modified, either by the -SH modifying compound, p-chloromercuribenzoate or in the ribosomes of streptomycin-resistant mutants of E. coli which contain a mutant S12. Reconstituted ribosomes with S12 totally missing are most active in translating poly(U) in the absence of elongation factors and GTP. Factor-free translation does involve the ribosomal translocation mechanism even in the absence of EF-G and GTP. Factor-free translation also does not require protein L7/12 at the GTP-ase and EF-G ribosomal binding site (Bermek, 1978; Petterson and Kurland, 1980). Thus the translocation mechanism is inherent within the ribosome and is thermodynamically permitted by energy released in the transpeptidation (~7 kcal/mole) during which the high-energy ester bond in peptidyl-tRNA is replaced by an amide (peptide) bond. The elongation factors and GTP, then, do not create new principal mechanisms, but simply enhance the mechanism inherent in the ribosome itself.

The phenomenon of factor-free translation has only been observed with synthetic templates and has not been shown in any eukaryotic cell-free systems.

Termination of poly(U) translation is unlike that of natural mRNA in that it does not possess the termination

codons. It resembles more closely premature termination which occurs when an aminoacyl-tRNA is unavailable to bind at the ribosomal A-site. In this case, EF-Tu in the presence of releasing factors (RF) causes slow hydrolysis of the nascent peptidyl-tRNA at the P-site. Nascent poly(phe)-tRNA remains bound to the 70S ribosomes until RF-1 and the stop (codon) trinucleotide UAG are added (Caskey et al., 1969). The poly(phe)-tRNA bound to the ribosome exists in two states: one, at the P-site, reacts with puromycin; the other, at the A-site, cannot react with puromycin until elongation factors and GTP are added (Skoultchi et al., 1969).

1.2.3 Fidelity of Protein Synthesis

The accuracy with which the genetic code is translated into protein products is still an open question. Studies attempting to measure the fidelity of translation have been done both in vivo and in vitro, both approaches having benefits and pitfalls.

Errors of gene expression in vivo can result from many factors: faulty tRNA, mRNA synthesis, codon-anticodon recognition, initiation, mistranslocation, premature termination, etc. In vivo measurements of error frequency of protein synthesis may be underestimates due to rapid cellular degradation of aberrant proteins (Capecchi et al., 1975; Goldberg and St. John, 1976). The first estimate of fidelity of protein synthesis in vivo was made by Loftfield (1963) who

tried to demonstrate valine for isoleucine substitutions in tryptic peptides of ovalbumin normally not containing valine. An error rate of 1 per 3000 (3×10^{-4}) amino acid residues presented a maximum error level. In a more sensitive assay, Loftfield and Vanderjagt (1972) reported that in rabbit hemoglobin valine substituted for isoleucine in two positions with a frequency of $2\text{-}6 \times 10^{-4}$ residues. By detecting isoleucine in the α- and β-chains of human hemoglobin, the genes of which contain no codons for isoleucine, Popp et al., (1976) determined the average substitution frequency to be 3×10^{-5} locus. At this level of substitution, 8.5% of the protein chains would contain one incorrect amino acid if the substitution frequency was the same for each codon. Popp et al., also showed a significant increase in substitution frequency in persons who had been previously exposed to γ-radiation fallout. It is difficult to ascertain whether the error frequencies measured in vivo are the result of mistranslation or whether they reflect a low level of genetic mutation.

Misreading of the genetic code specifically at the ribosomal level has been studied predominantly in E. coli. Edelmann and Gallant (1977) have measured faulty incorporation of cysteine into flagellin, reflecting specific misreading of the CG^{u}_{c} arginine codons as UG^{u}_{c} cysteine codons, at a rate of 10^{-4}/codon. Rel^{-} mutants of E. coli which exhibit increased codon misreading particularly upon specific amino acid

starvation have been used extensively (Parker et al., 1978; Gallant and Palmer, 1979; Gallant and Foley, 1980; Parker and Friesen, 1980) to study the nature of ribosomal ambiguity in E. coli. Amino acid starvation has also been employed in conjunction with 2-D gel electrophoresis (O'Farrell, 1975) to detect specific codon ambiguities in aminoacyl-tRNA synthetase mutants of CHO cells (Parker et al., 1978) and to induce errors at specific codon sites.

Several of the above techniques have been employed in assessments of translational fidelity with respect to aging and error catastrophe. Popp et al., (1976) failed to find a significant increase of isoleucine into human HbA with advanced age, but did find some increase in error-containing hemoglobin from old rodents (Hirsch and Popp, 1973). Buchanan and Stevens (1978) have reported the frequency of misincorporation of methionine into chromosomal protein, H1, to increase from 7×10^{-5}/codon in early-passage fibroblasts to 2×10^{-4}/codon in late passage. This correlates to findings of age-related changes in H1 during aging (Medvedev and Medvedeva, 1978). Wilson et al., (1978) failed to detect aberrant proteins in aged nervous tissue using 2-D gel electrophoresis. However, Harley et al., (1980) have shown a significant decrease in mistranslation rates in aging human fibroblasts using 2-D gel electrophoresis and amino acid starvation technique. They estimate that the error frequency of protein synthesis declines from about 2×10^{-4}

in early passage to 0.5×10^{-4} in late passage.

In vitro studies of protein synthetic fidelity face the obvious criticism that translation is examined in an artificial milieu. Mistranslation rates in vitro are usually at least 10 x greater than in vivo estimates. The intention of the following work is to study the fidelity of a very specific kind of translation involving only elongation on one codon (UUU) under conditions which do not limit protein synthetic rate and which allow measurement of total misincorporation ie inthe absence of proteolytic degradation of synthesis products. Measurements of synthetic fidelity are carried out with the specific purpose of investigating the Orgel hypothesis by testing many of its predictions.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Biochemicals

The following tritiated (^{3}H) amino acids and nucleotides were obtained as sterile solutions from New England Nuclear (specific activity in curies/mmole is shown in brackets): aminoisobutyric acid (10), glutamic acid (51), glycine (15), leucine (120-160), lysine (72), phenylalanine (17.8 - 21.2), tyrosine (60.3), polyuridylic acid (15.1), thymidine (6.7) and uridine (22.2). DL-^{3}H-p-fluorophenylalanine (2.2) was obtained from Amersham. ^{35}S-methionine (510-1048) was also obtained from New England Nuclear. All amino acid stock solutions were kept sterile prior to use and neutralized with potassium hydroxide for use in protein synthesis studies.

Adenosine triphosphate (ATP) and guanine triphosphate (GTP) were obtained as the di- and tri-sodium salts, respectively, from Boehringer-Mannheim. Creatine phosphate, creatine phosphokinase, polyuridylate (potassium salt), micrococcal nuclease and Tris were also obtained from this source. Calbiochem supplied HEPES, histidinol and spermine.

The following compounds were obtained from Sigma: amino acids, colchicine, cycloheximide, EGTA, polyphenylalanine, puromycin, RNase A, TPCK, trypsin (grade 4) and vincristine.

Rabbit globin mRNA was obtained from Miles Laboratories and poly(U,G) (potassium salt) from P-L Biochemicals. Diphtheria toxin was the generous gift of Dr. R. Gupta, McMaster University.

2.2 Poly(U)-directed Protein Synthesis

2.2.1 Cell Culture

The following solutions were routinely used for the growth and propagation of cells. Minimum essential medium A (MEMA) was Eagle's minimum essential medium with non-essential amino acids and Earl's salts (GIBCO) supplemented with D-glucose (15 g/l), sodium pyruvate (0.11 g/l) and ferric nitrate (0.1 mg/l). The pH was adjusted to 7.4 and 2.2 g/l sodium bicarbonate added.

Regular growth medium (RGM) was MEMA supplemented with 15% fetal bovine serum (FBS) (GIBCO).

Cells were routinely washed with phosphate-buffered saline (PBS) and harvested with 0.125% trypsin (Difco) in PBS.

All solutions used in the culture of cells were prepared from double-distilled water and rendered sterile by passage through a .22 micron Millipore filter.

2.2.1.1 Mass Culture

Information concerning the tissue source, maximum in vitro lifespan and donor age and sex of all fibroblast strains used in this study is listed in Table 2-1. The cells were propagated to the desired passage level at 37°C on plastic dishes (58 cm^2, Corning) with RGM in an atmosphere

of 95% air: 5% CO_2. The cells were then inoculated into roller bottles (490 cm^2, Corning) containing 75 ml RGM at a 1:8 split ratio. Cells exhibiting slower growth (WS2 as well as very late passage normal cells) were split at a 1:4 ratio. Bottles were gassed with 95% air: 5% CO_2, sealed and rolled at 1 rpm. The growth state of the culture was monitored microscopically and the cells were harvested two to three days after reaching confluence. (Cells were propagated in roller bottles with extreme care to ensure that sterility was maintained).

2.2.1.2 Clonal Culture

A confluent culture of A_2 at MPD 17 was inoculated into 100 mm dishes at low density (approximately 100 cells/dish). After 10-14 days, colonies ranging in diameter from 0.5 - 1.5 cm were removed by trypsinization within cloning cylinders and re-inoculated separately into 35 mm dishes. At confluence, the clonal cultures were split into a 100 mm dish and upon reaching confluence, a total of 20 MPD was added to the cultures cumulative MPD from the time of low-density to seeding. The cells were propagated to late passage by serial subcultivation in dishes and inoculated into roller bottles at mid passage (45-53 MPD) and late passage (60-70 MPD). The mean number of days to confluence, the cell density and accumulated debris were determined by microscopic examination of all clonal cultures. Estimates of the maximum in vitro lifespan of each clone were made by

TABLE 2-1

Cell strain	Tissue source[a]	In vitro lifespan[b]	Donor Age	Donor Sex	Reference
Normal cells					
A2	skin	70	12	M	Harley and Goldstein 1978
A25	skin	65	8	F	unpublished
JO69	skin	58	67	M	Goldstein etal 1979
Progeria cells[c]					
P5	skin	50	8	M	Goldstein and Moerman 1975
P18	skin	60	4	F	Goldstein etal 1979
WS2	skin	37	37	M	Yatscoff etal 1978
Fetal cells					
MRC5[d]	lung	75	-		Jacobs etal 1978
Transformed cells					
SV MRC5[d]	-		-		Yatscoff etal 1978

[a]Normal and progeroid cells were obtained by anterior forearm biopsy and established as reported by Goldstein and Littlefield (1969).

[b]In vitro lifespan denotes the cumulative number of mean population doublings ±5 from a value of 10 at the time of biopsy until the culture could no longer reach confluence after two weekly refeedings.

[c]P denotes a cell strain derived from a patient with diagnosed progeria; WS denotes Werner syndrome.

[d]Obtained as a gift from Dr. R. Holliday, Mill Hill, England.

rating the cumulative MPD reached by the culture when it did not become confluent after two bi-weekly refeedings.

2.2.1.3 Post-mitotic Culture

A confluent mass culture of A_2 at 67 MPD was inoculated at a 1:16 split ratio into roller bottles containing 150 ml RGM. Upon reaching confluence, after approximately 16 days, the culture was refed at monthly intervals by adding 40 ml of fresh RGM to the roller bottles. Cultures were harvested for error frequency assays (section 2.2.3) at 0, 6, 10 and 16 weeks post-confluence. Cells from each harvest were inoculated into dishes for thymidine labelling index (section 2.2.4) and protein synthetic rate (section 2.2.5) determinations.

2.2.2 Extract Preparation

The following solutions were used in the preparation of cell-free extracts:

Homogenization Buffer (HB) - 15 mM Tris-Cl, pH 7.4, 1.5 mM $Mg(OAc)_2$, 10 mM KCl and 6 mM β-mercaptoethanol.

10 x Buffer - 20 mM Hepes-K^+, pH 7.4, 1.2 M KCl, 50 mM $Mg(OAc)_2$ and 6 mM β-mercaptoethanol.

Energy Mix (EM) - stock solutions of 50 mM ATP: 10 mM GTP: .3 M creatine phosphate: 1.0 mg/ml CPK were mixed 1:1:1:1, pH 7.4.

Dialysis Buffer (DB) - 20 mM Hepes K^+, pH 7.4, 120 mM KCl, 2.5 mM $Mg(OAc)_2$ and 6 mM β-mercaptoethanol.

Cells were grown in roller bottles until 2-3 days

post-confluent as determined by microscopic examination. RGM was decanted and the cells washed with 20 ml PBS per bottle. The PBS was decanted and 10 ml 0.125% trypsin (Difco) added per bottle. The bottles were rolled at 37°C, 2 rpm until the cells detached (10-15 minutes). The action of trypsin was stopped with 12 ml cold RGM and the resulting suspension placed on ice. All subsequent work was carried out at 0-4°C except where noted. An aliquot of the resulting cell suspension was removed for Coulter counting and sizing. The cells were pelleted by centrifugation at 500 x g for 20 minutes. The cell pellet was resuspended in PBS and spun again at 500 x g for 10 minutes. The volume of the cell pellet was noted. Three volumes of HB were used to swell the cells for 30 minutes. The swollen cells were disrupted in a Dounce homogenizer fitted with a tight plunger until free nuclei were visible microscopically. To the lysate was added one-tenth volume of 10 x buffer to restore isotonicity. The lysate was centrifuged at 30,000 x g for 30 minutes to remove nuclei and cellular debris. The supernatant (S-30) was carefully removed and subjected to ribosomal runoff by incubation with 1/50 volume of EM at 37° for 45 minutes. After the ribosomal runoff, the S-30 was dialyzed in a spinning dialysis unit for 2 hours against 4 changes of DB. Absorbance at 260 nm was measured on a Gilford model 2400 spectrophotometer and the S-30 was adjusted with DB to an RNA concentration of 10 A_{260} units/ml.

The S-30 was used immediately for error frequency determinations (section 2.2.3) or stored frozen at -70°C for other use.

2.2.3 Error Frequency Assays

Optimum ionic conditions for the translation of poly(U) (section 3.1.1.2) were determined using cell strain A_2 at early passage. The S-30 was diluted with ½ volume of Optimum Adjusting Buffer (OAB) containing 34.7 mM Hepes-K^+, pH 7.4, 282 mM KCl, 15.53 mM $Mg(OAc)_2$ and 37.3 mM each of phenylalanine, leucine and lysine. Upon addition of energy mix, radiolabelled amino acids and poly(U), the standard reaction mixture contained: 6 A_{260} units/ml of S-30, 20 mM Hepes-K^+, pH 7.4, 140 mM KCl, 5.5 mM $Mg(OAc)_2$, 6 mM β-mercaptoethanol, 10 μM phenylalanine, 10 μM leucine, 10 μM lysine, 1 mM ATP, 0.2 mM GTP, 6 mM CP and 20 μg/ml CPK. Where appropriate, the concentration of poly(U) was 0.5 mg/ml. The reaction mixture was portioned into testtubes which contained either ^{3}H-phe (20 μCi/ml), ^{3}H-Leu (140 μCi/ml) or ^{3}H-Lys (140 μCi/ml), each with and without poly(U). Each tube was immersed in a 37°C waterbath. Aliquots removed at 0, 10, 20 and 30 minutes were spotted onto glass fibre discs (GFC, Whatman) and immersed immediately into 5% TCA containing 0.5% casamino acids. The discs were washed once with 5% TCA with casamino acids, heated at 90°C for 20 minutes and washed with TCA-casamino acids again. The discs were then washed with ethanol and diethylether,

air-dried and their radioactivity measured by liquid scintillation counting in a solution of 0.05 g POPOP and 4.0 g PPO per litre of toluene.

2.2.4 Thymidine Labelling Index

This procedure was adapted from Cristofalo and Sharf (1973).

Cells were seeded onto sterile coverslips (in triplicate) at a density of 0.25 - 0.5 x $10^4/cm^2$ (approximately 1:4 split ratio) and allowed to attach in RGM for 24 hours. The medium was removed and fresh RGM containing 0.1 µCi/ml ^{3}H-TdR (specific activity 6.7 Ci/mmole) added. The cells were incubated for a further 30 hours. The labelling medium was then aspirated and the cells washed 5 times with PBS. The cells were fixed in glacial acetic acid ethanol (1:3 v/v) for 10 minutes, rinsed twice with absolute ethanol and air-dried. The coverslips were mounted, cell-side up, onto slides. When dry, the slides were dipped in Kodak NTB2 nuclear track emulsion at 45°C and allowed to dry. The emulsion was exposed in a light-proof box for 7 days at 4°C, and subsequently developed with Kodak D-19 for 6 minutes, fixed with Kodak acid fixer for 5 minutes, rinsed twice in distilled water and air-dried. The thymidine labelling index was expressed at the number of nuclei with more than 10 grains ÷ the total number of nuclei.

2.2.5 Cellular Protein Synthetic Rate

Cells were grown in 10 replicate 60 mm dishes until 2 days post-confluent. Two dishes were trypsinized and the total number of cells/dish determined. The medium was aspirated from the remaining dishes and fresh RGM containing 1.0 μCi/ml ^{3}H-phe (specific activity 5.15 Ci/mole) previously equilibrated to 37°C and 5% CO_2 was then added and the dishes returned immediately to the incubator. Protein synthesis was arrested at 0, 20, 40 and 60 minutes by removing the labelling medium, rinsing cells twice with ice-cold PBS and precipitating the protein with 2 washes of ice-cold 5% TCA containing 1 mM phenylalanine. The dishes were washed quickly with ice-cold distilled water and the precipitated protein dissolved in 0.4 M NaOH/ 0.4% DOC. Labelled protein was determined by liquid scintillation counting (in PCS made acidic with 20 λ/ml of 1.0 N HCl to prevent precipitation of NaOH/DOC). Counting efficiency was 35-38%. Protein synthetic rates were determined after adjusting for dilution of labelled phenylalanine by cold amino acid concentration in RGM (0.2 mM phenylalanine) and expressed as femtomoles phenylalanine incorporated per cell.

2.2.6 Cellular Proteolytic Degradation Rate

Cells used in these studies were grown in 60 mm dishes until 2 days post-confluent. The growth medium was aspirated and the cells pulsed with RGM (this time supple-

mented with only 10% FCS) containing ^{3}H-phenylalanine at 10 μCi/ml. After the pulse period, the dishes were put on ice and the cells rinsed five times over 25 minutes with ice-cold MEMA (with HEPES 20 mM, pH 7.4 substituted for $NaHCO_3$) containing 20 mM phenylalanine (HB-MEMA-phe). The dishes were placed in a 37°C water bath oscillating at 60 cps through an amplitude of 4 cm. The HB-MEMA-phe was withdrawn after 15 minutes and largely contained ^{3}H-phe which was unincorporated into protein during the pulse. To begin the chase, 3 ml of fresh HB-MEMA-phe previously warmed to 37°C was added to each dish. The entire volume of chase medium was replaced by fresh chase medium at hourly intervals. Radiolabel released from the cells was measured in an aliquot of the chase medium by liquid scintillation counting of the aliquot directly in PCS. After 8 hours, the last aliquot of the chase medium was removed and the cell protein precipitated with ice-cold 15% TCA containing 20 μM phenylalanine. The precipitated monolayer was washed twice with 5% TCA with 20 μM phenylalanine, rinsed quickly with water and dissolved in 0.4 M NaOH/0.4% DOC. Labelled protein in this solution was counted in acidified PCS.

The percent proteolysis was determined as:

$$\% \text{ proteolysis} = 100 \times \sum_{n=1}^{8} X_n / \text{Total after n hours}$$

$$\text{Where total} = \sum_{1}^{8} X_n + X_i.$$

Where X_n = dpm in chase aliquot at the nth hour

X_i = dpm in TCA-insoluble material after the 8th hour.

2.3 Endogenous mRNA-directed Cell-free Protein Synthesis

2.3.1 Extract Preparation

Cells were grown and harvested as in section 2.2.1. Extracts were prepared as described in section 2.2.2 with the exception that the ribosomal runoff procedure was omitted. Thus the S-30 was dialyzed directly without pre-incubation with EM at 37°C. After dialysis, extracts were not diluted to 10 A_{260} units/ml but were used directly for cell-free protein synthesis.

2.3.2 Cell-free Protein Synthesis

To freshly prepared S-30 (500λ) which had not been subjected to ribosomal runoff, was added 60λ EM and 40λ ^{35}S-Met (100 μCi/ml translation grade, NEN). The mixture was immersed in a 30° waterbath. The synthetic rate was determined by removing aliquots (10λ) from the mixture at 0, 10, 20 and 30 minutes. The aliquots were applied to glass fiber discs and treated and prepared as described in section 2.2.3. After 30 minutes, 1.0 ml of cold 10% TCA with 1% casamino acids was added to the mixture. The precipitated protein was pelleted by centrifugation at 2,000 x g at 4°C. The pellet was washed three times with 10% TCA with 1% casamino acids and stored frozen at -70°C.

2.3.3 Two-dimensional Gel Electrophoresis

This procedure was adapted from O'Farrell (1975). The acid-insoluble pellet containing ^{35}S-labelled

protein was dissolved in a buffer containing 9.5 M urea, 2% w/v NP-40, 1.6% 4-6 ampholines (LKB) and 0.4% 3-10 ampholines (LKB), 5% β-mercaptoethanol to a concentration of 1 mg protein/ml. (S-30, prepared as in section 2.3.1, had a protein concentration of 3-4 mg/ml). A portion of this solution (10-50λ) containing at least 100,000 dpm of ^{35}S was applied to pre-run isoelectric focusing tube gels (4.0% 4-6 ampholines and 1.0% 3-10 ampholines) and electrophoresed at 400 volts. After 20 hours, the gels were removed from the tubes and swirled in a buffer containing 10% w/v, glycerol, 5% v/v β-mercaptoethanol, 2.3% w/v SDS and 62.5 mM Tris, pH 6.8, for 30 minutes. The tube gels were fixed atop slab gels containing 12% polyacrylamide and electrophoresed through the second dimension for 9 hours at 100 volts. Bromophenol blue was employed as a front marker.

Upon completion of the electrophoresis, the slab gel was removed and rinsed thoroughly 3 times in a solution of water:methanol:acetic acid (5:25:1). The slab gels were then dried at 100°C under gentle suction onto filter paper. The filter paper containing the gel was exposed to Kodak XR-1 X-ray film for 4-7 days and subsequently developed (see section 3.2.3).

CHAPTER THREE

RESULTS

This chapter falls into two parts; the first concerns the use of the synthetic mRNA, poly(U), in studies of mistranslation in cell-free extracts while the second section deals with preliminary attempts to translate natural mRNA with the cell-free system.

3.1 Poly(U)-directed Protein Synthesis

3.1.1 Characteristics of Cell-free extracts from HDF

Since this is the first comprehensive report on the nature of cell-free extracts of human diploid fibroblasts and their use in protein synthetic studies, it is felt that a detailed description of the in vitro synthetic system is in order.

3.1.1.1 Developing an Assay for Synthetic Activity

Of necessity, the preliminary work on this thesis involved developing a protein synthetic system from human diploid fibroblasts. Essentially all of the experiments carried out until an efficient protein synthetic system was developed were performed with extracts of early-passage cells of A_2 strain between generations MPD 25 and 40.

Initial experiments attempted to achieve poly(U)-directed protein synthesis by removing the cell's plasma membrane with the non-ionic detergent NP-40 (Austin and Kay, 1975), thus rendering the cell permeable to an exogenous template. It was hoped that the cellular protein synthetic

apparatus would be able to translate poly(U) and that ^{3}H-phe incorporation into TCA-insoluble material would be stimulated. Although higher concentrations of NP-40 (5% in 10 mM Tris-saline) alone stimulated incorporation of ^{3}H-phe, this effect was unchanged with the addition of poly(U).

A similar permeabilization was later attempted following the method of Burrone (1978). Isotonic buffer containing 0.1% toluene had no effect on protein synthesis (^{3}H-phe incorporation) of cells exposed for less than 5 minutes at 0^oC. Exposure to toluene for 10 minutes inhibited protein synthesis markedly. Under no conditions did poly(U) stimulate ^{3}H-phe incorporation in toluene-treated fibroblasts.

Lysis of the cells by disruption in a Dounce homogenizer was then attempted. Preliminary evidence (C. Harley and G. Csullog, unpublished results) showed that douncing of fibroblasts previously swollen in hypotonic buffer (HB), diminished protein synthetic activity. Furthermore, it was found that dounced fibroblasts almost completely inhibited poly(U)-stimulated phenylalanine incorporation in rabbit reticulocyte lysates as shown in Figure 3-1. Notably the addition of NP-40 alone did not inhibit poly(U)-stimulated phenylalanine incorporation in rabbit reticulocyte lysates.

Removal of membranous and nuclear debris from the fibroblast lysate by centrifugation at 30,000 x g_{max} markedly enhanced poly(U)-directed protein synthesis as shown in

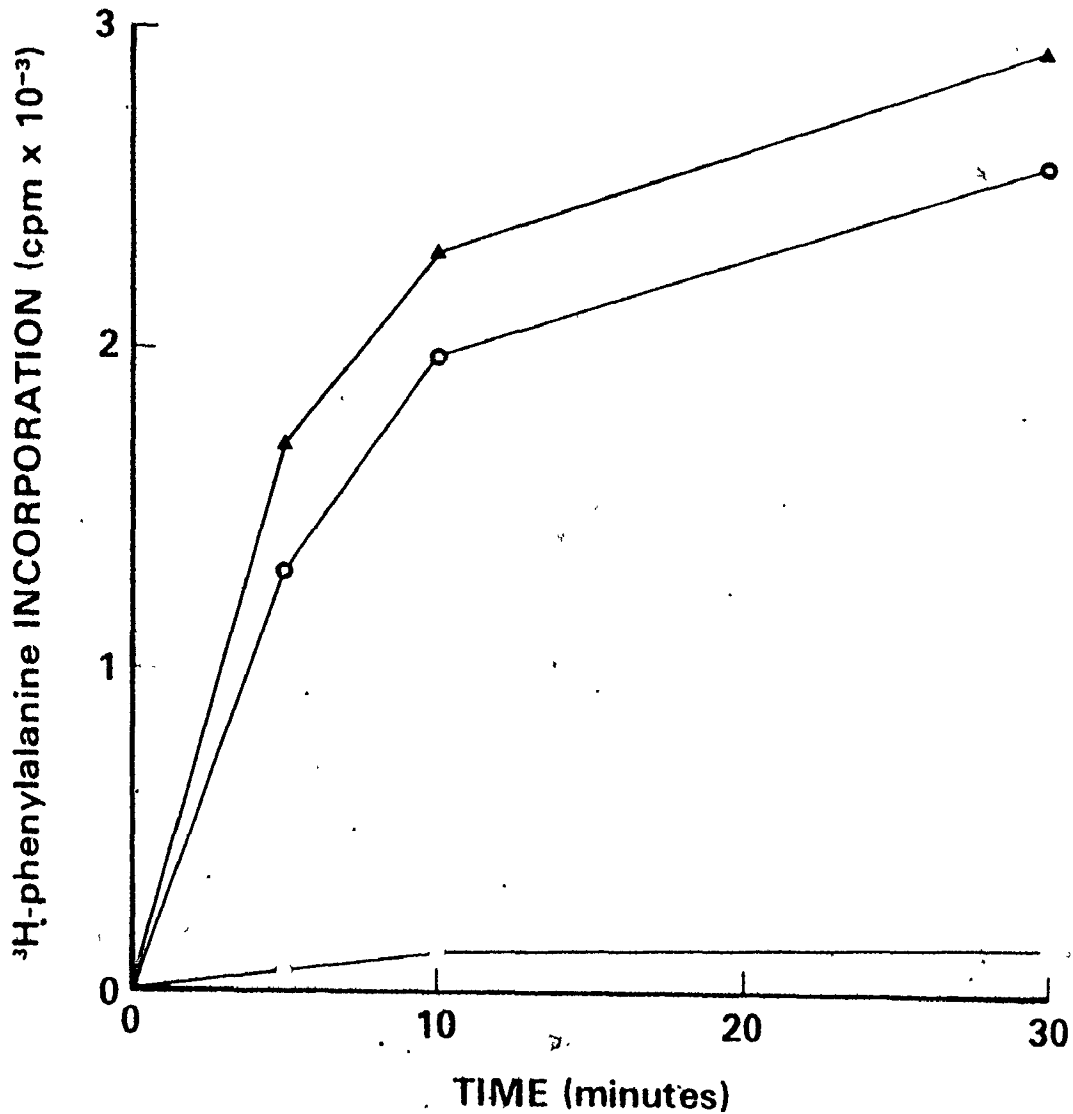


Figure 3-1 The poly(U)-stimulated incorporation of ^{3}H-phenylalanine into TCA-insoluble material by rabbit reticulocyte lysate. RRL (Pelham and Jackson, 1976) was incubated with 2/5 volume of HB alone(●), 1% NP-40 in HB(▲) or a dounced lysate of cell strain A_2 in HB(○).

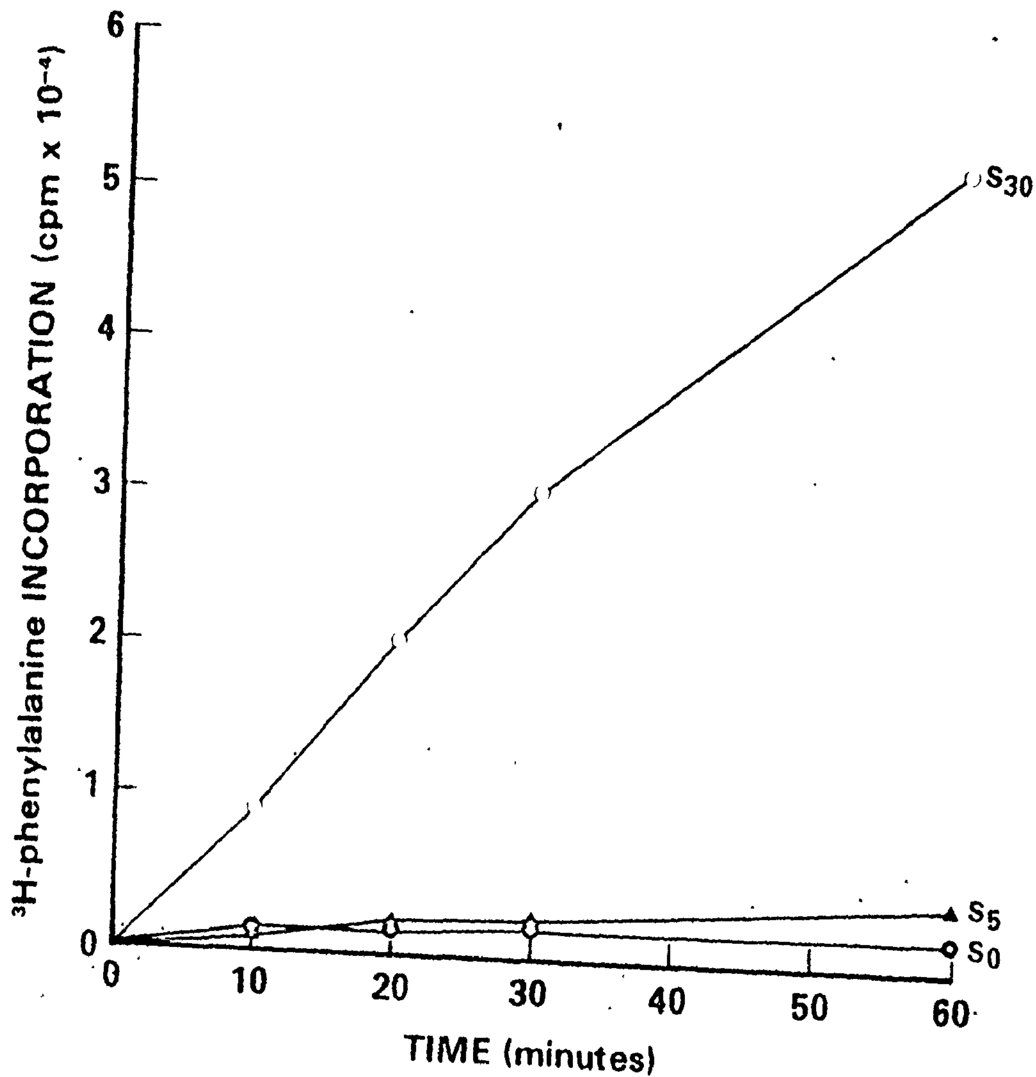


Figure 3-2 Poly(U)-directed polyphenylalanine synthesis in extracts of human diploid fibroblasts. Dounced cell preparations were subject to centrifugation at 0(●), 5(▲) or 30(○) thousand x g with subsequent ribosomal runoff and dialysis. Each extract was incubated with ^{3}H-phe(32μCi/ml), poly(U) (250 mg/ml), amino acid mix (phe= 10μM) with K^+ and Mg^{+2} concentrations of 120 and 4.1 mM, respectively. Time points represent approximately 10^6 cells of strain A_2 at 53 MPD.

Figure 3-3 Effect of diluting S-30 containing DNA on protein synthetic activity.

Runoff, dialysed S-30 from A_{25} at 20 MPD contained DNA (A_{260}= 25 ml^{-1}). the S-30 was diluted with DB to the A_{260} concentration shown. ^{3}H-phenylalanine incorporation is shown after 30 minutes incubation at 37°C.

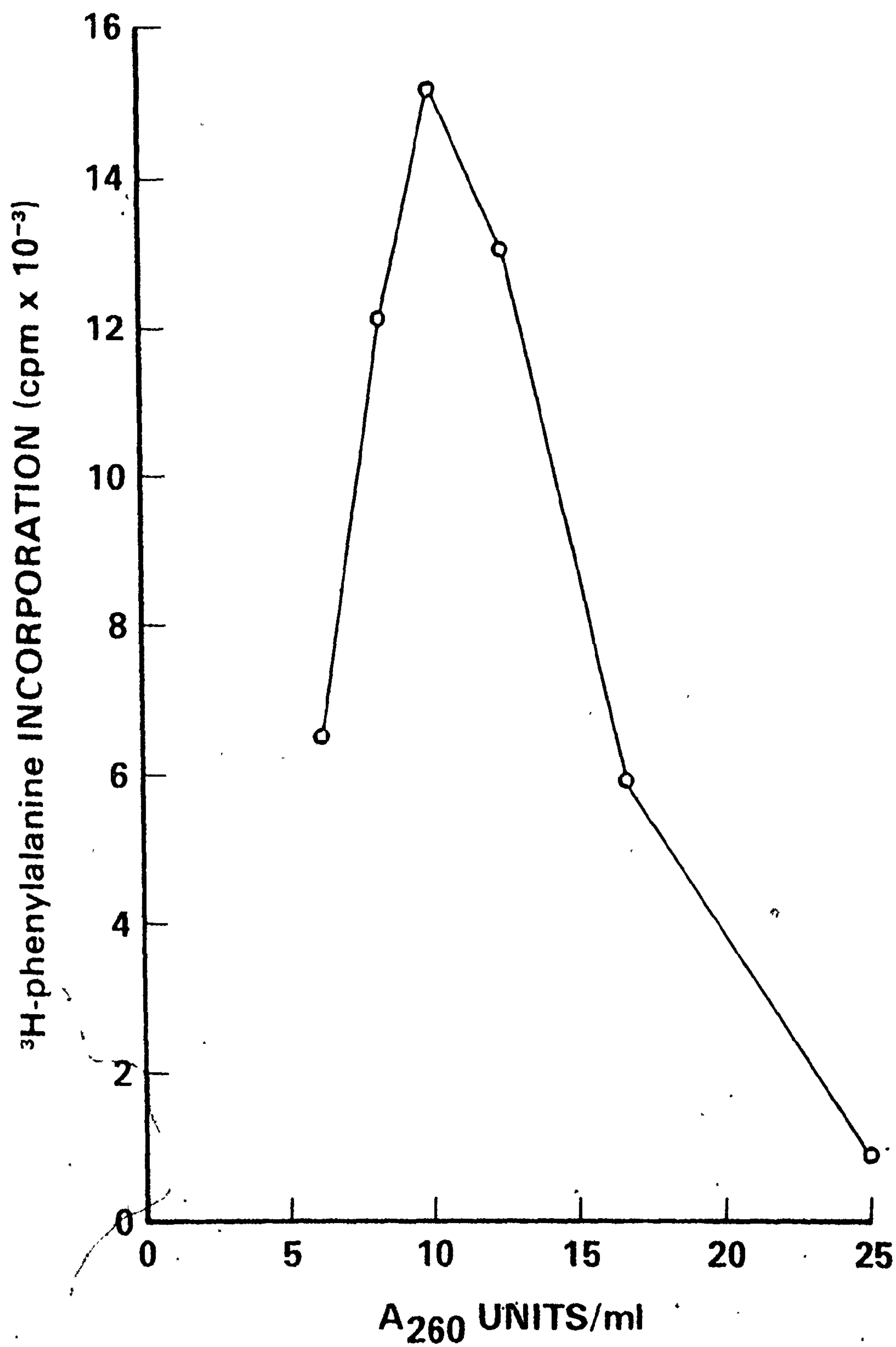


Figure 3-3 see legend opposite.

Figure 3-2. Poly(U) translation was linear for up to one hour in the runoff and dialyzed S-30 fraction. In our experience, substitution of rapid dialysis by chromatography over Sephadex G-25 invariably resulted in loss of poly(U)-stimulated protein synthesis.

The final protocol developed (see section 2.2.2) for the preparation of the cell-free extracts yielded extracts active in the translation of poly(U); however, each step had to be carried out carefully to ensure activity. Of particular importance was the extent to which cells are disrupted. If cells were swollen in HB to the point at which the nuclei become fragile, rupture of the nuclear membrane and release of nuclear contents into the S-30 occurred and inhibited poly(U)-directed synthetic activity. Figure 3-3 shows the effect of dilution of an S-30 which was extracted from severely disrupted cells. The S-30 contained DNA as judged by its viscoelastic properties and by absorbance at 260 nm. S-30's prepared in this vigorous manner appeared to contain inhibitory nuclear factors which apparently could be diluted to restore poly(U)-directed synthetic activity.

Not all cells harvested yielded extracts active in the translation of poly(U). As shown in Figure 3-4, extracts of early-passage cells in the middle of logarithmic growth phase translated poly(U) very poorly. Although there was some activity in extracts of cultures just reaching confluence, maximal activity occurred 2-3 days after confluence had been

Figure 3-4 Poly(U)-dependent protein synthesis in extracts of cells in various growth states.

Cells of strain A_2 at 30 MPD were inoculated into roller bottles at 2-day intervals, harvested simultaneously after the number of days shown and assayed for poly(U)-stimulated polyphenylalanine synthesis. The activity of the extracts on the right axis. In parallel, cellular growth kinetics were measured by inoculation of the same cells into dishes, harvesting duplicate dishes and determining cell number on successive days.

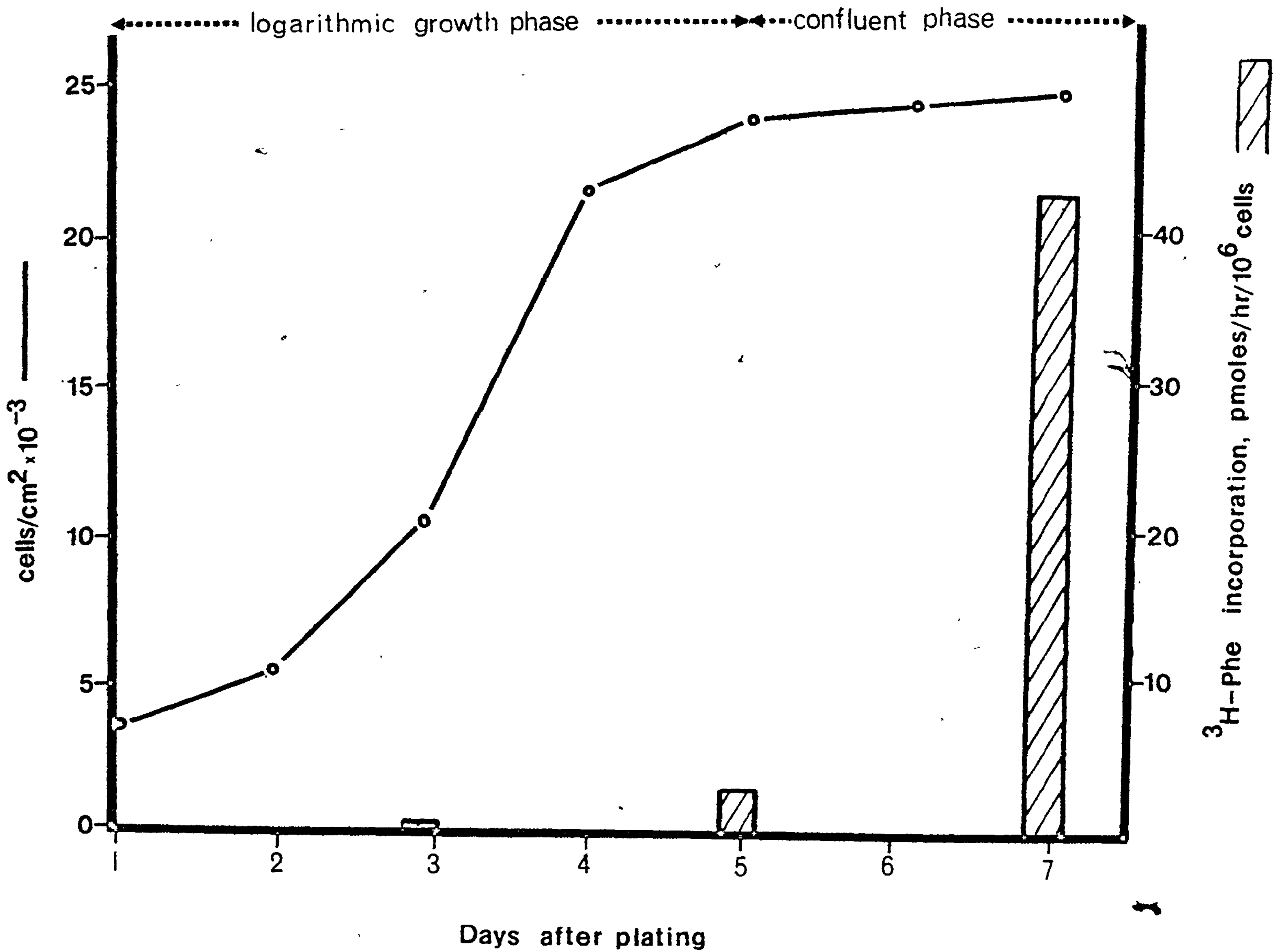


Figure 3-4 see legend opposite.

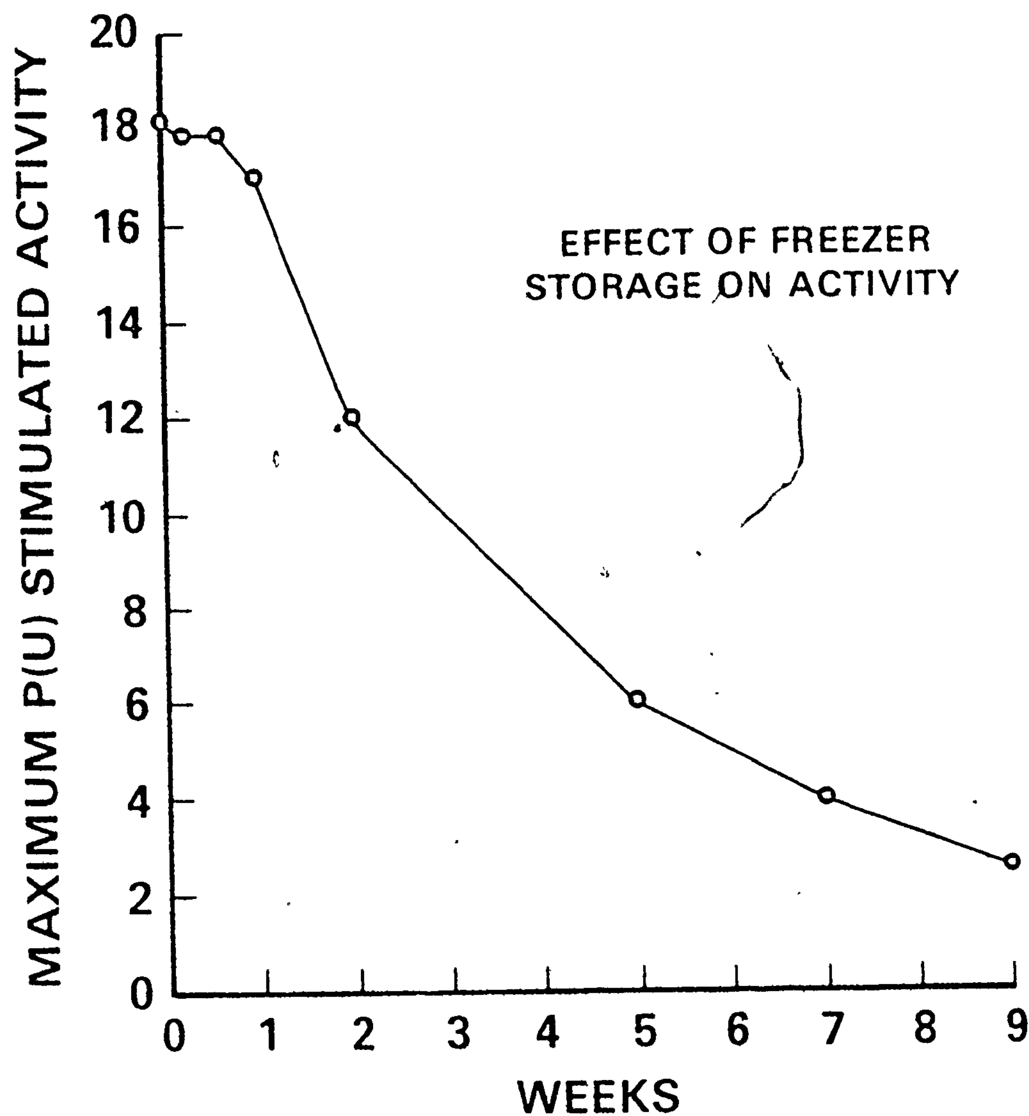


Figure 3-5 Effect of freezer storage on poly(U)-directed protein synthetic activity.
Aliquots of S-30 from strain A_2 at 35 MPD where flash frozen at -196°C.,stored at -70°C. and thawed after the intervals shown. Poly(U) stimulation of phenylalanine incorporation was assayed as in section 2.2.3. Synthetic activity is shown as pmoles Phe/hr/10^6 cells.

reached. Other experiments (see section 3.1.4) indicated no further decline even if held in stationary phase for long periods of time.

Cell-free extracts could not be stored in the frozen state at either -70°C of -196°C without immediate and continuous loss in synthetic activity (Figure 3-5). This loss of activity was thought to be due primarily to the loss of the reducing agent, β-mercaptoethanol, by slow oxidation, since addition of 1 mM DTT to thawed extracts restored synthetic activity partially. However, freezing in 1 mM DTT had no effect on S-30 storage lifetime at -70°C.

3.1.1.2 Optimization of Synthetic Rate

To increase the sensitivity of error frequency assays (section 3.1.2) by maximizing the rate of phenylalanine incorporation, optimum ionic conditions for poly(U) translation were determined. All initial optimization experiments were carried out with extracts of early-passage cells of strain A_2. When optimum conditions of poly(U) translation were established with respect to Mg^{+2}, K^{+2} and poly(U) concentration, these optima were tested in cell extracts of late-passage A_2 cells in subsequent experiments. The optimum concentration of Mg^{+2} ion was determined first. Figure 3-6 shows a Mg^{+2} optimum for A_2 at 30 MPD of 5-6 mM. In a later experiment under conditions optimized for K^+, poly(U) and phenylalanine, the Mg^{+2} optimum in an extract of late-passage A_2 (66 MPD) (Figure 3-6) was unchanged (~5.5 mM) although the

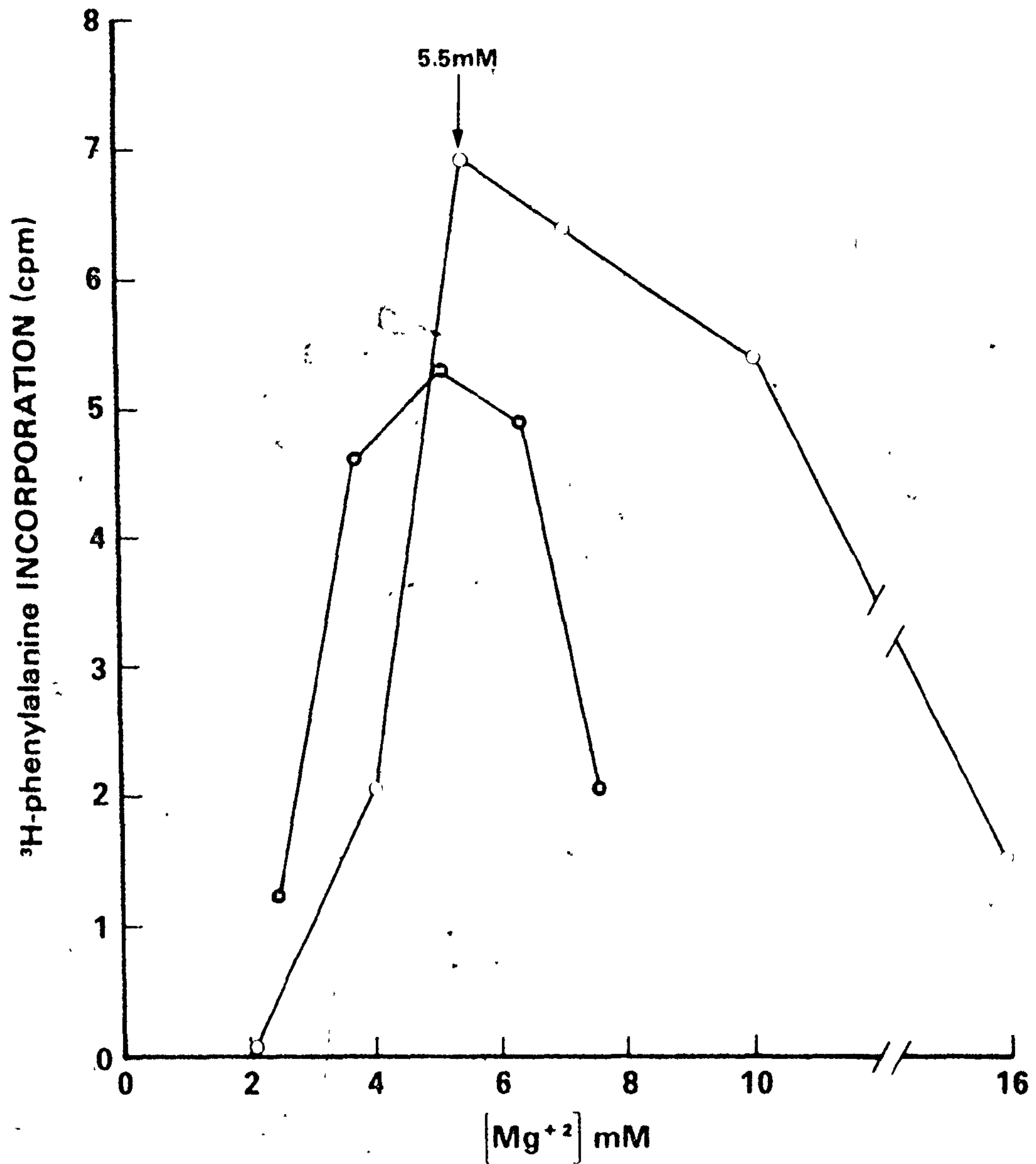


Figure 3-6 The effect of Mg^{+2} concentration on poly(U) translation. The ordinate shows ^{3}H-phenylalanine incorporation after 30 minute incubation at 37°C. in cpm x 10^{-3} for A_2 at 30 MPD(**O**) and in a later experiment cpm x 10^{-4} for A_2 at 66 MPD(O) at various concentrations of $Mg(OAc)_2$.

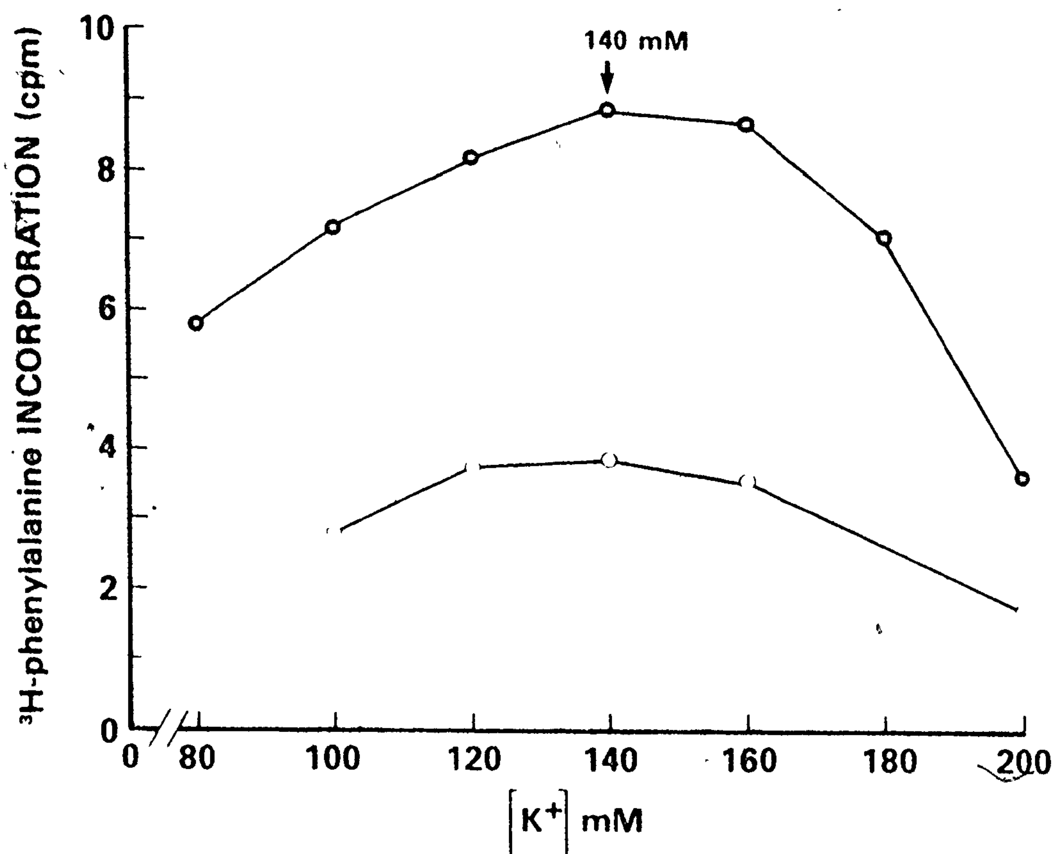


Figure 3-7 Optimum potassium ion concentration for poly(U) translation. Poly(U)-dependent synthesis as a function of KCl concentration in extracts of strain A_2 at 30 MPD(**O**, ordinate cpm x 10^{-3}) and at 66 MPD(O, ordinate cpm x 10^{-4}).

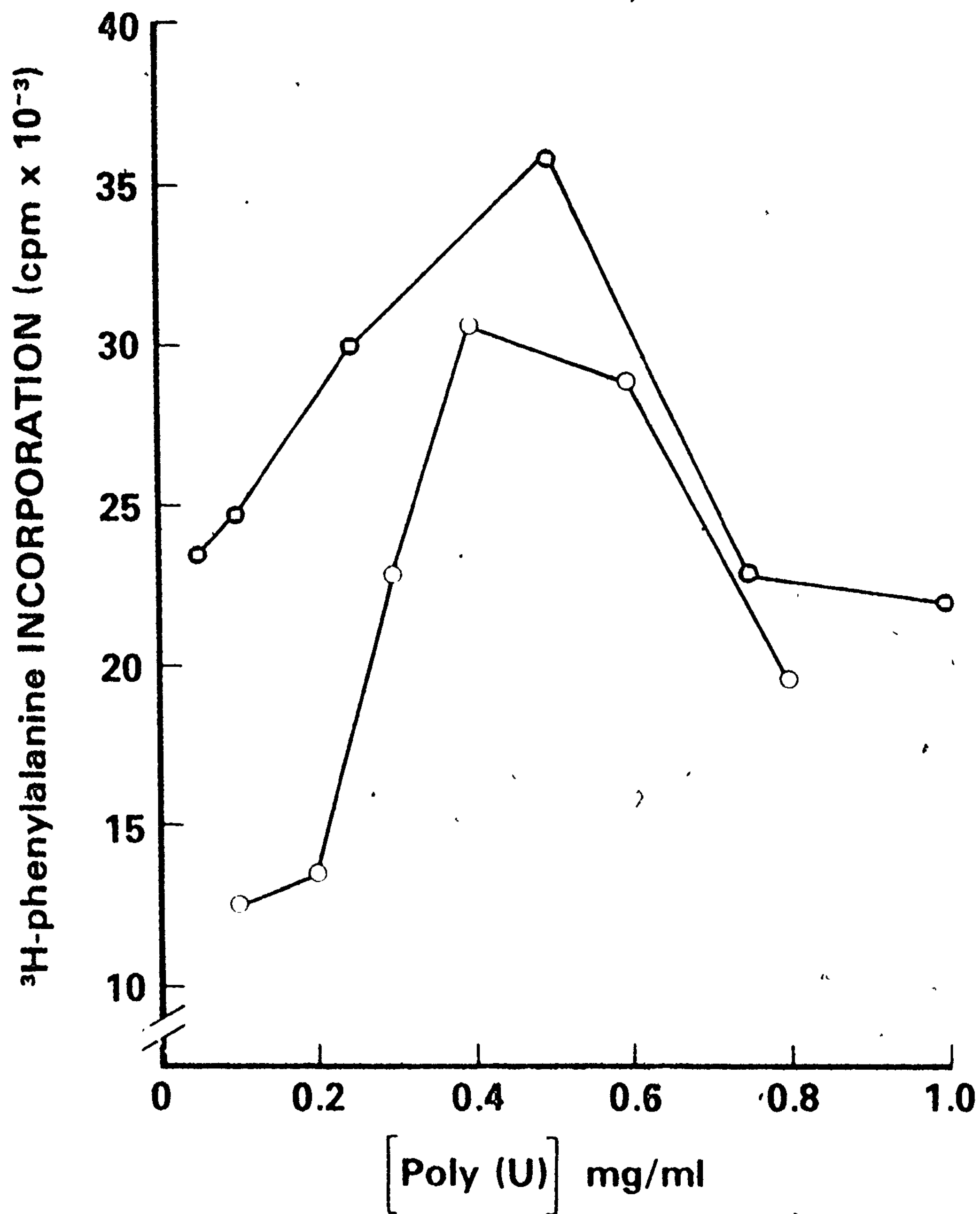


Figure 3-8 Optimum poly(U) concentration.

Extracts of strain A_2 at 30 MPD(**O**) and at 66 MPD (O) were assayed for phenylalanine incorporation at various poly(U) concentrations.

overall curve was broader. An improvement of synthetic rate of approximately 10-fold under K^+, poly(U) and phenylalanine optima was also noted. Curiously in two attempts, dialysis of the S-30 during extract preparation against a buffer containing 5.5 mM magnesium acetate gave extracts of low synthetic activity; thus, magnesium concentration during dialysis was kept at 25 mM.

With the magnesium optimum established at 5.5 mM, potassium ion concentration was determined to be optimal at 140-150 mM (Figure 3-7). This optimum was also unchanged in late-passage cell extracts.

Poly(U) concentration was optimal at 0.5 mg/ml (Figure 3-8). This optimum concentration was also the concentration used in experiments to determine Mg^{+2} and K^+ optima. As with the other optima, the optimal poly(U) concentration does not change at late passage.

In all preliminary experiments, phenylalanine was present only at the concentration of radiolabel added. To ascertain whether this low concentration of phenylalanine (~0.85 μM) was limiting the rate of poly(U) translation, the rate of phenylalanine incorporation stimulated by poly(U) was monitored in two ways, increasing amounts of (a) radio-labelled ^{3}H-phe or (b) cold phenylalanine solution to increase the phenylalanine concentration. Both methods showed that phenylalanine concentration was rate-limiting below 4-8 μM of added phenylalanine (Figure 3-9). Furthermore, it was

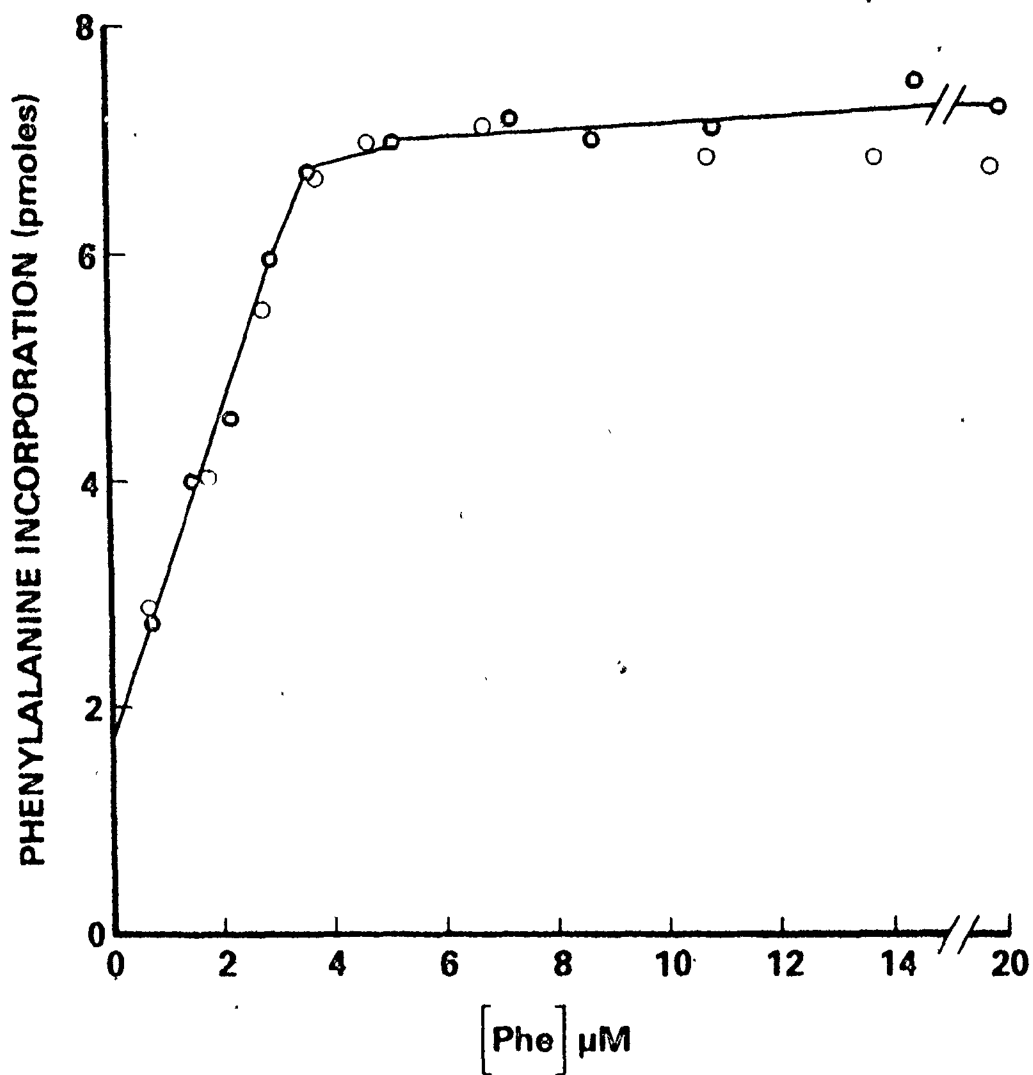


Figure 3-9 The dependence of poly(U) translation on phenylalnine concentration.

To an extract of A_2 at 31 MPD was added increasing amounts of ^{3}H-phe(20 Ci/mmole, **O**) or cold phenylalanine(50μM, O) both in neutralized solution, pH 7.4.

revealed that some residual phenylalanine (1.0 - 1.5 μM) remained after dialysis. Attempts to remove all of the phenylalanine by G-25 Sephadex column chromatography resulted in extracts which were inactive in protein synthesis.

Thus, optimal concentrations of Mg^{+2} (5.5 mM), K^+ (140 mM), poly(U) (0.5 mg/ml) and phenylalanine (10 μM) were determined for the translation of poly(U) in extracts of early-passage cultures of strain A_2. These optima did not change appreciably during serial subcultivation of A_2 into late passage. The above optima were used in all subsequent experiments in which standardized conditions were essential.

These standardized conditions were not optimum for each cell strain assayed, however (see section 3.1.2). For example, the Mg^{+2} optimum of extracts prepared from strain JO69, derived from old donors, occurred at ~9.5 mM (Figure 3-10) as opposed to ~5.5 mM optimum for strain A_2. The effect of this difference on synthetic rate and error frequency is discussed in sections 3.1.2.3 and 4.1.1.

The effect of other variables on poly(U) translation rate was also examined. Conditions for poly(U) translation included a standard incubation temperature of 37°C. However, as seen in Figure 3-11, although ^{3}H-phenylalanine incorporation into cold TCA-precipitable material was almost linear with respect to temperature ($Q_{10} \simeq 2.0$) up to 37°C, the rate of phenylalanine incorporation into hot-TCA precipitable material was essentially the same at 30° and 37°C. Other experiments

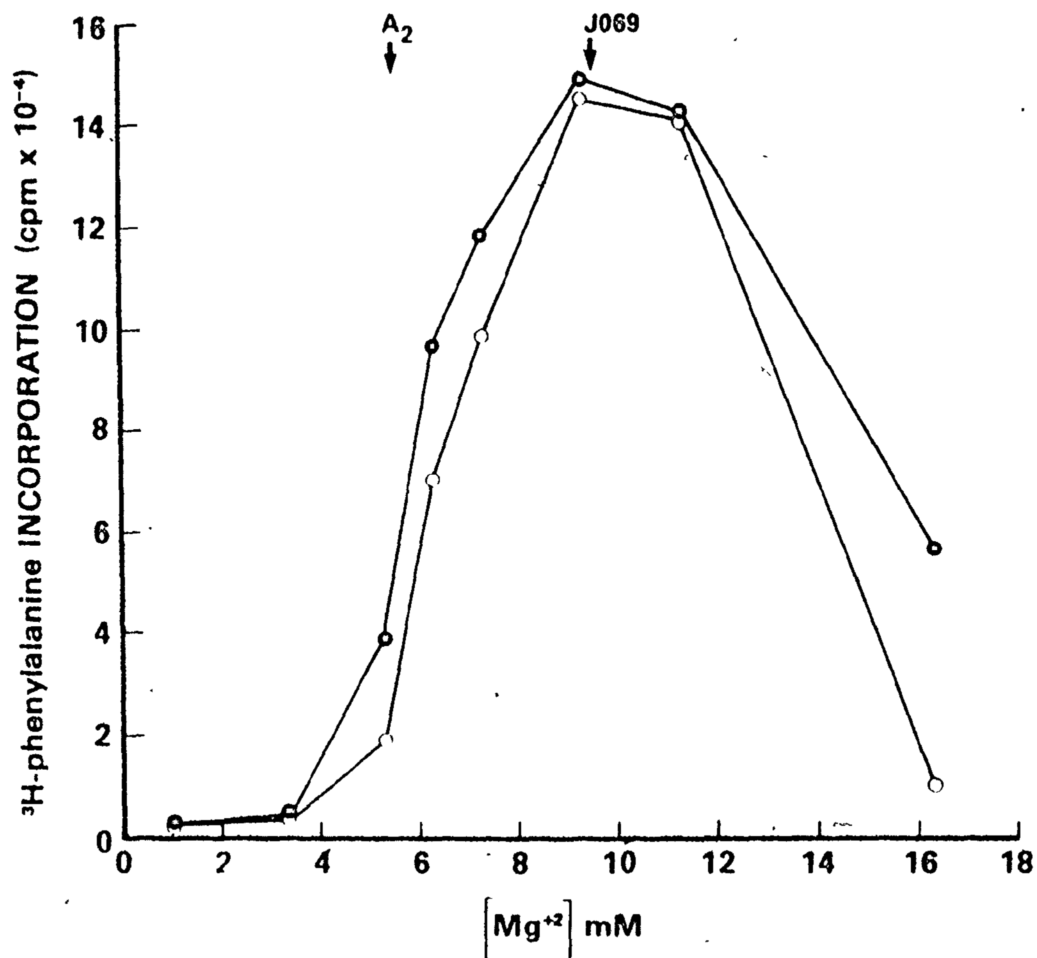


Figure 3-10 Poly(U) translation as a function of magnesium concentration in extracts from cells of an old donor. An extract of strain J069 at 53 MPD was incubated at various concentrations of $Mg(OAc)_2$ at 37° C. for thirty minutes and aliquots removed. One set of aliquots was treated with 5% TCA at 90° C. for 30 minutes (○); while the other set of aliquots rinsed with cold 5% TCA (**o**).

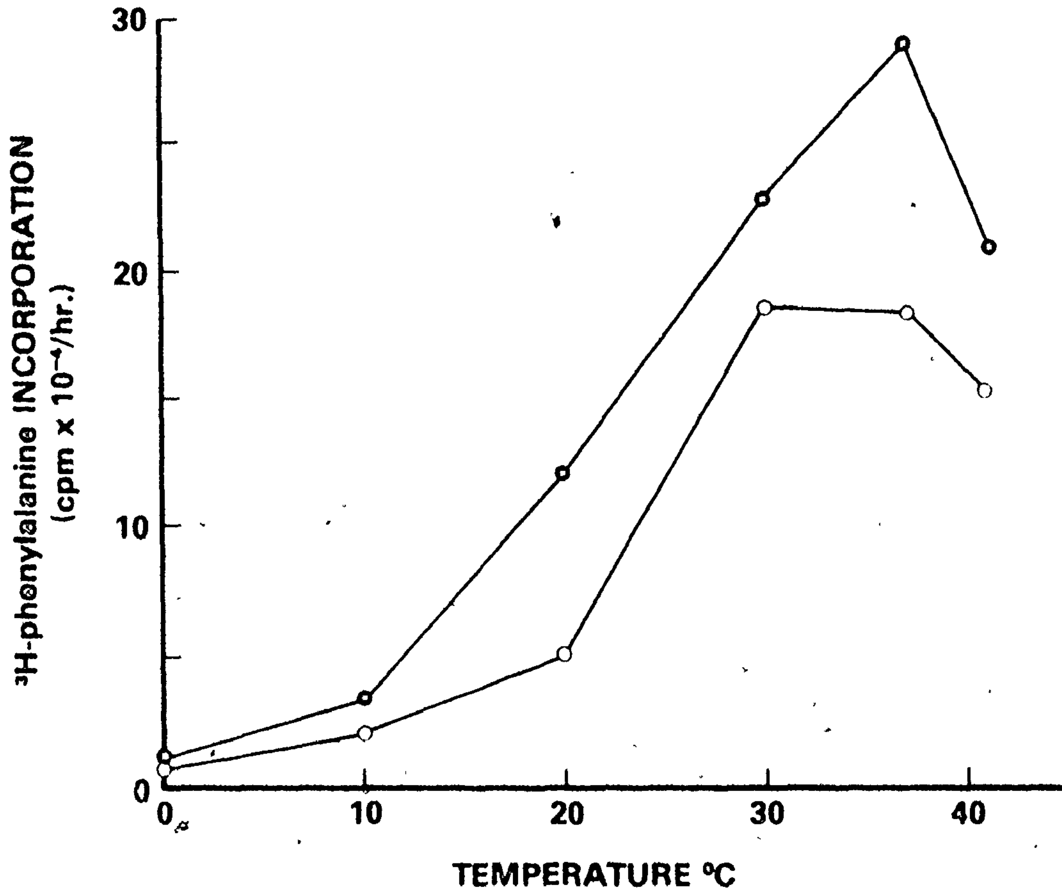


Figure 3-11 Temperature dependence of poly(U) translation.
A cell extract from a culture of A_2 clone 14 at 53MPD was incubated at the temperatures indicated under standard conditions of poly(U) translation (except ^{3}H-phe = 40 μCi/ml). Duplicate aliquots were removed at 0, 10, 20 and 30 minutes and incorporation rates of phenylalanine into hot(○) or cold(**O**) TCA-insoluble material determined.

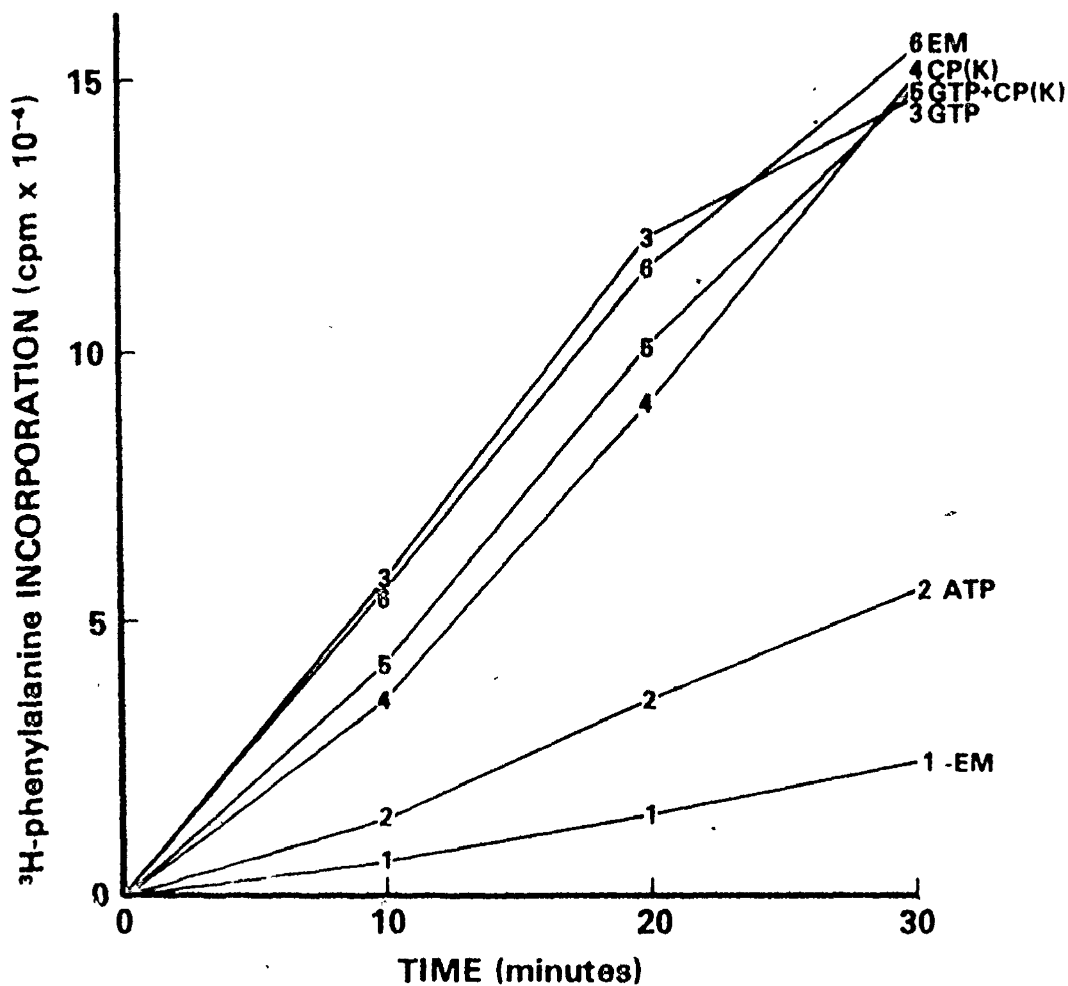


Figure 3-12 Components of the energy mix.

A cell extract from a culture of A_2 clone 30 at 51 MPD was incubated under standard poly(U) translation conditions except that no energy mix was added. To portions of S-30 were added appropriate amounts of 1- water, pH 7.4, 2- ATP, 3- GTP, 4- CP and CPK, 5- GTP and CP(K) and 6- complete EM. Aliquots from each mixture were removed at 0, 10, 20 and 30 minutes.

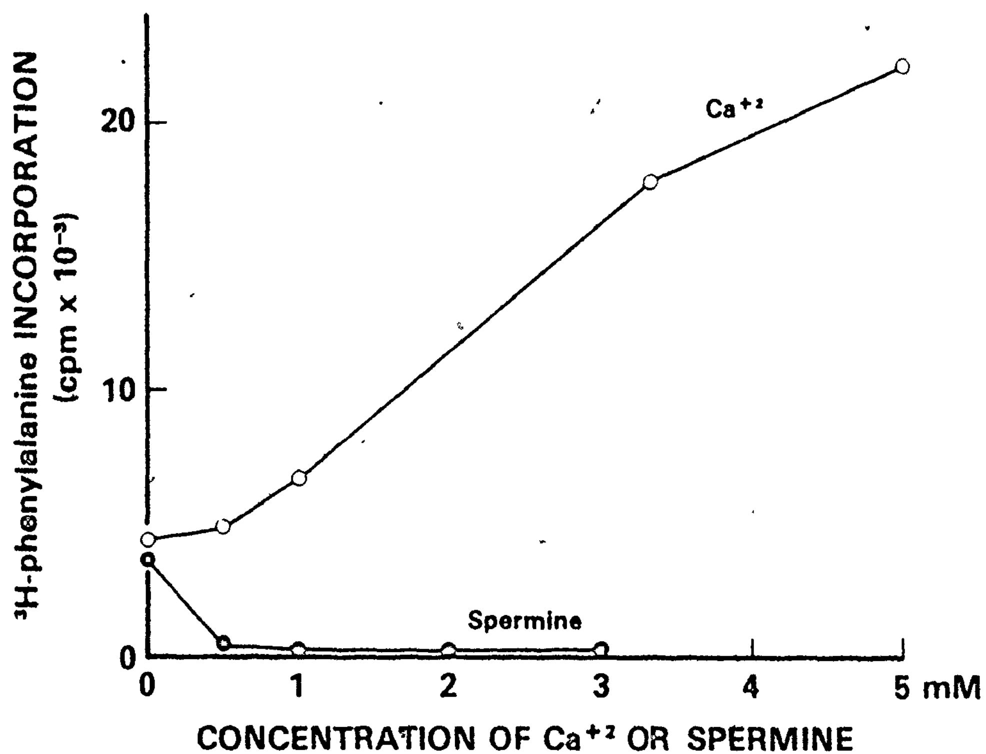


Figure 3-13 The effect of Ca^{+2} and spermine on poly(U) translation rate.

Cell extract of a culture of A_2 clone 10 at 53 MPD was assayed under standard conditions with added $CaCl_2$(O) or neutralized spermine dihydrochloride(**O**). The ordinate displays the synthetic rate (per hour) as determined over a time course of 0, 10 20 and 30 minutes.

showed that little improvement in synthetic rate was realized at temperatures between 30° and 37°C.

How the various components of the energy mix (EM) affected the synthetic rate was determined by omitting components one at a time and maintaining poly(U)-stimulated ^{3}H-phenylalanine incorporation (Figure 3-12). Considerable synthesis was found even without exogenous energy sources. Both GTP alone and CP(K) alone or the two combined had essentially as much stimulatory effect (~6 fold) on poly(U) translation as the complete energy mix. ATP alone on the other hand, stimulated synthesis to a much lesser extent. Furthermore, in other experiments, ATP was shown to inhibit synthetic activity enhanced by GTP alone.

Contrary to results in other cell-free systems (Jelenc and Kurland, 1979), addition of polyamines, such as spermine had a markedly inhibitory effect on poly(U) translation under standardized conditions (Figure 3-13). The addition of another divalent cation, Ca^{+2}, however, had a significant stimulatory effect.

3.1.1.3 Inhibition of Protein Synthesis

Protein synthetic activity in cell-free extracts of HDF was found to be affected by known inhibitors of eukaryotic protein synthesis. Cycloheximide inhibited poly(U)-directed phenylalanine incorporation into protein as much as 90% at 2×10^{-3} M (Figure 3-14a). Further inhibition was not possible due to difficulty in dissolving the cycloheximide in

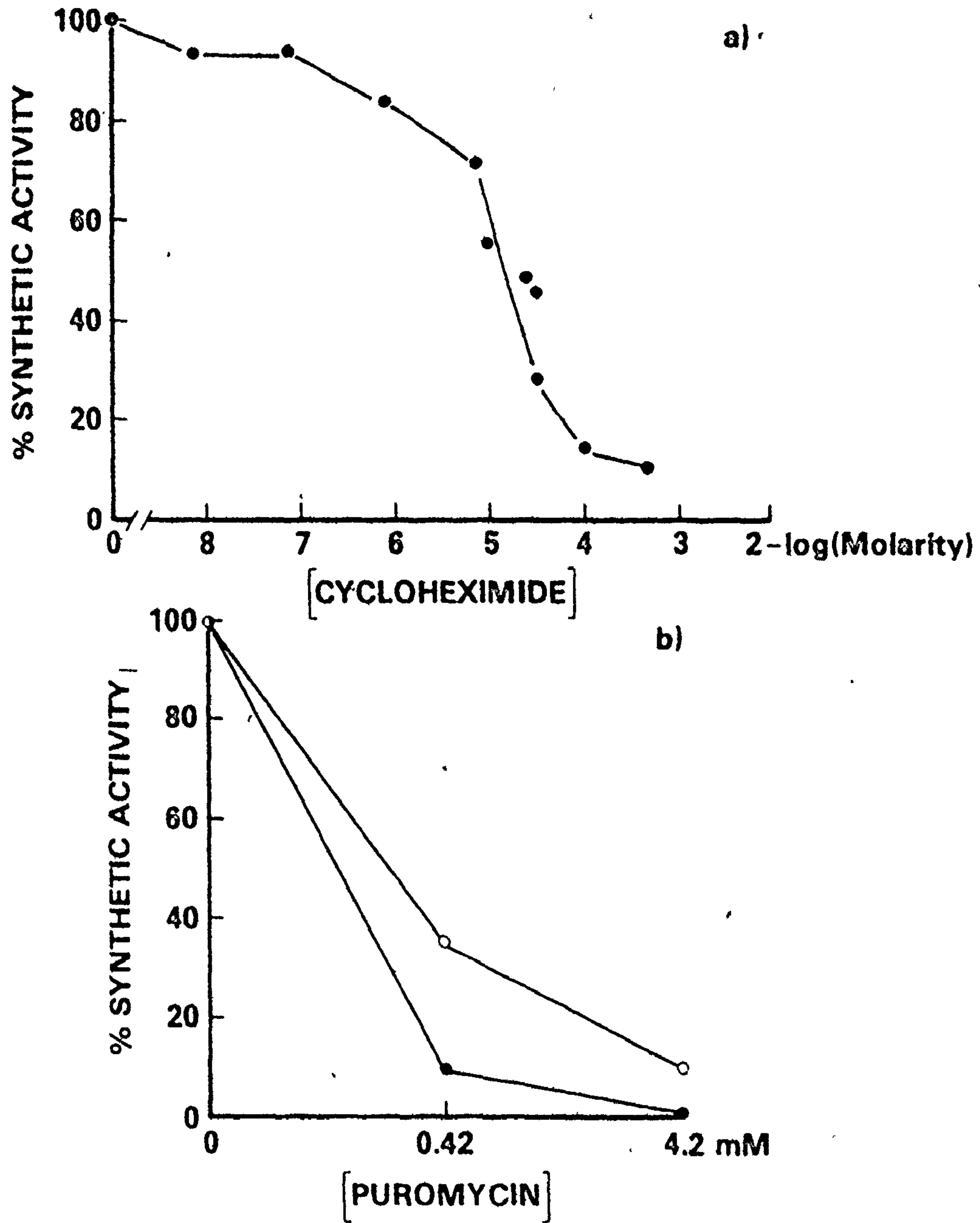


Figure 3-14 Inhibition of poly(U)-directed protein synthesis by cycloheximide(a) and puromycin(b).
Incorporation of ^{3}H-phenylalanine into hot-TCA insoluble material was measured after 30 minutes incubation with increasing concentrations of inhibitors of protein synthesis, with absence of inhibitor being considered 100% synthetic activity. Inhibition of endogenous (i.e. -poly(U)) incorporation of leucine(O) was not as marked as inhibition of phenylalanine incorporation(●).

buffered solution. Half-maximal inhibition occurred at a concentration of 10^{-5} M cycloheximide.

Puromycin (Figure 3-14b) inhibited both poly(U)-directed incorporation of phenylalanine and also low-level endogenous protein synthesis remaining after ribosomal run-off. The latter synthetic activity was not as sensitive (90% inhibition of 4.2 mM) to puromycin as was poly(U)-directed protein synthesis (99.5% inhibition at 4.2 mM).

Poly(U)-directed protein synthesis was almost completely inhibited by diphtheria toxin (Figure 3-15). Cell extracts from strain A_2 were found to be more sensitive to diphtheria toxin than extracts from strain A_{25}. Half-maximal inhibition occurred at 0.06 and 0.3 LF/ml for extracts from A_2 and A_{25}, respectively. In other experiments, the A_{25} cell strain, interestingly, grew well in RGM containing diphtheria toxin of a concentration of 1 LF/ml, whereas a dose of 0.1 LF/ml was enough to cause growth cessation in strain A_2.

p-Tosylphenylchloroketone (TPCK), an irreversible inhibitor of EF-G in prokaryotic cell-free systems, had no effect on the poly(U) translation rate. Chloramphenicol, at concentrations inhibitory to 70S-ribosomal dependent synthesis, was also shown not to have any effect on poly(U) translation. Paradoxically, the amino acid analogue, histidinol, a specific inhibitor of histidinyl-tRNA synthetase, was shown to inhibit poly(U)-directed protein synthesis, even at moderate concentrations (4 mM) (data not shown).

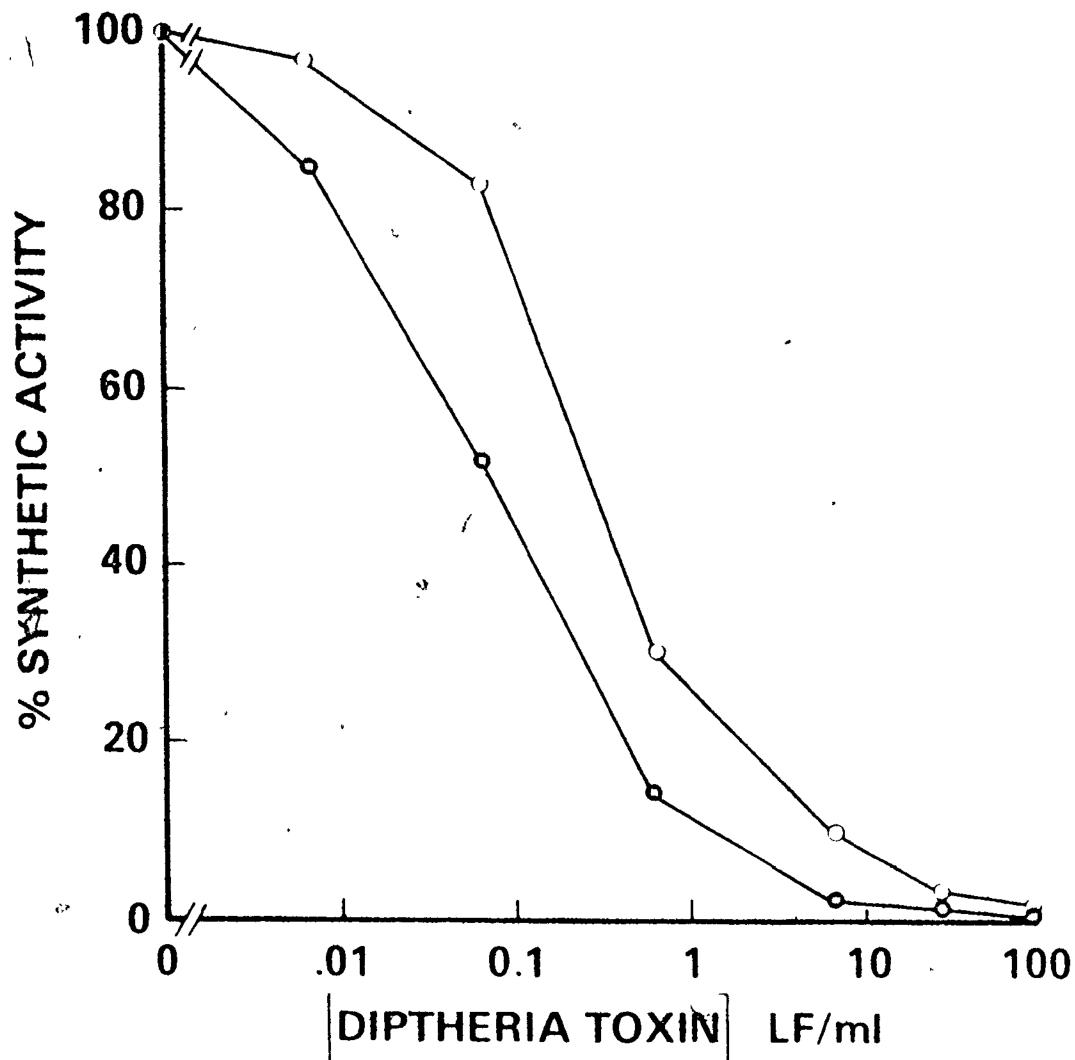


Figure 3-15 Diphtheria toxin sensitivity of poly(U)- directed protein synthesis.

Cell extracts from cultures of A_2 at 36 MPD(**O**) and A_{25} at 25 MPD(O) were assayed under standard conditions of polyU translation with diphtheria toxin (1 LF/ml = 3.3 μg protein/ml) at the concentrations indicated. Phenylalanine incorporation was determined in duplicate after a 30 minute incubation.

3.1.1.4 RNase and Proteolytic Activity

To determine whether cell-free extracts which are active in poly(U) translation also possess ribonuclease activity (which may degrade poly(U) and/or ribosomal RNA), two assays of RNAse activity were carried out in these extracts. In the first, ^{3}H-poly(U) was incubated with an extract whose protein synthetic activity was assayed in a separate aliquot. As shown in Figure 3-16a, the aliquot containing ^{3}H-phenylalanine and cold poly(U) showed considerable protein synthetic activity, the aliquot containing ^{3}H-poly(U) showed no decrease in TCA-insoluble cpm over 60 minutes. In the second method, tritiated cellular RNA was prepared by pulsing a confluent dish of cells (strain A_2 at 22 MPD) with RGM containing ^{3}H-uridine (1 μCi/ml) for 24 hours. The cells were disrupted and an S-30 extract prepared. The S-30 containing ^{3}H-RNA plus residual ^{3}H-uridine was added to a cell extract known to be active in poly(U)-directed protein synthesis (Figure 3-16b). This mixture exhibited no RNA degradation, in fact a slight increase in TCA insoluble counts was observed, reflecting a small level of RNA synthesis in these extracts. When pancreatic RNase A (20 μg/ml) was added to this mixture, however, rapid degradation of the labelled RNA ensued (approximately 35% of the RNA present in the original mixture existed in a core of ribosomal RNA which was resistant to RNase A).

Attempts were also made to ascertain whether any

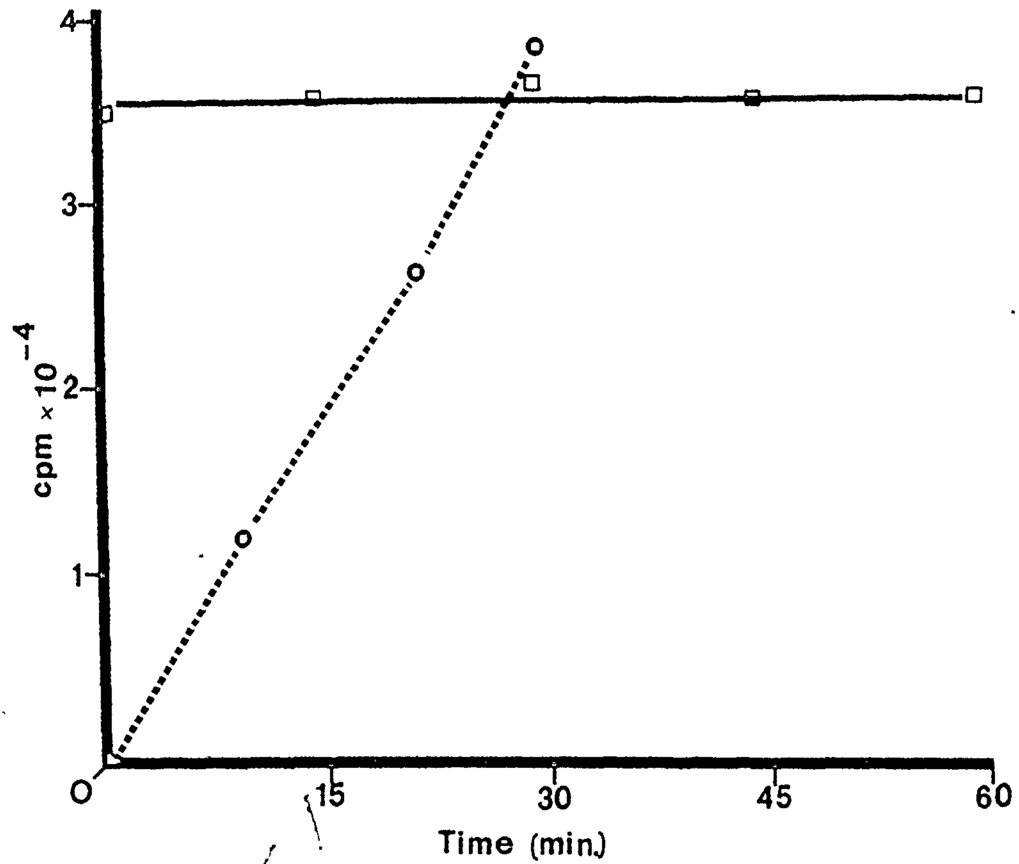


Figure 3-16a The absence of ribonucleolytic activity under conditions of in vitro protein synthesis.

An extract of strain A_2 at 37 MPD was prepared for poly(U) translation in the usual way(section 2.2.3) except that to one portion was added ^{3}H-phe(20 μCi/ml, ...) and to the other ^{3}H-poly(U)(2.5 μCi/ml, ———). Hot TCA-insoluble cpm were determined after the times indicated.

Figure 3-16b Absence of ribonuclease activity in extracts under standard conditions of protein synthesis. Cells of strain A_2 at 22 MPD were pulsed with ^{3}H-uridine (1 µCi/ml) for 24 hours and an extract prepared as in section 2.2.2. This extract was added (1:1) to an extract of A_2 at 30 MPD whose activity in poly(U) translation was separately confirmed. The combined extracts were assayed for TCA-insoluble cpm (^{3}H-RNA) with(■) and without(●) added pancreatic ribonuclease A.

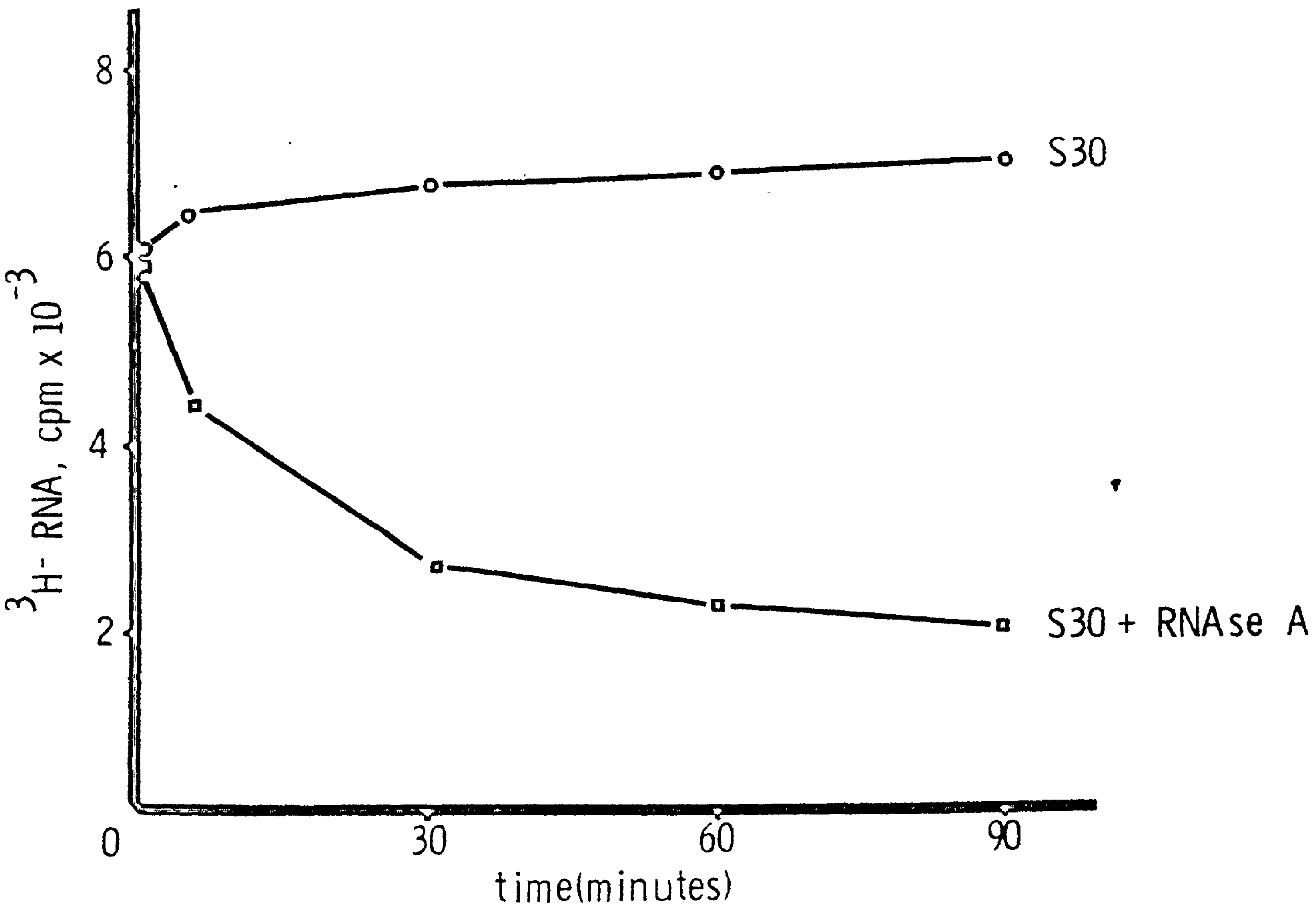


Figure 3-16b see legend opposite.

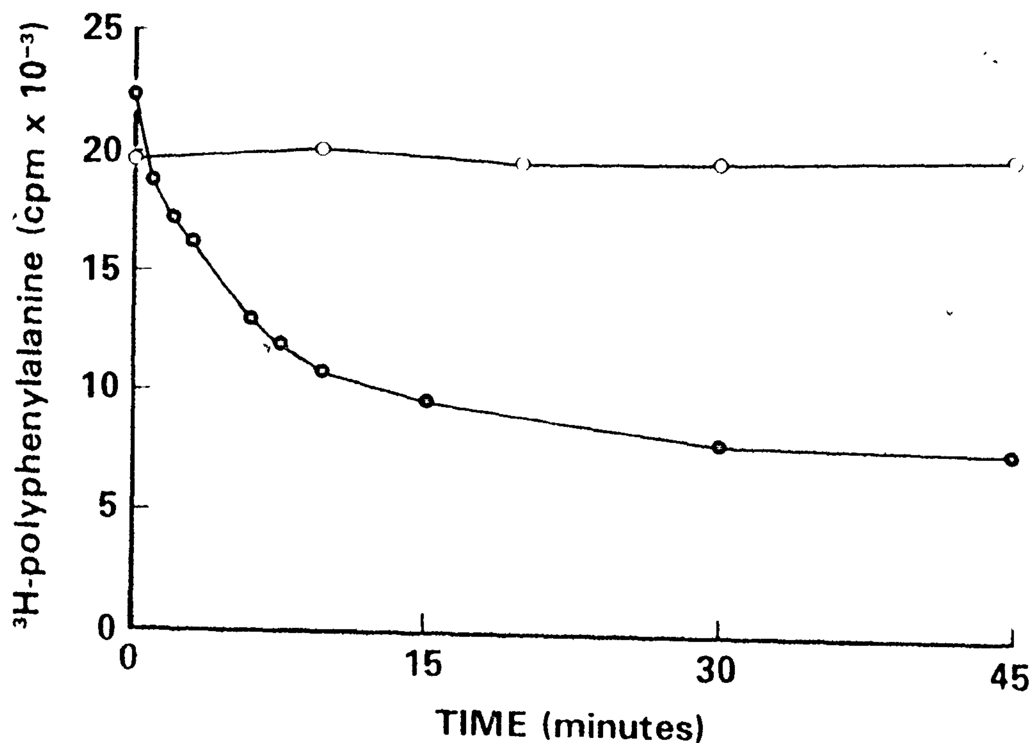


Figure 3-17 Absence of polyphenylalanine degradation under standard conditions of protein synthesis.

An extract of strain A_2 at 66 MPD was incubated for 30 minutes under conditions standardized for poly(U) translation. After dialysis, the mixture contained 6×10^3 cpm/λ of TCA-insoluble material. This mixture was added to 2 volumes of fresh extract and incubated under standard conditions again in the absence(○) or presence(●) of grade IV trypsin. Duplicate aliquots were removed from each assay at the times indicated and remaining TCA-insoluble radioactivity was determined.

proteolytic activity was present in cell-free extracts of human fibroblasts under the standardized conditions of protein synthesis. ^{3}H-polyphenylalanine was prepared by incubating a cell-free extract from a culture of A_2 with ^{3}H-phenylalanine and poly(U) under standard conditions. After 30 minutes, the mixture was subject to extensive dialysis to remove radio-label unincorporated into poly(phe). (In a separate error frequency assay (section 2.2.5) of this extract, it was shown that the poly(phe) prepared in this manner contained 0.08% leucine.) ^{3}H-poly(phe) was then incubated with another portion of the same S-30 which, although it had been frozen for 1 day, was still active in poly(U) translation. Figure 3-17 shows that the S-30 alone, under standard conditions of protein synthesis, did not degrade poly(phe). When trypsin (grade IV) was added to a concentration of 0.1%, the poly(phe) was rapidly degraded.

3.1.2 Error Frequency Determination

Once conditions were optimized for poly(U) translation, studies to determine the fidelity of cell-free protein synthesis were initiated. Figure 3-18 shows the results from a typical error frequency assay after ribosomal runoff.

Phenylalanine incorporation in the absence of poly(U) occurred at very low levels, (ranging from undetectable to 120 fmoles/hr/10^{6} cells). Addition of poly(U) resulted in a marked stimulation (on the order of 1000-fold) of phenyl-alanine incorporated into acid-insoluble material. Since

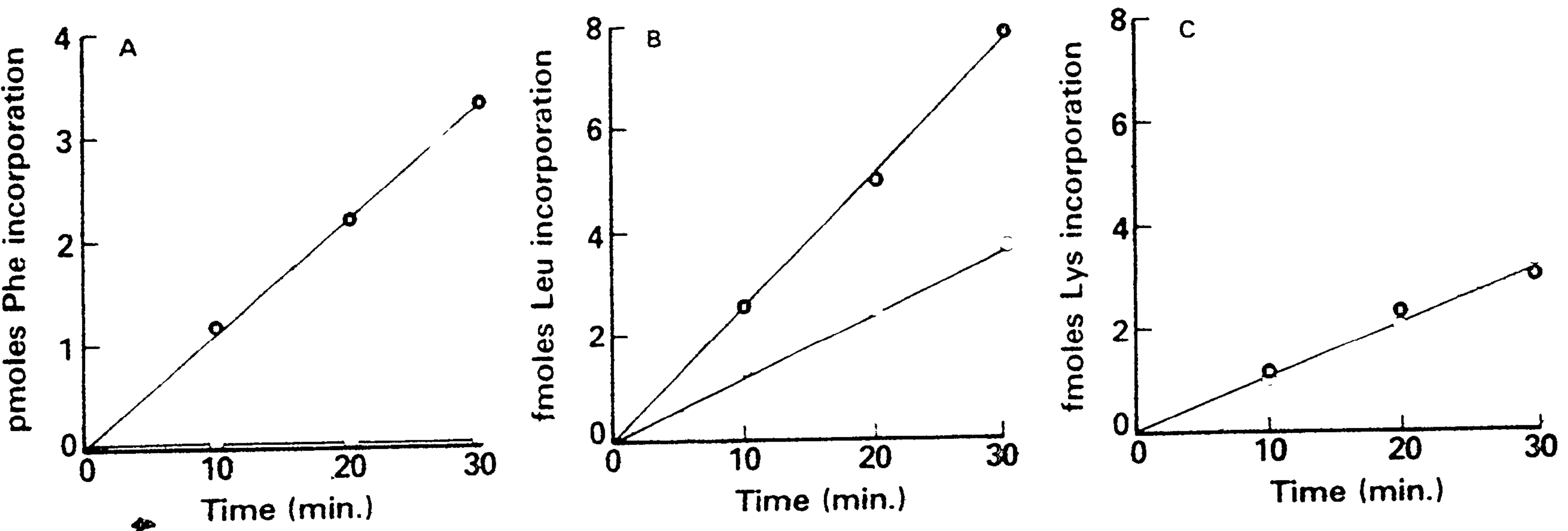


Figure 3-18 Error frequency determination.

Standardized conditions developed for error frequency determination (section 2.2.3) were used to measure phenylalanine(A), leucine(B) and lysine(C) incorporation into TCA-insoluble material in the absence(O) or presence(**O**) of poly(U). Each time point represents the equivalent of 2.5×10^5 cells of strain A_2 at 28 MPD.

the ^{3}H-leucine used in error frequency assays was present in 7-8 x the specific activity of the ^{3}H-phenylalanine 140-160 μCi/ml vs 20 μCi/ml, respectively, a low level of leucine incorporation in the absence of poly(U) was always detected. Poly(U) was found to stimulate leucine incorporation in every extract that showed protein synthetic activity. Leucine incorporation in the absence of poly(U) was inhibited 90% by puromycin (Figure 3-14b), by Ca^{+2} - dependent micrococcal nuclease (Pelhem and Jackson, 1976) and by histidinol (data not shown) an inhibitor of histidinyl-tRNA synthetase, indicating that low levels of protein synthesis remained after ribosomal runoff. When endogenous phenylalanine incorporation was measurable, the ratio of endogenous phe/leu incorporation varied from 2.6 - 7.7 (with a mean of 4.5). Since poly(U) is known to inhibit protein synthesis in some systems (Chatterjee et al., 1979) the effect of poly(U) on endogenous incorporation (of leucine) was monitored by including a control amino acid, lysine, as part of the assay to serve as a measure of endogenous protein synthesis. Rarely did the presence of poly(U) affect lysine incorporation ($\pm$ 3%). However, when poly(U) was shown to inhibit lysine incorporation significantly, inhibition of endogenous leucine incorporation was also corrected for.

In all assays, the error frequency of poly(U) translation was expressed as follows:

$$\% \text{ error frequency} = \frac{\text{Leu}}{\text{Leu + Phe}} \times 100$$

(approximately, $\% \text{ error frequency} = \frac{\text{Leu}}{\text{Phe}} \times 100$

or:

$$\frac{\left[\text{leucine(+p(U))} - \left[\frac{\text{lysine(+p(U))}}{\text{lysine(-p(U))}} \times \text{leucine(-p(U))}\right]\right]}{\left[\text{phenylalanine(+p(U))} - \text{phenylalanine(-p(U))}\right]} \times 100$$

3.1.2.1 Normal Cells

Cell-free extracts of cell strains established from normal donors (Table 2-1) were assayed for fidelity of protein synthesis under standard conditions (section 2.2.5) and the results are shown in Table 3-1. Several findings are noteworthy. Cell strain A_2, in extracts of 3 different lots of early-passage cells, exhibited an error frequency of 0.25% declining to 0.048% in extracts of late-passage cells. This five-fold decline in error frequency was also evident in extracts of cell strain A_{25} in which the error frequency declined from 1.16% at early passage to 0.227% at late passage. Cell strain J069, derived from an old donor, exhibited a two-fold decline from mid to late passage. It is evident from Table 3-1 that the rate of protein synthesis in extracts prepared in the standard protocol was highly variable ranging, for example, from 16.7 to 99.8 pmoles phenylalanine/hr/10^6 cells in extracts of A_2 at late passage. Also, there was no correlation between synthetic rate and the error frequency measured in fresh extracts. It was observed, however, that while extracts which were frozen even for one day had only

Table 3-1

Error Frequencies in Extracts of Normal Fibroblasts

Cell Strain	Passage MPD	Synthetic Rate pmoles/hr/10^6 cells	Error Frequency (%) mean ± S.D. (a)
A2 early	26	25.8	0.263
	29	17.4	0.222, 0.212
	31	18.9	0.269
			0.250 ± 0.028
A2 late	64	99.8	0.040
	66	75.8	0.078
	66.5	16.7	0.027
			0.048 ± 0.027
A25 early	22.5	24.2	1.04, 1.22
	27.5	24.8	1.10, 1.30
			1.16 ± 0.049
A25 late	50	35.8	0.285, 0.265
	53	67.5	0.16, 0.19
	58	39.6	0.236, 0.227
			0.227 ± 0.046
J069 mid	27	3.93	0.135
	30	25.2	0.125, 0.135
			0.133 ± 0.005
J069 late	49	20.2	0.060, 0.070
	53	8.1	0.055, 0.063
			0.062 ± 0.004

(a) Mistranslation rates were determined as in sections 2.2.3 and 3.1.2 and reported as % error frequency. Where appropriate, duplicate determinations are reported. The mean error frequency ± standard deviation for each group of cells is also shown.

slightly lower (90%) synthetic activity, error frequency was increased two- to ten-fold. Although protein synthetic rate was highly variable, error frequencies determined with these extracts were quite reproducible as seen by the close range of duplicate determinations within any one experiment and the small standard deviation between experiments with extracts prepared from different lots of cells of the same strain.

Comparing the mean error frequencies of each cell strain using the "Student's" t-test (Table 3-2), one sees that early- and late-passage cells within normal strains exhibited significantly different error frequencies ($p \leq 0.005$ in all strains tested). Cell strain A_{25} had a significantly higher error frequency than A_2 and JO69 at both early and late passage. It was doubtful however whether error frequencies determined for A_2 and JO69 were different, since no significant difference was found at late passage and since the youngest JO69 cells harvested for error assay were already midway through their tissue culture lifespan. Thus, the t-statistic generated for JO69 at mid passage and A_2 at early passage is not a valid comparison. When the more stringent Behrens-Fisher modification (Fisher, 1939; Hoel, 1962) of the t-test (for small sample sizes from a normal population of unknown variance) was used, essentially no change in the significance levels was observed.

As mentioned earlier (section 3.1.1.2) standard conditions of the error frequency assay were optimal for

Table 3-2

Differences in Error Frequency of Normal Cell Strains

	A2 early	A2 late	A25 early	A25 late	J069 mid	J069 late
A2 early	-	0.005	0.005		NS	
A2 late	7.24	-		0.01		NS
A25 early	9.7		-	0.005	0.005	
A25 late		4.52	16.2	-		0.025
J069 mid	4.26		21.4		-	0.005
J069 late		0.52		3.59	10.92	-

Significance levels (p)

The "Student's" t-test for comparing independent means (from Table 3-1) was used to generate t-statistics listed in the lower left half of the block table. Significant differences are shown in the upper right half of the table.

NS. - Not significant.

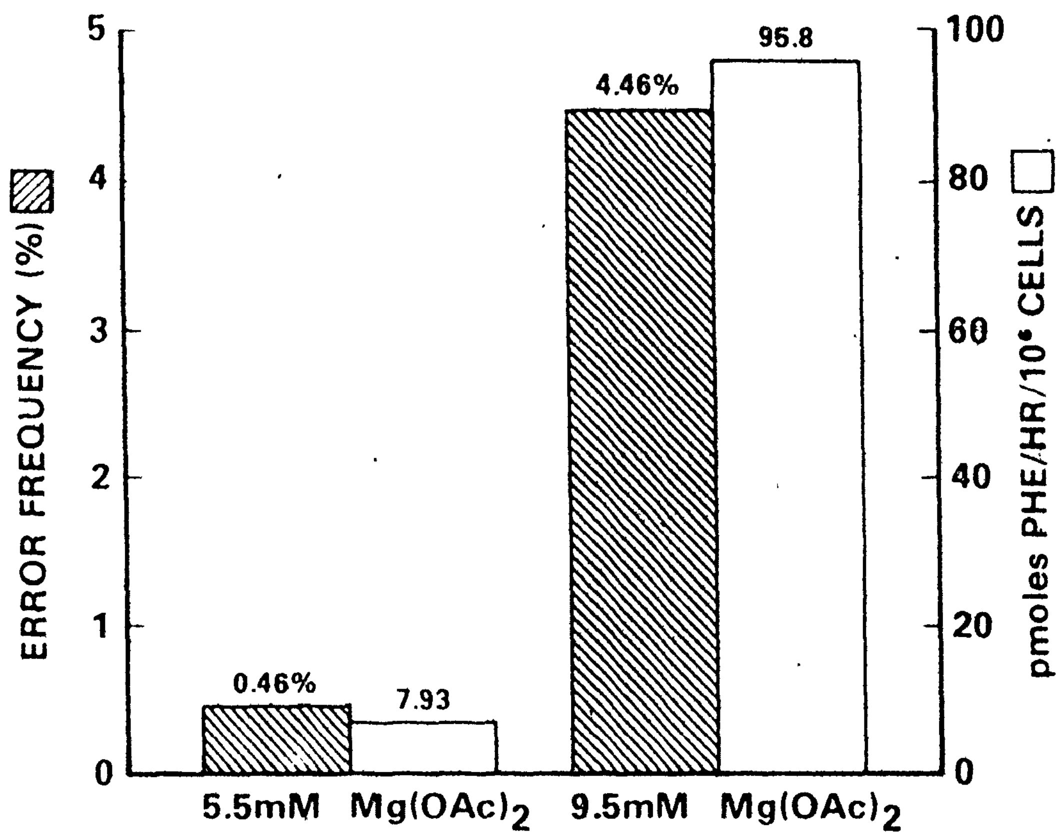


Figure 3-19 The effect of high magnesium concentration on error frequency.

A frozen extract of strain J069 at 53 MPD was assayed for error frequency under standard conditions (5.5 mM Mg^{+2}) and with added $Mg(OAc)_2$. The mixtures were incubated for 30 minutes and triplicate aliquots measured for radioactivity.

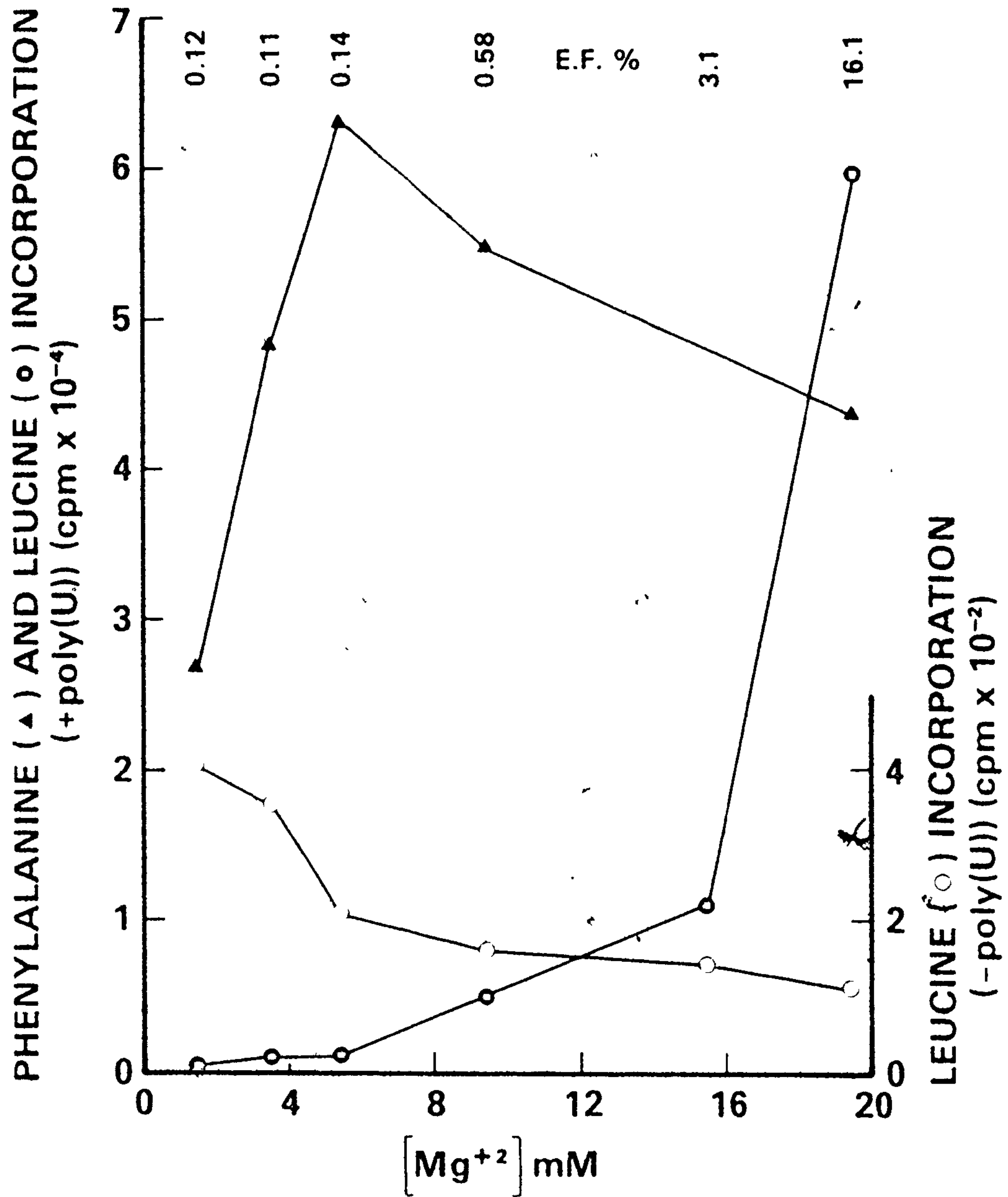


Figure 3-20 The effect of magnesium concentration on phenylalanine and leucine incorporation.

An extract of strain A_2 at 35 MPD was assayed for phenylalanine(▲- plus poly(U)) and leucine(●- plus poly(U), ○- minus poly(U)) incorporation at various concentrations of $Mg(OAc)_2$ after a 30 minute incubation.

poly(U) translation in extracts of strain A_2 at early passage and were not necessarily optimum for extracts of other cell strains. For example, in extracts of J069 at 53 MPD, the optimum concentration of Mg^{+2} was 9.5 mM rather than 5.5 mM as for strain A_2. The error frequency determined at the 9.5 mM Mg^{+2} optimum for J069 was almost ten-fold higher than under standard conditions (Figure 3-19).

The effect of magnesium concentration on amino acid incorporation rates in a fresh extract of strain A_2 at 35 MPD (Figure 3-20) showed that a) phenylalanine incorporation was optimal at 5.5 mM and b) endogenous leucine incorporation declined whereas poly(U)-stimulated leucine incorporation increased rapidly with increasing Mg^{+2} concentration. Thus, error frequency continues to increase at higher Mg^{+2} concentrations as well.

3.1.2.2 Progeria and Werner Syndrome

Cell strains, P_{18} and P_5, established from two patients with classical progeria and one strain, WS_2, from a patient with Werner Syndrome were used for determination of error frequency in prematurely aging cells. Although these cells showed a distinctly slower growth rate compared to their normal counterparts, they grew reasonably well; P_5 and P_{18} reaching confluence in about 7-8 days, after a 1:8 split into roller bottles. The WS_2 strain reached confluence in about 7-8 days after a 1:4 split.

As seen in extracts of normal cells, the in vitro

Table 3-3

Error Frequency in Extracts of Progeroid Cells

Cell Strain	Passage MPD	In Vitro Synthetic Rate pmoles Phe/hr/10^6 cells	Error Frequency % mean ± S.D.
P18	36	7.48	0.224, 0.213
	35	28.1	0.216, 0.236
	29	10.8	0.20, 0.258
			0.225 ± 0.020
P5	31	16.54	0.194, 0.195
	34	215	0.25, 0.238
			0.220 ± 0.029
WS2	30	4.85	0.216, 0.208
	28	28.0	0.12
			0.166 ± 0.065

synthetic rate in extracts of progeroid cells was highly variable (Table 3-3). The synthetic activity of 215 pmoles phe/hr/10^6 cells in one extract of P_5 at 34 MPD was one of the highest activities found in any extract. There was no obvious decrement in synthetic capability of extracts from progeroid cells. In five separate extracts (3 of P_{18} and 2 of P_5), the error frequency determined for progeric cells was 0.22% with no difference between the two cell strains. From two separate error frequency determinations WS_2 exhibited a mean error frequency of 0.166%. However, the relatively large standard deviation made its error frequency insignificantly different from that of progeric cells.

Comparing the error frequencies of extracts from progeroid cell strains to those of normal population, it is seen that error frequencies of all of P_5, P_{18} and WS_2 fall within the broad normal range (Figure 3-21).

3.1.2.3 <u>Transformed Cells</u>

A fetal cell line, MRC-5, and its SV-40 transformed counterpart, SVMRC-5, were grown and assayed for error frequency in vitro. The untransformed cell strain grew more rapidly than either A_2 or A_{25} at corresponding passage levels reaching confluence in 4 days (early passage) to 7 days (late passage) after a 1:8 split. The transformed cell line grew even more rapidly. As this cell line rendered the growth medium highly acidic within 3-4 days, roller bottles inoculated with SVMRC-5 were not gassed with 5%

Table 3-4

Error Frequency in Fetal and Transformed Cell Extracts

Cell Strain		Passage MPD	In vitro Synthetic Rate pmoles/hr/10^6 cells	Error Frequency % mean ± S.D.
MRC-5	early	29	34.5	0.44, 0.48
		34	36	0.39
				0.425 ± 0.049
	late	50	55.8	0.115, 0.130
		54	36.0	0.101, 0.098
				0.111 ± 0.015
SV-MRC-5			16.3	0.33, 0.37
			24.5	0.29, 0.27
				0.315 ± 0.044

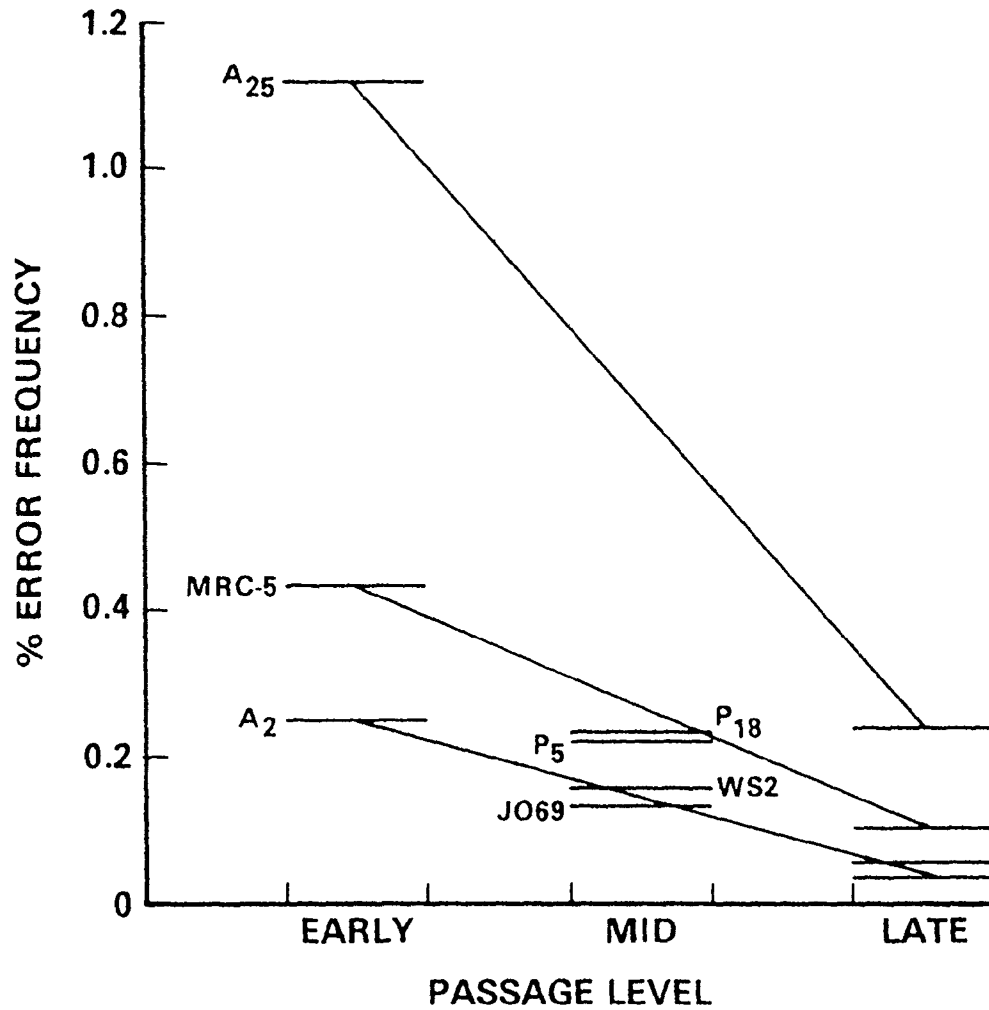


Figure 3-21 Comparison of error frequency in strains of normal and progeroid cells.
Data from Tables 3-1,3 and 4 are depicted graphically with lines joining error frequencies of normal cell strains at different passage levels.

CO_2 prior to rolling. After confluence was reached in 3-4 days, it was apparent that many cells had detached and remained suspended in the growth medium. Extracts prepared from these suspended cells had no poly(U) translating capacity even though a considerable number of the cells were still viable (high density plating efficiency ~50%). Cells which remained attached to the plastic roller bottle yielded extracts with considerable synthetic activity.

As seen in the other normal cell strains, MRC-5 also exhibited an apparent decline in error frequency from early passage (0.43%) to late passage (0.11%). As with the other normal strains, this four-fold decline in error frequency was significant ($p \leq 0.005$).

3.1.3 Decline in Error Frequency as a Function of Passage

3.1.3.1 The A_2 Series

To determine the significance of the apparently lower error frequency observed in extracts of late passage normal cells compared to their early passage counterparts, the error frequency in extracts of one normal cell strain, A_2, was determined at several stages throughout its tissue culture lifespan. All cells used in this series of experiments were serially subcultivated from a single lot of strain A_2 at 17 MPD. Error frequencies were determined at 13 sequential passage levels from MPD 22 to MPD 74. The results are shown in Figure 3-22.

Figure 3-22 Decrease of error frequency as a function of passage.

A single lot of cells of strain A_2 was serially subcultivated to the end of its in vitro lifespan and assayed for error frequency at 13 passage levels. Least-squares analysis shows the regression line $y = -0.0046x + 74.6$ fits the data with a correlation coefficient of $r = -0.93$. Horizontal bars show the range of error frequencies determined in duplicate.

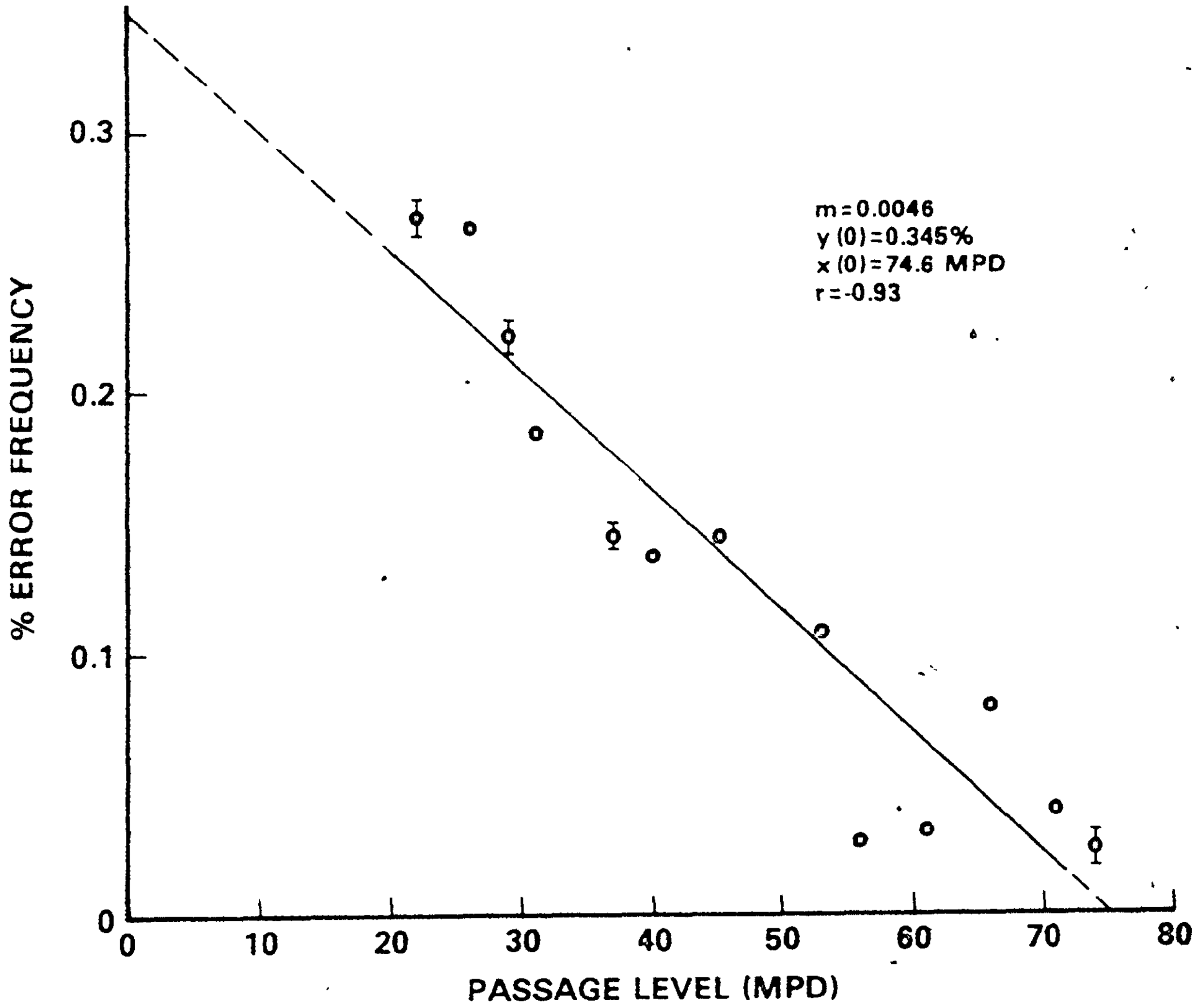


Figure 3-22 Legend opposite.

The error frequency of poly(U) translation exhibited a ten-fold decline in a sequential manner from 0.274% at MPD 22 to 0.025% at MPD 74. Linear regression analysis of the data (by the method of least squares) showed that the line $y = -0.0046x + 74.6$ fits the data with a correlation of $r = -0.934$. Intercepts of this line are $y(0) = 0.346\%$ and $x(0) = 74.6$ MPD. Testing the null hypothesis showed that the slope of the line was significantly different from zero ($t = 8.67$, 11 df, $p \leq 0.001$).

Other measurements of protein synthetic activity did not show any significant trend as a function of passage. The rate or phenylalanine incorporation directed by poly(U) (Figure 3-23) had a mean of 22.7 pmoles/hr/10^6 cells ranging from a low of 110 fmole/hr/10^6 cells in an extract of cells at 71 MPD to a high of 76 pmoles/hr/10^6 cells in an extract of cells at 66 MPD. Similarly, residual levels of protein synthesis varied considerably in each extract as shown by the incorporation of leucine and lysine in the absence of poly(U) (Figure 3-24). Consistently endogenous leucine incorporation was higher than lysine incorporation by an average of 1.5-fold.

The cells used in this study grew well in roller bottles, taking progressively longer times, however, to reach confluence (Figure 3-25). The youngest cells at 22 MPD grew rapidly to confluence in about 4 days at a 1:8 split ratio. Cells were inoculated into roller bottles at a 1:8

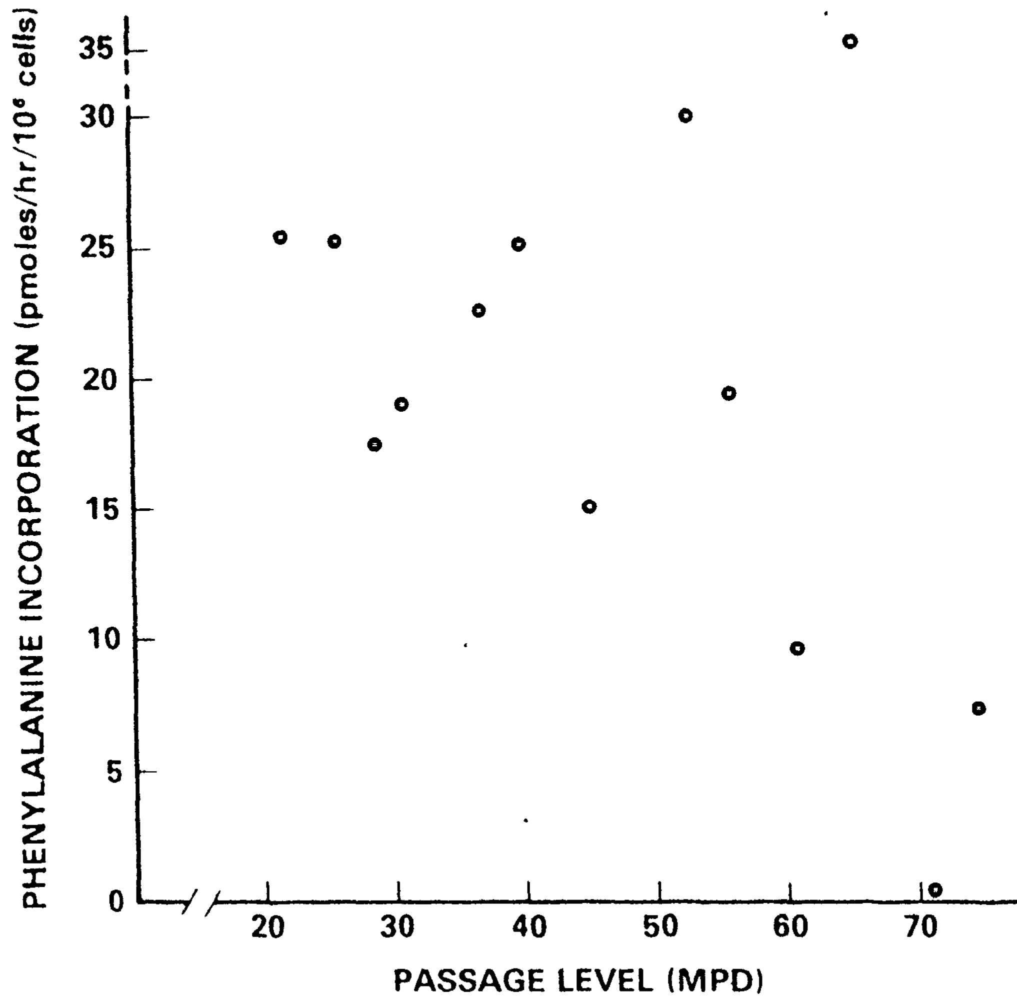


Figure 3-23 Synthetic activity in extracts of the A_2 series.

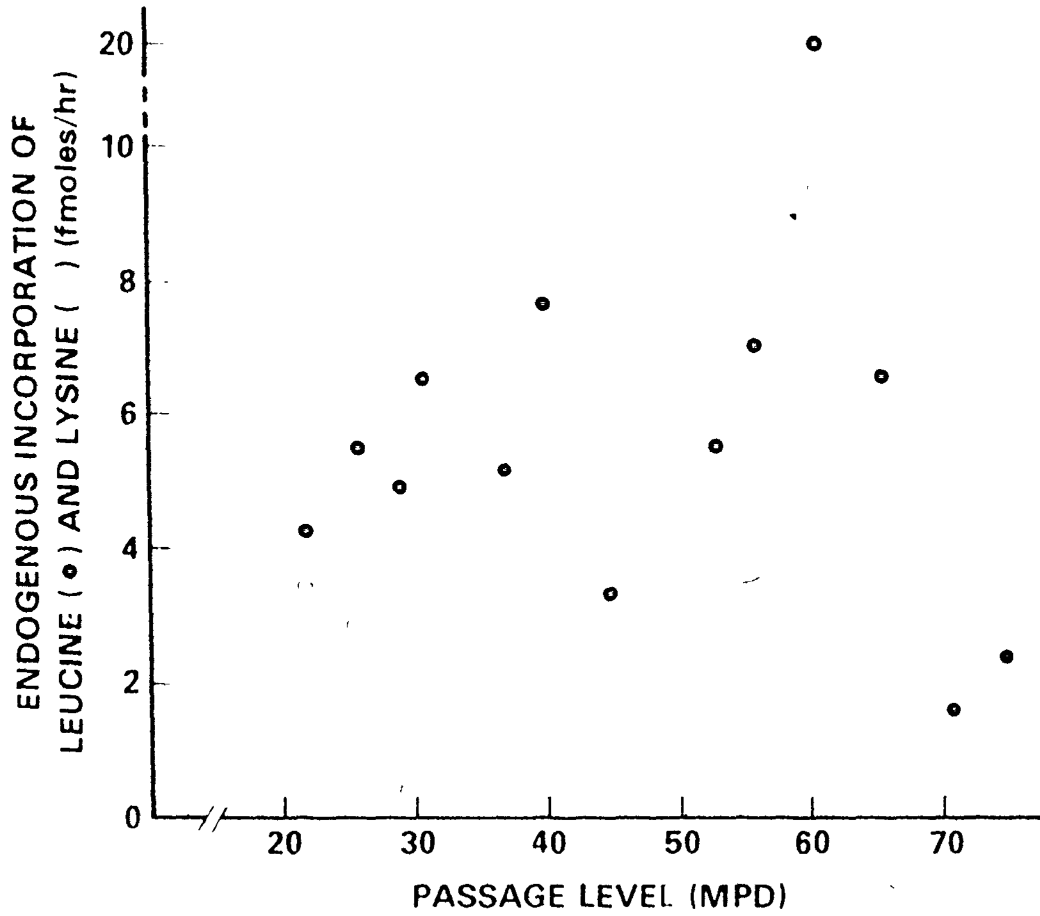


Figure 3-24 Residual endogenous incorporation of leucine and lysine at various passage levels.

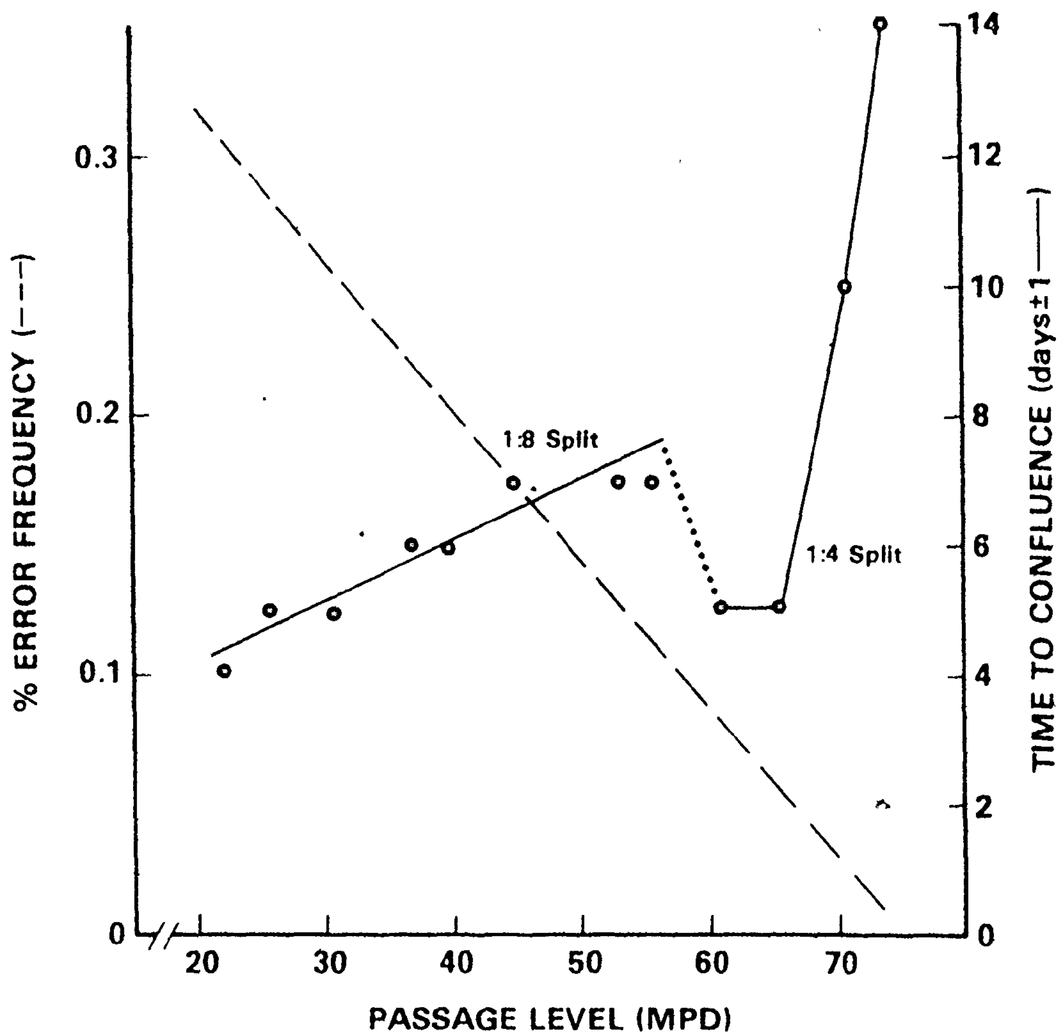


Figure 3-25 Inverse correlation of error frequency and growth rate. The number of days to confluence was determined microscopically for cells of strain A_2 at a 1:8 split ratio (22-56 MPD) or a 1:4 split ratio(61-74 MPD). The regression relation showing decreasing error frequency with passage level from figure 3-22 is shown as the broken line.

split ratio until 60 MPD when the inoculum was increased to a 1:4 split ratio. A sharp increase in the time to confluence was observed between 66-70 MPD and at 74 MPD the culture did not reach confluence after 14 days when it was refed by adding 20 mls of fresh RGM. Cells at 74 MPD were harvested 3 days after refeeding although it was difficult to be certain that confluence was attained.

3.1.3.2 Effect of Cellular Growth Rate on Error Frequency

To determine whether the decreasing growth rate in late passage cells was responsible for the observed decline in error frequency as a function of passage level, the growth rate of early passage cells was inhibited by growth in lower concentrations of fetal calf serum (FCS). The growth rates of early passage A_2 in RGM containing varying amounts of FCS is shown in Figure 3-26. In 10% serum, the cells grew rapidly reaching confluence in about 5 days (logarithmic doubling time ~1 day). After confluence was attained a slight loss in cell number was observed. Cells grown in 7.5% FCS, showed no appreciable decline in growth vigor (data not shown). At 2.5 and 5% FCS, a considerable increase in the doubling time to about 2½ to 3 days was observed, confluence being reached 10 days after seeding. Late passage cells in 15% fetal calf serum also had a doubling time of 3 days and reached confluence only after 10 days.

Although young cells grown in low serum approximated

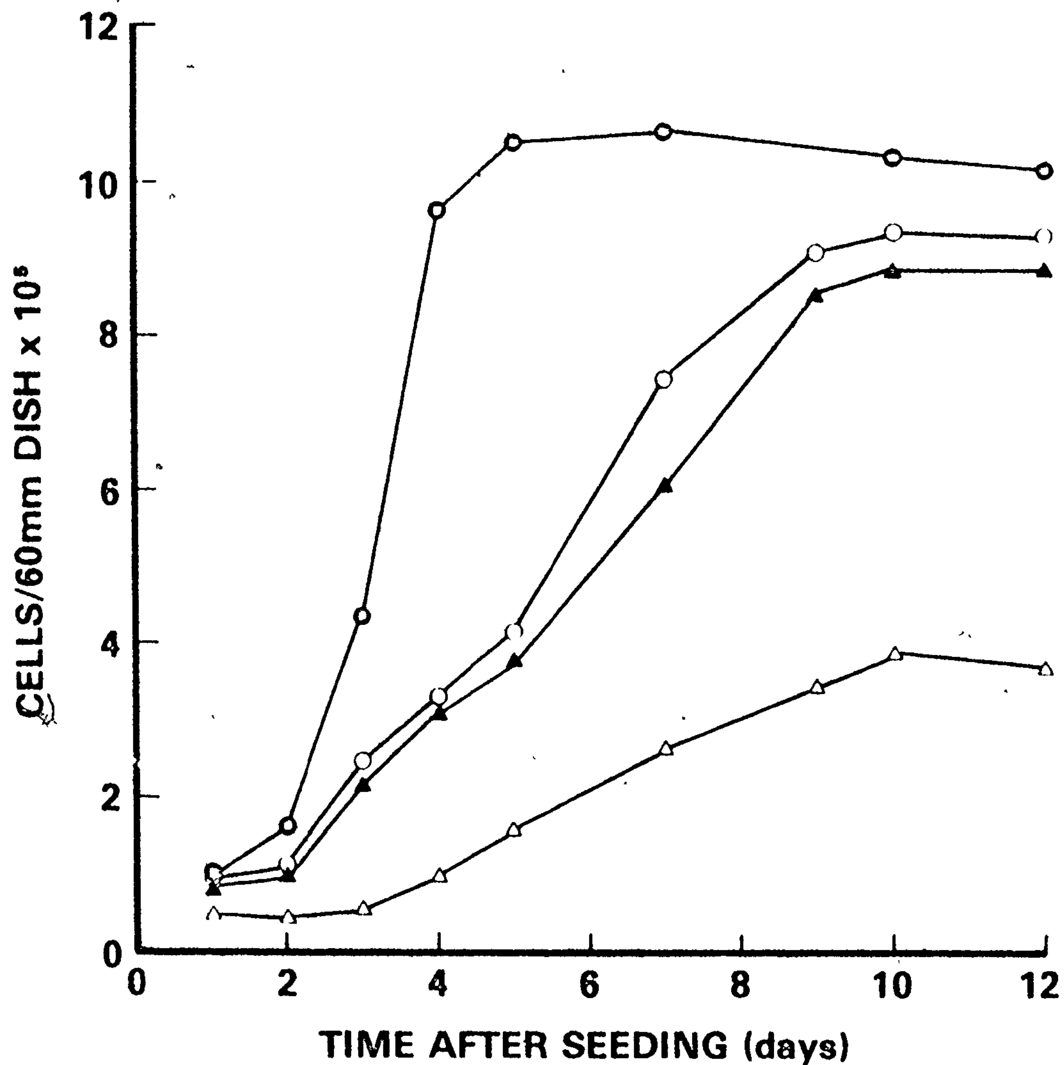


Figure 3-26 Growth of A_2 cells under low serum conditions. Cells of srain A_2 were inoculated at 28 MPD into 60 mm dishes containing MEM supplemented with 10%(**O**), 5%(O) and 2.5%(▲) FCS. Strain A_2 at 63 MPD (△) was grown in 15% FCS. Duplicate dishes were harvested and the total number of cells/dish determined at the specified intervals after inoculation.

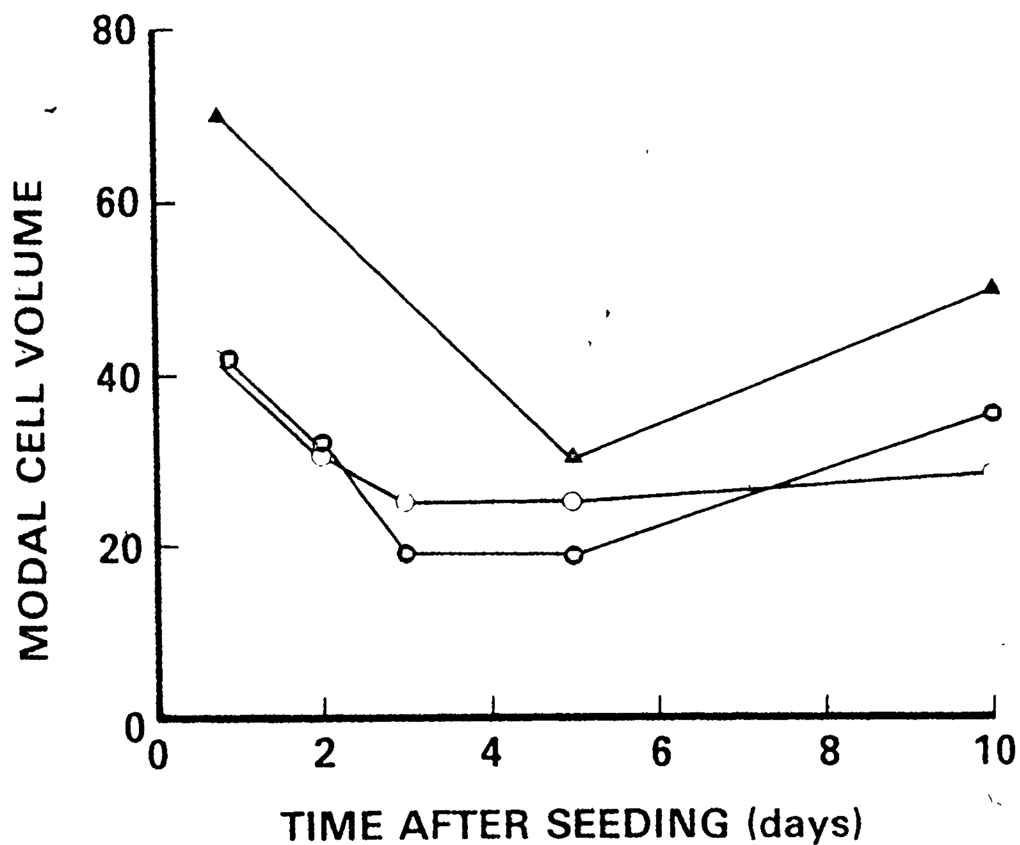


Figure 3-27 Cell volume during growth.

Strain A_2 at 28 MPD was inoculated into 60 mm dishes each containing MEMA supplemented with 10%(●) or 2.5%(○) FCS. Strain A_2 at 63 MPD was inoculated into 15% FCS(▲). Cells were harvested at the times indicated and the modal cell size was determined on a Coulter Chanalyzer.

the doubling time of old cells grown in 15% serum, differences in the growth characteristics of young and old cells still persisted. For example, actual cell numbers still differed markedly; there were still more than four times the number of young cells than old cells at confluence (Figure 3-26). Also cell volume at all stages of growth differed (Figure 3-27). About 16 hours after inoculation all cells had become larger than in the confluent state. During periods of growth, a substantial decrease in cell size was noted, the smallest decrease being in young cells in low serum. These cells however, failed to increase in volume at confluence. The late passage cells were the largest at all stages of growth.

To harvest cells grown in different serum concentrations, early- and late-passage cells were inoculated into roller bottles containing 2.5% and 15% FCS respectively and 5 days later cells at early passage were inoculated into 10% FCS.

All cells were harvested and extracts prepared and assayed on the same day. Table 3-5 shows results of error frequency assays in these cells in two separate experiments using two separate lots of strain A_2. In both experiments, the error frequency determined in extracts of slowly-growing early passage cells (2-2.5% FCS) was identical to that of cells grown rapidly. As before, poly(U)-directed protein synthetic rate was highly variable among the extracts. Another parameter, protein synthesis in the intact cells, was measured in the second experiment. Cells grown in 2% FCS exhibited the same

Table 3-5

The Effect of Serum Limitation on Growth Rate and Error Frequency

	Cell Passage	% Serum	Days to Confluence	In Vivo Synthesis (a)	In Vitro Synthesis (b)	Error Frequency
Experiment 1 A2	30	15	5	n.d.	39.6	0.36±0.02
A2	30	2.5	10	n.d.	52.4	0.33±0.01
Experiment 2 A2	35	10	5	8.4	19.1	0.25±0.04
A2	35	2.0	12	8.6	16.3	0.27±0.02
A2	63	15	10	7.6	46.5	0.03±0.005

(a) Cellular protein synthetic rate was determined as distributed in section 2.2.7, fmoles phenylalanine/hr/cell.

(b) Poly(U)-directed phenylalanine incorporation in extracts, pmoles phenylalanine/hr/10^6 cells.

n.d. - not determined.

rate of protein synthesis as cells grown in 10% FCS. Late passage cells had a slightly slower rate of protein synthesis. On a per cell basis, the protein synthetic activity of extracts was found to be less than 1% of the synthetic activity in the intact cells.

3.1.3.3 Clonal Selection

To observe the effect of serial subcultivation on the error frequency of a clonally pure culture of cells compared to that of a clonally mixed mass culture, several clonal cultures of strain A_2 were established from the parental strain. Because of the nature of clonal culture, clones could only be assayed for error frequency in the final 15-25% of their tissue culture lifespan. Parental cells were taken from the frozen state at MPD 14 and 3 MPD added upon reaching confluence. A total of 500 cells at 17 MPD were then inoculated into dishes. The plating efficiency at low-density inoculation was only 15-20% for A_2, thus only about 100 cells attached. Of these only one-half (50) gave colonies large enough for picking. There was much heterogeneity in the colonies that formed i.e. size of colony (0.5 - 1.5 cm), number of cells in the colony, size of cells, and the density of cells in the colony varied from one colony to the next. Of the 50 clones that were picked most were able to eventually fill at least one 100 mm dish. At this point 20 MPD were added to the passage level of the cells at seeding; thus, a cumulative 37 MPD. Most of the clones

did not grow well enough to survive the establishment period of 8 MPD. Clones which did not reach confluence at 1:8 split after a weekly refeeding were not carried. Only 10 clones were established representing only 2% of the initial number of cells used.

Two separate lots of the parental strain were used, clones 2 and 8 from one lot and clones 10-36 from another. A total of 12 clones were assayed for error frequency. Clones were subcultivated in dishes for later inoculation into roller bottles at desired passage levels.

3.1.3.3.1 Error Frequency

Extracts of clonal cultures were prepared as before and the standard error frequency assay carried out on each extract. Error frequencies were determined at the earliest passage level and as close to the terminal passage level (determined in dishes, carried in parallel) as possible. Error frequency assays were carried out at an intermediate passage level in some of the clones as well. Parameters of in vitro protein synthesis in extracts of clones are listed in Table 3-6.

As in previous experience, all clones yielded active extracts, although considerable variability in activity of the extract i.e. the rate of poly(U) translation was observed. There were also considerable changes in endogenous amino acid incorporation in these extracts with the lysine:leucine ratio varying markedly. Although the range of error frequencies

Table 3-6

Error Frequency Determinations in
Clones of Strain A2

Clone	Passage (MPD)	Phenylalanine incorporation +P(U) pmoles/hr/10^6 cells	Endogenous Amino Acid Incorporation fmoles/hr/10^6 cells		Error Frequency % ± range
			Leucine	Lysine	
2	45	70.4	104	98	0.085
	48	56.0	107	135	0.076±0.001
	55	88.5	99.2	115	0.070
	63	70.5	109	140	0.081
	66	89.9	130.5	116	0.073±0.001
8	45	43.5	105	129	0.075
	48	65.4	99	135	0.084
	55	78.0	126.5	103	0.097
	63	63.0	120	98	0.227
	66	61.5	119	110	0.257
10	53	119.0	66	32	0.026
	61	90.6	61	17	0.041
12	53	168.2	78.5	21	0.23±0.02
	61	112.5	86	52	0.215±0.03
13	53	72.8	100	n.d.	0.145±0.012
14	53	156	226	36	0.059±0.007
	59	31.9	79	23	0.045±0.009
	66	17.2	107	29	0.25±0.000
	68+6 wks.	31.5	177	39	0.245±0.040
18	53	175	232	400	0.40±0.02
	59	28.0	81	27	0.408±0.01
	64	35.9	44	22	0.394±0.002
26	53	38.5	100.1	62	0.063

Continued/...

Table 3-6 Continued

Clone	Passage (MPD)	Phenylalanine incorporation +P(U) pmoles/hr/10^6 cells	Endogenous Amino Acid Incorporation fmoles/hr/10^6 cells		Error Frequency % ± range
			Leucine	Lysine	
29	53	17.2	59	15	0.170
	61	71.4	72	55	0.173
30	51	74.0	101	80	0.135±0.015
	62	70.3	77	86	0.148±0.013
32	53	132.8	141	280	0.30±0.02
36	51	75.9	33	n.d.	0.100±0.007
	62	31.0	198	33	0.106±0.002

Figure 3-28 Error frequencies in clones of strain A_2. Twelve(12) clones were selected from the parental mass culture (see text) and assayed for error frequency at various passage intervals. Shown are the means of duplicate determinations, the range indicated as bar graphs, or as singlicate determinations. The mean of the error frequencies of the clones at MPD 53(**X**) was $0.169 \pm 0.103\%$. The regression line from Figure 3-22 is shown as the dashed line.

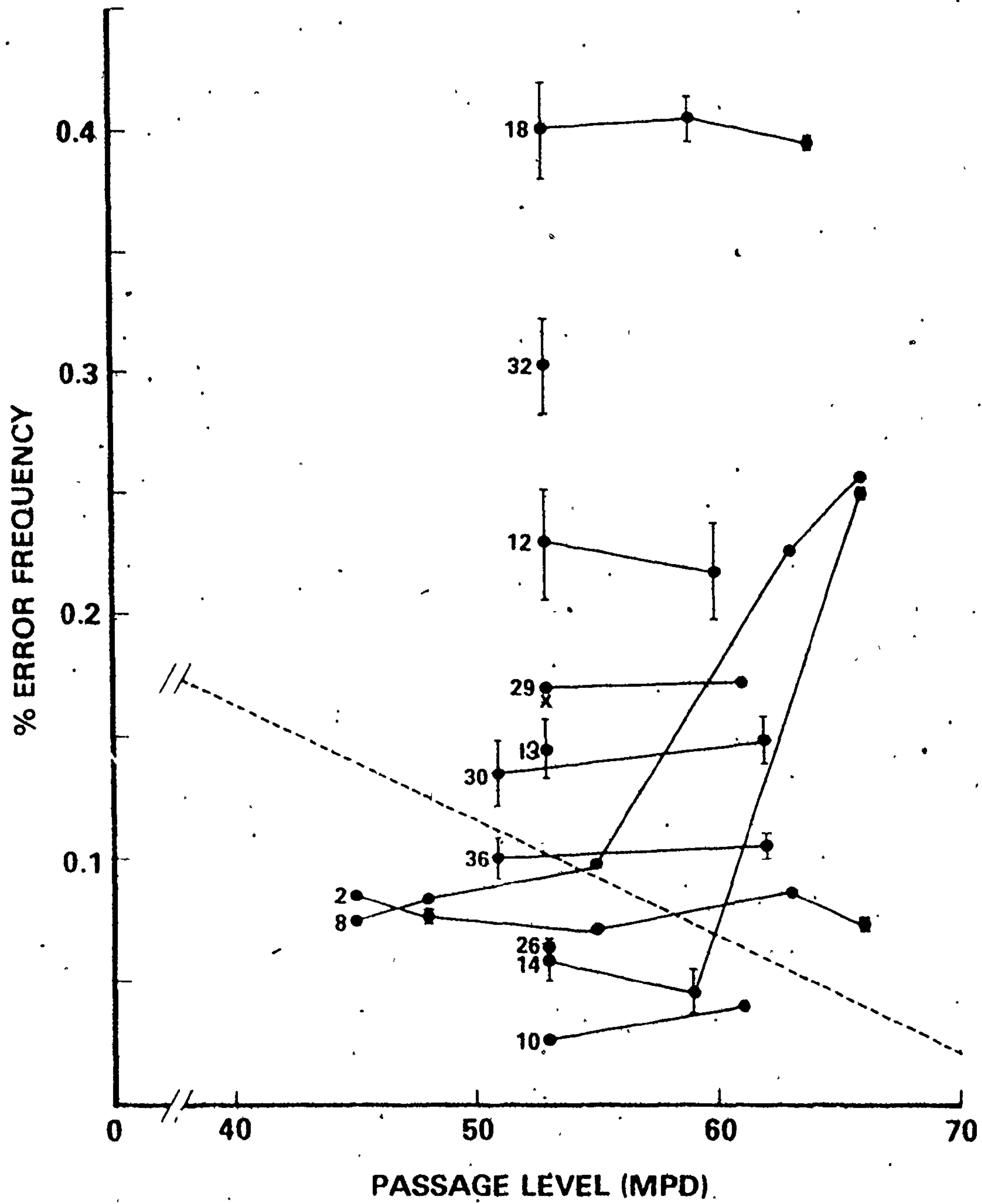


Figure 3-28 Error frequencies in clones of strain A_2. legend opposite.

measured in a single extract was somewhat larger than previous experience, duplicability was still good.

Important observations can be made concerning the error frequency of clones (Figure 3-28).

1. Clones varied markedly in error frequency at any particular passage level. At MPD 53 for example, error frequencies ranged from 0.026% in clone 10 to 0.40% in clone 18, with the rest of the clones falling within this large range. The mean interclonal error frequency was calculated to be 0.169 $\pm$ 0.103 (% $\pm$ s.d.) at MPD 53 (median, 0.12%). Thus the distribution of clonal error frequencies was not normal at MPD 53 but rather slightly skewed in favour of the lower error frequency clones.

2. Unlike the mass culture, none of the clones exhibited a significant decline in error frequency in later passage. There were two groups of clones however: Group A, seven clones (2,10,12,18,29,30,36), in which the error frequency stayed more or less constant in later passage i.e. the slope of the line was not significantly different from zero. This statement is an assumption for clone 10 and clone 29, however, since they were assayed in singlicate at earlier- and later- passage; and Group B, 2 clones (8 and 14) in which an abrupt increase in error frequency at later passage was observed. N.B. This was the only case in which an increase in error frequency with passage was observed in all error frequency determinations that had been done. In

clone 8 the increase continued from MPD 63 to MPD 66. In clone 14, the error frequency remained at the elevated level, i.e. did not increase or decrease, when this clone was held in a post-mitotic state (see section 3.1.4.2) for a period of 6 weeks.

For the purpose of analyzing the data on error frequency in clones and comparing it to the decline of error frequency with passage of the mass culture, the two Groups A and B were considered separately.

Clone 2 of Group A was deemed representative of clones in group A since it was assayed over the greatest passage interval. The error frequency of clone 2 varies with passage level with a slope, i.e. regression co-efficient of $b = -0.000255$ with 95 and 99% confidence limits of ± 0.000367 and ± 0.0007677 respectively. Clearly, zero slope is contained within even the 95% confidence range. Correlation coefficient of this slope was calculated as $r = -0.385$. The decline of error frequency in the mass culture is described by the line $y = -0.0046x + 74.6$, where y = % error frequency and x = passage level in MPD. The correlation coefficient for this least-squares line was found to be $r = -0.934$. Thus the 95 and 99% confidence limits of the regression coefficient of $b = -0.0046$ are ± 0.00108 and ± 0.00153 respectively. Clearly there is no overlap of the ranges of the regression co-efficients of clone 2 and the mass culture at even the 99% confidence limits.

Another approach was to analyze clone 2 and the mass

culture as two groups with regression coefficients b_1 and b_2 respectively. The residual variance of each group S_i^2 is given as

$$S_i^2 = \frac{\sum (y - y_1)^2}{\sum (n_1 - 2)}$$

where y = the observed value and y_1 = the value predicted by regression group i. Thus for 2 groups, a pooled estimate may be obtained (similar to a 2-sample t-test) as

$$S^2 = \frac{\sum (y - y_1)^2 + \sum (y - y_2)^2}{n_1 + n_2 - 4}$$

Comparing b_1 and b_2 as

$$\text{var}\ (b_1 - b_2) = S^2 \left[\frac{1}{\sum (x - x_1)^2} + \frac{1}{\sum (x - x_2)^2} \right]$$

where x = the observed value and x_1 = the mean x value of group i, the difference is tested by using the t-statistic

$$t = \frac{-0.00435}{0.0014} = -3.104.$$

Testing the hypothesis that the slopes are equal against the hypothesis that they are not,

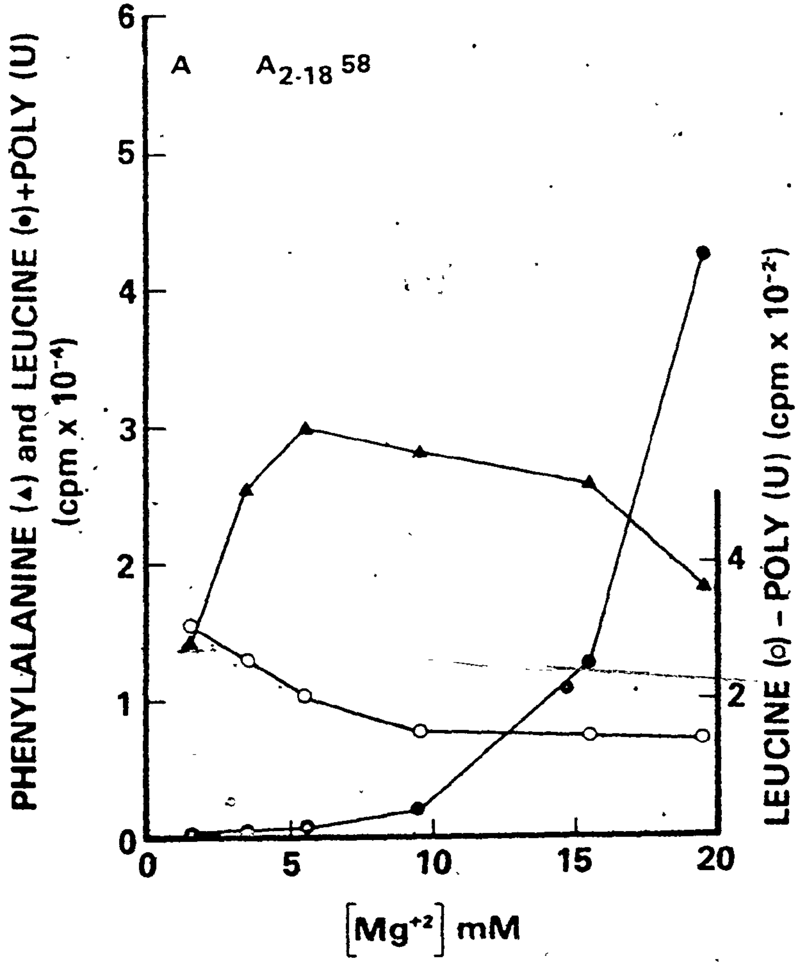
$$H_0 : b_1 = b_2 \text{ against } H_1 : b_1 \neq b_2$$

The critical region is $t_{.005,\ 14} = 2.977$, and thus we can conclude that the slopes are not equal ($p < 0.01$).

The mean of slopes of all clones in group A was found to be $b_A = +0.000333$. Pooling the clones of group A in this manner did not change the regression coefficient appreciably. Meanwhile the increase in the degrees of freedom

Figure 3-29 The effect of magnesium concentration on amino acid incorporation in extracts of clones.

Thawed extracts of clones 18 a) and clone 10 b) both at 58 MPD were assayed for the incorporation of phenylalanine (+poly(U)) and leucine(±poly(U)). Thirty-minute incorporation of phenylalanine(▲) and leucine(●) in the presence of poly(U) is shown on the left ordinate and leucine(○) incorporation in the absence of poly(U) on the right.



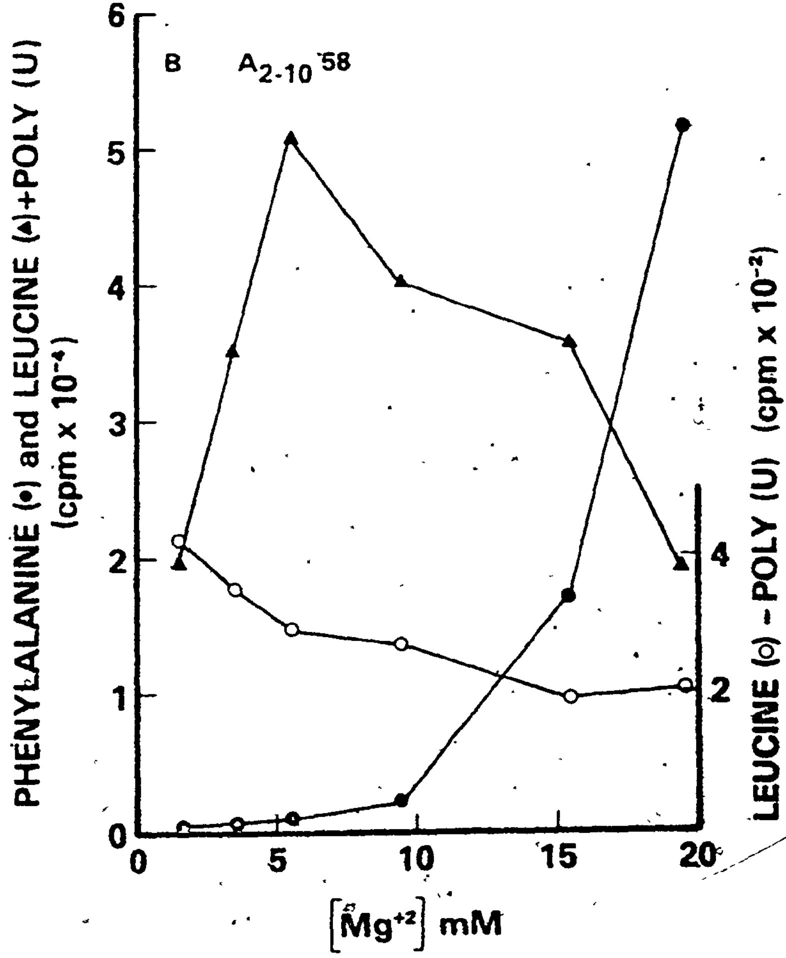


Figure. 3–29 see legend opposite.

made the difference between the regression coefficients of the mass culture and the clones even more pronounced. Furthermore, omitting clones of group B from the statistical analysis led to a conservative estimate of the difference between the mass culture and clones, since both clones 8 and 14 have large positive regression coefficients.

As shown previously (Figures 3-19), the concentration of magnesium in the extract affected the error frequency. To determine whether, clone 18 (E.F. = 0.4%) and clone 10 (E.F. 0.03%) have different magnesium optima, the error frequency was measured at various Mg^{+2} concentrations. Clone 18 at 58 MPD (Figure 3-29a) showed a broad curve with an optimum for phenylalanine incorporation at about 5.5 mM $Mg(OAc)_2$. Clone 10 at 58 (Figure 3-29b) MPD also showed a magnesium optimum at 5.5 mM. Also, in both clones poly(U)-stimulated leucine incorporation rose dramatically as in the A_2 mass culture (Figure 3-20), resulting in an error frequency of 25-30% at 20 mM $Mg(OAc)_2$.

3.1.3.3.2 Growth Characteristics

During serial subcultivation in dishes, each clone exhibited growth characteristics which made it unique i.e. no two clones grew in exactly the same manner. These differences were readily detectable by microscopic examination and subjective estimates of the clone's growth vigour could be made (Table 3-7). Plating efficiency varied markedly as did the time to reach confluence. Upon reaching confluence, the cell density and

Table 3-7

Growth Characteristics of A2 Clones

Clone 2	Very good growth vigor, grew as well as the mass culture, although cells were larger. Difficulty reaching confluence at MPD 66 (10 d, 1:4 split), with good density and little debris. Max. MPD ∿ 68 est.
Clone 8	Very good growth vigor, growing slightly slower than mass culture. At confluence MPD 66 (12 d, 1:4) with good density, some debris. Max MPD ∿ 68 est.
Clone 10	Good growth vigor. Good plating efficiency until 57 MPD. Good density and little debris until 60 MPD. 8 days to confluence (1:8) at 53 MPD, 12 d (1:4) at 66 MPD. Max MPD 63-65.
Clone 12	Average growth vigor. Good plating efficiency throughout, poor density, little debris. Phase III suddenly at max MPD 60-63.
Clone 13	Poorest growth vigor. Poor plating efficiency and cell density at confluence. Much debris deposited max. MPD 55. Cells have cuboid appearance.
Clone 14	Good growth vigor. Average plating efficiency, good density, much debris after 60 MPD. Confluence 8 d (1:8) at 53 MPD, 14 d (1:4) at 66 MPD. Max. MPD 66-68.
Clone 18	Best growth vigor. Good plating efficiency and cell density throughout. Little debris. Confluence 7 d (1:8) at 53, 10 d (1:4) at 63. Phase III sudden, max MPD 66-68.
Clone 26	Poor growth vigor. Average plating efficiency and cell density, much debris. Confluence 10 d (1:8) at 50 MPD, 18 d (1:4) at 55 MPD. Max. MPD 55-56.
Clone 29	Poor growth vigor. Average plating efficiency, poor density, very much debris. Confluence 10 d (1:14) at 52 MPD, 16 d (1:4) at 61 MPD. Max MPD 63-64.
Clone 30	Average growth vigor. Good plating efficiency, good density, some debris. Confluence 8 d (1:8) at 51 MPD, 12 d (1:4) at 62 MPD. Max. MPD 63-65.
Clone 32	Average growth vigor. Average plating efficiency, poor density, very much debris. Confluence 8 d (1:8) at 50 MPD, 12 d (1:4) at 55 MPD. Max MPD 56-58.
Clone 36	Good growth vigor. Good plating efficiency, cell density and very little debris. Confluence 6 d (1:8) at 53 MPD, 12 d (1:8) at 62 MPD. Max. MPD 64.

Table 3-8

Growth Characteristics of Clones of Strain A2 used for Extract Preparation

Clone	Passage Level (MPD)	Days to Confluence	Split Ratio	Cell Density $x10^{-4}/cm^2$	Cell Size μ^3
2	45	6	1:8	1.42	n.d.
	48	8	1:8	1.53	n.d.
	55	8	1:8	1.25	n.d.
	63	12	1:8	1.01	n.d.
	66	12	1:4	1.06	n.d.
8	45	6-7	1:8	1.23	n.d.
	48	7	1:8	1.34	n.d.
	55	8	1:8	1.22	n.d.
	63	10	1:8	1.08	n.d.
	66	11	1:4	0.98	n.d.
10	53	10	1:8	0.96	40
	61	10-12	1:4	0.78	40
12	53	9	1:8	1.03	27
	61	10	1:4	0.90	38
14	53	6-7	1:8	2.06	31
	59	10	1:8	1.03	42
	66	14	1:4	1.05	35
	68+6 wks.	-	-	1.23	40
18	53	7	1:8	2.12	33
	59	10	1:8	1.04	31
	64	12	1:4	1.04	39
26	53	9	1:8	1.71	35
29	53	12	1:4	0.91	41
	61	20	1:4	0.78	48
30	51	9	1:8	1.21	32
	62	12	1:4	1.32	36

Continued/....

Table 3-8 continued

Clone	Passage level (MPD)	Days to Confluence	Split Ratio	Cell Density $x10^{-4}/cm^2$	Cell Size μ^3
32	53	12	1:4	1.96	43
36	51 62	8 16	1:8 1:4	1.17 0.79	27 40

n.d. - not determined.

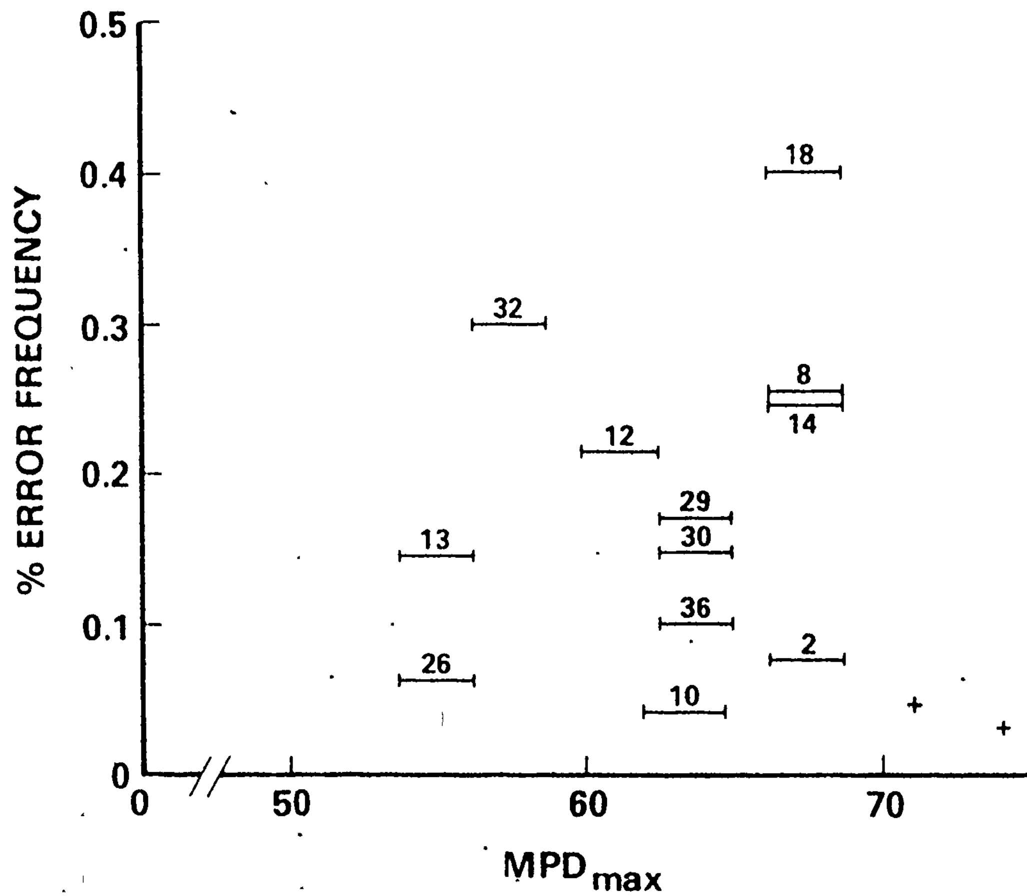


Figure 3-30 Clonal error frequency vs. maximum tissue culture lifespan. Estimates of MPD_{max} for clonal cultures were made by serial subcultivation until cultures could no longer reach confluence after two weekly refeedings, and are shown as bar graphs ± 2 MPD. Two unique estimates of mass culture MPD_{max} are shown as the symbol(+).

even the gross morphology of the clones was highly variable. The amount of debris accumulated by the clonal culture was also considered as an indication of the culture's growth vigor. Clones 10-36 were carried until maximum MPD and it was noted that some clones entered Phase III and reached MPD_{max} suddenly (i.e. clone 18) or remained in Phase III for several generations before MPD_{max} (i.e. clone 36).

Most clones grew as well in roller bottles as in dishes, taking on the average 2 days longer to reach confluence, however. Information on cell density and size of these cells used in extract preparation is outlined in Table 3-8. Comparing Tables 3-6,-7 and -8 one can see that none of the growth parameters measured for clones correlates with error frequency measured in their extracts. Most importantly perhaps the MPD_{max} of the clones does not correlate with the error frequencies determined (as seen in Figure 3-30).

Comparing growth of the clones t- that of the mass culture, it was noted that in general clones grew much more poorly than the mass culture - larger cells, longer doubling times, as well as lesser cell density and more debris at confluence. A few clones (2,8,14 and 18) were possible exceptions. Significantly, none of the clones lived as long in tissue culture as did the mass culture ($p<0.01$).

3.1.3.3.3 Synthetic and Proteolytic Rates

To ascertain whether there were any differences in the rate of cellular (in vivo) protein synthesis between clones with high or low error frequency, the protein synthetic rate in intact cells was measured in some of the clones and compared to the synthetic rate of the mass culture.

As shown in Figure 3-31a the incorporation of phenylalanine was essentially linear for one hour; however, although the mass culture had a very short lag period (i.e. period before protein synthetic rate becomes linear), clone 14 and 18 had a lag period of about 7-8 minutes. The synthetic rate was determined by calculating the slope that best fit the data giving rate as fmoles incorporation/hr/cell. Protein synthetic rate on a per cell basis was essentially unchanged in early- and late- passage cells of the mass culture. It was also observed that there was no difference in the protein synthetic rate of clone 14 before and after the increase in error frequency occurred. Other clones also showed no changes in the cellular protein synthetic rate at different passage levels; however, some differences between clones was observed (Figure 3-31b). Clone 10 had the lowest apparent synthetic rate of the clones assayed.

Comparing the clones collectively, to the mass culture, it was found that the mean of the clonal synthetic rates ($x = 4.46 \pm 1.05$) was significantly lower ($p < 0.001$) than the

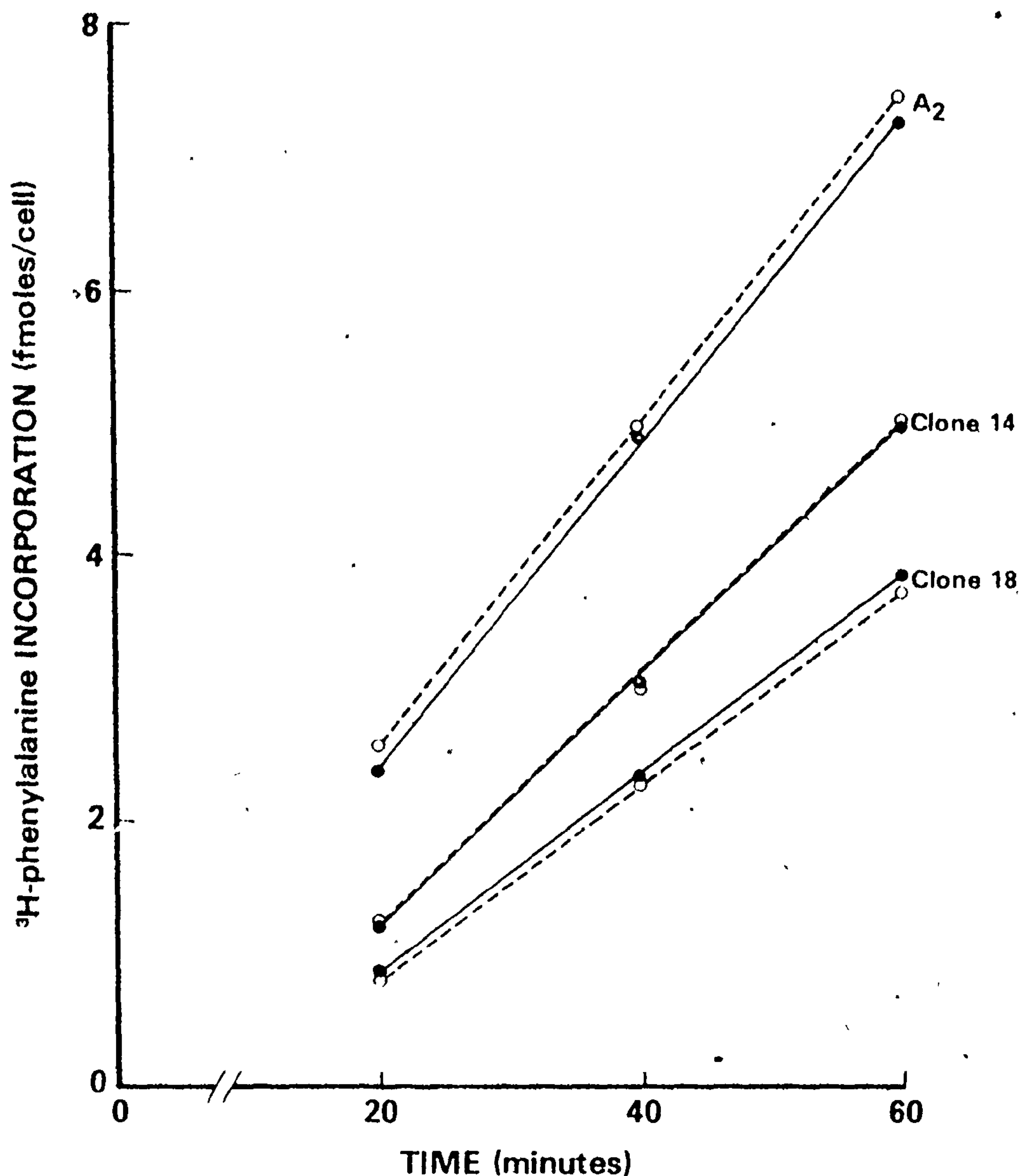


Figure 3-31a Protein synthetic rate determination.
Strain A_2 at 30(●) and 60(○) MPD and clones 14 and 18 both at 53(●) and 66(○) MPD were assayed for cellular protein synthetic rate (section 2.2.7). Incorporation of ^{3}H-phenylalnine was corrected for specific activity and normalized on a per cell basis.

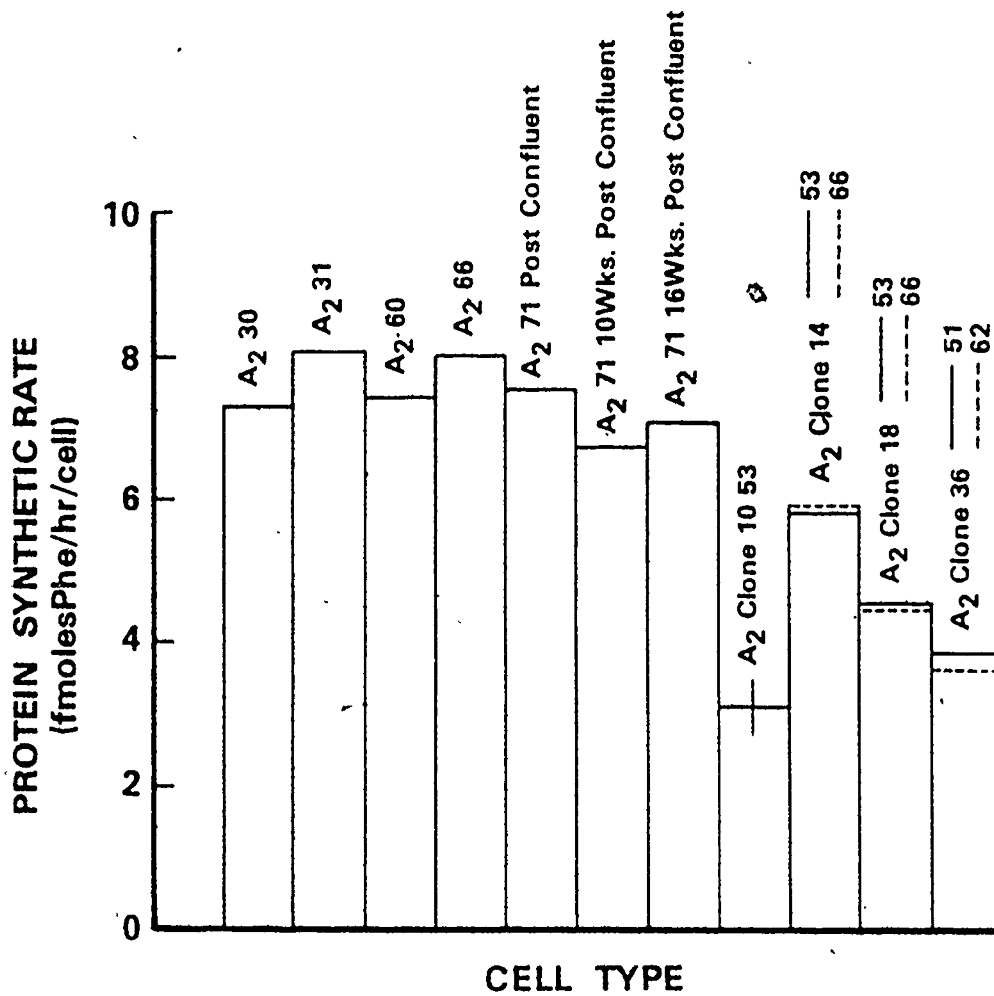


Figure 3-31b Protein synthetic rate in mass culture and select clones of strain A_2.

Cellular protein synthetic rate was determined for mass cultures of A_2 at early and late passage as well as various times in postmitotic culture. Clones 14, 18 and 36 were assayed at two passage levels and clone 10 at 53 MPD only. The bar superimposed on the clone 10 histogram shows the range of a duplicate determination.

synthetic rates of the mass culture ($x = 7.36 \pm 0.55$).

To determine whether protein degradation differed between clones, cellular proteolysis was measured in clone 10 (low error frequency) and clone 18 (high error frequency) using the method of intermittent perfusion (section 2.2.8). Proteolysis in clonal cultures measured in this manner was compared to proteolysis in mass cultures of strain A_2 (Figure 3-32). Chasing a 2-hour pulse with ^{3}H-phenylalanine at 10 μCi/ml in RGM revealed that after 8 hours (Figure 3-32), there was a small difference in the extent of proteolysis between early- and late-passage cells. Although this difference is small, previous work has shown that late-passage cells have consistently about 5-15% greater proteolysis than early-passage cells (Elliot, 1980). Clone 10 at 53 MPD had the highest proteolysis (32% after 8 hours) and clone 18 the lowest (28% after 8 hours), contrary to expectations.

Analysis of variance (2 factor design, repeated measures on one factor; Winer, 1962; Bruning and Kintz, 1977) of the data has revealed that the difference in proteolytic rate between early- and late-passage mass cultures was <u>not</u> statistically significant (at the $p = 0.1$ level). The difference in proteolytic rate between high error frequency clone 18 and low error frequency clone 10 was significant ($F = 23.6$; $p < 0.01$), however.

Essentially identical results were achieved in an experiment using a one hour pulse of ^{3}H-phe at 20 μCi/ml.

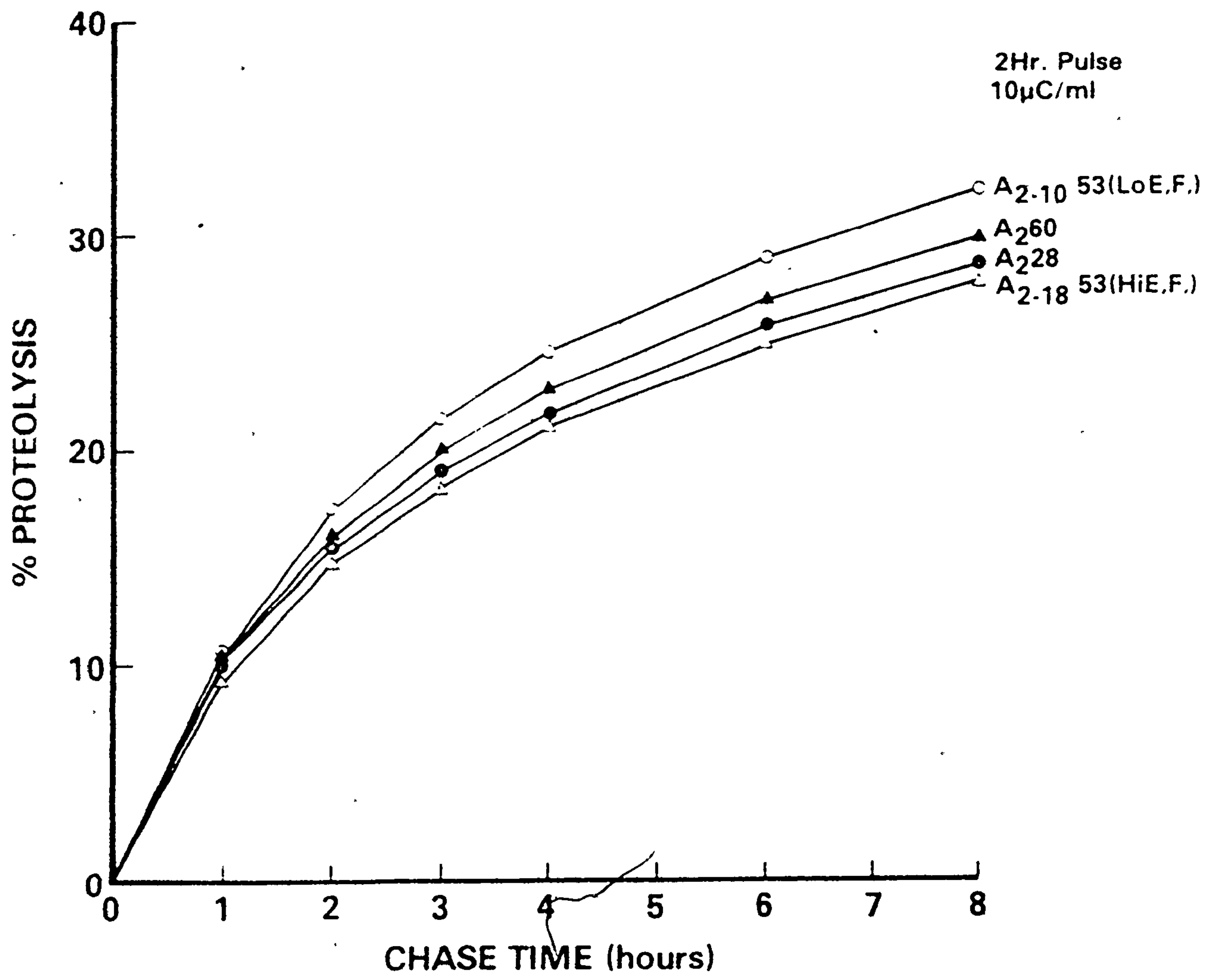


Figure 3–32 Cellular proteolysis in early and late passage mass culures as well as in high and low error frequency clones. (Section 2.2.6)

3.1.4 Post-mitotic Cells

To ascertain whether error frequency changed over a long period of time under conditions which minimized cell selection, a study of mistranslation rates in post-mitotic cells was initiated. A mass culture of strain A_2 at terminal passage was employed so that results could be compared to previous work.

3.1.4.1 Growth Characteristics

Cells of A_2 at 67 MPD which were inoculated into roller bottles at a 1:16 split ratio grew well and reached confluence in about 16 days (final MPD 71). The cultures were kept alive for 4 months by monthly refeeding. The condition of the cells was closely scrutinized by weekly microscopic examination. After 6 weeks, extensive microscopic examination revealed no mitotic figures even after refeeding. As the cultures stayed in a confluent state, considerable debris was released rendering the medium cloudy. Cells were harvested at 0, 6, 10 and 16 weeks post-confluence (in a sterile fashion) and an aliquot of the harvested cells re-inoculated into dishes for ^{3}H-TdR index (section 2.2.4) and protein synthetic rate determinations (section 2.2.7). By the criterion that these cells could no longer reach confluence after biweekly refeeding, these cells were at terminal passage; however, after several weeks in dishes, foci of growth resembling clonal outgrowths could be seen (Plate I).

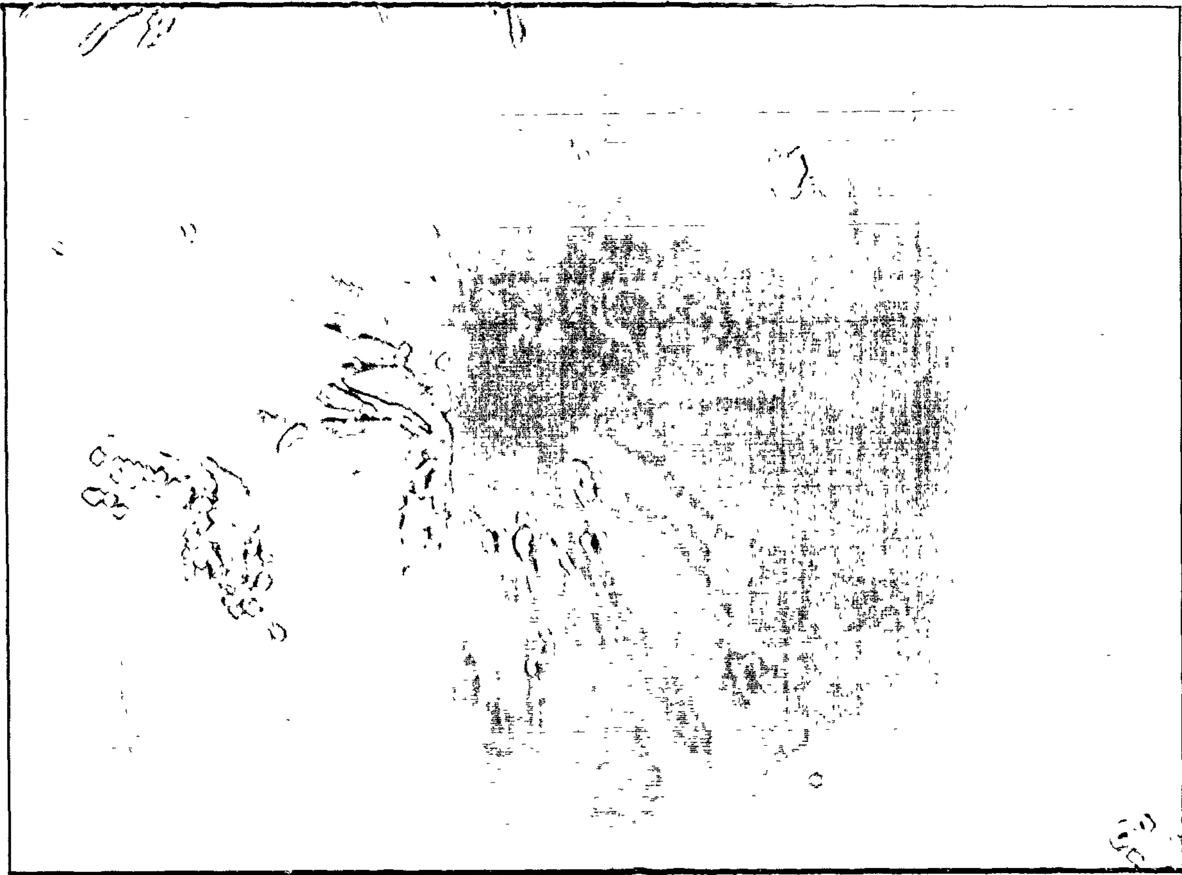


Plate I Growth activity in Phase III cultures.
Cells of strain A_2 at 71 MPD could not reach confluence after two weekly refeedings. Some cells however showed some limited growth potential and formed foci (colonies) of a few dozen cells.

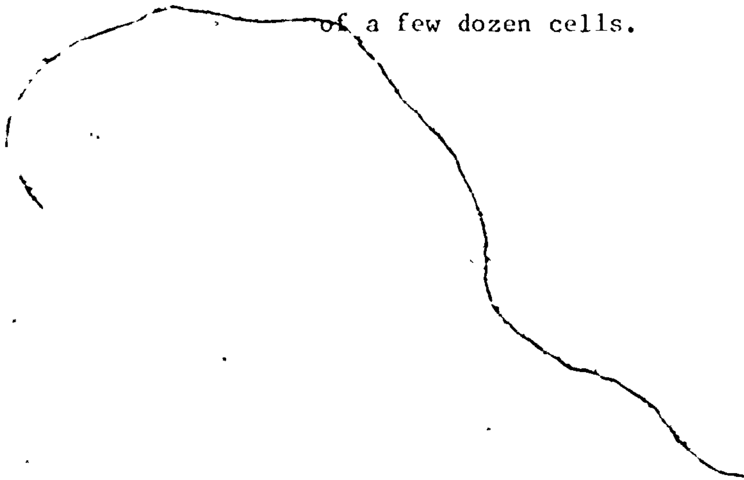


Table 3-9

Post-mitotic Cells - Culture Characteristics

	Weeks Post-replicative	Cell Density[a]	^{3}H-TdR Index %	Cellular protein synthetic rate[b]	Multi nucleated cells %
A_2 71	0	10.1	11.1	7.54	2
	6	10.5	15.3	n.d.	9
	10	10.3	13.1	6.71	9
	16	10.2	9.5	7.05	17
A_{2-14} 68	6	9.8	43	8.0	0.5

n.d. - not determined

(a) density of cells grown in roller bottles - $10^3/cm^2$

(b) protein synthetic rate in intact cells - pmoles/phenylalanine/hr/cell.

Plate II Multinucleated cells in postmitotic cultures.
With increasing time in postmitotic culture an increasing proportion of cells exhibited dysmorphic nuclei and multinucleation. Shown are cells of strain A_2 after 16 weeks in the postmitotic state. Cells were fixed in EtOH:HOAc(3:1).

Table 3-9 shows growth characteristics of these post-mitotic cells and one late-passage culture (A_2 at 66 MPD) still able to replicate. Cell density was constant over the 4 month period and although there was difficulty distinguishing ^{3}H-TdR incorporation due to DNA replication and DNA repair, the thymidine labelling index remained universally low. The in vivo protein synthetic rate did not change considerably even after 16 weeks in the confluent state. One significant trend was seen, however, in the number of cells containing more than one nucleus (Plate II). At the beginning of the post-replicative period, 2% of the cells were binucleate and after 16 weeks, 17% of the cells were binucleated, with about 1% being trinucleate.

3.1.4.2 Error Frequency

Cells harvested at all intervals of the post-mitotic phase yielded active extracts (Table 3-10). All extracts of this series had unusually high residual endogenous protein synthesis activity as well. Although the range of error frequencies determined in each experiment was small, considerable variation in calculated error frequency occurs even though no obvious increase (or decrease) with time was observed.

One clone of A_2 - clone 14, which showed an increase in error frequency at late passage, was kept in a post-mitotic state for a period of 6 weeks. The error frequencies determined for clone 14 rose from a low 0.05 ± 0.002% at 59 MPD to 0.25 ± 0.005% at 66 MPD. After 6 weeks in the post-mitotic state at

Table 3-10

Post-mitotic Cultures - Error Frequencies

Weeks	Error Frequency % ± range	Synthetic Rate fmoles amino acid/hr/10^6 cells Phe +Poly(U)	Leu -Poly(U)	Lys -poly(U)
0	0.030±0.004	36	60	40
6	0.048±0.004	70.5	81	120
10	0.004±0.004	51.3	51	53
16	0.023±0.006	88.2	164	98
		($x10^3$)		

68 MPD, an active extract had an error frequency of 0.242 $\pm$ 0.01%. Thus the rapid increase in error frequency observed in the replicative phase of this clone did not continue in the post-replicative phase.

3.2 Endogenous Cell-free Protein Synthesis

With minor modifications to the protocol of preparing cell-free extracts of fibroblasts, endogenous protein synthesis directed by cellular mRNA may be examined. By omitting the ribosomal runoff, considerable endogenous protein synthetic activity is retained. The results described below are of a preliminary nature and it must be emphasized that no optima were established for endogenous cell-free protein synthesis and most reactions were carried out under optima established for poly(U) translation.

3.2.1 Characteristics

It was initially observed that the incubation temperature used in poly(U) assays (i.e. runoff conditions) caused rapid completion of endogenous protein synthesis in 15 minutes (Figure 3-33). It was also observed that endogenous leucine incorporation was inhibited (55%) by the addition of poly(U). Incorporation of phenylalanine in the presence of poly(U) was also inhibited initially but continued over 45 minutes, possibly due to a small level of polyphenylalanine synthesis. At 30°C, amino acid incorporation continued over a longer period (Figure 3-34). Cold acid insoluble material representative of label present in protein plus charged tRNA showed a rapid rise, while hot acid insoluble material (protein only) had a considerable lag period. Both curves began to deviate considerably from linear incorporation after about 20 minutes. The difference between the hot- and

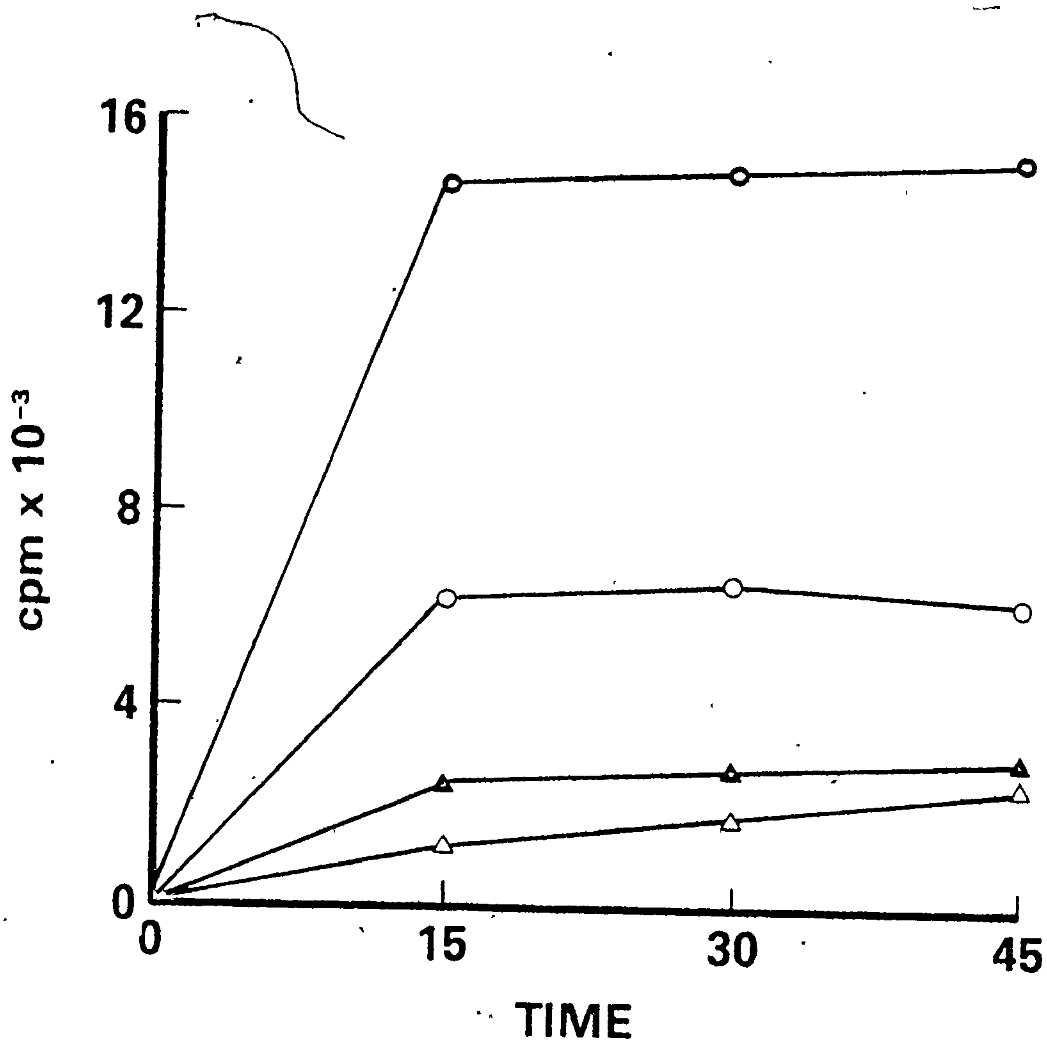


Figure 3-33 Endogenous incorporation of amino acids in cell-free extracts.
An extract of strain A_2 at 32 MPD was incubated at 37°C with ^{3}H-leucine (circles) or ^{3}H-phenylalanine (triangles) in the presence(open symbols) or absence(closed symbols) of poly(U).

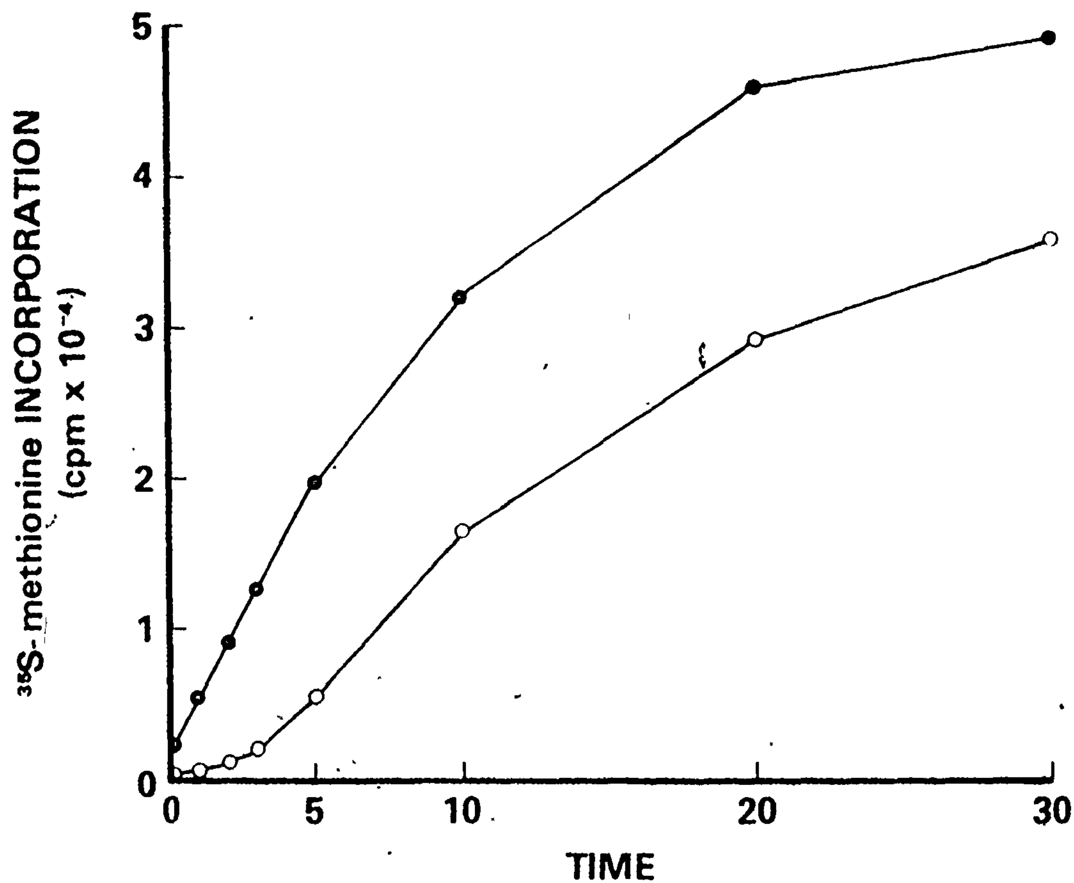


Figure 3-34 Endogenous incorporation of ^{35}S-methionine at 30°C. A cell-free extract of strain A_2 at 26 MPD was incubated with ^{35}S-methionine (172μCi/ml) and aliquots equivalent to 0.42×10^6 cells were removed at the times shown. Duplicate aliquots were spotted onto GFC's with duplicates being worked up with(○) or without(⊙) heating at 90°C in 5% TCA for 30 minutes.

cold-acid insoluble material remained essentially constant over the last 25 minutes.

The incorporation of several amino acids was monitored and compared to the presence of these amino acids in the proteins of intact fibroblasts (Table 3-11). The proportions of amino acids incorporated into protein by the cell-free extract closely resembled their proportions in cellular protein ($\chi^2 = 1.09$, $p < 0.05$).

Material synthesized by these extracts is sensitive to proteolytic agents such as trypsin (Figure 3-35) even at 0^oC whereas no proteolytic activity was detected in the extract alone.

Protein synthetic activity in these extracts was inhibited by puromycin (data not shown) and histidinol. Extracts were also more susceptible to loss of synthetic activity by freezing than extracts prepared for poly(U) translation.

Amino acid analogues are also incorporated into protein during endogenous protein synthesis (Table 3-12). The time-dependent incorporation of α-aminoisobutyric acid (AIB) was observed in an extract of strain A_2 at 32 MPD, although its incorporation was barely above background. Incorporation of AIB did not exceed background counts in an extract of A_2 at late passage. The phenylalanine analogue, p-fluorophenylalanine, was incorporated in substantial amounts. The ratio of p-fluorophenylalanine: phenylalanine declined from 9.26%

Table 3-11

Amino Acid Incorporation directed by Endogenous Cell-free protein synthesis

Amino acid	Incorporation pmoles/hr/10^6 cells (a)	Relative Incorporation (b)	Relative Presence in Fibroblasts (c)
glu	6.1	1.79	1.50
gly	8.0	2.35	2.08
leu	8.6	2.53	3.26
lys	6.4	1.88	1.98
met	1.8	0.53	0.38
phe	3.4	1.00	1.00

(a) Incorporation rates were determined in a fresh extract of strain A2 at 32 MPD. Counts per minute were corrected for specific activity of the label; rates were determined over a time course of 30 minutes.

(b) Phenylalanine incorporation = 1.00

(c) From Harley (1980).

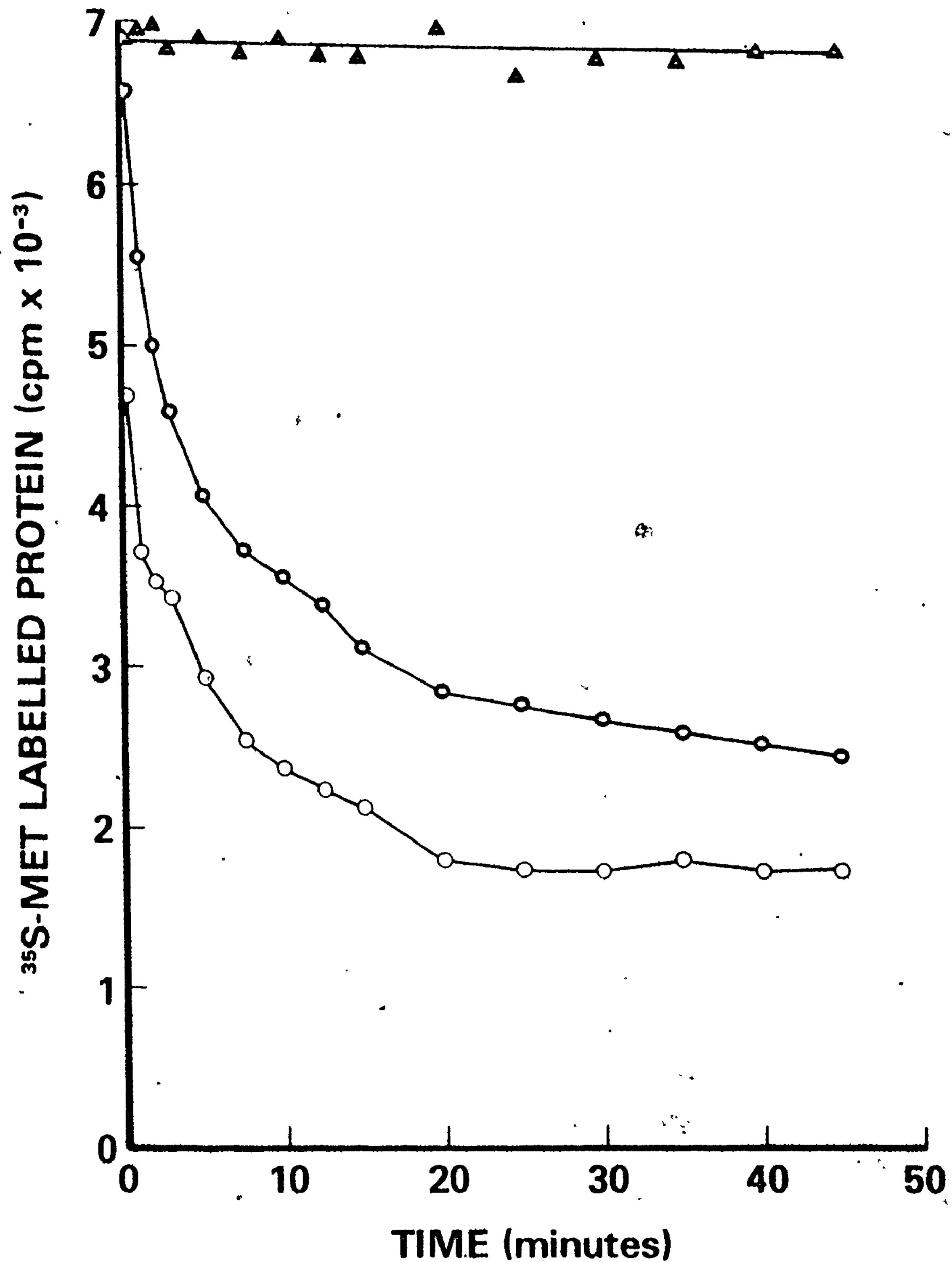


Figure 3-35 Proteolytic degradation of endogenous cell-free synthesis products.

An extract of strain A_2 at 41 MPD prepared for endogenous cell-free synthesis was labelled with ^{35}S-Met(170μCi/ml) for 30 minutes. The mixture was freeze-thawed five times then dialyzed to remove unincorporated label. The dialyzed extract was treated with 0%(▲), 0.1%(◉) and 0.2%(○) trypsin. Aliquots were measured for TCA-insoluble cpm.

Table 3-12

Incorporation of Amino Acid Analogues by

Endogenous Cell-free Protein Synthesis.

		Amino acid incorporation, pmoles/hr/10^6 cells				
Cell/Passage		Lys	AIB	Phe	F-phe	$\frac{\text{F-Phe}}{\text{Phe}}$
A2	32	2.78	0.0008	1.23	0.114	9.26%
A2	71	2.09	n.d.	0.78	0.019	2.44%

Cell-free extracts of early- and late-passage fibroblasts were prepared for endogenous protein synthesis (section 2.3.1) and molar incorporation of amino acid analogues AIB (α-aminoisobutyric acid) and F-Phe (p-fluorophenylalanine) was determined.

at early passage to 2.44% at late passage (c.f. section 3.1.2.2).

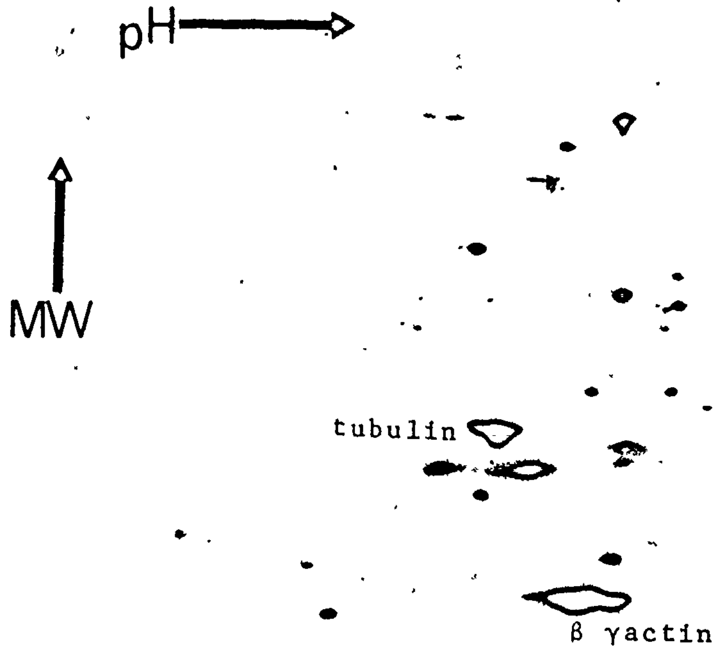
3.2.2 Two-dimensional Electrophoresis

To determine whether amino acid incorporation into acid-insoluble material was truly represntative of protein synthesis, extracts were labelled with translation-grade ^{35}S-methionine and the products were analyzed by two-dimensional electrophoresis (section 2.3.3). Plate III(top) shows the 2-D protein pattern obtained from an extract of strain A_2 at 35 MPD. It is noted that several proteins are synthesized implying that complete proteins are being synthesized. Comparing the 2-D pattern obtained in vitro with that obtained by pulsing intact cells with ^{35}S-methionine for one hour (Plate III(bottom)), one can illustrate the presence of major cellular proteins, such as the two isomers of actin and tubulin, in the cell-free products. (Since the two gels were run under different conditions, a firm assignment was impossible.) The anomalous pattern of spots in the tubulin region illustrates that cellular and cell-free protein synthesis are somewhat different however.

3.2.3 Translation of globin mRNA

To determine whether extracts prepared for endogenous protein synthesis could translate a natural, though exogenous, mRNA, attempts were made to stimulate protein synthesis by the addition of rabbit globin mRNA. Under conditions Standard-

a



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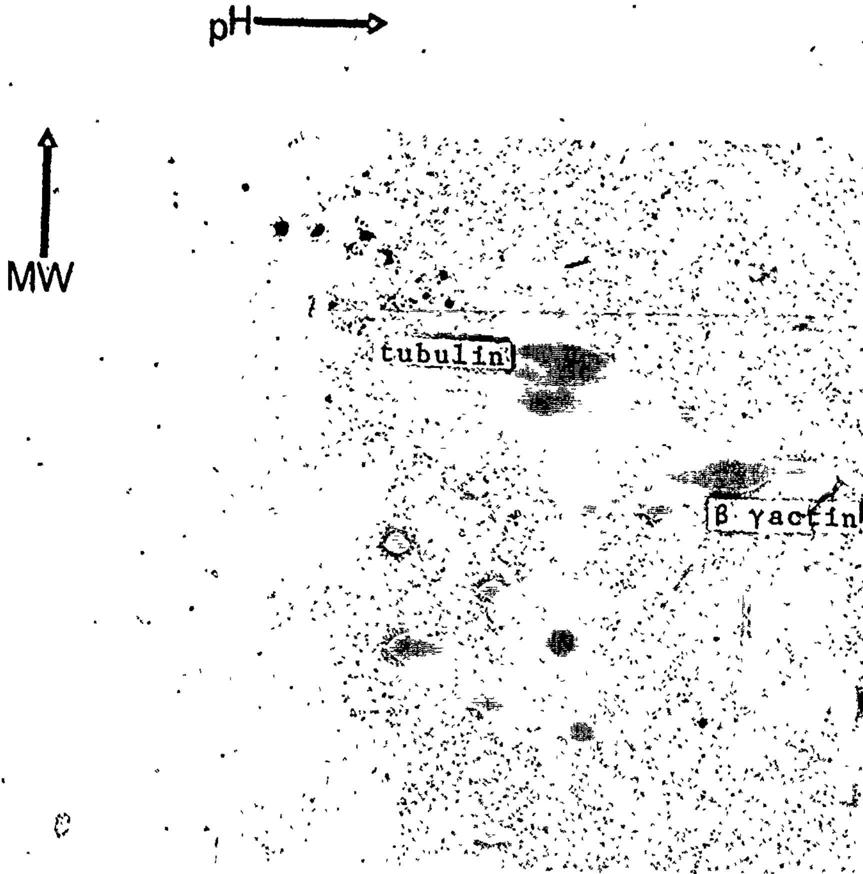


Plate III Two-dimensional gel electrophoresis of human diploid fibroblast proteins synthesized in vivo(a) and in vitro(b). a– from Harley(1980).

ized for poly(U) translation and endogenous protein synthesis ($[Mg^{+2}]$ = 5.5 mM), there is no stimulation of leucine incorporation by the addition of poly(U). However, in extracts prepared for endogenous protein synthesis, globin mRNA stimulates leucine incorporation approximately 3-fold at lower magnesium concentration ($[Mg^{+2}]$ = 2.5 mM) (Figure 3-36). For possible future developments of this assay see Chapter 5 --- Prospective Studies.

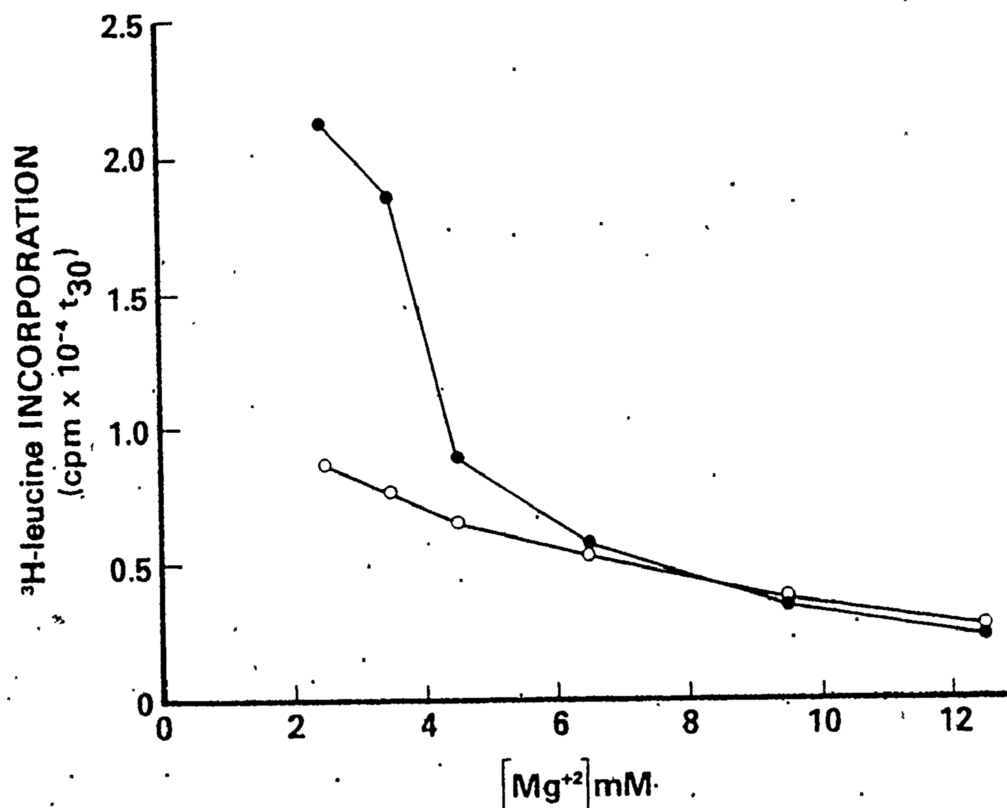


Figure 3-36 Stimulation of in vitro leucine incorporation by rabbit globin mRNA.

An unfrozen extract of strain A_2 at 35 MPD was prepared as before (section 2.3.1) and incubated at 30°C with ^{3}H-leucine in the presence(●) or absence(○) of rabbit globin mRNA(23µg/ml, 0.61 A_{260}/ml) at various concentrations of $Mg(OAc)_2$. Aliquots were assayed for hot TCA-insoluble cpm at 0, 10, 20 and 30 minute time points. The 30-minute time point vs. Mg^{+2} concentration is plotted above.

CHAPTER FOUR

DISCUSSION

Although there have been previous reports of cell-free protein synthetic systems derived from such human cells as HeLa (McDowell et al., 1972; Weber et al., 1975) and KB (Wilhelm et al., 1978), these are aneuploid lines and do not exhibit in vitro senescence. This is the first comprehensive report on the development and characteristics of an in vitro system capable of cell-free protein synthesis that has been derived from normal human diploid fibroblasts. The system has been used to explore the fidelity of protein synthesis during the in vitro senescence phenomenon characteristic of human diploid fibroblasts. (Recently, a report on poly(U) translation in extracts of human fibroblasts has appeared from another group (Buchanan et al., 1980) and comparisons between their findings and ours will be made in the appropriate sections of this Discussion.)

4.1 Poly(U)-directed Protein Synthesis

4.1.1 The Human Diploid Fibroblast Cell-Free System

The final protocol which was developed consistently yielded extracts of considerable protein synthetic activity (Wojtyk and Goldstein, 1980). During the initial stages of development it became evident that broken cell preparations contain materials inhibitory to protein synthesis (Figure 3-1) and that these factors could be removed by centrifugation at 30,000 xg (Figure 3-2). The runoff step was essential in

studying poly(U)-directed protein synthesis since considerable endogenous protein synthesis activity remained if the runoff step was omitted (Figure 3-33). In our hands, ribosomal runoff decreased endogenous protein synthesis to minute, indeed barely detectable, levels.

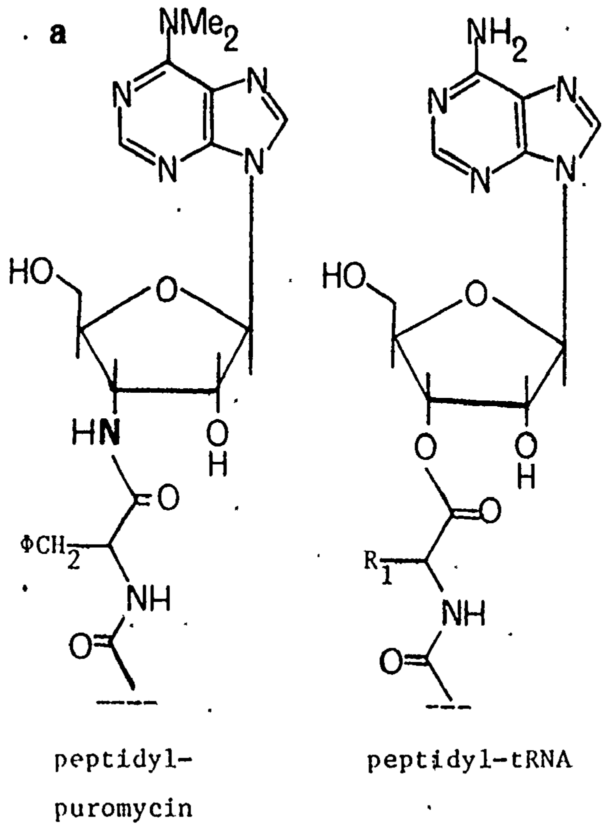
There are several indications that poly(U)-stimulated phenylalanine incorporation represents true protein synthetic activity. Products from poly(U)-directed protein synthesis are susceptible to proteolytic degradation by the exogenous protease, trypsin (Figure 3-17). However, since trypsin cleaves peptide bonds specifically at the carboxyl end of lysine and arginine residues, initial degradation of the synthetic product by trypsin would not lead to appreciable loss in TCA-insoluble material. In preparing the grade of trypsin used, TPCK-inactivation of chymotrypsin was not involved. Therefore, most of the proteolytic activity observed was probably due to contaminating amounts of chymotrypsin which has specificities for Phe, Trp and Tyr residues.

The incorporation of phenylalanine into TCA-insoluble material depends on several sequential reactions. The temperature dependence of poly(phe) synthesis (Figure 3-11) shows that the rate of poly(U) translation approximately doubles with each 10°C rise in temperature. (Although the temperature coefficient, Q_{10}, does vary from one enzymatic reaction to another, most values are around Q_{10} = 2.0

(Lehninger, 1975; p. 196).)

The translation of poly(U) was shown to be affected by several inhibitors of protein synthesis. Puromycin (Figure 4-1a) inhibited poly(U)-directed phenylalanine incorporation (99% at 4.2 mM, Figure 3-14b). Thus, all of the poly(U) translation depends on the availability of free A-sites on the functional ribosomes. Puromycin blocks ribosomal A-sites by supporting transpeptidation of the nascent peptide chain to puromycin at the A-site and formation of peptidyl-puromycin. This peptidyl-puromycin does not have the required structure for translocation to the ribosomal P-site. The function of both 70S and 80S (i.e. prokaryotic and eukaryotic) ribosomes is inhibited by puromycin. Cycloheximide (Figure 4-1b) is an inhibitor of eukaryotic (80S) ribosomal function only, inhibiting both initiation and elongation (Oleinick, 1977). Poly(U)-directed phenylalanine incorporation was inhibited 90% by cycloheximide at 2 mM (Figure 3-14a). Further inhibition by cycloheximide was not possible due to difficulties in dissolving the inhibitor at higher concentrations.

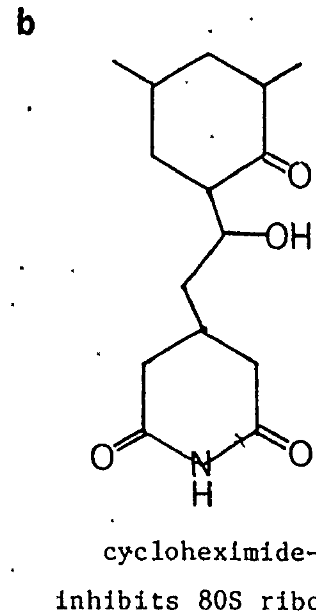
There is no protein synthesis in these extracts contributed by 70S ribosomes from mitochondria. The complete inhibition of poly(phe) synthesis by diphtheria toxin (Figures 4-1c and 3-15) shows that all poly(U) translation is dependent on EF-2, which is inactivated by ADP-ribosylation catalyzed by the toxin. Diphtheria toxin does not react



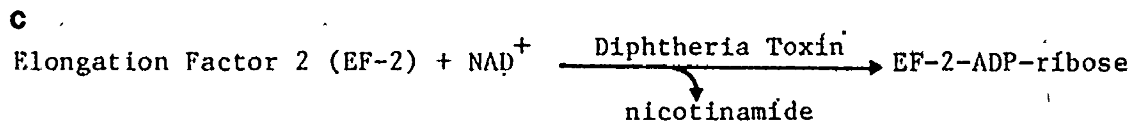
peptidyl-
puromycin

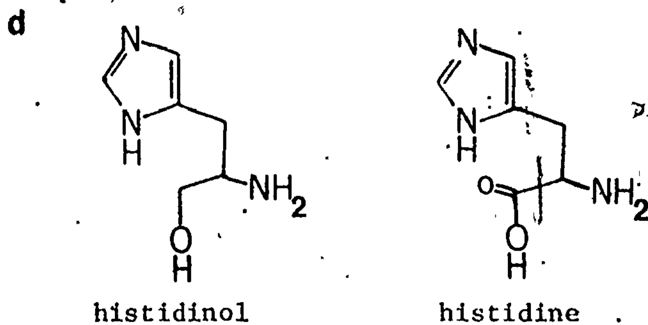
peptidyl-tRNA

puromycin resembles aminoacyl-tRNA
however its amide bond to the ribose
cannot be cleaved.



cycloheximide-
inhibits 80S ribosomes.





histidinol histidine

- structural similarities

Figure 4-1 Some inhibitors of eukaryotic protein synthesis.

with mitochondrial elongation factors (Weissbach and Ochoa, 1976). Similarly, there is little contribution of factor-free protein synthesis (see section 1.2.2) under these conditions. TPCK, which inhibits 70S ribosomal function by reacting with active -SH groups at mRNA-ribosomal binding sites of 70S ribosomes has no effect on poly(U) translation in this system (data not shown).

Although some protein synthesis occurs in the absence of an added exogenous energy source, this simply reflects the extract's inherent ability to generate trinucleotides spontaneously (Figure 3-12). The presence of GTP seems to be most important in the translation of poly(U). Other components of the energy mix may simply act by regenerating GTP from its dinucleotide.

Thus it is concluded that cell-free extracts of human diploid fibroblasts are capable of translating poly(U) in a manner involving all the components of the normal elongation process on 80S ribosomes alone.

Compared to other cell-free protein synthetic systems, human diploid fibroblasts yield extracts which are highly active in the translation of poly(U) (Figure 4-2). On the basis of RNA concentration (A_{260} ml^{-1}), extracts of HDF are amongst the most active for poly(U) translation, only exceeded by rabbit reticulocyte lysates (Gupta et al., 1968).

Even though these extracts are very active synthetically, these are the results of the first such study and there

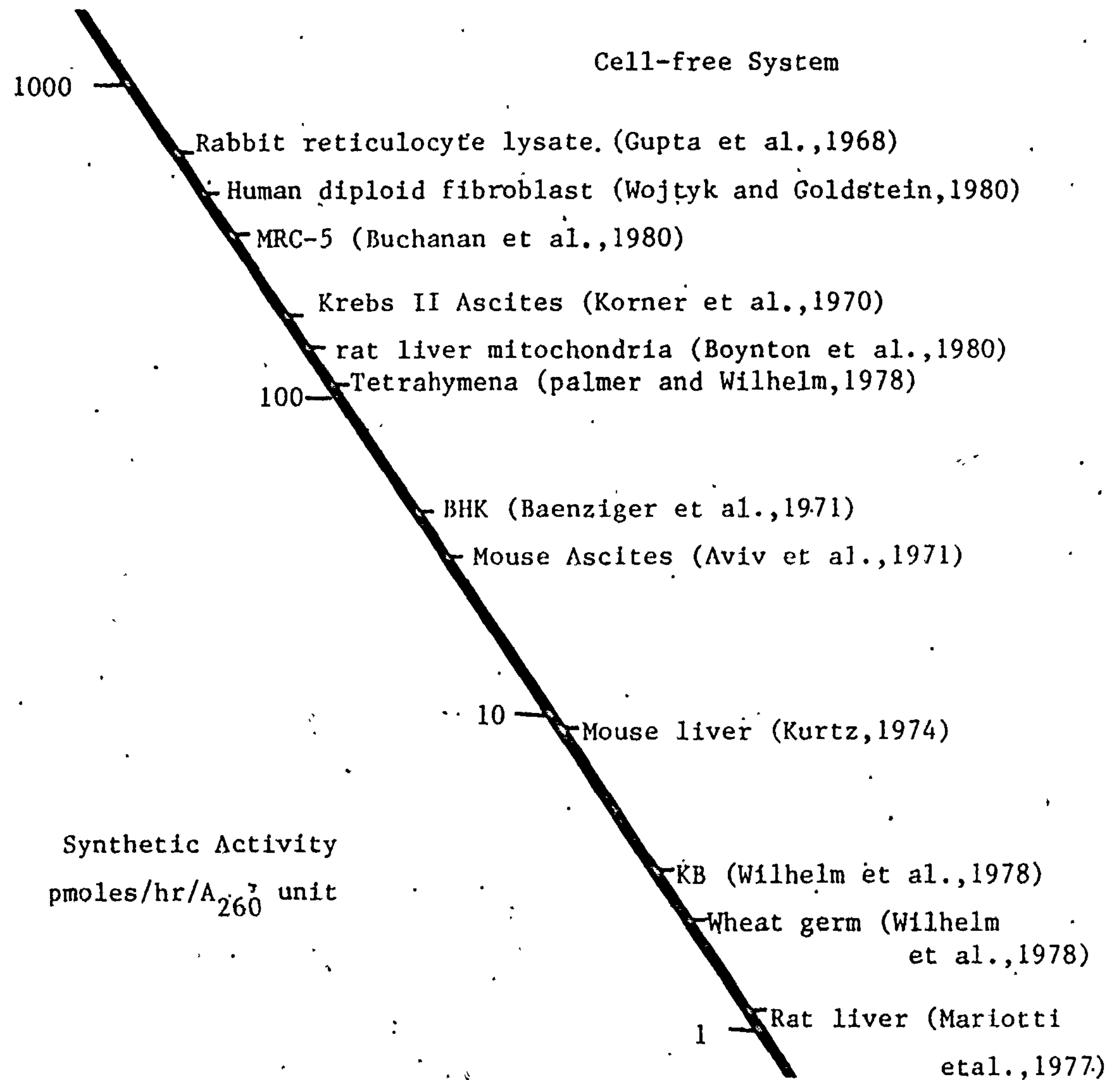


Figure 4-2 Protein synthetic activity of various eukaryotic cell-free systems with poly(U) as template.

is probably room for improvement. A standard protocol for extract preparation and for error frequency assay was adopted.

The standard conditions used for all error frequency determinations were optimal for poly(U) translation with respect to K^+, Mg^{+2}, poly(U) and phenylalanine concentration as determined in extracts of early-passage cells of strain A_2. These optima did not change in late-passage cell extracts. This is in contrast to studies of Kurtz (1975) and Mariotti and Ruscitto (1977) who reported an increase in optimal magnesium concentration in extracts of mouse and rat liver, respectively. Mainwaring (1969) found no age-related change in magnesium optima with age in mouse liver extracts. The magnesium optimum for extracts of strain A_2 was constant throughout passage and also in two selected clones of A_2 (Figures 3-6 and 3-29). This may indicate that there is relative consistency in the optimum conditions for cell-free protein synthesis amongst cells within any particular strain. This is not true however in comparing optimum conditions between strains. Extracts of J069 and A_2 have very different magnesium optima (9.5 and 5.5 mM, respectively), likely reflecting fundamentally different ribosomal microenvironments. As discussed in section 1.2.2 the translation of poly(U) at low magnesium concentrations is dependent upon initiation factors (eIF-2A, eIF-4C, eIF-4D and eIF-5) as well as elongation factors (EF-1 and EF-2). At higher

magnesium concentrations, initiation factors are not essential and at even higher concentrations so-called factor-free translation may occur (Spirin, 1978). It has been shown in extracts of rabbit reticulocytes (Shafritz and Anderson, 1970; Crystal et al., 1974) that, in the absence of the required initiation factors, the Mg^{+2} optimum is about 10 mM. In the presence of all but one of the required factors, there is no change in the Mg^{+2} optimum. However, when all the required factors are present in saturating amounts, the Mg^{+2} optimum falls to 4-6 mM, with total activity increasing. This "poly(U)-Mg^{+2} shift assay" has also been used to demonstrate dependence of IF-1 and IF-2 in prokaryotic cell-free translation of poly(U) at low Mg^{+2} concentrations. From this we can conclude that extracts of JO69 are deficient in one or more of the initiation factors mentioned above whereas extracts of A_2 possess these in saturating quantities. Whether this deficiency in initiation factors is a general property of normal cells from old donors was not determined.

Almost all cells studied gave extracts active in the translation of poly(U), the notable exception being cell cultures harvested while still in the growth phase. Extracts of early-passage cells of strain A_2, both in log phase (exponentially growing) or in late-log phase (almost confluent, no longer growing exponentially) did not translate poly(U) (Figure 3-4). Only after cultures were (2-3 days) post-

confluent were active extracts obtained. This activity did not change appreciably in cells kept in the confluent state for up to 16 weeks (Table 3-10). This is a puzzling phenomenon since previous reports (eg. Levine et al., 1965; Engelhardt, 1971; Rudland, 1974; Engelhardt and Sarnoski, 1975) have invariably shown that the apparent rate of protein synthesis is enhanced during growth of various cells compared to their quiescent state. Using intact cells, Harley (1980) has shown that true synthetic rate remains unchanged, rather proteolysis is decreased during growth. It has also been reported that as fibroblasts enter stationary phase, although there is a two-fold decrease in the total number of ribosomes within the cell, there is a disaggregation of cytoplasmic polysomes (Stanners and Becker, 1971). Thus, whereas nearly all of the free ribosomes are bound to mRNA (polysomes active in protein synthesis) during growth, one-third remain unbound to polysomes in stationary phase. The poly(U) assay may, then, require this subpopulation of free or unbound ribosomes. This explanation of the afore-mentioned phenomenon may be too simplistic however since the ribosomal runoff procedure, also meant to disaggregate polysomes, is effective only in extracts of confluent cells.

One of the major goals in our study of error frequency in extracts of fibroblasts was to eliminate proteolytic degradation under the conditions of protein syn-

thesis. This is unlike the in vivo situation where protein synthesis and degradation occur simultaneously within the cell and proteolytic surveillance (Capecchi et al., 1975; Goldberg and St. John, 1976) may degrade abnormal (i.e. error-containing) proteins before they are detected. Our results show that under conditions for poly(U) translation there is no proteolytic degradation of poly(phe) (Figure 3-17) or of endogenous protein products (Figure 3-35). However, extracts prepared in a similar manner (Elliot and Goldstein, in preparation) do exhibit ATP-dependent neutral protease activity at higher ATP concentrations (ATP, 50 mM; pH 7.4). In this light, limited proteolysis, undetectable by TCA-precipitation may still occur under protein synthetic conditions (ATP, 1 mM). The proteolytic activity reportedly associated with functional ribosomes (of HeLa cells) (Korant, 1977) was not found in these extracts.

4.1.2 Nature of Errors in Poly(U) Translation

Being a homopolymer, poly(U) is useful in the detection of errors in a very few specific steps of the overall translation mechanism. Neither initiation nor termination errors can be studied due to lack of the required codons. Only errors in elongation and the acylation of $tRNA_{phe}$ are detectable; even still, frame shift errors would also go unnoticed. Thus errors in the translation of poly(U) arise solely from faulty codon-anticodon recognition and/or misacylation of $tRNA_{phe}$.

The first evidence that polynucleotide-directed polypeptide synthesis proceeded with an inherent ambiguity came from work in the early 1960's with the study of poly(U)-directed protein synthesis in extracts of E. coli (Bretscher and Grunberg-Manago, 1962; Matthei et al., 1962). Besides promoting the incorporation of phenalanine into protein, poly(U) also stimulated the incorporation of other amino acids such as leucine. Initially, it was thought that codon assignments were not specific, eg. UUU could code for leucine as well as phenylalanine. This situation was somewhat clarified by the realization that the 3'-nucleotide of the triplet codon could undergo pairing interactions with a corresponding nucleotide on the anti-codon of an incoming aa-tRNA which did not follow the original Watson-Crick rules for nucleotide base-pairing (Figure 4-3) (Crick, 1966). This "wobble" hypothesis thus allowed for a natural level of infidelity in the protein synthetic apparatus. In normal in vivo conditions, this inherent level of infidelity is quite small, whereas the unnatural conditions in vitro may further reduce ribosomal fidelity. Even higher levels of miscoding which violate the "wobble" rules as well may be brought about by further perturbation of the ribosome either by mutation or by the use of antibiotics.

In the case of leucine misincorporation on a poly(U) template, the UUU triplet may be misread as UUC (which also codes for phenylalanine and therefore is undetectable),

codon 3'-nucleotide	Anticodon 5'-nucleotide	
	Watson Crick pairing	wobble base-pairing
U	A[1]	G I
C	G	G I
A	U	U I[2]
G	C	C U[3]

1. Adenine never occurs in the wobble (5') position of the tRNA anticodon.
2. Topal and Fresco (1976).
3. Suppressor tRNA (anticodon UUA) reads the terminator codon UAG (Gorini, 1974)

Figure 4-3 Base pairing according to the Wobble Hypothesis.

UUA or UUG due to misreading at the 3'-(or wobble) nucleotide or may be misread as CUU miscoding at the 5'-nucleotide. The nature of the ambiguity is not known due to the existence of multiple isoaccepting species of $tRNA^{Leu}$, each with its specific anticodon (Singhal and Fallis, 1979). In E. coli, a likely candidate for misreading poly(U) is $tRNA_3^{Leu}$ (Thompson and Stone, 1977) whose anticodon GAG would pair correctly with the 3' and middle base of the UUU triplet while the 5'-base would have to undergo a non-wobble U-G base pair. Numerous studies of ribosomes programmed with various templates have shown that isoaccepting species $tRNA_4^{Leu}$ and $tRNA_5^{Leu}$ (Kan et al., 1970; Blank and Soll, 1971; DiNatale and Eilat, 1976) bind to poly(U)-ribosomes most efficiently and may be the species involved in poly(U) mistranslation. Although the anticodon sequences of $tRNA_4^{Leu}$, $tRNA_5^{Leu}$ are not known, they recognize both UUA and UUG leucine codons and yet have been shown <u>not</u> to insert leucine into positions normally occupied by either UUU- or UUC-phenylalanine codons of MS2-RNA (Holmes et al., 1978; Goldman et al., 1979; Goldman and Hatfield, 1979).

Considerable evidence now exists of an editing function (Yarus, 1979) which scrutinizes the codon-anticodon interaction at the A-site of the ribosome. The mechanism is dependent on the hydrolysis of GTP (Thompson and Stone, 1977). Thirty to sixty GTP's are required to bind one mismatched tRNA (such as Leu-$tRNA_3^{Leu}$ on poly(U)) to the A-site, whereas

only 1 GTP is required to bind Phe-$tRNA^{Phe}$ (Yarus, 1979). By developing an optimal cell-free system from E. coli, Jelenc and Kurland (1979) have attained levels of ribosomal fidelity a thousand times greater than prevously reported. This low level of mistranslation depends strongly on an efficient energy-regenerating system. Some mispairs, such as Leu-$tRNA_1^{Leu}$ on poly(U), do not result in excess GTP hydrolysis however and must therefore be excluded by some other mechanism.

The ribosomal P-site may also affect codon-anticodon interaction at the A-site since noncognate tRNAs bound at the P-site have been shown to inhibit the binding of cognate tRNA at the A-site (Luhrmann et al., 1979; Wurmbach and Nierhaus, 1979). Furthermore, peptidyl-tRNA dissociation may occur after the mismatched codon-anticodon pair has been translocated to the P-site (for a discussion, see Yarus, 1979).

Errors in poly(U) translation may also occur if $tRNA^{Phe}$ is misacylated with a noncognate amino acid. Although misacylation of $tRNA^{Phe}$ occurs at appreciable rates (~1%) for amino acid analogues such as p-fluorophenylalanine, (Davies, 1969; Shmookler-Reis, 1976), mischarging with natural amino acids which are sterically and chemically different is quite rare occurring at a rate of 10^{-6} to 10^{-8} (Savageau and Freter, 1979). This remarkable accuracy of tRNA acylation results from an energy-dependent proofreading mechanism inherent in both the cognate and non-cognate

aa-tRNA synthetases (Yarus, 1972; Hopfield et al., 1976; Fersht and Dingwall, 1979) which hydrolyzes non-cognate aa-tRNA much more rapidly than the correct cognate aa-tRNA.

Mischarging of $tRNA^{Phe}$ with chemically unrelated amino acids such as glutamic acid (GAA) and lysine (AAA) is possible only when $tRNA^{Phe}$ is modified chemically (Davies, 1969).

By far the majority of work with the in vitro translation of synthetic messengers has been carried out with the E. coli cell-free systems. Weinstein et al., (1966) concluded that the fidelity of protein synthesis in extracts of eukaryotic cells was greater than generally found in prokaryotic systems. Experiments using heterologous systems of aa-tRNAs from mammalian sources and ribosomes from bacteria and vice-versa indicated that the ribosomes from mammalian cells were responsible for increased fidelity. Ribosomal ambiguity can be increased in mammalian systems by agents which similarly cause ambiguity in bacterial extracts. These include Mg^{+2} concentration, pH (Lamfrom and Grunberg-Manago, 1967) and antibiotics (Wilhelm et al., 1978; Buchanan et al., 1980). It remains questionable whether mammalian ribosomes which typically show a leu/phe ratio of 10^{-2} with poly(U) are more faithful than bacterial ribosomes since the report of Jelenc and Kurland (1979) describes a very active and precise cell-free system which exhibits a mistranslation rate of leu/phe = 10^{-4}. It has

yet to be seen whether fidelity of poly(U)-directed protein synthesis in eukaryotic systems can be improved by modifications similar to those used by Jelenc and Kurland.

4.1.3 Testing the Orgel Hypothesis

The standard assay for measuring the fidelity of protein synthesis involves detection of the misincorporation of a wrong amino acid, leucine, into polyphenylalanine synthesized from a poly(U) template. The activity of extracts of HDF in translating poly(U) (Figure 4-2) allowed the measurement of this misincorporation to a sensitivity of leu/phe ~0.01% (10^{-4}). Tests of the Orgel hypothesis were then undertaken under standardized conditions (Goldstein et al., 1980; Wojtyk and Goldstein, 1980).

Broadly stated, the error theory of cellular senescence requires that an auto-catalytic augmentation of errors occurs in the apparatus responsible for the processing of genetic information. Positive error feedback loops can exist in any component of information transfer but it was the fidelity of protein synthesis, i.e. translation, that was studied in this thesis. The theory makes numerous predictions, the simplest being that the fidelity of protein synthesis should be diminished in old cells. It would be predicted, therefore, that the error frequency (leu/phe ratios) of poly(U) translation would be greater in extracts of late-passage cells compared to early-passage cell extracts. In three normal cell lines, the error frequency

of protein synthesis decreased from early to late passage (Figure 3-21). This decline was highly significant ($p < 0.005$) (Table 3-2) due in part to the small variance of error frequency determinations within each group (Table 3-1). One of the normal cell strains, J069, derived from an old donor, did not show an increase in leu/phe ratio at late-passage (rather it showed a decrease) and also did not have a higher error frequency than cells from young donors again, both contrary to Orgel's hypothesis. One fetal cell strain, MRC-5, also showed a decrease in error frequency at late passage.

The normal cell strains exhibited a 5-fold range in error frequencies throughout their tissue culture life-span with A_{25} defining the upper part of the range. It is not known, since only 4 normal cell strains were examined, what the distribution of error frequencies is for a normal population. There is some evidence that A_{25} may not be entirely normal since it shows striking resistance to diphtheria toxin both in vivo and in vitro (Figure 3-15). This may reflect an abnormally high level of EF-2 present in these cells or may reveal a mutant form of EF-2 such as observed in diphtheria toxin-resistant mutants of Chinese Hamster ovary (CHO) cells (Gupta and Siminovitch, 1978; Moehring et al., 1980).

Cell strains established from patients with premature aging syndromes contain a host of abnormal proteins

(Goldstein and Moerman, 1976), and this has given some support to the theory of cellular aging since these strains also exhibit shortened in vitro lifespans. Extracts of these cells, however, showed no increase in error frequency of poly(U) translation when compared to normal cells (Table 3-3), even if A_{25} is omitted from the normal group. This suggests that the primary defect in cells from premature aging syndromes resides not in the elongation process and that the aberrant proteins found in these cells do not arise by errors in protein synthesis, but rather by some other alteration of protein structure, such as post-translational modification (Kahn et al., 1978).

Although not specifically stated by Orgel, a corollary of the error hypothesis is that cells with infinite lifespan should possess a remarkably low error frequency. Thus the error frequency of a transformed cell line should be lower than the error frequency of the mortal parental cell strain. The error frequency in extracts of SV-40 transformed MRC-5 was found to be between the values obtained for MRC-5 at early and late passage (Table 3-4). Thus no significant difference between transformed and non-transformed cells could be shown.

This work, then, contradicts the error catstrophe theory in the following ways:

i) the fidelity of poly(U)-directed protein synthesis in extracts of normal fibroblasts does not decline

at late passage,

ii) cells from an old donor do not exhibit an elevated error frequency,

iii) the error frequency in extracts of cells established from progerics and Werner Syndrome patients falls within the normal range of values, and

iv) a permanent line does not show any significant decrease in error frequency compared to its mortal parental strain.

How well error frequency of poly(U) translation reflects synthetic fidelity in vivo is, as yet, an unanswered question. If the error frequency determined for HDF on the order of 0.1% (10^{-3}) reflected synthetic fidelity in the intact cell, an average protein 300 residues in length would have $(0.999)^{300}$ = 74% chance of being synthesized correctly if the error frequency were the same at each codon. Thus, 26% of aberrant proteins may be compatible with cell survival. Error rates of 1-20% have been observed in rel^{-} mutants of E. coli (see Yarus, 1979) and are still compatible with survival.

There is some evidence that the data presented on error frequency in the translation of poly(U) reflect proportionately the error frequency of protein synthesis in the intact fibroblast. Essentially identical results contrary to the Orgel hypothesis have been obtained in vivo following the induction of mistranslation by amino

acid starvation (Harley, 1980; Harley et al., 1980). Although error frequency of protein synthesis estimated by this method is 10-fold lower than that determined by the in vitro poly(U) assay (10^{-4} vs 10^{-3} respectively) no significant increase in the error frequency of late-passage cells, cells from old donors and from progerics and Werner syndrome was found ($p < 0.05$). Amino acid starvation did show a significant increase ($p < 0.001$) in error frequency of transformed cells compared to their non-transformed counterparts. The discrepancy between this observation and that observed in the poly(U) assay has not been resolved.

The fidelity of poly(U) translation has been studied in the extracts of aging tissues by several groups. The results have been most confounding since all possible predictions have been borne out. Kurtz (1975) compared the leu/phe ratio in extracts of mouse livers from animals 1 to 30 months of age and showed a slight decrease in this ratio implying that fidelity of poly(U) translation increases with the animal's age. Mariotti and Ruscitto (1977) found the exact opposite --- a 2-to 3-fold increase in leu/phe ratio in extracts of rat livers from animals 3 and 12 months of age. Also, the fidelity of poly(U) translation was found unchanged with age in extracts of mouse liver (Mori et al., 1979) and chick brain (Yang et al., 1977). It has been shown that the fidelity of protein

synthesis in extracts of animal tissues can be affected by the animal's nutrition (Shmookler-Reis, in preparation) and this may account for the disparate results above.

The accuracy of poly(U) translation in extracts of MRC-5 has been recently reported by others (Buchanan et al., 1980). This group reported error frequencies of 1-2% (5-20 times greater than ours) in these extracts with no increase in error frequency at late passage. However, the significant decreases in error frequencies reported by us, were not observed. Buchanan et al also found that the antibiotic parmomycin profoundly increased the error frequency in these extracts. There was no difference in this stimulation of errors in extracts from early or late-passage cells.

It has been proposed (Kurland, 1977; Kurland, 1980) that the fidelity of protein synthesis is directly proportional to the rate at which the ribosome translates the mRNA; i.e. the faster the rate of mRNA translation, the more likely a non-cognate aa-tRNA will occupy the ribosomal A-site and be accepted into the nascent chain. If we assume that, since our extracts have a constant concentration of ribosomes (A_{260}/ml) and that each ribosome (or the same proportion of ribosomes in each extract) participates in poly(U) translation, there is no correlation between synthetic rate and error frequency in our system.

4.1.4 Decline of Error Frequency with Passage

Initial studies showed decline of error frequency

at late passage, and further investigations were undertaken into the character and significance of this decline. There were three possibilities. First, does the error frequency remain high and decline only at late passage (Figure 4-4a), much like thymidine labelling index (Cristofalo, 1976)? Second, could the drop in error frequency be restricted to an early passage culture (Figure 4-4c) with constant error level being maintained throughout mid- and late-passage cultures? The third alternative is that decline in error frequency occurs steadily throughout serial subcultivation (Figure 4-4b).

A single lot of cells of strain A_2 was assayed for in vitro error frequency at 13 stages of passage from very early (22 MPD) to terminal passage (74 MPD). The decline in error frequency occurred steadily (Figures 3-22 and 4-4b), as an apparently linear function of passage at a rate of -0.0046% MPD^{-1} with an overall 10-fold decrease in error frequency. There was no deviation from linearity even at very late (close to terminal) passage. This strikingly significant decline in error frequency did not correlate with the highly variable synthetic rate found in the extracts. Trivial explanations of this observation such as some time-dependent change in solutions (buffer, energy mix or radio-label stock) seem highly unlikely since all stock solutions were periodically renewed. Thus the decline in error frequency as a function of passage is most likely a true

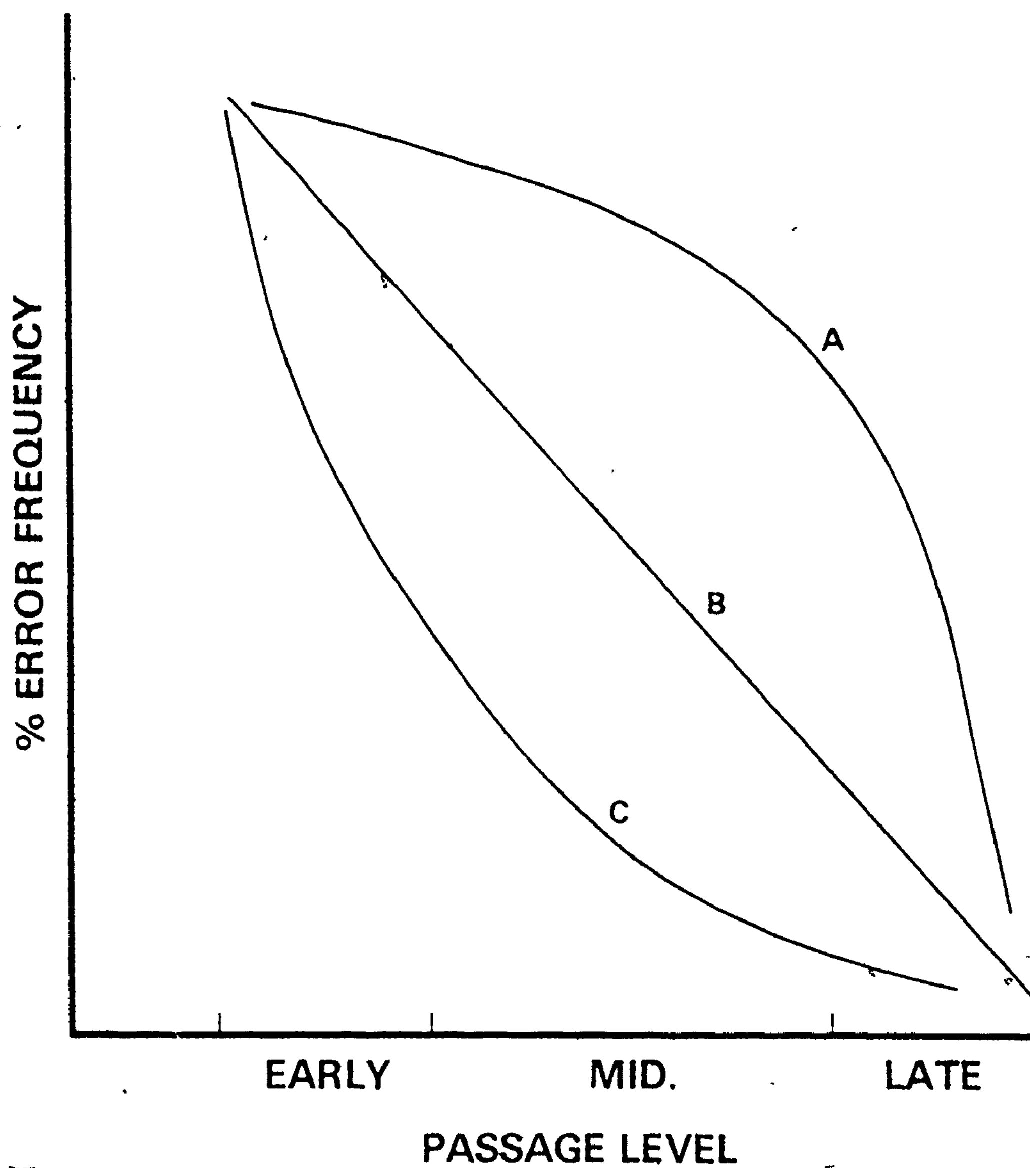


Figure 4-4 Error frequency as a function of passage.
The decline of error frequency throughout the tissue culture lifespan of human diploid fibroblasts may be manifest as a rapid decrease in late passage (A) or in early passage (C). The decline may also occur steadily throughout subcultivation.

biological phenomenon which stands in direct contradiction to the Orgel Hypothesis.

An Orgelian error catastrophe may still be a factor in the survival of post-mitotic cells, such as found in nerve or muscle tissue, and may also explain the loss of cells no longer capable of division in fibroblast cultures. The finite replicative lifespan of a fibroblast culture may be divided into 3 phases (section 1.1.1), establishment (Phase I), rapid proliferation (Phase II), and the slower proliferation leading to loss of proliferative capacity (Phase III). Cells can exist in this post-replicative phase for long periods of time and still remain viable (Matsumura et al., 1979b).

By arbitrary measure, a culture is deemed post-replicative if it will not reach confluence after 2 or 3 weekly refeedings. However, increasing heterogeneity in cellular growth rates in Phase III (Macieira-Coelho, 1974) make it difficult to ensure that a post-mitotic culture at terminal passage is truly post-replicative since some cells may still possess the capacity to undergo a few more cell cycles. A culture of HeLa cells totally devoid of DNA replicative ability was only recently established by Burmer and Norwood (1980) by UV-irradiation of cells which had incorporated the photosensitizing nucleotide bromodeoxyuridine into DNA. This technique is not 100% efficient in removing mitotic cells from diploid fibroblast

culture, and, as yet, a completely post-mitotic and post-replicative (i.e. truly senescent) culture of diploid fibroblasts has to be reported.

Undaunted, cell cultures were maintained in a post-mitotic state and subsequently assayed to determine whether the error frequency increases. Cultures of A_2 at 71 MPD were capable of being maintained in stationary phase for long periods of time after becoming confluent; however, they were deemed to be at terminal passage since they were unable to survive a subsequent subcultivation at a (1:4 split, i.e. they did not become confluent after 3 weekly refeedings). The post-mitotic cultures accumulated increasing amounts of debris in the growth medium and also within the cells. No mitotic events were observed and cell density remained constant even after 16 weeks in stationary phase.

Upon harvesting cells for error frequency determination, a portion of cells was plated into dishes for assay of other growth parameters. There was no obvious decrement in plating efficiency compared to late-passage cells. Most of the cells adhered but did not divide. Only a few foci of growth could be seen after several weeks indicating that the post-mitotic culture contained a small sub-population of cells with some remaining replicative capacity. Cellular protein synthetic rate of cells from post-mitotic cultures was not significantly different from that of late-passage cells (Table 3-6). This result contrasts with the finding

that post-mitotic cultures of WI-38 fibroblasts synthesized proteins at much slower rates than early-passage cells (Razin et al., 1977). The number of multinucleated cells increased progressively with time in stationary phase from 2% at the beginning to 17% after 16 weeks. This increase in multinucleates has been previously reported in cultures of WI-38 kept post-mitotic up to 25 weeks (Matsumura et al., 1979a).

Curiously the thymidine labelling index of these cells remained higher than expected (~10-15%). The number of grains in labelled nuclei exceeded expectations due to DNA repair processes (Goldstein, 1971). This may represent cells having passed S phase during the thymidine labelling. Both uni- and multi-nucleated cells were labelled. The binucleated cells had approximately equal numbers of grains in each nucleus indicating that both nuclei were capable of some DNA synthesis.

All of the post-mitotic cultures yielded active extracts. There was no increase in the error frequency observed in these extracts as a function of time (Table 3-7, Figure 4-5). There was also no progressive change in the in vitro synthetic rate in these extracts.

It seems, therefore, that cells show a decrease in error frequency during their replicative lifespan and upon entering the post-replicative phase are not subject to further improvement in translational accuracy, nor do they

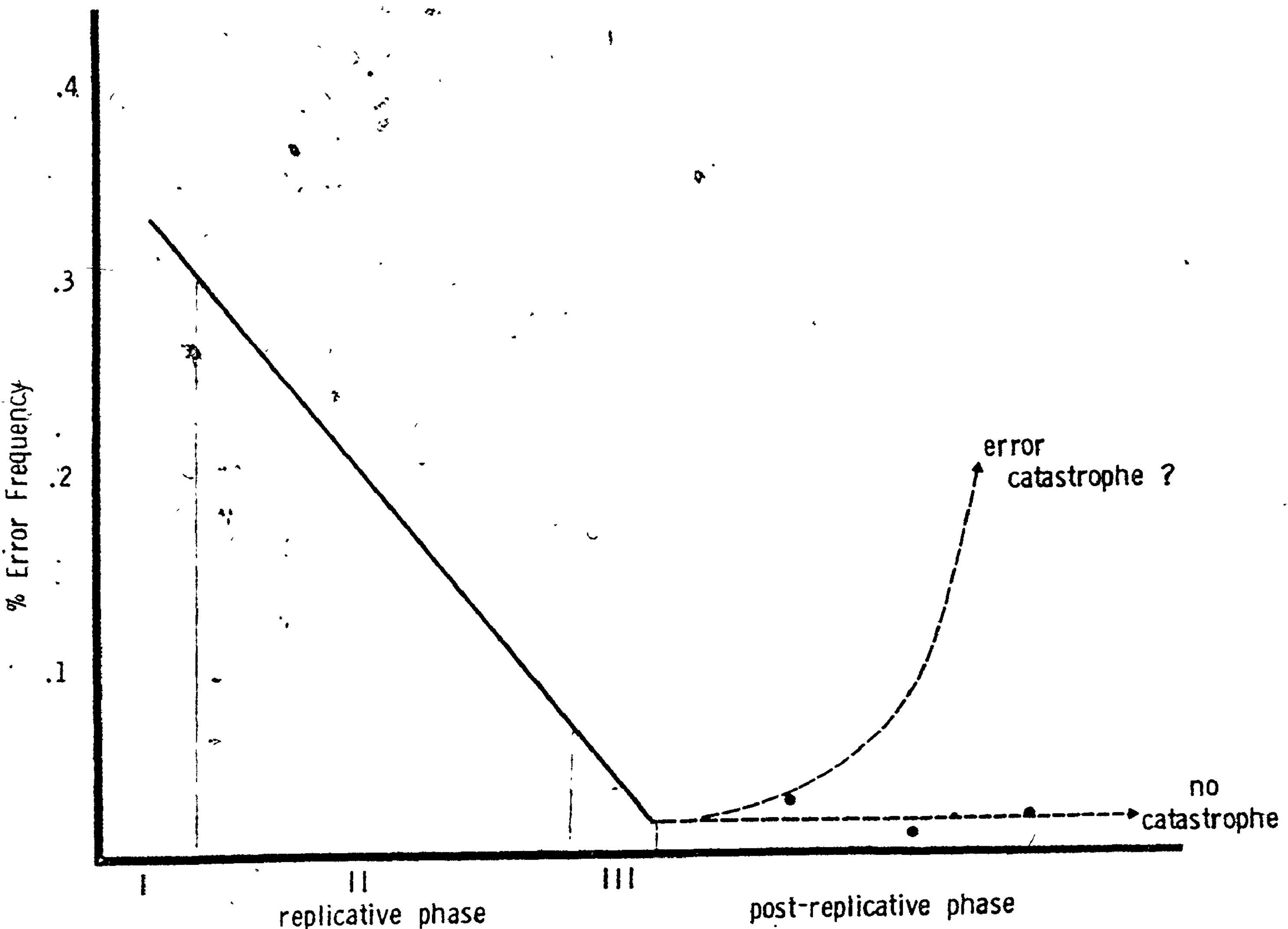


Figure 4-5 Absence of error catastrophe in the post-replicative phase of fibroblast culture.

suffer error catastrophe. This may have physiological importance to a variety of cells in the body, particularly those which become differentiated early in life. For example, epithelial stem cells destined to become nervous tissue may have high error frequency during early development. The error frequency of these cells (neuroblasts) would decline throughout their proliferative phase to ensure a minimum error frequency in cells before entering their post-replicative and differentiated states as mature neurons, thus ensuring a more efficient translation apparatus needed for precise differentiated function.

Two possible mechanisms to account for the decrease of in vitro error frequency as a function of passage were investigated (Figure 4-6). Since the data from the longitudinal A_2 series of error frequency assays showed an inverse correlation between the error frequency and the number of days for cultures to reach confluence (Figure 3-25), the accuracy of the translation apparatus may have been affected by cellular growth rate. More directly error frequency may have been diminished at slower protein synthetic rates (Kurland, 1977) although there was no evidence that actual synthetic rates change during aging of cells in vitro (Harley, 1980; Figure 3-31). If the error frequency is growth-related, and if the growth of early-passage cells is slowed to mimic the growth rate of late-passage cells, a concomitant decrease in the in vitro error rate should be

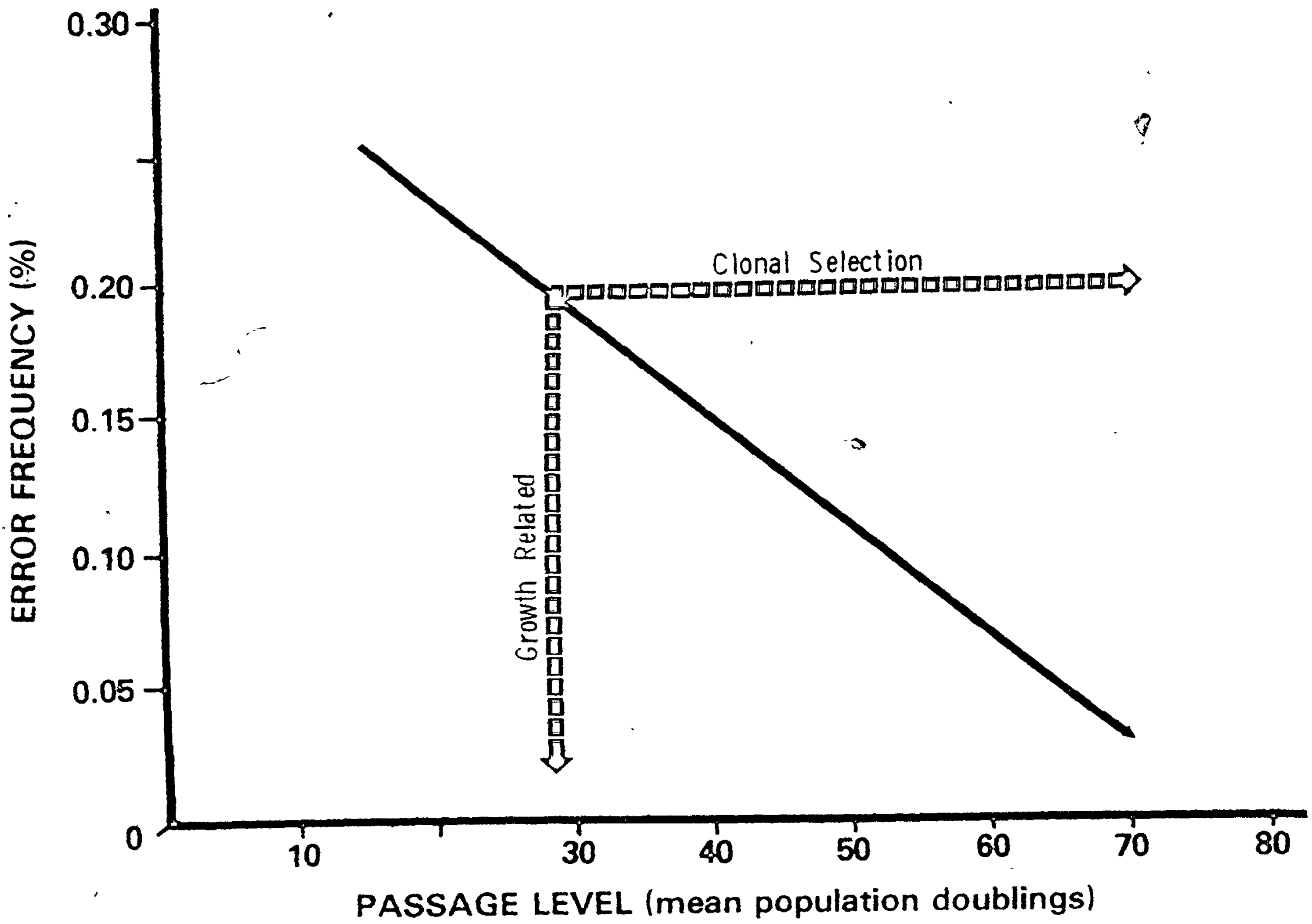


Figure 4-6 Tw› possible mechanisms for decrease in error frequency at later passage.

observed.

The results of error frequency assays on cells grown in low serum containing medium show that, although early-passage cells in low serum grew at the slower rate of late-passage cells in regular growth medium, there was no effect of growth rate on the in vitro error frequency. Also, although cells grew more slowly in low serum medium, their cellular protein synthetic rate was equal to that of rapidly growing cells (Table 3-5). This requires that the cellular protein degradation rate be elevated in the slow-growing cells; indeed, it is in late-passage cells (Elliot, 1980; Harley, 1980). It should also be noted that error frequencies measured in these experiments (0.35 and 0.26% for A_2 at 30 and 35 MPD respectively) are higher than predicted from the original A_2 series (0.21 and 0.18%, respectively). Since each experiment employed a different lot of strain A_2, perhaps different lots of the same strain behave uniquely.

It is concluded that slowing the growth of early-passage cells has no effect on the error frequency of poly(U) translation, and the decrease in error frequency as a function of passage is not related to cellular growth rate.

Since much work has been done testing the other possible mechanism, clonal selection, it is discussed more fully in the following section.

4.1.5 The Clonal Selection Hypothesis

The error frequency determined in cell extracts is actually the mean of an error frequency distribution. Since a mass culture is a heterogeneous mixture of individual clonal populations, another mechanism which could accurately explain the decrease in error frequency as a function of passage is clonal selection. If, throughout serial subcultivation, cells of high error frequency were selectively eliminated from the mass culture only the progeny of low error frequency cells would survive to late-passage. The result would be a late-passage culture consisting of a mixed population of clones having low error frequency.

The elimination of cells with high error frequency from the mass culture can be accomplished if a) the cells with high error frequency grow as well as low error frequency cells but possess a shorter in vitro lifespan, or b) the high error frequency cells possess less growth vigour and are less able to compete with cells having low error frequencies. Thus the "clonal selection" hypothesis invokes some testable predictions.

1. a clonal culture of cells should exhibit a constant error frequency throughout the remainder of its tissue culture lifespan and not a decrease in error frequency with later passage (Figure 4-5).
2. there should exist a range of clonal error frequencies which reflects the range of error frequencies exhibited

by the mass culture during its replicative lifespan.

3. there should be a demonstrable inverse correlation between error frequency of a clone and either its maximum in vitro lifespan and/or parameters of growth vigour.

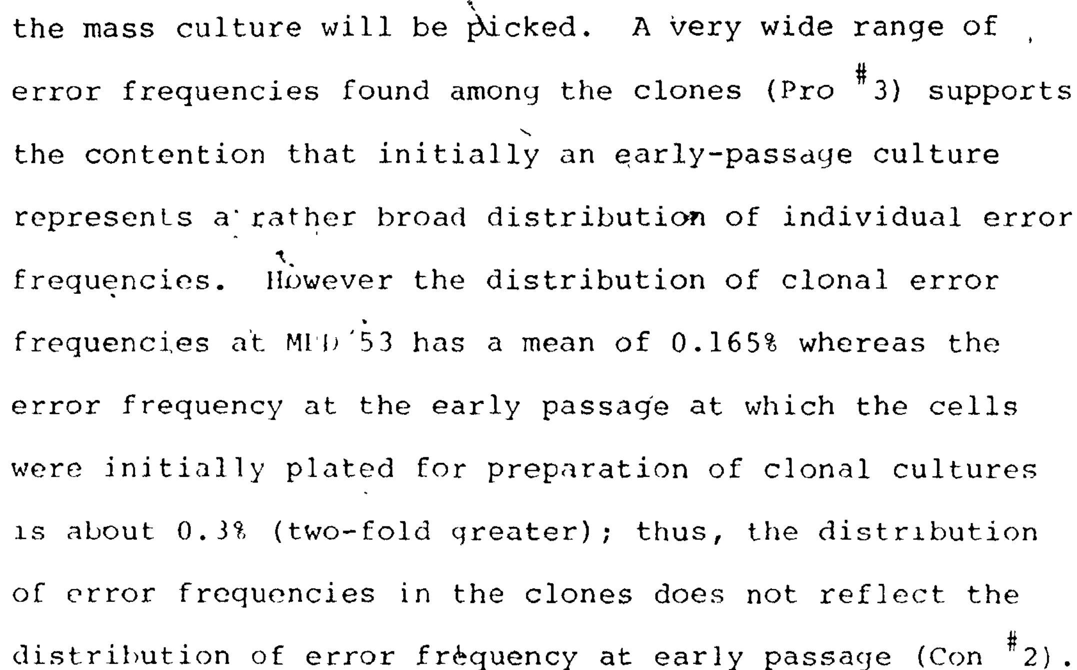
The study of clones has yielded information both supporting and contradicting the clonal selection hypothesis. (Table 4-1). The error frequency stayed constant with passage in some clones (Group A) while in others (Group B) it showed an abrupt increase at late passage (Figure 3-28). Both groups were significantly different from the mass culture. The constant error frequency demonstrated by Group A clones is the strongest evidence for clonal selection (Pro#1). None of the clones studied showed a decrease in error frequency with passage (Pro#2). Clearly a phenomenon occurring within the mass culture is absent in Group A clonal cultures. Since the major difference in the two conditions is the clonal heterogeneity of the mass culture, it follows that clonal heterogeneity is a precondition and clonal selection the possible mechanism for the observed decrease in error frequency during passage of the mass culture.

About half of the clones comprising a mass culture should have an error frequency higher than that of the mass culture. If the error frequency of clones remained constant throughout serial subcultivation, there is good chance that some clones in which error frequency is higher than that of

Table 4-1

Clonal Selection - Pros and Cons

Pros	Cons
1. Group A clones exhibit constant error frequency with passage.	1. There is no correlation between error frequency and growth parameters.
2. No clone showed a significant decrease in error frequency.	2. Distribution of clonal error frequencies does not reflect distribution at early passage.
3. Clones have a wide range of error frequencies.	3. High error frequency clone exhibits decreased proteolytic rate.
4. Many clones exhibit shortened in vitro lifespan - clonal succession.	
5. Post-mitotic cells (no clonal succession) show constant error frequency.	



the mass culture will be picked. A very wide range of error frequencies found among the clones (Pro #3) supports the contention that initially an early-passage culture represents a rather broad distribution of individual error frequencies. However the distribution of clonal error frequencies at MPD 53 has a mean of 0.165% whereas the error frequency at the early passage at which the cells were initially plated for preparation of clonal cultures is about 0.3% (two-fold greater); thus, the distribution of error frequencies in the clones does not reflect the distribution of error frequency at early passage (Con #2). Although seemingly contrary to the clonal selection hypothesis, it must be remembered that the clones which were finally studied represented only 2% of the possible number of clones. The remaining 98% were selected against during establishment of the clonal cultures either by non-attachment, poor growth or shortened in vitro lifespan. Thus upon low-density plating of cells from a mass culture and clonal subcultivation, the (hypothetical) normal distribution of error frequencies at early passage became skewed in favour of the low error frequency clones. This selectively skewed distribution remained constant throughout passage and was reached in the clones at MPD 53. Serial subcultivation, involving the high-density plating of cells, may have similar selection properties in skewing the error distribution toward lower error frequencies.

Several data support the concept of clonal succession during in vitro aging of a cell culture. Heterogeneity in growth characteristics among clonal populations such as we observed has been reported by several groups (Merz and Ross, 1969; Cristofalo and Sharf, 1973; Absher et al., 1974; Smith and Hayflick, 1974; Martinez et al., 1978). It has been shown also that a large proportion of cells in a mass culture possess very limited proliferative capacity (Smith et al., 1978) although continuous subcultivation of a mass culture at low densities has no effect on in vitro lifespan (Smith and Braunschweiger, 1979). There is good evidence that the maximum MPDs of clones which make up the mass culture are bimodally distributed (Smith and Hayflick, 1974; Smith and Whitney, 1980), with one subpopulation having low replicative potential and the other reaching higher maximum MPD; the highest MPD observed in clones was equal to that of the mass culture, a finding consistent with our data. Our data conflict however with reports (Smith et al., 1978; Smith et al., 1980) that the colony size of clones is an accurate predictor of remaining in vitro lifespan. At mid-passage, we have found that considerable variability exists in the manner in which clones enter Phase III, some grow well until 2-3 generations prior to terminal passage, whereas others grow poorly for 10 generations or more. By using slight chromosomal rearrangements as markers, Salk et al., (1980) have elegantly shown that certain clones predominate

in an early-passage culture and are then replaced (succeeded) by previously minor clones which come to predominate in late passage.

Although not directly supporting the concept of clonal selection, the fact that clonal succession occurs in the mass culture indicates that fewer clones are represented in the mass culture with each successive generation, i.e. clonal attenuation (Pro#3). If the decrease in error frequency as a function of the passage level of a mass culture depends on clonal attenuation, then under conditions where clonal succession is impossible, the error frequency of a mass culture should not decline. In cultures of post-mitotic cells, in which clonal succession is effectively halted, the error frequency remains constant (Pro#5).

Despite the ways in which the data support the clonal selection hypothesis, significant negative evidence is also revealed. Most importantly, no correlation between error frequency and several growth parameters could be established. For example, of the Group A clones, regarding growth vigor, clone 18 with the highest error frequency grew as well as clone 10 which had an error frequency ten times lower. Futhermore no decrease in the maximum MPD was seen with increasing error frequency in the clones. Certainly, there was no way one could predict the growth of the mass culture by knowing the growth characteristics of the set of clones. Also, one could not predict the decline in

error frequency in the mass culture by the data on clonal error frequencies summarized in Figure 3-28. Clearly the trend of error frequencies in the mass culture could be predicted from the clonal data, only if a selection pressure against the high error frequency clones could be demonstrated.

Since no deficit in growth vigor or potential was seen with high error frequency, it is difficult to ascertain the relevance of poly(U)-directed error frequency to the growth of the cell. If the error frequency of poly(U) translation reflected the error frequency of protein synthesis in vivo, then the high error frequency clone may be expected to have an increased proteolytic rate. The exact opposite was found (Con #3). Thus, either error frequency determined by this method is not relevant in describing cellular function or selective pressures on high error frequency cells are too slight to be readily detectable above the inherent variability of growth potential among clones. Although the relation between increased proteolysis and abnormal proteins exists (Capecchi et al., 1974; Goldberg and St. John, 1976), the association is tenuous, however.

The clones of Group B further complicated the clonal selection hypothesis. Why the error frequency increases abruptly at late passage is inexplicable. It is doubtful whether the increase in error frequency represents an Orgelian catastrophe since (a) the error frequency does not continue

to increase in clone 14 after 6 weeks in the post-mitotic state, and (b) no decrement in growth vigor was associated with the error increase.

However, other explanations are possible. Variation inherent in the clones may allow discrete levels of error frequency to exist. The error frequency may increase to higher levels and remain stable, i.e. not continue to increase in the Orgelian sense. Induction of errors in bacteria through the use of antibiotics (Edelmann and Gallant, 1977a; Gallant and Palmer, 1979) caused error frequency to increase to a new stable level and also failed to cause an error catastrophe. Thus, although the error feedback mechanism may itself exist, it is not sufficient to cause an error catastrophe.

The increase in error frequency may be the result of a mutational event altering the fidelity of the translational apparatus. Such a mutation would have to occur with a relatively high frequency however to be observed in 2 out of 9 clones.

It is possible, too, that the cultures which increase in error frequency may not be clonally pure. If two cells plated near one another during clonal seeding, the resulting biclonal colony would be considered monoclonal. If the two clones comprising this colony have distinctly different error frequencies and growth characteristics a change in error frequency would be observed if the ratio of clones

changed abruptly at late passage. If the lower error frequency clone grew more quickly it would dominate the culture until its terminal passage. The other clone would then take over the culture and express its error frequency until terminal passage. However, the fact that an increase in error frequency occurs Group B clones argues against clonal selection of this type explaining decreasing error frequency in late-passage mass cultures.

Clonal selection is perhaps the final way in which the Orgel hypothesis could have been used to explain in part the senescence of human diploid fibroblasts in vitro. The crucial link between error frequency and senescence, that is, of decreased in vitro lifespan and/or detrimental growth with elevated error frequency, could not be established.

4.2 Translation of Natural mRNA

Natural messenger RNA can also act as a template for protein synthesis in extracts of human diploid fibroblasts. Unlike using poly(U) the use of natural template involves proper initiation and termination and thus circumvents many of the arguments disfavouring the use of artificial templates in the study of protein synthesis.

4.2.1 Endogenous (Cellular) RNA

In extracts in which the ribosomal runoff step is omitted, endogenous protein synthesis remains active. Contrary to the reports of Buchanan et al., (1980) who studied poly(U)-directed synthesis in non-runoff extracts,

in our hands, synthesis in these extracts was inhibited by poly(U) (Figure 3-33). It is apparent therefore that only after runoff were ribosomes available for poly(U) translation.

Several data show that the incorporation of amino acids into acid insoluble material represents true protein synthesis: (a) incorporation is inhibitable by puromycin and histidinol (data not shown) known inhibitors of protein synthesis; (b) the proportion of amino acids incorporated accurately reflects the proportion of amino acids present in the intact cell (Table 3-11); thus, the mRNAs translated in vitro must be similar to those found in the intact cell and; (c) the product is very sensitive to proteolytic degradation (Figure 3-35).

As shown by the similarities of the 2-D gel electrophoretic patterns of proteins synthesized in vivo and in vitro (Plate 3), cell-free extracts properly terminate the translation of cellular mRNA. The protein synthesis may simply involve completion of the elongation process on already existing polysomes. It is difficult to ascertain whether initiation occurs under the conditions employed since ribosomes which have been subject to 45 minute runoff, initiate onto endogenous mRNA very poorly, enabling endogenous synthesis to be minimized during poly(U) translation. Cell-free synthesis directed by endogenous mRNA is linear for about 30 minutes however, implying that reinitiation does

occur, although at a much diminished rate compared to in vivo initiation.

The incorporation of amino acid analogues AIB and p-fluorophenylalanine (Table 3-12) illustrates that misacylation of tRNA may be assayed in HDF extracts. The decline with passage in misacylation rate observed for p-F-phe/phe is contrary to results of Holliday (1969) and Lewis and Tarrant (1972) who observed an increase in the incorporation of amino acid analogues in late passage MRC-5 fibroblasts.

4.2.2 Exogenous mRNA

The addition of rabbit globin mRNA to extracts prepared for endogenous cell-free protein synthesis, further stimulated synthetic activity. The ionic requirements for natural mRNA and poly(U) are different since globin mRNA could only stimulate protein synthesis at lower magnesium concentration (Figure 3-36). This suggests that initiation onto globin mRNA is strongly dependent on eIF-2 (Merrick, 1979) and hence occurs at an AUG initiator codon. It is not known however whether the product of rabbit globin mRNA translation in these extracts is indeed rabbit globin (see Chapter 5 --- Prospective Studies).

CHAPTER FIVE

PROSPECTIVE STUDIES

As with many novel methodologies, the most significant results arising from this work may not be what has already been accomplished (i.e. this thesis) but rather what future experiments with the cell-free protein synthetic system will show. The cell-free extract may be used in the study of protein synthesis in many ways (Figure 5-1). It affords to date the only system to study protein synthesis of normal, (diploid) human cells grown under carefully controlled and defined conditions. Studies into aging and its effects on protein synthesis can also be continued although the emphasis may not necessarily be directed in this way.

Several protocols for further experimentation into the fidelity of poly(U) translation were elaborated during the course of this work.

i) The general nature of declining error frequency as a function of passage may be tested in fibroblast strains from old donors and progeric patients. If the clonal selection hypothesis is tenable, perhaps profound differences in clonal selection in aged cells may be seen in what amounts to, essentially, a clonal selection assay.

ii) The cellular protein synthesis machinery may be dissected and its parts mixed to ascertain whether, for

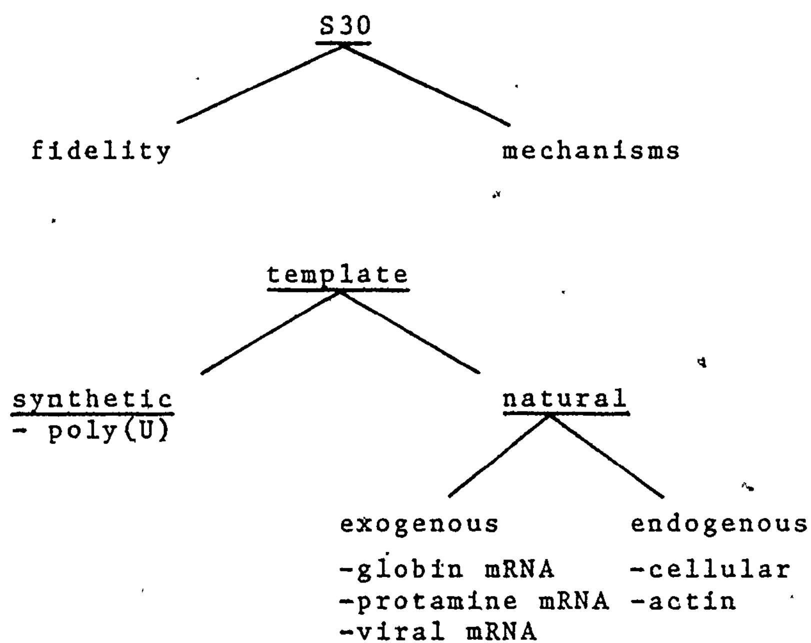


Figure 5-1 Possible uses of extracts from human diploid fibroblasts.

example, the ribosomal or S-100 fraction is solely responsible for declining error frequency. Further analysis could implicate a particular ribosomal subunit or synthetic factor.

iii) A similar mixing technique could shed light on why strain A_{25} has such a high protein synthetic error rate — whether this is due to ribosomal or S-100 components. Taken together with the apparent high level of diphtheria-toxin resistance found in A_{25}, this cell strain may be an EF-2 mutant, or have a very high frequency of spontaneous mutation. The characteristics of other protein synthetic mutants may also be studied with the cell-free system.

iv) Changing the components of the assay system can be used to modulate the error frequency. The effect of magnesium concentration on both synthetic rate and error frequency can be used to compare in vitro protein synthesis in young and aged cells. Antibiotics are known to affect mistranslation rate and the effect of these on mistranslation in extracts of human cells can be studied. Induction of errors by the method of Gallant and Palmer (1979) can test the concept of error feedback.

The fidelity of protein synthesis can be studied using natural mRNA as well, after conditions have been optimized. Initial studies on the translation of rabbit globin mRNA (section 3.2.3) should be continued. Since

premature or incorrect termination, as well as out-of-frame reading due to faulty initiation could occur. It must be shown that the product of globin mRNA translation is, indeed, globin. This may be done physically, by 2-D gel electrophoresis, and antigenically, by reacting the product with anti-globin antibody. Two of the human globin chains (α- and β-globin) have no isoleucine in their primary sequence (Figure 5-2a) (Dayhoff, 1972). Thus, an estimate of isoleucine incorporation into the globin molecule, or the trypsin peptides thereof, would give an estimate of the error frequency of translation in a completely human system.

Another potentially useful template for studies of synthetic fidelity is the readily available protamine mRNA (Gedamu et al., 1978). Although not a human mRNA, its protein product has several amino acids not represented (Figure 5-2b), and consists of predominantly arginine residues. Thus, the sensitivity to which error frequencies may be determined is much greater with this template.

Finally, these extracts may be used to study the general nature of protein synthesis in human fibroblasts. Natural mRNA may be used to study mechanisms of initiation and termination in vitro. The extracts may be utilized to translate any natural mRNA eg. viral mRNA in studies not directly related to protein synthesis as well.

a) ● HEMOGLOBIN ALPHA CHAIN - HUMAN, CHIMPANZEE AND GORILLA

```
            5         10        15        20        25        30
  1 V L S P A D K T N V K A A W G K V G A H A G E Y G A E A L E
 31 R M F L S F P T T K T Y F P H F D L S H G S A Q V K G H G K
 61 K V A D A L T N A V A H V D D M P N A L S A L S D L H A H K
 91 L R V D P V N F K L L S H C L L V T L A A H L P A E F T P A
121 V H A S L D K F L A S V S T V L T S K Y R

                    5                   10                  15
  1 VAL LEU SER PRO ALA ASP LYS THR ASN VAL LYS ALA ALA TRP GLY
 16 LYS VAL GLY ALA HIS ALA GLY GLU TYR GLY ALA GLU ALA LEU GLU
 31 ARG MET PHE LEU SER PHE PRO THR THR LYS THR TYR PHE PRO HIS
 46 PHE ASP LEU SER HIS GLY SER ALA GLN VAL LYS GLY HIS GLY LYS
 61 LYS VAL ALA ASP ALA LEU THR ASN ALA VAL ALA HIS VAL ASP ASP
 76 MET PRO ASN ALA LEU SER ALA LEU SER ASP LEU HIS ALA HIS LYS
 91 LEU ARG VAL ASP PRO VAL ASN PHE LYS LEU LEU SER HIS CYS LEU
106 LEU VAL THR LEU ALA ALA HIS LEU PRO ALA GLU PHE THR PRO ALA
121 VAL HIS ALA SER LEU ASP LYS PHE LEU ALA SER VAL SER THR VAL
136 LEU THR SER LYS TYR ARG
```

COMPOSITION

21 ALA A	1 GLN Q	18 LEU L	11 SER S
3 ARG R	4 GLU E	11 LYS K	9 THR T
4 ASN N	7 GLY G	2 MET M	1 TRP W
8 ASP D	10 HIS H	7 PHE F	3 TYR Y
1 CYS C	0 ILE I	7 PRO P	13 VAL V

MOL. WT.= 15,126 TOTAL NO. OF RESIDUES = 141

THE SEQUENCE SHOWN IS HUMAN.

b) ● PROTAMINES - RAINBOW TROUT

IRIDINE IA
PRO ARG ARG ARG ARG SER SER SER ARG PRO VAL ARG
ARG ARG ARG ARG PRO ARG ARG VAL SER ARG ARG ARG
ARG ARG ARG GLY GLY ARG ARG ARG ARG

IRIDINE IB
PRO ARG ARG ARG ARG ARG ARG SER SER SER ARG PRO
ILE ARG ARG ARG ARG PRO ARG ARG VAL SER ARG ARG
ARG ARG ARG GLY GLY ARG ARG ARG ARG

IRIDINE II
PRO ARG ARG ARG ARG SER SER SER ARG PRO VAL ARG
ARG ARG ARG ALA ARG ARG VAL SER ARG ARG ARG ARG
ARG ARG GLY GLY ARG ARG ARG ARG

Figure 5-2 Primary sequence and amino acid composition of human α-globin(a) and trout protamine(b).

CHAPTER SIX

SUMMARY AND CONCLUSIONS

The initial objective of this research was to develop an in vitro protein-synthesizing system from human diploid fibroblasts. This was attained summarily with ultimate employment of the system to directly test the Orgel hypothesis of cellular senescence. Under conditions which allowed accurate measurement of the fidelity of the fibroblast translation apparatus, data were obtained from cells existing in three different states:

1. Mass culture
2. Clonal culture
3. Post-mitotic culture.

The data obtained from mass culture experiments contradicted the Orgel hypothesis in that neither late-passage cells, progeric cells or cells from an old donor manifested (the expected) higher error frequency when compared to suitable controls. The highly significant decline of error frequency in extracts of a normal strain also spoke strongly against the general predictions of the Orgel hypothesis.

Mechanistic explanations for the decline in error frequency with passage showed that the phenomenon was not related to the growth rate of the cells but was due to the clonal heterogeneity of the mass culture and further implied cell selection throughout the replicative lifespan. Since Orgel (1963) originally proposed that error catastrophe could

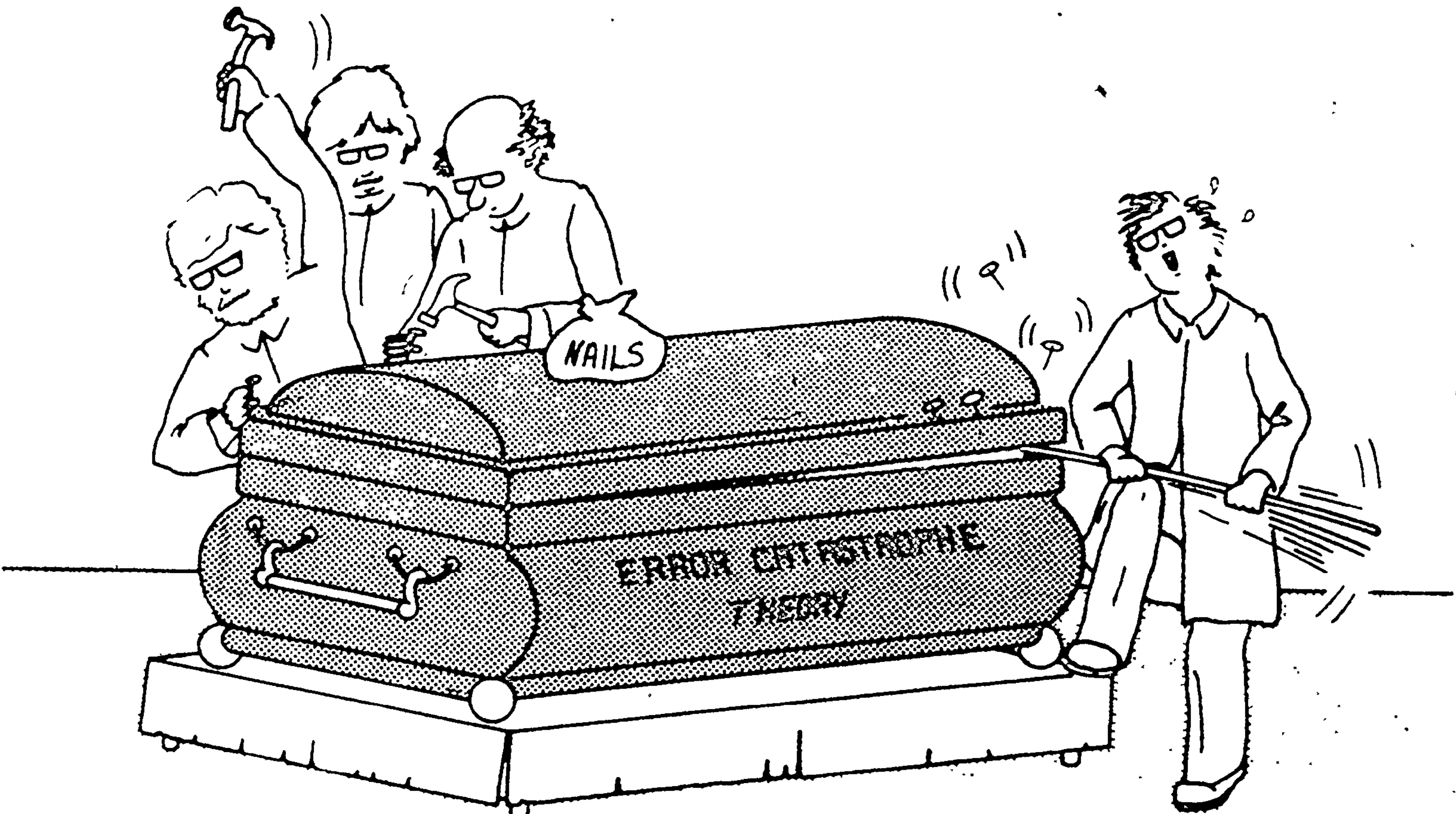
only occur in a system devoid of cell selection, it would seem that fibroblast culture was not suitable to test the Orgel hypothesis. However, in a series of experiments in which cells were maintained under conditions in which there was no replication and therefore no cell selection, no increase in error frequency was observed. Furthermore, the error frequency of numerous clonal populations of cells was not a factor in influencing cell growth or maximum lifespan. Over the range of error frequencies studied, an increase in translational errors was not detrimental. Also, in no instance was the phenomenon of error feedback observed (with the possible exception of two (Group B) clones which showed an innocuous increase in error frequency at late passage).

Although the nature of Orgel's theory has been tested and contradicted, it must be remembered that the cell-free system under study related only to a specific kind of error among the numerous possible errors of the translation machinery. The fidelity of poly(U)-directed in vitro protein synthesis only measured ribosomal ambiguity, tRNA-mRNA mismatching, therefore it would be presumptuous to extrapolate these results and generally dismiss the hypothesis. Tests available today may, as yet, be too crude and mini-catastrophes may still occur, the resulting damage hastening cell death.

A final note. The Error Catastrophe Theory has proved a very good hypothesis in terms of generating numerous predictions, experimental approaches, and tests. As duly noted

in the 1000-word equivalent on the next page, this author feels that with the overwhelming contrary evidence, it is time to close the lid on this theory; this work being another nail in the coffin. The theory has also exposed a phenomenon common to (aging) research in that to every point found a counterpoint is also made, with the research community divided into camps.

There often exist (a group of) men who tenaciously resist the weight of evidence. It is often difficult to assess whether this kind of conservatism acts to prevent the hasty abandonment of an idea before all the facts are in, or to deter the advancement of new ideas by keeping alive ideas that should have been buried long ago. This author awaits the prybar but trusts the nail to hold.



DON'T WORRY LESLIE, HOLLIDAY WILL GET YOU OUT !

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