

THE UNIVERSITY OF MANITOBA

ON THE INVOLVEMENT OF A NOVEL PROTEOLYTIC SYSTEM
IN L-GLUTAMINE CONTROL OF POLYPHOSPHATE AND NUCLEIC ACID METABOLISM
UNDER HYPERTHERMIC CONDITIONS IN MAMMALIAN CELLS TRANSFORMED BY
Ha-ras ONCOGENE, SV40 VIRUS AND 9,10-DIMETHYL-1,2-BENZANTHRACENE.

BY

ABDULAI MOHAMED DEFFIE

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FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

Department of Microbiology

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June 1987

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

My parents and teachers, who showed me the way;

My wife, Marie, who guided me through the way;

My daughter, Yewa, who will lead me into the future;

and

Dr. LeJohn, who has shown me the way, guided me through,

and led me into the future.

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ABSTRACT

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A novel dinucleoside polyphosphate (HS3) had previously been described in various mammalian cell lines and reported to show distinct patterns of synthesis for transformed and normal cells in response to glutamine starvation (Goh 1979). Those studies were done using nonisogenic transformed and untransformed cell lines.

The studies described here have used freshly transformed cells and isogenic pairs of transformed and untransformed cell lines to show that, regardless of the manner by which mammalian cells are transformed, there is a corresponding and uniform change in the pattern of HS3 accumulation by the cells in response to glutamine starvation. The change in HS3 production pattern was observed to occur only a few cell generations after the cells were transformed, thus suggesting a direct, causal relationship between cell transformation and a change in HS3 synthesis pattern. Using somatic cell fusion techniques, it was shown that the transformed cell pattern of HS3 synthesis may be recessive to the untransformed pattern.

There was a temperature-dependent aspect in the HS3 synthesis pattern of transformed cells. While untransformed cells displayed an indifference to availability of glutamine in their HS3 metabolism pattern at normal (37°C) or hyperthermic (42°C) temperatures, transformed cells switched from glutamine dependence at 37°C to an 'apparent' glutamine independence when kept for 4h at 42°C. This was the case for several isogenic pairs of transformed and untransformed cell lines studied. The temperature effect in transformed cells did not seem to be due to a generalised impairment of metabolic activity of the cells at the hyperthermic temperature since nucleic acid

(DNA and RNA) biosynthesis occurred optimally and, like HS3 synthesis, with an 'apparent' independence on supplied glutamine at 42°C. Furthermore, in the presence of certain drugs (for example, protease inhibitors or methionine sulfoximine in the growth medium), HS3 (as well as nucleic acid) biosynthesis occurred at 42°C in a glutamine-dependent manner similar to that observed at 37°C. Glutaminase (a glutamine deaminating enzyme) also enabled transformed cells to synthesise HS3 at 42°C. Transformed cells, but not untransformed cells, were shown to have increased intracellular levels of glutamine at 42°C compared to the levels at 37°C. Direct measurement of glutamine and protease activity in the growth medium confirmed that transformed cell lines, but not their untransformed counterparts, had enhanced levels of proteolytic activity with a concomitant generation of glutamine in their growth medium at 42°C; no glutamine was generated in cultures at 37°C.

The heat-induced proteolytic activity, as well as glutamine generated, were sensitive to a wide variety of protease inhibitors and to cycloheximide and actinomycin D. Efforts to identify the putative heat-induced protease(s) by polyacrylamide gel electrophoresis and autoradiographic techniques were unsuccessful. Attempts to identify possible substrate(s) for the heat-induced protease(s) showed that secreted proteins as well as authentic serum proteins may be substrates for the heat-induced protease(s).

The results presented in this thesis was consistent with the hypothesis that transformed cells respond to hyperthermia by secreting protease(s) which break down proteins external to the cells, to generate free amino acids in the medium; the generated glutamine act to control HS3 metabolism in a manner similar to the effect at 37°C.

The significance of this novel proteolytic activity is unknown, but it may have nutritional and homeostatic significance, as well as useful implications for cancer therapeutic modalities that involve hyperthermia.

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ABBREVIATIONS

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AAF	N-2-acetylaminofluorene
ACT. D	actinomycin D
ADP	adenosine 5'-diphosphate
α -MEM	alpha-minimal essential medium
ALS	antilymphocyte serum
ALV	avian leukosis virus
AMP	adenosine 5'-monophosphate
APRT	adenine phosphoribosyl transferase
APRT ⁻	adenine phosphoribosyl transferase deficient
AraC	1- β -D-arabino-furanosylcytosine
ATP	adenosine 5'-triphosphate
Benzam.	benzamidine
BP	benzo(a)pyrene
BrdU	5-bromodeoxyuridine
BSA	bovine serum albumin
cAMP	cyclic 3',5'-adenosine monophosphate
CFE	colony-forming efficiency
CHX	cycloheximide
CHO	Chinese hamster ovary
Ci	curie
cm	centimetre
c-oncs	cellular oncogenes
cpm	counts per minute
CSP	cell surface protein
DFBS (DFCS)	dialysed fetal bovine (calf) serum

DMBA	dimethyl-1,2-benzanthracene
DMS	double minute chromosomes
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DNAse	deoxyribonuclease
Σ -ACA	Σ -amino-n-caproic acid
EDTA	ethylenediamine-tetraacetic acid
EGF	epidermal growth factor
FBS (FCS)	fetal bovine (calf) serum
FGF	fibroblast growth factor
Fig (fig)	figure
g	gram
GAT ⁻	glycine/adenine/thymine-deficient
GDP	guanosine 5'-diphosphate
Gln (gln)	glutamine
Glnase	glutaminase
GPAG	growth-promoting -globulin
GTP	guanosine 5'-triphosphate
HAT	hypoxanthine/aminopterin/thymidine-supplemented medium
HCl	hydrochloric acid
HGPRT	hypoxanthine-guanine phosphoribosyltransferase
HGPRT ⁻	hypoxanthine-guanine phosphoribosyltransferase-deficient
³ H-PDBU	³ H-phorbol dibutyrate
h (hr, hrs)	hour
HSPs	heat shock proteins
HSRs	homogeneously staining regions
id	intradermal

IgM	immunoglobulin M
im	intramuscular
ip	intraperitoneal
iv	intravascular
l	litre
LETS	large, external, transformation-sensitive protein
LTRs	long terminal repeats
M	molar
med	medium (growth medium)
MFO	mixed-function oxidase
mg	milligram
min	minute
ml	millilitre
mm	millimetre
mM	millimolar
m mol	millimole
MS	methionine sulfoximine
MSF	multiplication stimulating factor
MW	molecular weight
NADP ⁺	nicotinamide adenine dinucleotide phosphate
nm	nanometre
NPGb	nitrophenyl-p-guanidinobenzoate
ODC	ornithine decarboxylase
OER	oxygen enhancement ratio
PA	plasminogen activator
PAHs	polycyclic aromatic hydrocarbons
PDGF	platelet-derived growth factor

PE	plating efficiency
PEG	polyethylene glycol
PEI	polyethyleneimine
Pi	inorganic phosphate
PI	phosphatidylinositol
PMSF	phenylmethylsulfonyl fluoride
PPi	inorganic pyrophosphate
RNA	ribonucleic acid
RNase	ribonuclease
RSV	Rous sarcoma virus
SAM	S-adenosylmethionine
sc	subcutaneous
SCE	sister chromatid exchange
SDS	sodium dodecyl sulfate
TAME	N α -p-tosyl-L-arginine methyl ester
TCA	trichloroacetic acid
TdR	thymidine
TER	thermal enhancement ratio
TGF	therapeutic gain factor
TGF	transforming growth factor
TK	thymidine kinase
TK ⁻	thymidine kinase-deficient
TLCK	N α -p-tosyl-L-lysine-chloromethylketone
TLNP	trypsin-like neutral protease
TPA	12-O-tetradecanoylphorbol-13-acetate
Tris	tris (hydroxymethyl) amino methane
ts	temperature-sensitive

uCi	microcurie
UDP	uridine 5'-diphosphate
ug	microgram
ul	microlitre
uM	micromolar
UV	ultraviolet
v-oncs	viral oncogenes
WT	'wild type'
(-)	<u>not</u> supplemented (unless otherwise defined in text)
(+)	supplemented (unless otherwise defined in text)

INTRODUCTION

INTRODUCTION

The answer to the question of what causes a normal cell to assume transformed, and ultimately cancerous, characteristics remains enigmatic. It is clear now that during the process by which a normal cell is converted to the neoplastic state, many diverse structural, biochemical and genetic changes occur in the cell. It is the hope of many researchers in the area of cancer cell biology to be able to identify differences between normal and neoplastic cells that could be exploited to achieve a selective therapeutic advantage for the treatment of cancer. It is, however, somewhat disappointing that, despite many efforts and significant advances, there is as yet no single marker that categorically distinguishes a normal cell from its neoplastic counterpart. This may be due partly to the great diversity of cell types, differentiated states, and of cellular processes in mammalian organisms, and partly to the diversity of the agents of cell transformation.

It is becoming increasingly clear that the final effect of the myriad biochemical, structural and genetic changes that accompany cell transformation is a generalised deregulation of cell growth. In general, neoplastic cells seem to be unable to regulate their rate of growth as well as their maximum growth potential. This is in contrast to most normal cells which seem capable of regulating most aspects of their growth features. It is, therefore, imperative that great effort should be spent towards understanding and controlling the growth features of neoplastic cells.

In 1975, three unique dinucleoside polyphosphates, designated HS1, HS2, and HS3, were reported to exist in various fungi. One of them,

HS3, was partially characterised as consisting of a glutamyl-ADP-sugar moiety covalently linked in an unknown manner to a UDP-mannitol-tetraphosphate. Various biochemical and genetic studies showed that these three dinucleoside polyphosphates may be involved with cellular regulation during adverse growth conditions.

HS3 was found to be the only one of the three polyphosphates that accumulated in mammalian cells when the cells were starved of glutamine. Detailed physiological studies and studies involving mammalian somatic cell mutants clearly indicated that HS3 was intimately involved in the regulation of nucleic acid biosynthesis. Also, and perhaps more important, it was observed that the response of cells to the glutamine starvation condition (with respect to HS3 accumulation) was different for transformed (or ~~permanent~~ cell lines in culture as compared to normal diploid cell lines: transformed cell lines accumulated HS3 only when de novo purine biosynthesis was blocked (by glutamine deprivation, drugs or mutation); on the other hand, normal diploid cell lines accumulated uniform high levels of HS3 whether or not growth conditions were optimal. These observations were made in a study that utilised a variety of permanent and primary cell lines. Based on the involvement of HS3 in macromolecular biosynthesis, it became suggestive that HS3 may be involved in an explanation of the differential growth potential of transformed compared to normal cells. Another implication of these observations is that the pattern of HS3 accumulation may be used as an indicator of immortality of cells in culture.

Two other biochemical or physiological characteristics have been found to show distinctive patterns that can be used as markers of neoplastic change: these are, firstly, the differential heat sensitivity of normal and neoplastic cells; and secondly, the ability of neoplastic

cells to secrete enhanced levels of proteases under normal growth conditions when compared to their normal counterparts. Both of these features have, individually, formed the basis for development of new cancer treatment modalities and have also been used in explaining many features of malignancy. As is the case with most of the other known features of neoplastic cells, the problem that confronts many researchers is how to combine knowledge accumulated for these two properties to develop rational protocols for controlling, or at least explaining, neoplasia.

The results described in this thesis represent an attempt to combine three separate areas of research into differences between normal and neoplastic cells, namely, HS3 synthesis in response to glutamine starvation, hyperthermic responses, and protease secretion. The aims of this research are threefold: firstly, to establish a direct and causal relationship between HS3 response pattern to glutamine availability with in vitro cell transformation; secondly, to study the effect of moderate hyperthermia on HS3 response patterns in transformed and untransformed cells; and thirdly, to study the pattern of protease secretion under hyperthermic conditions and how it relates to HS3 synthesis. It is evident from these studies that the patterns of HS3 metabolism, protease secretion, and the response to hyperthermia are integrated in a novel way that can be used to distinguish neoplastic cells from their normal counterparts. Perhaps this thesis will form a basis on which multiple characteristics of neoplastic cells can be studied together and used in a unified way to understand the mechanisms of neoplasia and how to control neoplastic disease.

1. GROWTH OF CELLS IN CULTURE

A. Development of Cell Culture Techniques

Cell culture techniques have provided convenient systems for performing controlled studies on the structure, metabolism, differentiation, and development of normal mamalian cells, and of abnormal processes associated with the diseased state. The basic objective of any cell culture system is to be able to serially maintain a live population of cells derived from a single parental cell. Early attempts at this objective were hampered mainly by the unavailability of a proper growth-supporting medium.

During the early 1900s, the initial reports of successful culture of vertebrate cells were published. First, Carrel (1912) reported his success at keeping a chicken muscle explant viable and proliferating in a growth medium consisting of Ringer's salt solution (Ringer 1882), chicken plasma and muscle extract. Four years later, Rous and Jones (1916) also cultured bits of chicken tissue in a growth medium derived from blood clots and modified Ringer's solution; they reported successful serial passage of vertebrate cells in culture. The medium used in these early studies, although complex, could not support the growth of sparse cultures necessary to give rise to clones of cells. Because of the undefined nature of the medium the precise components of the medium necessary for growth of the cells were unknown. Following the discovery of antibiotics, there was a rapid development of cell culture. It became possible to maintain cells for longer periods of time free of bacterial contamination.

A major advance in cell culture was reported in 1948 when Sanford, Earle and Likely cloned L-cells in very small volumes of medium that had been "conditioned" to allow growth of sparse cultures (Sanford et al 1948). Another approach to generating clonal growth was developed by Puck and Marcus (1955) who used an irradiated layer of "feeder" cells to clone HeLa cells (Gey et al 1952). These two results suggested that specific, definable components in the growth medium were essential for supporting growth of sparse cultures. Subsequently, efforts to develop chemically defined growth media started. Eagle, working with HeLa and mouse L cells (Eagle 1955a, 1955b) first showed that the need for undefined complex body fluids could be satisfied with as little as 1% of dialysed horse serum in an otherwise defined mixture of nutrients; the defined portion of the medium consisted of thirteen amino acids, vitamins, cofactors, salts and carbohydrates. Several modified forms of Eagle's medium have since been developed, most of which are designed to meet the nutritional needs of specific cell lines.

Prior to 1960, only limited success was achieved at propagating diploid primary cells with a stable genome. Diploid cell cultures are important because their stable genome allows for more precise genetic studies than those involving unstable heteroploid karyotypes typical of HeLa and mouse L cells used in earlier studies. Hayflick and Moorhead (1961) were the first to serially propagate normal human fibroblast cells for long periods of time in culture. Their studies showed that the cells maintain their fibroblastic morphology and diploid karyotype for prolonged culture period. They also showed that primary cultures have a limited life span in culture and are subject to senescence after

a maximum number of cell divisions. This latter phenomenon was reported earlier by Swim and Parker (1957).

Eagle's medium (Eagle 1955a, 1955b) or other variations of it, were normally supplemented with a non-defined portion in the form of dialysed serum. Other poorly defined biological fluids or extracts that have substituted for serum include spinal or amniotic fluid, lymph, plasma, milk or colostrum, and embryo extracts (Barnes and Sato 1980). Because of availability and ease of storage, serum is the most commonly used supplement. Serum supports growth and survival of cells in culture by providing hormones, mitogens, nutrients, binding proteins and attachment factors necessary for propagation of cells in vitro (Barnes and Sato 1980).

In spite of the growth-promoting effects of serum, there are instances where serum and other poorly-defined biological fluids are not suitable or sufficient for certain kinds of work. Firstly, their undefined and complex nature makes serum and other biological fluids unsuitable for studies of the interactions of hormones and drugs with cells, and of studies of nutrition and secretion at the cellular and molecular levels. Secondly, serum-containing medium may be an incomplete substitute for the in vitro environment in many cases. For example, normal thyroid follicle cells may not grow in serum-containing medium, since these cells require a much higher concentration of thyrotropin than that found in normal blood (Barnes and Sato 1980). Similar situations may arise when other differentiated cells are cultured. Thirdly, serum may be toxic at some concentrations for many cell types. Serum contains many substances which normally do not come

in contact with most cells in vivo (Gospodarowicz et al 1979) and the toxicity of high levels of serum for most cell types in culture is well known (Barnes and Sato 1980). Because of the above reasons, efforts have been made in recent years to develop defined components that can replace serum in the growth medium. Any substitutes for serum must, at least in principle, provide the three main classes of factors supplied by serum, namely, the nutritional, hormonal (or mitogenic), and attachment factors. The efforts at finding substitutes for serum have been approached in two different ways. The first approach, pioneered by Sato and Hayashi (Sato 1975; Hayashi and Sato 1976), has shown that cells with a particular hormonal requirement will grow in serum-free medium provided that hormone, among others, are present. They conclude that a set of hormones, some defined and some undefined are the active molecules of serum. The use of mixtures of hormones and growth factors to replace serum for growth of cultured cells has gained rapid popularity since their report. Barnes and Sato (1980), have reviewed the status of serum-free cell culture; they provide lists of established cell lines which can be grown in serum-free media, as well as the hormones, binding proteins and attachment factors used as supplements. The second approach, promoted by Hamilton and Ham (1977), reports that serum-free basal medium supports growth of Chinese hamster cells if the medium is "optimised" with a large number of trace metals. Ham (1982) has compiled a list of the various optimised media available and the cell lines (established and primary lines) that can be cultured in them. The differences in these two approaches may be related to the different cell lines used in the studies. A large number of cell lines may need to be tested by both methods. Alternatively, the hormone preparations of Sato's group can be checked for any possible

contamination by trace elements, or the trace elements used by Ham's group may be checked to see if they mimic the effects of hormones.

Efforts in the development of cell culture techniques should appropriately be directed towards simplifying the growth medium without affecting growth potential of cells. Such media will provide minimal interference between growth media components and drugs and other exogenous materials that are investigated. Paradoxically, there is an equal need for the growth medium to be designed so that it will allow growth of as many cell types as possible, some yet unculturable; such a broad-based growth medium may require more complexity.

B. Factors That Affect Growth of Normal Cells in Culture

In recent years, mammalian cell culturists have paid particular attention to two main areas in the rapidly developing cell culture technology. Firstly, a goal towards the development of completely defined media that can sustain as many diverse cell types as possible; and secondly, the ability to control the rate of growth as well as the growth potential of cultured mammalian cells. Although physical, chemical and biological factors can influence the growth of cells in culture, the principal control is effected by components of the growth medium.

(a) Tissue Culture Media

All growth media routinely used for cell culture consist of a nutritional part and a mitogenic part. The nutritional part is the

components in the growth medium that are used within the cell as metabolic substrates or cofactors; it constitutes the defined part of the growth medium. The mitogenic part is supplied by serum, other biological fluids or a defined mixture of hormones and growth factors. Growth factors should not be used by cells as nutrients (Gospodarowicz and Moran 1976) and they should be active at very low concentrations. The distinction between nutrients and non-nutrients is particularly important when studying the effects of growth factors in vitro since the growth of cells in culture can be limited by a lack of certain nutrients, and the readdition of those nutrients can stimulate growth. However, in spite of the distinctiveness between nutrients and non-nutrients, there is much data that suggest that interactions between these two components are important in controlling normal cellular processes (Holley 1975; McKeehan 1982a; Rozengurt 1982).

(i) Nutritional Components

The medium formulated by Eagle (Eagle 1955a; 1955b) for mouse L cells and HeLa cells is considered adequate for the nutritional requirements of most normal diploid fibroblast cells as well as heteroploid lines in culture (Mellman 1971). It consists of 13 L-amino acids (arginine, cystine, glutamine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, tyrosine and valine) vitamins and cofactors (choline, folic acid, nicotinamide, pantothenate, pyridoxal, riboflavin and thiamine), D-glucose or one of several other hexoses (galactose, mannose, maltose) and certain ions in the form of salts (Na^+ , K^+ , Ca^{2+} , Mg^{2+} , Cl^- and HPO_4^{2-}); the medium also contains penicillin and streptomycin to control bacterial growth, and

phenol red as an indicator of pH changes. A variety of other formulations are currently used for various cell lines, but they are all modified forms of Eagle's medium. It has been noted (Eagle and Piez 1962) that additional nutrients are required when cells are grown below a critical level. These so-called "superior" media are recognised for their effects on the clonability of cells, which suggest that there are population-dependent requirements of cells in culture.

A balanced amino acid requirement for cells in culture has been specified by Eagle (1955a; 1955b). An altered balance between the amino acids in the culture medium has a marked impact on cell proliferation (Ham 1974). Of all the amino acids specified by Eagle, at least five are considered non-essential to the mammalian organism. The essential need of these five amino acids for cells in culture implies that certain specific enzymic pathways are shut off in cell cultures, or it may mean that there is a higher requirement for these amino acids in cell cultures as compared to the whole organism. The unusual requirements for certain amino acids in cell cultures may also be due to amino acid-destroying factors in cultures. In the case of arginine, Kihara and DeLaFor (1968) have noted that fetal calf serum contains significant heat-stable arginase activity which may destroy arginine in the medium; however, this may not be a significant factor since their results also show that sera that did not show arginase activity also needed arginine in the medium just as much. Another explanation may come from the possibility that mycoplasmal contamination may deplete the growth medium of certain amino acids as has been observed for arginine by Schimke and Barile (1964).

Of all the amino acids in the growth medium, glutamine is required at the highest concentration for most mammalian cells (Eagle 1955a; 1955b); in fact cell growth is rapidly arrested on removal or depletion of glutamine in the growth medium, and glutamine is the only amino acid that shows such rapid effect (during first cell cycle after withdrawal) among all the amino acids (Zetterberg and Engstrom 1981). LeJohn and coworkers have also reported that glutamine is the only amino acid whose withdrawal from the growth medium leads to an accumulation of a unique polyphosphate in mammalian cells (Goh et al 1977; LeJohn et al 1979). Glutamine is the amino acid in highest concentration in plasma (Scriver and Rozenberg 1973) and in many mammalian tissues (Hamilton 1945), yet the uptake of glutamine from the diet is not any higher than other amino acids. This suggests that significant glutamine synthesis takes place in mammalian cells. The amount of glutamine found in tissues is determined by a balance between distinct synthetic and degradative pathways. The major synthetic reactions are catalysed by glutamine synthetase (Iqbal and Ottoway 1970) whereas the degradative reactions are mainly catalysed by glutaminase, a deaminating enzyme (Hartman 1972), but glutamine is also involved in several other metabolic pathways (Patterson 1972). Glutamine synthetase has been shown to be highly regulated in many tissues (Tate and Meister 1971; Cook et al 1978). In a recent study using L6 mouse cells, Smith et al (1984) show that glutamine synthetase activity is regulated by concentrations of substrate, product, or a number of different hormones. Glutaminase activity, on the other hand, is not influenced by any of the above factors. One probable reason for the lack of regulation of glutaminase

is because the enzyme is restricted to the mitochondrial space (Smith et al 1984, unpublished results) and access of the enzyme by glutamine requires the uptake of glutamine by mitochondria (Joseph and Meijer 1981), a process which could itself be regulated. The basis for the high requirement of glutamine in cell cultures has been vigorously investigated. Glutamine is involved in a number of metabolic pathways including protein synthesis, de novo synthesis of nucleotides, as a respiratory fuel, synthesis of coenzymes, synthesis of other amino acids, and in several other aminating reactions (Kitos et al 1962; Patterson 1972; Kovacevic and Morris 1972; McKeehan 1982; Zielke et al 1976). Work by Zetterberg et al (1982) has shown that the requirement for glutamine for cell proliferation is limited to the interval that coincides with G₁ and early S of the cell cycle, which is consistent with the hypothesis that glutamine mainly controls cell proliferation by acting as a precursor to nucleosides which are used in de novo synthesis of nucleic acids. This has recently been extended by Engstrom and Zetterberg (1984) who have shown that adenine and adenosine and their metabolites hypoxanthine and inosine, can counteract the inhibitory effect of glutamine depletion in proliferating and serum-stimulated cells. Goh and LeJohn (1977) also show that the polyphosphate accumulation induced by glutamine starvation of cells was abolished by purines; pyrimidines were less effective. All these studies emphasize that the role of glutamine in growth control is most likely at the level of its involvement in de novo nucleic acid biosynthesis. However, the fact that pyrimidines (Goh and LeJohn 1977; Engstrom and Zetterberg 1984) and guanosine (Engstrom and Zetterberg

1984) are not effective at counteracting the effect of glutamine withdrawal shows that other mechanisms might be involved. One such mechanism that has been proposed is that glutamine and adenine exert their regulatory action at the cell surface (Engstrom and Zetterberg 1984); this is in line with the observation that adenine binds to specific cell surface receptors in a wide variety of cell types (Rozengurt 1982). Finally, another possible reason that may account for the requirement of a large supply of glutamine in growth media is the observation that changes in pH and temperature can influence the spontaneous decomposition of glutamine to pyrrolidone carboxylic acid and ammonia (Tritsch and Moore 1962).

Most of the vitamins and cofactors in Eagle's medium are involved in regulating the activities of various metabolic enzymes. They are all needed in trace amounts in the growth medium. Other vitamins added to other formulations of culture media may be involved in specific roles essential for cell growth. Ascorbic acid has been used in human diploid fibroblast cultures as a requirement for collagen synthesis which may be essential for cell attachment (Mellman 1971). Vitamin B₁₂ and biotin are also essential in certain cultured cells for methionine biosynthesis (Mangum et al 1969). The requirement for inositol is particularly interesting. Inositol was first described as a dietary essential in mammals and was classified as a vitamin (Wooley 1940). A requirement for exogenous inositol has been ascribed to many, but not all, mammalian cell lines in vitro (Eagle et al 1957; Furuya et al 1971); inositol is not included in Eagle's medium (Eagle 1955a; 1955b) probably because the cell lines he studied at the time did not require inositol for

growth. The classification of inositol as a vitamin has been questioned (Jackson and Shin 1982) on the grounds that, unlike other vitamins, it is required in relatively large quantities and that it has no known role as an enzyme prosthetic group or cofactor in intermediary metabolism. Rather, they suggest that inositol plays a role of growth factor as measured by their effect on plating efficiency and the growth curves of BHK21, Nil8 and mutant CHO-K1 cell lines. However, if we accept the definition of growth factor suggested by Gospodarowicz and Moran (1976), inositol cannot qualify as a growth factor because it is a building block of phosphatidyl-inositol, an essential membrane phospholipid; also, inositol starvation does not interfere with other cellular growth processes such as RNA and protein synthesis - the typical pleiotypic response elicited by growth factors (Jackson and Shin 1982). These two facts would qualify inositol as a nutrient rather than a growth factor. Obviously, more work is needed to clarify the role of inositol in cultures, as well as the varying responses of cells to inositol starvation.

D-glucose or any of the other hexoses included in culture media is used primarily as an energy source. Glycolysis and aerobic respiration, utilising glucose as substrate, are fundamental and essential to survival and growth of cells. In a study comparing the utilization of glucose, mannose or galactose, Cristofalo and Kritchevsky (1965) showed that in WI-38 cells, glucose and mannose were utilized similarly, whereas fructose and galactose were used somewhat slower; it is not reported whether the difference can be accounted for by any uptake differences. Eagle et al (1958), however, noted earlier in nine

different mammalian cell lines that the rates of utilization of all four hexoses were comparable. Culture conditions may account for some of the differences in utilization of the hexose. For example, growth of WI-38 cells occurred with glucose in the pH range of 6.8 - 7.4 and with galactose only at pH 6.4 - 6.8; addition of 0.1% glucose or 0.05% pyruvate permitted growth with galactose at pHs above 7.0 (Baugh et al 1967). The critical factor in this pH control is not known. Glucose is the preferred sugar during rapid proliferative phase of growth of WI-38 cells in culture (Kruse and Miedema 1965) but galactose becomes superior to glucose during the post-confluent phase of cell growth (Baugh and Tytell 1967); the reason for this is not known. Some of the differences in the utilization rates of the various hexoses may be accounted for by enzymatic differences. Human diploid fibroblast cells have much lower galactokinase activity than hexokinase (Mellman 1971, unpublished data). Besides glycolysis and respiration, glucose and other hexoses are involved in other metabolic pathways in cultured cells.

The physiological importance of some of the ions in mammalian tissues was first recognized by Ringer in 1882. The available data indicate that most physiological processes require some ions as cofactors. The monovalent cations Na^+ , K^+ and H^+ are involved in maintaining membrane electrical potentials, plasma membrane integrity and as effectors of growth factors and other mitogenic stimuli. The divalent cations Ca^{2+} and Mg^{2+} perform similar functions, but in addition they are more directly involved in control of cell proliferation (Rubin et al 1978; 1979). At certain optimal

concentrations, calcium triggers cascades of enzyme activations and structural changes which, among other things, end up in DNA synthesis and cell division (Means et al 1982). Calcium withdrawal from the growth medium results in cessation of growth in G_0/G_1 (Boynton et al 1976; Ristow and Paul 1979) and near the G_1/S transition (Whitfield 1982) of the cell cycle. The other phases of the cell cycle are unaffected by the calcium reduction but the daughter cells get arrested in late G_1 phase if the calcium withdrawal is continued (Whitfield 1982). A more direct role for calcium in control of cell proliferation is the observation that supranormal concentrations of calcium is mitogenic for quiescent 3T3 cells (Dulbecco and Elkington 1975). It has been suggested that this mitogenic effect is mediated by formation of extracellular insoluble complexes of Ca^{2+} , PP_i and HPO_4^{2-} (Rubin and Sanui 1977), which stimulate sugar uptake and thymidine incorporation into 3T3 cells. Another aspect of the control of cell proliferation by calcium is its role in the cAMP response (Rasmussen 1970). Finally, two sets of data have suggested that there is an interplay between calcium and magnesium in growth control: firstly, the inhibition of DNA synthesis in 3T3 cells by calcium starvation is prevented by high concentrations of magnesium (Rubin et al 1979); and secondly, calcium starvation leads to a decreased intracellular magnesium concentration without much effect on intracellular calcium concentration (Moscatelli et al 1979). These data suggest that magnesium is the mediator of the mitogenic effects of calcium, which means that calcium exerts its growth regulatory effects by modulating the availability of magnesium ions. Indeed several reactions following mitogenic stimulation have

been found to depend on the intracellular magnesium concentration (Rubin et al 1978). Magnesium is required as a cofactor by more intracellular enzymes than any other inorganic cation (Mahler 1961). It activates several intracellular enzymes including those involved in key phosphate transfer reactions, the Na^+/K^+ transport ATPase, and the Ca^{2+} transport ATPase (Mahler 1961; Wacker 1969). However, unlike calcium, magnesium is not a suitable sensitive cascade triggerer of cellular events since the level of free magnesium lacks specific binding proteins equivalent to calmodulin and troponin C for calcium. It seems rather, that magnesium exerts its effects via the various enzymes with which it interacts as cofactor. The role of magnesium in cell proliferation and transformation has been reviewed (Sanui and Rubin 1982). Phosphate is another ion that is involved in some key aspects of cell metabolism, such as the numerous phosphorylations/dephosphorylations that occur in cells and which in turn regulate several metabolic processes. Phosphate ions are also more directly involved in the regulation of cellular proliferation in vitro, as Holley and Kiernan (1979) have shown that 3T3 cells can be arrested in G_1 (or G_0) phase of the cell cycle by drastically reducing phosphate concentration in the culture medium, and that readdition of phosphate results in reinitiation of DNA synthesis. This contradicts an earlier observation that the rapid increase in phosphate uptake which follows mitogenic stimulation by serum is not necessary for the subsequent initiation of DNA synthesis and that an increase in the intracellular pool of phosphate is not a prerequisite for initiation of proliferation (Greenburg et al 1977). A more recent report (Engstrom et al 1982) does affirm that phosphate may indeed be

important in initiation of DNA synthesis after alkaline treatment, a mitogenic condition (Zetterberg and Engstrom 1981). The growth-stimulatory role of phosphate still remains to be elucidated.

Although lipids are part of the nutritional needs of cells in culture, they are normally not part of the definable components of the growth medium. Rather, lipids are supplied by the serum component. In a study by Bettger and Ham (1982), several transformed, highly adapted cell lines could multiply for extended periods in media devoid of lipids whereas the animals from which these cells were derived required fatty acids as well as fat-soluble vitamins. Whether this is due to the cell culture condition per se or to the transformation feature is not quite clear. Holmes et al (1969) have identified a cholesterol requirement for human diploid fibroblasts derived from both cartilage and skin. The cells did not survive when the serum was delipidated. Cholesterol synthesis from acetate was not detectable in the diploid fibroblast cell lines, whereas it was found functional in heteroploid cell lines. Other workers however, did find some cholesterol formation in WI-38 cells but estimated that lipid production was not sufficient to sustain growth (Howard and Kritchevsky 1969). Cells in culture, nevertheless, do show their need for exogenous lipid supply since they readily utilize the lipid present in serum lipoproteins in preference to de novo biosynthesis, and many cell lines show a growth response to supplemented lipid when serum supplied lipids are limiting or absent (Spector et al 1981).

(ii) Mitogenic Components

In addition to the basal nutrient mixture of amino acids, sugars, vitamins and cofactors, and salts, nearly all mammalian cells in culture require a supplement of poorly-defined biological fluids or extracts for proliferation; this requirement is somewhat reduced for many transformed cells. The most commonly used biological fluid is serum. Serum provides not only the growth-stimulating factors but also nutritional and hormonal components (Temin et al 1972; Sato 1975; Barnes and Sato 1980), binding proteins that stabilize, transport or somehow modulate the activity of nutrients and hormones in the culture medium (Crichton 1975; Kaufmann et al 1977), basement membrane components such as fibronectin (Yamada and Olden 1978), and attachment factors (Knox and Griffiths 1979; Hewith et al 1980). Fetuin is the first macromolecular serum fraction that was isolated (Lieberman and Ove 1957; Fisher et al 1958) and it was shown to be essential for promoting cell attachment and spreading on tissue culture substrata. When purified fetuin is added to cultures that attach poorly to plates, the cells attach much better and growth is improved (Brunet et al 1981; 1982).

Efforts to purify the growth-stimulating factors from serum have been slow due to several reasons: firstly, serum contains very low concentrations of the growth factors; secondly, serum is a complex mixture of several growth factors which are relatively similar with regard to charge and size, and hence purification by conventional techniques is difficult; thirdly, inactivity resulting from disassociation of growth factor from carrier molecule, may occur during

the purification process; and finally, assays for purified material may prove confusing and inaccurate since many of the factors are synergistic in their actions. In spite of these difficulties, several growth factors in serum have been purified and characterized biologically and chemically (for reviews see Gospodarowicz and Moran 1976; Barnes and Sato 1980; Ross et al 1982; Heldin and Westermark 1984). The growth factors in serum are distinguishable by several characteristics such as molecular weight and structure, receptor specificity, target cell specificity, immunological reactivity, and mobility in gels.

One problem in identifying growth factors in serum is the need to demonstrate that a purified sample of the material has growth-promoting effects in vitro. Gospodarowicz and Moran (1976) have proposed the following hierarchy of criteria for the evaluation of growth factors in vitro: the putative growth factor should induce, 1) initiation of DNA synthesis in cells, 2) one cycle of division in confluent cultures, 3) several division cycles in sparse and confluent cultures, and 4) clonal growth. Most of the serum growth factors identified so far meet only the first criterion but not the others (Gospodarowicz and Moran 1976). However, some non-serum growth factors, notably multiplication stimulating factor (MSF) obtained from conditioned medium of rat liver cells (Dulak and Temin 1973), epidermal growth factor (EGF) purified from mouse salivary glands (Cohen 1962), fibroblast growth factor (FGF) from bovine pituitary (Gospodarowicz 1975), and transforming growth factors (TGFs) from transformed cells (DeLarco and Todaro 1978; Marquardt et al 1983), have been shown to fulfil at least three, if not all, of the above criteria when tested on a variety of cell types.

The most potent growth-stimulating factor in serum is derived from platelets. The initial evidence for this came from the observation that chicken plasma was less effective than chicken serum for promoting the growth of chicken muscle fibroblasts (Balk 1971). This has been confirmed with many other systems (reviewed by Ross and Vogel 1978; Balk 1980). Subsequently, the growth-promoting factor, called cationic platelet-derived growth factor (PDGF) has been purified and characterized (Antoniades et al 1979; Antoniades 1981). Other platelet-derived growth factors such as two anionic growth factors (Heldin et al 1977) connective tissue-activating peptide (Castor et al 1979), an uncharacterized growth factor distinct from cationic PDGF (Eastman and Sirbasku 1980) and platelet-derived transforming growth factors (Childs et al 1982) have also been identified. A growth-promoting α -globulin (GPAG), which greatly stimulates DNA, RNA and protein synthesis in L cells and human lung fibroblasts has been identified in serum; GPAG is taken into cells by pinocytosis (Michl and Spruna 1975). GPAG determines whether or not cells will continue to divide (Michl 1977) probably through a specific growth-enhancing protein termed "pleiotropin", which enters cells by pinocytosis (Michl and Spruna 1976). It is of interest to note that PDGF has been shown to stimulate pinocytosis and cellular proliferation in Swiss 3T3 cells (Davies and Ross 1978); this suggests that the rate of pinocytosis may be coupled to growth regulation mediated by growth factors. Serum also contains growth factors not derived from platelets (for review see Gospodarowicz and Moran 1976), but since these factors are also present in plasma, their growth-promoting activity is at best not significant by

themselves for cells in culture, considering the inferior nature of plasma relative to serum in cell culture.

The mechanism(s) of action of growth factors has been studied. The first step in the action of many growth factors is to bind specific receptors on the cell surface. Subsequent to this, two main lines of events are initiated in the cell: firstly, specific phosphorylations/dephosphorylations of cellular proteins occur which may or may not involve CAMP (discussed in a later section). Secondly, there is serum or mitogen-induced modification in the rates of many activities (coordinate response) including uptake of all classes of nutrients, protein synthesis and nucleic acid synthesis (Rubin and Sanui 1979). Of particular interest is the increase in ionic fluxes following mitogenic stimulation. There are several reports indicating that a primary and early effect of serum and certain growth-promoting factors is to increase Na^+ entry into cells (Hulser and Frank 1971; Rozengurt 1982; Leffert 1982). This effect has been noted in a wide variety of cell types (reviewed by Rozengurt 1982). Villereal and Owen (1982) have further reported that, in human fibroblasts, serum actually activates a normally inoperative Na^+ influx system rather than merely increasing the turnover rate of Na^+ transport pathways already operative in serum-deprived cells. From studies using calcium ionophores, intracellular calcium antagonists, and calmodulin antagonists, it is deduced that an early step in the serum-induced influx of Na^+ is an elevation of intracellular free Ca^{2+} concentration, thus suggesting that Ca^{2+} may be acting as a second messenger in this process (Villereal and Owen 1982). The origin of the calcium is both intracellular and

extracellular. Na^+ influx is thus coupled with movement of other ions. A $\text{Na}^+ - \text{Ca}^{2+}$ exchange system is also present across the mitochondrial membrane of many cell types (Carafoli and Crompton 1978; Al-Shaikhally et al 1979); this may be important in the role Ca^{2+} plays in the Na^+ influx process. Also, Na^+ movement is coupled to movement of K^+ by activation of the $\text{Na}^+ - \text{K}^+$ pump after mitogenic stimulation (Rozengurt 1982). Smith and Rozengurt (1978) showed that the increased pump activity in 3T3 cells is caused by a prior increase in net Na^+ influx. Finally, Na^+ entry is coupled to proton exit in many cell lines (Moolenaar et al 1981; Schuldiner and Rozengurt 1982). The $\text{Na}^+ - \text{H}^+$ antiport system is potentially important since it may allow a maintenance of alkaline intracellular pH, a condition which has been shown to be mitogenic (Zetterberg and Engstrom 1981).

It follows from the above discussion that there is a complex interaction between the mitogenic factors and the nutritional factors of the growth medium. Indeed, McKeehan (1982) has classified nutrients according to their kinetic relationships with serum growth factors.

(b) Control of Growth by pH

Tissue culture conditions are designed to maintain the pH of the growth medium within narrow limits: the medium has a buffering capacity, and a continuous mixture of air and CO_2 is passed through to help keep the pH constant. The importance of the extracellular pH in control of growth of cells has been recognized for some time (Fischer 1925). Various mammalian cell types have different pH optima for normal cell growth, and the final population density obtained depends on pH (MacKenzie et al 1961; Ceccarini and Eagle 1971); these studies have

also shown that cells are more tolerant of acid pH than of alkaline pH. Studies by Zetterberg and Engstrom (1981) and Engstrom et al (1982) have revealed that brief alkaline pH treatment of quiescent cells is mitogenic. The mitogenic effect depends on the population density: with 3T3 cells, the competency to respond decreases when confluency is attained. (There is no data available on the alkaline stimulation of confluent, contact-uninhibited, highly transformed cells in order to assess the role of normal contact inhibition of cells in this effect.) The time course for entrance of cells into S phase from the state of quiescence is identical for both alkaline stimulation and serum stimulation of growth, implying that both types of stimuli activate an equally long chain of reactions preceding initiation of DNA synthesis. The difference between the two types of stimulatory situations, however, is that, in contrast to growth stimulation induced by serum, alkaline treatment only has a temporary effect since cells only progress through one cell division cycle after which they re-enter quiescence (Engstrom et al 1982), much like the stimulatory effect induced by proteolytic enzymes (Burger 1970).

At least two different modes of action of alkaline pH in growth stimulation have been proposed: (1) alkaline pH may act directly on the cell surface, similar to the observation that proteolytic enzymes stimulate quiescent cells to undergo one cell cycle by a direct action on the cell surface (Stoker 1973). One mechanism by which this might occur is by formation of growth-stimulating complexes of insoluble $\text{Ca}_3(\text{PO}_4)_2$ at the cell surface, and alkali pH promotes precipitation of such complexes (Rubin and Sanui, 1977). Another possible mechanism of

action of alkaline pH at cell surface is by extraction of plasma membrane proteins. This has been demonstrated in bacteria (Rothfield 1971) and isolated endoplasmic reticulum (Glaumann 1970), and growth-inhibitory proteins have been extracted from quiescent fibroblasts by urea (Natrav and Datta 1978). Furthermore, alkaline pH can exert its effect on cell surface by altering transmembrane transport properties. The permeability of chicken embryo cells to 2-deoxyglucose is altered by changes in medium pH (Rubin 1971). (2) Extracellular alkaline pH may act primarily at an intracellular site(s). Most mammalian cells vary their internal pH as a function of extracellular pH (Gillies 1981). An increase of intracellular pH could activate pleiotropic second messengers such as cyclic nucleotides and ions, which in turn could activate intracellular enzymes that are pH-dependent (example, phosphorylations of glycolytic enzymes) (Gillies 1981, for review).

(c) Population Density and Geometric Factors

Under standard conditions of cultivation, the growth rate of cells decreases when a critical cell density, characteristic for each cell type, is reached. This phenomenon was first described in the 3T3 cell line (Todaro and Green 1963) and was called density-dependent inhibition of growth (Stoker and Rubin 1967). Dulbecco and Elkington (1973) have shown that for epithelial cells in culture, the dish surface restriction is the predominant factor stopping growth, probably related to the in vivo role of this cell type in basement membrane formation where they must form a monolayer. On the other hand, for fibroblast cells exhaustion of medium components, particularly serum, is more limiting

than restriction of growth space. In this latter cell type, however, available surface also becomes important at high cell densities.

Three major hypotheses have been proposed to account for density-dependent inhibition of growth. The first hypothesis suggests that cell-cell contact is a necessary requirement for growth inhibition (Todaro et al 1964; 1965) as evidenced by the observation that confluent normal cells show little or no growth, whereas subconfluent cultures proliferate rapidly. This hypothesis is also supported by wound-healing experiments (Todaro et al 1965; Dulbecco 1970). The second hypothesis suggests that depletion of growth factors in the bulk medium (Holley and Kiernan 1968) or in a microdiffusion barrier surrounding the cells (Stoker 1973) results in growth inhibition. Support for this comes from their observation that the final density of 3T3 cells in a confluent culture is directly proportional to the concentration of serum in the medium. Also, Smets (1971) has shown that used medium could be restored by dialysis against fresh medium and by addition of low molecular weight constituents. The third hypothesis proposes that soluble or membrane-bound growth inhibitors released into the medium accumulate to a sufficiently high concentration to act on cells (Harel et al 1978). Such inhibitory factors have been isolated and characterized as glycoproteins in a number of cell culture systems (Wang et al 1982; Harel et al 1983). Harel et al (1984) have recently shown that Rous Sarcoma virus (RSV) - transformed 3T3 cells produce the growth inhibitory substance, but they are much less sensitive to the inhibitor than their normal counterparts. The growth-inhibitory glycoproteins may be the "chalcones" discussed in a short review by

Potter (1983); chalcones have characteristics expected of surface active macromolecules required for normal cell-cell interactions leading to density-dependent quiescence as presented for the contact receptor-chalone hypothesis of Allred (1978). It should be emphasized that the three hypotheses presented above are not mutually exclusive. Indeed growth factors may interact with growth-inhibitory molecules either through competition or down regulation of cell surface receptors, or through generation of antagonistic intracellular biochemical signals. In addition, it is possible that the same molecule can exert its effects anchored on the cell surface and released into the medium. Whatever the mechanism, density-dependent growth inhibition seems to be an important means of control of normal cell proliferation.

The influence of geometry on control of cell growth has been studied by Folkman and coworkers (Folkman and Greenspan 1975; Folkman and Moscona 1978). Two geometrical factors seem to be important in growth control: the geometry of the individual cell, and the geometry of the cell population. According to Folkman and coworkers, the geometry of the individual cells becomes important when one compares growth of normal cells in suspension to that of a monolayer. While other workers (Stoker et al 1968) think that it is the anchorage phenomenon that is the key element in this kind of comparison, Folkman and coworkers have the view that it is the difference in cell shape under the two conditions that is critical. Normal cells in suspension are rounded or spherical whereas cells attached to plastic or glass surfaces are flat and spread, and this correlates with the fact that normal cells do not grow in suspension but they grow when attached to

glass or plastic. Folkman and Greenspan (1975) show that normal diploid cells whose growth is inhibited in suspension culture, can escape this inhibition if they are allowed to stretch out on tiny glass beads or glass fibrils suspended in soft agar or methylcellulose. Furthermore, by using the plastic of soft contact lens which allows cell attachment but not cell spreading, Folkman and Moscona (1978) varied the adhesiveness of tissue culture dishes by applying different concentrations of the plastic; with this experiment they showed that a correlation exists between cell shape and DNA synthesis and growth. The flatter the cells, the more they grew.

Transformed cells have escaped the restriction of cell geometry on growth since transformed cells can proliferate equally well in the flat state or the spherical state. However, when transformed cells grow in agar suspension, they are subject to the second type of geometrical control, namely the geometrical restriction on the cell population. The growth of transformed cells attached on a flat surface occurs in only two dimensions, in which case they will grow indefinitely provided they have sufficient space and nutrients. Hence in two-dimensional growth of transformed cells, there is no self control. But when transformed cells are grown in agar, growth is in three dimension, and under this condition there is self control since colonies can only attain a certain maximum size containing a maximum number of cells irrespective of the availability of growth space and nutrients. This level of geometric control can be partly understood in terms of diffusion gradients, but the geometric control on individual cells cannot be explained on that basis.

C. Cell Cycle Pattern of Cells in Culture

The life cycle of normal eukaryotic cells consists of a period of DNA replication (S) separated from mitosis (M) by two intervening growth phases called G_1 and G_2 ; G_1 is the prereplicative period and G_2 is the postreplicative period (Howard and Pelc, 1951). The cell cycle of mammalian cells can also be divided into a chromosomal phase (which spans S, G_2 , and M) and a growth phase consisting of G_1 (Mitchison 1971; Stancel et al 1981). On physiological basis, the cell cycle can be divided into two areas: cell cycle initiation and cell cycle progression (Hochhauser et al 1981). While these divisions have been demonstrated in a wide variety of cell types, it must not be regarded as invariable for all cell types. An examination of the available data shows that the boundaries between the cell cycle phases are not absolute and the duration of the phases can vary considerably among different types of cells (for reviews, see Prescott 1976; Hochhauser et al 1981; Yanishevsky and Stein 1981).

Traditionally, the cell cycle is presumed to start in G_1 , just after the creation of two new cells by mitosis. The G_1 period is assumed to contain a succession of events that lead to the initiation of DNA replication. An early clue to the importance of G_1 came when it was observed that G_1 is far more variable in length than S, G_2 , or M (Sisken and Kinosita 1961). Variability in G_1 is observed under conditions of partial inhibition of protein synthesis and changes in culture conditions such as serum or nutrient deprivation and high cell density. G_1 variability accounts for alterations of cycle times of cell populations, synchrony decay, and for variations in cycle times among

different cell types or in the same cell type growing in vivo or in vitro (Prescott 1976; Hochhauser et al 1981). It is suggested that variableness in the mass of individual cells at the beginning of the G_1 period, due to the failure of cytokinesis to divide the cells into two equal-sized daughters, accounts for the variability of G_1 among individual cells of a given population (Killander and Zetterberg 1965); this assumes that exit of the cells from G_1 phase into S phase is dependent upon the attainment by the cell of a crucial mass or some other specific component of growth that increases in parallel with cell mass. Any hypothesis designed to explain the significance of the G_1 period must also take into account those situations in which the G_1 period is absent. G_1 -less cycles have been observed in unicellular organisms, in certain normal and tumorous cells from multicellular organisms, and in some cultured cells (Prescott 1976). Within multicellular organisms, G_1 appears to be absent in cells that are engaged in very rapid proliferation, such as cells in the cleavage stages of embryos (Gamow and Prescott 1970), and normal adult bone marrow cells of the erythrocyte series also lack G_1 (Alpen and Johnston 1967). Two cultured cell lines without a G_1 have also been described (Robbins and Scharff 1967; Liskay 1977). The observation of G_1 -less cells has raised questions about the importance of that phase in the life cycle of cells, especially with cells growing in rich media. There are suggestions that G_1 is merely an interruption between two chromosomal phases when cell growth fails to keep pace with the chromosomal phase (Mitchison 1971; Stancel et al 1981).

The S phase is the period during which DNA replication occurs.

Numerous observations with polynucleated cells have shown that cytoplasmic factors are involved in the initiation of DNA synthesis (Church 1967; Graves 1972); cell fusion studies show that such cytoplasmic factors are not species-specific (Johnson and Harris 1967). These studies also show that the S phase, once initiated, is carried out independently in each nucleus both for heterokaryons and homokaryons; this asynchronous progression, and hence termination, of S phase has sometimes erroneously led to the interpretation that the initiation event is not synchronous in different nuclei. Replication of mitochondrial DNA and nuclear DNA are not synchronous with each other; in fact replication of individual mitochondria within the same cell is asynchronous (Meyer and Ris 1966). Also, whereas nuclear DNA synthesis occurs only in S phase, mitochondrial DNA synthesis occurs throughout the cell cycle (Bosmann 1971).

G_2 is the period of preparation for cell division, but there are instances where G_2 is absent (Gaulden 1956; Lawrence 1968). The position of transition between G_2 and M cannot be identified precisely since that transition is based on observation of chromosome condensation and condensation begins sometime before it can be identified microscopically. The transition is not marked by any identifiable change in cell metabolism, such as RNA and protein synthesis (Prescott 1976).

(a) Growth Control Points in the Cell Cycle

Under unfavourable growth conditions, cycling normal cells can arrest at two different points in the cell cycle: in late G_1 and G_2 (Gelfant 1981). The G_1 control point(s) seem to be very important

since cycling cells which are induced to enter quiescence by nutrient deprivation or other means, whether in vivo or in vitro have a G_1 DNA content, and upon re-entering the cycle, replicate their DNA before entering mitosis (Baserga 1976; Pardee et al 1978). Pardee (1974) has suggested that the provision of a control point sensitive to environmental conditions in the cell cycle could be an evolutionary adaptation to allow cells to sample their environment and decide whether or not to proceed through the cell cycle.

Based on the kinetics with which G_1 -blocked cells resumed proliferation, it has been proposed by Pardee (1974) that a single major regulatory event occurs in G_1 that permits a cell to complete another round of the cell cycle. Pardee introduced the term restriction, or R, point to describe the block point in G_1 . According to the R point hypothesis, G_1 cells prepare for DNA synthesis while continuously monitoring their environment; if conditions are favourable, the cells pass through the R point and become committed to complete the remainder of the cell cycle, but if conditions are not favourable they cease proliferation at the R point. The R point has been temporally mapped in mouse 3T3 cells at 2 to 3 hours prior to the onset of DNA synthesis (Yen and Pardee 1978). There is speculation that the R point, including the events that prepare cells to traverse the R point, constitute the cell cycle initiation stage, whereas the rest of the cycle constitute cell cycle progression. Yanishevsky and Stein (1981) have critically reviewed the evidences for and against the R point hypothesis in mammalian cells.

The mechanism of execution of R point in cycling cells has been

studied by several groups and the available data show that many different mechanisms may be operative. One proposed mechanism is that passage through the R point is dependent upon the cells attaining a critical size (Killander and Zetterberg 1965). A slightly modified version is that transit through the R point depends upon the ratio of cytoplasmic size to nuclear size (Frazer 1973), which implies the importance of cytoplasm in the initiation of DNA synthesis. Another way of looking at the relationship between cell growth and passage through the R point is to consider the activities that contribute to cell growth. Attention has been focussed on protein synthesis because of the abundance of proteins in the cell. The rate of cellular proliferation in several different mammalian cell types has been shown to be proportional to the rate of net protein accumulation (Robinson et al 1976; Baxter and Stanners 1978). As expected from this relationship, cells in stationary phase show little or no net accumulation of protein (Stanners et al 1979); also, decreasing the rate of protein accumulation in V79-8 cells (a G_1 -less derivative of a Chinese hamster line, isolated by Robbins and Scharff 1967) delays entry into S phase thereby creating a G_1 interval (Liskay et al 1980). Whether the requirement is for an increase in total protein synthesis or for synthesis of specific protein(s) has not yet been definitely clarified. Some reports indicate that specific proteins may be involved in the transit through the R point. Firstly, Chafouleas and coworkers have reported that increases in the intracellular concentration of calmodulin are coupled to the G_1/S transition of the cell cycle and that inhibition of the action of this protein by the anticalmodulin drug W13 inhibits

progression into S phase (Chafouleas and Means 1982; Chafouleas et al 1982). At least two specific functions which may be important at the G_1/S border that are mediated by calmodulin have been proposed: regulation of the cytoskeleton; and regulation of protein (especially histone) phosphorylation (Whitfield et al 1979; Tupper et al 1980; Chafouleas and Means 1982). Secondly, it has been proposed that the passage of cells through the R point is controlled by accumulation of a labile regulatory (R) protein, whose synthesis starts close to the beginning of the G_1 period and is maximal at the R point (Rossow et al 1979; Campisi et al 1982). The R protein acquires greater stability in transformed cells, which is consistent with the greater proliferative capacity of transformed cells as compared to their normal counterparts. A 53K protein, originally found bound to tumor (T) antigen in SV40-transformed cells (Smith et al 1979) and later detected in a variety of cells transformed by other means (Linzer and Levine 1979; DeLeo et al 1979), has been found to be identical to the R protein (Campisi et al 1982). In normal cells, high amounts are detected in rapidly proliferating cultures whereas low amounts are found in quiescent cultures (Dippold et al 1981). The protein is also found to be very stable in an SV40-transformed 3T3 derivative and quite labile in normal 3T3 cells (Oren et al 1981).

The above positive control mechanisms are supported by evidence from cell fusion studies that S phase cells can induce DNA synthesis in G_1 cells (Rao and Johnson 1970). Also, fusion of cells in early, middle and late G_1 to each other in various combinations results in accelerated entry into S phase of both nuclei in binucleates, and the acceleration

contributed by late G_1 nuclei is the greatest whereas that contributed by early G_1 nuclei is the least (Rao et al 1977). These reports indicate that cells accumulate an inducer as they go through G_1 .

Negative control mechanisms of cell proliferation have also been proposed (Pritchard et al 1969). This appears to be the mechanism operative for cells made quiescent due to senescence or terminal differentiation (Norwood et al 1974). It has been proposed that, in vivo, chalcones may play a role in acting as negative feedback inhibitors of tissue proliferation (Houck and Hennings 1973). This implies that tumour cells may fail to produce chalcones, or else that they have a decreased sensitivity to G_1 arrest by chalcones. The inhibitory molecules associated with density-dependent growth regulation (discussed earlier) may be important here.

In some instances cycling cells become arrested in G_2 (Gelfant 1977; Choie and Richter 1978), but generally, these G_2 -arrested cells account for only a small proportion of the quiescent population. G_2 arrest is more prevalent in plant cells (Rost 1977).

In an adult animal, most of the body cells remain viable for extended periods of time in the quiescent state. Even though these cells may not ordinarily reproduce in vivo, they can be stimulated to reproduce in culture. Kinetic studies have shown that these cells are held quiescent in early G_1 , and this was first termed G_0 by Lajtha (1963). Based on biochemical and kinetic studies, this type of G_1 block is distinct from the late G_1 block induced temporarily in cycling cells subjected to suboptimal growth conditions (reviewed by Yanishevsky and Stein 1981; Hochhauser et al 1981). In some cases G_0 -blocked cells

function as a reserve cell population that can enter the proliferating pool when they are needed (Gavosto and Pileri 1971). The balance between the proliferating and non-proliferating subpopulations is an important homeostatic mechanism for normal development as well as for pathological conditions such as the response to cellular injury and tumorigenesis.

(b) Ultrastructural and Biochemical Changes During the Cell Cycle

Ultrastructural and biochemical changes occurring during the cell cycle has been extensively reviewed by Hochhauser et al (1981). The ultrastructural changes are products, often the goals, of underlying biochemical changes.

The cell surface undergoes changes during the cell cycle that are temporally related to intracellular events (Porter et al 1973). Morphological and chemical changes on the cell surface are particularly apparent during mitosis. Interphase normal cells contain a prominent surface glycoprotein of molecular weight 250K, variously known as fibronectin, LETS (large, external, transformation-sensitive), CSP (cell surface protein), and a variety of lesser used names (reviewed by Hynes 1976); this protein is less accessible during mitosis and is absent or inaccessible in transformed cells (Hynes 1976). Fibronectin is involved in mediating the adherence of the cell to its substratum (Grinnell et al 1977) and its addition to cell cultures can partially restore normal morphology to some transformed cells (Olden et al 1979). Several other changes in surface proteins, glycoproteins, and glycolipids have been described (Hynes 1976), but their functional significance are not clearly known.

Although certain enzyme activities fluctuate with the cell cycle, synthesis of most high abundance proteins is not regulated as a function of cell cycle position. Milcarek and Zahn (1978) revealed very few cell cycle-dependent changes among the more than 300 HeLa cell polypeptides resolved by two-dimensional gel electrophoresis. One group of proteins that is cell cycle-dependent are the histones. Studies show that histone synthesis begins with the onset of DNA synthesis (Borum 1975; Pederson 1976). However, a later study by Groppi and Coffino (1980) disproved this and showed that histone synthesis occurs at equivalent rates in G_1 and S phases. Histone protein modifications are cell cycle-dependent. Methylation occurs in late S and G_2 (Tidwell et al 1968), whereas histone phosphorylation patterns during the cell cycle are different for the different kinds of histones (reviewed by Hochhauser et al 1981). Nonhistone chromosomal proteins also undergo shifts in synthesis and modification in a cell cycle-dependent fashion (Hochhauser et al 1981).

Changes in the activities of many enzymes have been measured in relation to the cell cycle. In many cases the changes have been shown to be due to degradation and synthesis of the particular enzyme (rather than activation or inactivation of existing enzyme molecules). DNA metabolic enzymes are synthesised in G_1 whereas enzymes essential for mitosis are synthesised in G_2 . The patterns of enzyme synthesis in relation to the cell cycle have been reviewed (Mitchison 1969; Prescott 1976).

Synthesis of all RNA stops during mitosis except for the synthesis of some 4S and 5S RNA (Klevecz and Forrest 1977). The rate of synthesis

of total RNA increases during interphase, but the increase is not restricted to one point in the cell cycle. All the major classes of RNA are synthesised continuously throughout interphase (Prescott 1976).

In conclusion, cell cycle phases are components of the cause and effect continuum of gene expressions that underlie the progression of the cycle. Cell cycle continuity is based on the sequential transcription and translation of sets of genes that are held in temporal order by the activation of one set of cell cycle genes by the function of an antecedently activated set of genes. To understand the regulation of cell proliferation and the loss of that regulation requires detailed knowledge of the molecular events that underlie the G_1 period, particularly those G_1 events concerned with arrest of cells. The use of cell cycle mutants may prove invaluable in such studies.

D. Cellular Senescence and Cell Transformation

(a) Senescence of Normal Cells in Culture

In 1957 Swim and Parker reported a limited life span for human embryonic and adult tissue fibroblasts in culture. Hayflick and Moorhead (1961), in a more extensive study, showed that cells from a variety of human tissues proliferated for variable periods of time and died. Later, Hayflick (1965) proposed that the limited replicative potential of human cells in vitro was an expression of senescence at the cellular level. In the same study, he showed that when normal diploid cells were frozen at any population doubling level and later recultured, the cells continued to divide until they reached the average total number of doublings characteristic of that line, thus indicating that

senescence in diploid cells in vitro is intrinsic to the cells and precisely timed. These observations have since been confirmed several times and reviewed (Cristofalo and Stanulis-Praeger 1982; Smith and Lincoln 1984).

There are three phases in the growth curve of normal diploid cell populations in culture (Hayflick 1965; Hayakawa 1969). Phase I starts immediately after cells are plated. In phase I, there is a poorly-defined lag period during which the rate of increase of cell population is very slow. Whether this is due to longer cell cycle times of individual cells in the population or to a reduced fraction of cycling cells, or both, is not known, but it may be partly related to the fact that normal cells often need to be anchored to their substratum before they can divide (Stoker et al 1968). Phase II is a period of rapid cell proliferation. The doubling time during phase II is constant (Hayflick 1965; Hayakawa 1969) which implies that the rapid increase in cell population during this period is essentially due to a higher fraction of cells that go through the cell cycle. The time period of phase II depends on the density of the cell inoculum. Phase III is a period of slower cell proliferation and a sharp decrease in the proportion of cells in the culture that synthesise DNA. Hayflick (1965) has proposed that phase III represents an in vitro model of senescence or aging. Senescent cells are in phase III, and therefore, most of the discussions in this section are concerned with cell populations in this phase of growth.

A fundamental question in the study of in vitro senescence is whether the life span of cultured diploid cells reflects a genetically

determined number of population doublings or whether it is a function of time spent in culture (chronologic time). The evidence by Hayflick (1965) involving freezing and reculturing of cells, together with several other reports (Cristofalo et al 1979; Nielsen and Ryan 1981) all indicate that cumulative population doublings are more important than chronologic time. However, other reports suggest otherwise (e.g. McHale et al 1971). During cellular aging, the cell cycle time increases while the fraction of cells participating in DNA synthesis decreases (Macieira-Coelho et al 1966).

Change in cellular morphology with age has been well documented. With normal cells, cell diameters and size heterogeneity increase with age; in contrast, cells of permanent lines are small, unimodal and do not change in size with age (Simons 1967). The correlation between size and ability to divide has generated contradictory results (Cristofalo and Stanulis-Praeger 1982; Mitsui et al 1981). Hence it seems that the results obtained depends on the experimental method used to measure cell growth. Nuclear size increases in parallel with increase in cell size as passage levels increase (Mitsui and Schneider 1976). Multinucleation (mostly binucleates) increase with population doubling (Kaji and Matsuo 1978).

Studies on changes in cell surface structure and function with in vitro aging has been reported (reviewed by Macieira-Colho 1983). Fibronectin is present in equal amounts on cells regardless of age (Courtois and Hughes 1976), but fibronectin released by late passage human diploid fibroblasts does not support normal cell-substratum interactions (Chandrasekhor and Millis 1980), suggesting that the

chemical nature of fibronectin changes during senescence. Cell surface structures such as filipodia and microvilli increase as the cells age (Shatos and Stanulis-Praeger 1980); this may represent cell-to-cell contact points that provide density-dependent conditions for growth cessation at lower absolute cell numbers. Gap junctions, which function as low resistance pathways for exchange of low molecular weight "information bearing" ions and metabolites, are sparse and of small diameter in phase III cells (Kelley 1976). The above data show that cell surface changes resulting from in vitro aging represents conditions that reduce proliferative capacity of cells, cell viability, and sensitivity to environmental conditions.

Various changes in organelles with cellular aging have been reported for cells in vitro, (reviewed by Robbins et al 1970; Cristofalo and Stanulis-Praeger 1982). Lysosomes, which contain numerous degradative enzymes, become larger, more numerous and contain greater amounts of various enzyme activities (Robbins et al 1970). No changes in mitochondrial number with age has been observed, but an increase in the proportion of defective mitochondria with age has been reported, (Lipetz and Cristofalo 1972). Glycolysis and respiration as well as activities of several respiratory enzymes do not change with age of cultures (Cristofalo 1970; Cristofalo and Stanulis-Praeger 1982). No reductions in the uptake and utilisation of glucose is observed with age (Cristofalo 1972). Other enzyme activities that decrease with age are superoxide dismutase (Glass and Gershon 1981) and glutamine synthetase (Viceps-Madore and Cristofalo 1978).

Macromolecular synthesis is affected in the senescing process.

Age-related changes in DNA, RNA and protein synthesis have been intensively studied. The most conclusive result from such studies is that all senescent cells, regardless of their tissue of origin or species, cannot replicate their DNA, a necessary requirement for continued cell proliferation. This is perhaps the most distinctive characteristic common to all senescent cells. Senescent cells can, under certain conditions such as cell fusion or viral infection, be induced to undergo a round of DNA replication but not sustained replication. Enzymes involved in DNA metabolism have been studied in senescent cells, and the major finding is that the activity and fidelity of DNA polymerase- α (the major DNA replication enzyme) decreases in aging cultures of diploid fibroblasts (Linn et al 1976; Murray and Holliday 1981); this has been generally attributed to the fact that in senescent cells there is a four-fold increase in the relative level of the 5S form of the enzyme, which has a higher error frequency than the 7S form (Murray and Holliday 1981). Some reports suggest the presence of a protein-mediated inhibition of DNA synthesis in senescent cells: when senescent cells are fused with young cells, neither cell in the dikaryon can initiate DNA synthesis, but brief treatment of the dikaryons by the protein synthesis inhibitors cycloheximide and puromycin can delay the inhibitory effect of the senescent cells by as much as 24-48 hrs (Drescher-Lincoln and Smith 1983; Burner et al 1983). The nature of this postulated inhibitory protein and its relationship with DNA polymerase α still remains to be determined. It may be of interest to determine whether this pressured protein inhibitor is similar to the DNA-binding protein which Stein (1975) calls "senescence"

protein that binds to single stranded DNA and inhibits DNA synthesis both during the non-DNA synthetic phases of the cell cycle in replicating cells, and during stationary phase in senescent cells. If DNA polymerase activity and fidelity are indeed reduced in senescent cells, then it is not difficult to see the obvious implications, that is, once DNA polymerase is affected, a number of other changes in macromolecules will follow. Another enzyme system connected with DNA metabolism that has been studied are the DNA repair enzymes. As an indication of the importance of DNA repair process in cellular aging, Hart and Setlow (1974, 1976) have reported a positive correlation between the life span of a species and its ability to carry out excision repair after UV damage. Other groups have noted defects in the repair mechanism of senescent cells (reviewed by Cristofalo and Stanulis-Praeger 1982). As a whole, the available data suggest that DNA repair capacity may be related to cellular aging but no causal relationship is evident from the data. Other aspects of DNA metabolism relevant to cellular aging such as activities of degradative enzymes associated with chromatin, role of somatic mutations, karyotype and chromatin modifications of senescent cells, have been discussed in recent reviews (Cristofalo and Stanulis-Praeger 1982; Smith and Lincoln 1984).

Early studies about the effects of cellular aging on RNA metabolism reported a marked reduction in the rate of RNA synthesis in old cultures of human diploid cells (Maciera-Coelho et al 1966). When this apparent decrease was reinterpreted in later studies on the basis of RNA synthesised per cell number, or per cellular DNA, it was found that the

net RNA synthesis is actually increased in old cultures (Schneider et al 1975); this increase is equally distributed among all RNA species (messenger, transfer and ribosomal). Enzymes involved in RNA synthesis have been compared between young and old cultures with the finding that both RNA polymerase I and II are found to be reduced in old cells, while polymerase II remains unchanged (Benson 1978).

Both protein synthesis and protein degradation decrease in senescent cells, though at different rates; the net effect is that protein content increases in senescent cells (Cristofalo and Stanulis-Praeger 1982). The decrease in synthetic rate cannot be attributed to defects in any of the individual components of the protein synthetic machinery (tRNA, ribosomes, cell extracts) since these have been shown to be indistinguishable on physical and biochemical grounds in young and old cells (Smith and Lincoln 1984). With regard to specific protein changes accompanying senescence, a study using two-dimensional gel electrophoresis compared 500 peptides and found that only two differed between young and old cells: greater quantities of these two peptides were produced by senescent cells (Engelhardt et al 1979). Hence only quantitative, not qualitative, changes are detected. On the other hand, other investigators (Sakagami et al 1979; Bravo et al 1981) have reported some qualitative differences in protein patterns between senescent and young cells. One of these proteins have been characterised as a proliferation-sensitive polypeptide and has been designated IEF49 cyclin (Bravo et al 1981). Whether or not these proteins are related to the presumed inhibitors or "senescence" proteins (discussed earlier) is not known.

All of the characteristics of senescent cells discussed so far do not point to a single cause of cellular aging. Rather, it seems that many changes occur simultaneously during senescence. Mechanisms of cellular aging that have been proposed are based on this pleiotropy of events involved in the process. Two general hypothesis concerning the mechanism of cellular aging have been proposed: a deterministic hypothesis and a stochastic hypothesis. The deterministic hypothesis is based on the concept that the decline in proliferative potential of cells in vitro is controlled by a genetic program that directs the cell to senescence (Cristofalo 1972). Support for this comes from the observation that each species has its own characteristic mean and maximal life span, and individual members of a species display parallel patterns of age change. The longevity of a species bears a direct relationship to the replicative life span of its normal fibroblasts in culture (Hayflick 1974; Rohme 1981). Cells in culture keep their replicative time by recognising the number of doublings completed rather than the time spent in culture. The molecular mechanism(s) involved is not known, but certain suggestions have been made. One mechanism could be via the inhibitors discussed earlier. Another mechanism proposes that specific enzymes sequentially modify bases in repeated sequences of DNA (Holliday 1975). This implies that the number of divisions undergone by the cell would be directly related to the number of such modifications in the DNA sequence. Other clock mechanisms operating by sequential mechanisms in macromolecular synthesis have also been proposed (Cristofalo and Stanulis-Praeger 1982). Gradual changes in DNA methylation patterns may also be involved in keeping the clock (Smith

and Lincoln 1984). The second class of mechanisms of cellular aging is the stochastic hypothesis. The most widely known hypothesis in this class is the "error catastrophe" hypothesis (Orgel 1963, 1973) which proposes that senescence results from the random accumulation of errors in macromolecular synthesis, and that this is positively reinforced by feedback of errors in protein and RNA synthesis. Supportive evidence for this hypothesis comes from studies of age-related heat-labile enzymes and loss of enzyme specificity (Holliday and Tarrant 1972), protein turnover (Shakespeare and Buchanan 1976) and loss of enzyme fidelity (Murray and Holliday 1981). Evidences against the hypothesis are that gel analysis have not found much evidence of protein synthetic differences between old and young cells (Harley et al 1980; Engelhardt et al 1981), and incorporation of amino acid analogues into proteins does not lead to a decrease in the life span of WI-38 cells (Ryan et al 1974). Other stochastic mechanisms that have been proposed include the somatic mutation hypothesis (Crowley and Curtis 1963), the free radical hypothesis (Harman 1956, 1981), and the cross-linking hypothesis (Bjorksten 1963). Burnet (1977) has attempted to link the deterministic and the stochastic hypotheses by proposing his "intrinsic mutagenesis" hypothesis which suggests that the mutability of key polymerases leading to error accumulation may be under genetic control.

(b) Cell Transformation - An Escape from Senescence

Cells can escape senescence and acquire the capability for indefinite proliferation in culture; such cells are referred to as transformed cells. Transformation of cells can occur spontaneously (Sanford et al 1954) at low frequency, or the frequency can be increased

by treatment of the cells with various oncogenic agents. The degree to which cells in culture undergo spontaneous transformation depends on the species from which they derive: chicken fibroblasts are stable and very resistant to transformation (Haff and Swim 1956; Lima and Macieiro-Coelho 1972), whereas mouse fibroblasts very often give rise to permanent lines in vitro (Todaro and Green 1963); human fibroblasts lie between mouse and chicken but they generally lack a tendency to transform spontaneously (Ponten 1976). Studies of the relationship between transformation and senescence suggest that old cells transform more readily than young (presenescent) cells (Summerhayes and Franks 1979).

Transformed cells are often abnormal in many respects, including abnormalities in their geometry, growth requirements, cell cycle progression, and of course their replicative life span. The remainder of this "Historical" section will be concerned with a review of the methods currently used to transform cells in culture, the genetic analysis of the transformed phenotype, and some of the myriad physical and biochemical changes that have been found to be associated with the transformed state.

2. IN VITRO CELL TRANSFORMATION BY CHEMICAL CARCINOGENS

Cancer is an ancient disease; it probably has been around since complex life evolved. The fossil record shows that osteomas and hemangiomas were present in dinosaurs at least eighteen million years ago; in addition, the appearance of plant tumours significantly predates that of animal tumours (reviewed by Selkirk 1980). Attempts to cure cancer are also ancient. Cancer therapeutic strategies have usually been based on an exploitation of some more sensitive aspects within the tumour cell's biochemical or genetic apparatus that is more labile than normal cells. This requires a thorough understanding of the biochemical pathways within the growing cell that transform it to the malignant state. Studies of this transformation process requires an ability to manipulate the transformation process as well as understand the mechanisms involved.

Epidemiological studies have shown that the majority of human cancers are caused by environmental factors (Higginson 1979). Many environmental agents have been tested and used in studies of in vitro cell transformation. Oncogenic agents are classified under three broad groups: chemical, viral and radiation. The level of each type of agent in the environment varies with time and place, but they all constitute a significant threat in the environment. All three classes have been used effectively for in vitro cell transformation.

A. Classes of Chemical Carcinogens

There are two groups of chemical carcinogens: direct acting chemicals and indirect acting chemicals which undergo metabolic activation by cells before they become carcinogenic. The former group includes biological alkylating agents, inorganic chemicals and radiochemicals, whereas the latter group consists of polycyclic aromatic hydrocarbons, aromatic amines, nitrosamines, hydrazines, hormones and related substances and a miscellaneous group of other chemicals (Slaga 1980). Many of these agents have been used in in vitro cell transformation systems, although some are more effective than others in terms of the time and incidence of induction of transformation. Inorganic carcinogens have not been studied as extensively as the organic carcinogens, but a few examples are well established (DiPaolo et al 1981; Harvey 1982).

The polycyclic aromatic hydrocarbons (PAHs), or polyarenes, are an important class of carcinogens because of their prevalence in air, water and soil; in addition, significant levels are detectable in many common foods (Harvey 1982). The involvement of PAHs in carcinogenesis was first noted in 1775 when Pott related the high incidence of scrotal cancer among Chimney Sweeps **to the soot** left in the chimneys after coal burning. This was later confirmed by more scientific experimentation, which led to the purification of the carcinogenic substance benzo(a)-pyrene (BP) from coal tar. Subsequently, other PAHs were discovered and many more were synthesised (reviewed by Selkirk 1980; Harvey 1982). Efforts have been made to correlate molecular structure to biological activity, and the main observation in this regard is that

the most active molecules have from four to six fused aromatic rings and an unsubstituted carbon-carbon bond flanked by two aromatic rings; this bond, known as the K-region, is distinguished by its relatively high electron density and olefin-like character. Introduction of methyl groups at other molecular sites can dramatically enhance or diminish activity, depending on the position of substitution, e.g. 7,12-dimethylbenz(a)anthracene (DMBA) is much more carcinogenic than its parent compound benz(a)anthracene (Huggins et al 1967; Pataki and Huggins 1969). Chen and Heidelberger (1969) have used various PAHs to transform cells in culture; they have described the effectiveness of various concentrations and other experimental variables in their study.

B. Multistage Carcinogenesis

(a) Stages in Carcinogenesis

Many studies suggest that chemical carcinogenesis is a multistage process (reviewed by Collins et al 1980; Weinstein et al 1984). The simplest model proposes an initiation followed by promotion. The initiation phase requires a single application of either a direct or indirect carcinogen at a subthreshold dose, whereas the promotion stage involves repetitive treatments of a noncarcinogenic compound after the initiation phase; there is evidence that the promotion phase is divided into several sub-phases (Boutwell 1974; Slaga et al 1980). A central dogma of chemical carcinogenesis holds that initiation is irreversible and promotion reversible; but recent reports have shown that promotion includes some irreversible steps (reviewed by Colburn 1980). It appears that the individual stages may involve qualitatively different mechanisms at the cellular and genetic levels; this predicts that the

establishment and maintenance of a malignant tumour involves multiple cellular genes and multiple types of changes in genomic structure and function. Hence, as Weinstein et al (1982) suggest, it is an oversimplification to speak of "the" cancer gene, or to assume that multistage carcinogenesis simply involves a successive series of random point mutations.

A comparison of the biological properties of initiators and promoters can be found in Iversen and Astrup (1984). Discussions of their probable sites of action and mechanisms follow.

(b) Initiation Stage

(i) Metabolic Activation of Chemical Carcinogens. The metabolism of chemicals leads to the formation of both biologically inert and highly reactive products. Most chemicals have to be metabolically activated to become cytotoxic, mutagenic or carcinogenic (reviewed by Wiebel 1980). Chemical carcinogens that are not electrophilic reactants may have to be converted metabolically into a chemically reactive electrophilic form that reacts with some critical macromolecule to initiate the carcinogenic process (Miller 1978; Miller and Miller 1976).

PAHs are metabolised principally on the microsomes of the endoplasmic reticulum. The principal catalysts are the microsomal mixed-function oxidase (MFO) enzymes (also known as monooxygenases, aryl hydrocarbon hydroxylase or microsomal drug-metabolising enzymes). This enzyme complex has been extensively reviewed (Gelboin 1980). MFO enzymes oxidise PAHs and a variety of other compounds to more polar derivatives, usually active epoxides (Sims and Grover 1974); these are

unstable intermediates which usually undergo secondary enzymic reactions such as hydration or conjugation. Although these secondary reactions are also involved in the activation process of some chemical carcinogens, they generally serve a detoxification role (Miller 1970; Gelboin et al 1972). A key determinant in the biological activity of a chemical is the steady-state level of its reactive intermediates. A change in the rate of any of the activation and deactivation reactions may alter the steady-state level of the reactive intermediates. However, the initial oxidation step seems to be the rate-limiting step in the metabolism of many chemical carcinogens (Wiebel 1980).

Chemical carcinogens alter MFO enzymes activity in several ways (Wiebel 1980). Such modulations in MFO activity play a role in the initiation process. Initiating carcinogens may differ in their degree of interference with MFO activity, their time course, the response of specific MFO forms, and tissue specificity. All of these affect manipulation of experimental models.

(ii) Modifiers of Chemical Carcinogenesis. There are a number of factors that can have an additive, synergistic or inhibitory effect on the carcinogenic activity of chemical carcinogens. The term cocarcinogenic action is used for agents that augment the carcinogenicity of chemicals when they are applied concomittantly (Slaga et al 1978). Carcinogens have influence on the initiation of the carcinogenic response through an effect either on the carcinogen (permeability, metabolism or repairability of the carcinogenic damage) or on the target cell. A wide variety of cocarcinogens are known (Slaga 1980). On the other hand, agents that decrease the initiation of

carcinogenesis, referred to as anticarcinogens, inhibit chemical carcinogenesis by counteracting metabolic activation of carcinogens, augmenting detoxification reactions, competitive inhibition, scavenging of active molecular species of carcinogens to prevent them from reaching their critical target sites in the cell, or effects on DNA repair systems (Slaga 1980; Wattenberg 1980). In vivo, additional factors such as age, sex, hormonal and immunological factors, trauma and diet, nutrition and lifestyle further modify chemical carcinogenesis (Slaga 1980).

(iii) Sites of Action of Chemical Carcinogens. Chemical carcinogens or their activated forms are strong electrophiles and they react non-enzymatically to form covalent bonds with nucleophilic groups of macromolecules in target cells (Miller and Miller 1976; 1981); these reactions are critical to the carcinogenic process. Most investigators consider DNA to be the critical target molecule in this initiation, but the importance of other target sites such as RNA and protein are not ruled out. The interaction of chemical carcinogens with DNA results in multiple chemical changes in the DNA. A central question in chemical carcinogenesis is which of these changes leads to mutations or changes in genetic expression. Such changes much persist through DNA replication cycles.

PAHs bind to DNA, RNA and protein (Kuroki et al 1971). The amount of hydrocarbon bound is proportional to the concentration of macromolecule in the tissue, with cellular protein binding most of the hydrocarbon, followed by RNA and then DNA (Selkirk 1980). DNA is considered the most critical binding molecule, but the large amount of

hydrocarbon that binds to RNA and protein indicates that only a small fraction of the applied carcinogen gets to the critical site. The question about which PAH derivatives are most efficiently bound to DNA, the type of chemical bonds formed, and the DNA bases involved are all major determinants of their effectiveness in cell transformation.

Another extensively studied carcinogen is N-2-acetyl-aminofluorene (AAF). The reactive metabolite of AAF binds covalently to nucleic acids (Kriek and Westra 1978; Grunberger and Santella 1983). At least three types of DNA adducts are formed, at different points on nitrogen and carbon atoms of guanosine residues (Grunberger and Santella 1983). Alkylating agents also bind to the oxygen atoms of guanosine residues. Pegg (1984) has reviewed the evidence that the O⁶ position of guanosine in DNA is the most likely initiating event in carcinogenesis by methylating agents, but another reviewer (Singer 1984) proposes that other sites are equally likely and important in initiation of chemical carcinogenesis. The roles of these modifications in carcinogenesis have been discussed (Singer 1984; Pegg 1984).

(c) Promotion Stage

The agents which are involved in the promotion stage of carcinogenesis are referred to as tumour promoters. It should be mentioned that certain PAHs can act as complete carcinogens without a need for promoters under certain conditions (Weinstein et al 1983; 1984). Presumably, under such conditions, the PAHs bind to DNA as well as induce membrane changes that alter growth and differentiation of cells.

(i) Tumour Promoters. Tumour promoters are compounds which have very weak or no carcinogenic activity by themselves, but which markedly enhance the carcinogenicity of other compounds (initiators) when applied repeatedly following a low or suboptimal dose of the initiator (Berenblum 1982). There is a wide range of chemicals that can act as tumour promoters (Slaga 1980; Berenblum 1982), but most of the information on the action of tumour promoters derives from studies on 12-O-tetradecanoylphorbol-13-acetate (TPA) and related phorbol esters. TPA and other phorbol esters are amphiphilic agents since they have both hydrophobic and hydrophilic domains. This domain structure has a bearing on the type of interaction these compounds make with the cell membrane.

(ii) In Vivo and In Vitro Studies. Evidence for multistage carcinogenesis has come from both in vivo and in vitro studies. The best studied in vivo system is the two-stage mouse skin tumorigenesis (reviewed by Van Duuren 1969; Boutwell 1977; Berenblum 1982), which utilises PAH as initiator and various compounds as promoters. Using this and other in vivo systems (Slaga 1980), the number of steps involved in carcinogenesis has been estimated at about six (reviewed by Colburn, 1980). Some of the steps may occur as part of alternate pathways.

In vitro studies of multistage carcinogenesis has been done with mouse, rat and Chinese hamster cells using PAH and other chemical carcinogens as initiator and TPA as promoter (Colburn 1980). In most of the studies the stages are identified as the sequential acquisition of transformed characteristics. Up to five stages in the carcinogenic

process has been observed by these studies.

Another approach is the hybrid in vivo - in vitro transformation system (Laerum and Rajewsky 1975; Steele et al 1978). In this type of study, the cells are exposed to the initiating agent in vivo, but the promotion stage is carried out with the cells in culture. The stages are identified by growth and morphologic criteria. The advantage of this system is that the in vivo initiation allows greater metabolic activation of the carcinogen by liver cells before the carcinogen gets to other target cells; the liver has a higher level of the activating enzymes than other organs of the body (Wiebel 1980).

(iii) Phenotypic Effects of Promoters. Tumour promoters exert pleiotropic effects on the growth, function and differentiation of a variety of cell types (reviewed by Colburn 1980; Weinstein et al 1984). Some of the effects are causal to the overall transformation effect, whereas other effects seem to be unnecessary or unconnected to promotion. Most of the phenotypic effects induced by tumour promoters in cell culture are dependent upon the continuous presence of the promoters (Weinstein 1981), but there are a few instances where the action of promoters led to irreversible effects (Weinstein et al 1984). Indeed, it is whether the promoter induces reversible or irreversible effect that determine if the process leads to a persistent transformed phenotype.

Several studies have shown that tumour promotion involves an inhibition of differentiation with an associated non-specific proliferation stimulus (reviewed by Colburn 1980). Also, biochemical characteristics of embryonic tissues such as low histidase activity

(Colburn et al 1975) are observed in cells after treatment with tumour promoters. In addition, certain features of differentiated cells disappear in response to TPA treatment (Krieg et al 1974). In most of the studies, suppression of differentiation was transient, with reversal occurring after removal of the promoter. In some cases, TPA delays rather than inhibit differentiation (Mufson et al 1978). Sometimes, the effect becomes irreversible (Raick 1974).

Tumour promoters have been found to transiently induce mimicry of transformation (Colburn 1980). This includes changes in morphology, growth characteristics, cell surface changes, and several biochemical changes. These changes are not observed when cells are treated with non-promoting (or weakly promoting) mitogens (Raick 1974; Raick and Burdzy 1973). Tumour promoters also enhance the expression of these characteristics after virus (Weinstein et al 1977) or chemical (O'Brien and Diamond 1978) transformation. One in vitro neoplastic characteristic which is not expressed in response to tumour promoters (without prior initiation) is anchorage-independence (Colburn, 1980). This may be due to the fact that anchorage-independence correlates very closely to actual in vivo tumorigenicity (Shin et al 1975), and since tumour promoters are not by themselves oncogenic, they may be unable to induce this characteristic.

In vivo and in vitro studies have also shown that there is a strong correlation between tumour-promoting activity and ornithine decarboxylase (ODC) activity for a number of phorbol esters (O'Brien et al 1975a; 1975b; Lichti et al 1978). However, another study shows that glucocorticoids, which inhibit tumour promotion, fail to inhibit

induction of ODC by promoters, thus suggesting a non-causal role of induced ODC in tumour promotion (Yuspa et al 1978).

The induction of certain proteases, including plasminogen activator (PA), has been proposed as a mediator of tumour promotion (Troll et al 1970; 1975). These studies are based on the effects of protease inhibitors on tumour promotion, but such evidence are not conclusive in view of the fact that protease inhibitors often have numerous unrelated effects on cell metabolism (Roblin et al 1975). Other studies have shown a correlation between tumour promoting activity and ability of the promoter to induce PA in cell cultures (Weinstein et al 1977). Another indication that PA may play a role in tumour promotion comes from the finding that promotion-inhibiting glucocorticoids also inhibit the induction of PA in hepatoma HTC cells by TPA (Viaje et al 1977).

Various other phenotypic effects of tumour promoters have been observed. These include enhanced prostaglandin levels, increased phospholipase activity, and an increase in the frequency of sister chromatid exchanges (Colburn 1980).

All these phenotypic effects of tumour promoters indicate that promotion is an inductive rather than a selective process. Colburn (1979) has provided experimental verification for that. The manifestation of these effects of promoters may actually represent different substages of the promotion phase. Radman and co-workers have identified protease induction and sister chromatid exchanges as irreversible steps in tumour promotion (Radman et al 1977; Kinsella and Radman 1978).

(iv) Mechanism of Action of Promoters. Whereas DNA is the postulated target molecule for initiators, the primary site of action for promoters is speculated to be the plasma membrane (Blumberg 1980; 1981; Weinstein et al 1984). Perturbations in membrane structure or function could trigger a series of physiological changes in the cell, mediated by influxes of ions and small molecules, activation/inactivation of enzymes, and ultimately activation or repression of genes.

The initial indication that tumour-promoting activity may be mediated by cell-surface interactions came from the work of Sivak et al (1972) who demonstrated changes in the activities of several plasma membrane enzymes. Since then a variety of other membrane effects have been observed (Blumberg 1980; 1981; Trosko et al 1983; Weinstein et al 1984). Using ^3H -phorbol dibutyrate (PDBU), several investigators have obtained direct evidence for specific high-affinity saturable "phorboid receptors" in membrane preparations and intact cells from avian, rodent and human tissues (Horowitz et al 1981; Hecker et al 1982). The abilities of a series of phorbol esters to compete with ^3H -PDBU for binding to cell surface receptors correlate with their tumour-promoting capabilities (Hecker et al 1982), providing evidence that the "phorboid receptors" mediate the biologic actions of the phorbol esters. The phorbol receptors are normally utilised by endogenous growth factors, which implies tumour promotion by phorbol esters may be closely related to the more general phenomenon of hormonal carcinogenesis (Weinstein et al 1983). Several findings suggest that there may be heterogeneity of "phorboid receptors", which could

contribute to the tissue specificity and pleiotropic effects of phorbol esters (Horowitz et al 1981). The amphiphilic nature of phorbol esters and some other tumour promoters allows them to bind to "phorboid receptors" as well as to other components of the cell membrane, so that multiple pathways of biochemical effects can be activated by these agents (Weinstein et al 1982; 1983).

One of the plasma membrane-mediated biochemical effects of tumour promoters is the stimulation of protein kinase C, a Ca^{2+} and phospholipid-dependent protein kinase that is activated also by diacylglycerol (Castagna et al 1982; reviewed by Nishizuka 1984a; Weinstein et al 1984); diacylglycerole is one of the primary products of membrane inositol phospholipid breakdown. Evidence that a phospholipid-protein kinase C complex is the receptor of tumour promoters has been obtained from several laboratories (for reviews see Kikkawa et al 1983; Weinstein 1983). Phorbol esters form a quarternary complex consisting of phospholipid, protein kinase C, Ca^{2+} , and tumour promoter (Weinstein 1983). This complex acts synergistically with Ca^{2+} to activate various cellular functions (Nishizuka 1984b). The critical protein targets phosphorylated by protein kinase C, in response to TPA stimulation, includes epidermal growth factor receptors and insulin receptors on serine and theonine, but not on tyrosine residues (reviewed by Weinstein et al 1982); the role of these protein modifications in tumour promotion is not known. Even though protein kinase C does not phosphorylate tyrosine, TPA does enhance the phosphorylation of tyrosine residues on a 43K protein, the same protein which is a target for the action of pp60c-src (Bishop et al 1983). It

appears that in addition to its effect on protein kinase C, TPA enhances, directly or indirectly, the activity of a cellular tyrosine-specific protein kinase. This links the tumour promotion process with oncogenes.

At the cellular level, tumour promoters interfere with intercellular communication, and this is believed to play a role in the mechanism of tumour promotion (reviewed by Trosko et al 1983). Many transformed cells are unable to communicate intercellularly in a proper fashion (Borek et al 1969; Steinberg and Defendi 1981). Many tumour promoters also inhibit intercellular communication in vitro (reviewed by Trosko et al 1983). Also, many transformation characteristics induced by tumour promoters could be connected with the loss of appropriate intercellular communication (Borek et al 1969). The importance of all these observations is based on the assumption that intercellular communication plays a major role in the regulation of cell proliferation and differentiation, so that any condition that disrupts this process potentially allows abnormal cells to gain a selective growth advantage. Hence tumour promotion can be viewed as a process by which the initiated cell can "escape" the normalising homeostatic growth/differentiation regulatory signals from its communicating neighbours (Trosko and Chang 1980). This implies that an initiated cell has a deficiency of some signal(s) **needed to regulate** its ability to divide and differentiate properly. **It also** implies that the initiated cell is sensitive to those regulatory signals and is capable of translating the molecular signals into biochemical responses. Four general areas of study have provided support for the view that proper intercellular communication between

normal and transformed cells inhibit tumour promotion. First, cell fusion between normal and tumour cells seems to lead, at least initially, to non-malignant hybrids (Klinger 1980). Second, in vitro neoplastic transformation can be suppressed under conditions of high cell density (Bertram 1979). Third, in vivo coinjection of transformed and normal cells can suppress the growth of the tumour cells (Newcomb et al 1978). Fourth, suppression or reversal of tumorigenicity of teratocarcinoma cells by injecting the cells into normal cell environment has been demonstrated (Mintz and Illmensee 1975). Although these observations indicate a role for intercellular communication in tumour promotion, they can also be explained by other mechanisms. Inhibition of intercellular communication may be a necessary but probably insufficient step in the tumour promotion process.

The phorbol ester tumour promoters can also induce changes at nuclear targets. Tumour promoters induce chromosomal aberrations, DNA damage and sister chromatid exchange (Troll et al 1982; Weinstein et al 1984). There is evidence that these effects are membrane-mediated (Cerrutti et al 1983). The tumour promoting agents stimulate arachidonic acid metabolism and lipid turnover that could generate high reactive forms of activated oxygen which lead to lipid peroxidation and other toxic effects including DNA damage (Weinstein et al 1983; Cerutti et al 1983). Most of the work is done with inflammatory cells which have specialised responses that involves high oxidative bursts in response to activators, hence the effect may be unrelated to tumour promotion. However, recent studies showing that DNA damaging agents are more effective than tumour promoters in enhancing the progression of

papillomas to carcinomas on mouse skin (Kennings et al 1983), suggest that the evolution of a fully malignant tumour multistage carcinogenesis may involve more than one cycle of damage to DNA. In any case, it seems unlikely that the critical events during tumour promotion involve DNA damage and chromosomal aberrations since the process of tumour promotion on mouse skin is often reversible (Berenblum 1982). Probably DNA effects are relevant only during the late promotion stages since chromosomal anomalies are more prominent during the late rather than early stages of carcinogenesis.

The observations of DNA effects of promoters poses questions about what the functional difference between initiators and promoters really is, since initiators are mutagens and promoters can induce chromosomal mutations. Some of the effects of promoters on DNA that have been reported may be artifacts derived from the experimental strategy used. For example, the induction of sister chromatid exchange is measured by the incorporation of bromodeoxyuridine; modulation of nucleotide pools by promoter enhancement of membrane permeability to the nucleoside might cause enhanced incorporation of bromodeoxyuridine and thereby lead to the misleading interpretation that promoters induced sister chromatid exchange (Trosko et al 1983). There is a need to determine whether or not the chromosomal effects of promoters are restricted to only certain cell types, whether they occur only under certain conditions, and whether they are actually associated with tumour promotion.

Another target of tumour promoters is the mitochondria. TPA impairs mitochondrial respiration in intact fibroblasts (Backer et al 1982), and also accelerates the hydrolysis of ATP by isolated rat liver

mitochondria (Backer and Weinstein 1983). The relevance of these in tumour promotion is not yet known.

Clearly, tumour promotion is mediated by several mechanisms. Tumour promoters represent a wide variety of chemical structures and they induce a pleiotropy of biological and biochemical responses. Many of the responses are those associated with cell division and differentiation. The responses to tumour promoters by initiated and uninitiated cells are similar (Trosko et al 1983). Hence the difference between normal and initiated cells might be in their abilities to restore their respective physiologies to the pre-promotion state after exposure to the promoting agent.

(d) Criticism of the Concept of Two-Stage Carcinogenesis

The two-stage theory of carcinogenesis predicts a synergism between the initiator and the promoter. The theory further implies that the synergism is observed only when the initiator and promoter are applied in a particular sequence, only at certain dose levels, and in sensitive animal-carcinogen systems only. Over the years the theory has come to be regarded as a paradigm, even though evidence suggests that it is probably relevant only for certain types of chemical carcinogenesis. Iversen and Astrup (1984) have reviewed some of the short-comings of two-stage theory of carcinogenesis. They conclude that most of the studies that suggest or assume the validity of the two-stage theory do not meet the requirements for a rigorous test of the theory.

One of the criticisms of the two-stage theory is that too often initiators and promoters act as complete carcinogens (Iversen and Astrup 1984) so that what constitutes initiation and promotion stages are not

always seen based on morphological or biochemical criteria. Iversen and Astrup (1984) note that given proper observation times, tumours have been produced by repeated applications of TPA alone or by small, single doses of polycyclic hydrocarbons. But in criticising the two-stage theory, one should also note that the long time periods required for carcinogenesis are, by themselves, indications that carcinogenesis is multistep.

Another criticism regards the sequence of application of initiation agents and promoters. The two-stage theory maintains that initiation must come first, and this has prevented effective experimentation on other sequence protocols. Many experiments are designed and the results interpreted based on the two-stage theory. Baba et al (1966) showed that when large doses of MCA and croton oil were used, it did not matter which of them was applied first, the tumour production was the same. This has also been shown with MCA and TPA (Iversen and Iversen 1982). Subsequent experiments have shown that the enhancement effect is not due to stimulation of DNA synthesis (induced by the short promoter treatment), that leads to more S phase cells being present when the carcinogen is applied (Iversen 1982a; 1982b).

C. Genetic and Epigenetic Mechanisms of Neoplastic Transformation

With the exception of certain embryonal tumours, the malignant phenotype is stable and heritable. Two different explanations of neoplastic transformation have been given: that the malignant phenotype arises from a change in DNA (genetic mechanism); or that it arises from a non-mutational change in the expression of cellular differentiation genes (epigenetic mechanism). The somatic mutation hypothesis predicts

a change in nucleotide sequence of DNA, presumably in genes that are crucial to carcinogenesis; the aberrant cellular differentiation hypothesis, on the other hand, predicts ~~two~~ things: that no change in DNA occurs, and that a qualitative or quantitative change in the spectrum of gene expression, as mRNA and/or protein molecules, should be present. The type of mechanism involved has an important bearing on the prospects of cancer therapy, especially therapeutic approaches based on hopes of reversing the transformed phenotype. The evidence for and against the genetic and epigenetic mechanisms of neoplastic transformation have been reviewed (Monnat and Loeb 1983).

Five recently developed techniques now facilitate detection and quantitation of genetic and epigenetic changes in transformation: recombinant DNA technology; DNA sequencing; monoclonal antibodies; analysis of cellular proteins by gel electrophoresis; and analysis of RNA sequences.

Recombinant DNA techniques have allowed a direct test of the somatic mutation hypothesis (reviewed by Monnat and Loeb 1983; Weinstein et al 1984). Mutated regions have been identified and the types of mutations determined by DNA sequencing. Two classes of genes have been especially investigated as critical mutation sites: genes whose products are required for cell replication (such as DNA polymerase genes) and genes belonging to the class referred to as oncogenes (Monnat and Loeb 1983). Other genetic sites are also studied (Weinstein et al 1984). There is evidence for a diminution in the fidelity of DNA polymerases purified from malignant cells (Chan and Becker 1979). The evidence that oncogenes are involved in carcinogenesis is enormous

(reviewed by Astrin and Rothberg 1983; Cooper and Lane 1984; Shih and Weeks 1984). Cell transformation by chemical carcinogens probably involves changes in the function of multiple oncogenes and other cellular genes, and multiple mechanisms involving different kinds of DNA changes are involved (Weinstein et al 1984).

D. Interaction of Chemical Carcinogens and Other Oncogenic Agents

An enhancement of chemical carcinogenesis, measured as a reduction in the latent period and/or increased incidence of cell transformation, has been observed when cells are pretreated with viruses or radiation (reviewed by Casto and DiPaolo 1973; Ullrich 1980; Yang and Tobias 1980; Fisher 1983). Similarly, pretreatment of cells with a subcarcinogenic dose of chemicals also enhance viral or radiation carcinogenesis. The interactions are complex, so that in many cases, it is difficult to distinguish between the primary carcinogen and the cocarcinogen. In most studies, the dosage levels and the sequence of application of the two agents determine which agent is considered to be the primary carcinogen.

(a) Cocarcinogenesis With Radiation

Interactions between chemicals and radiation in chemical carcinogenesis have been observed for both ionising and non-ionising radiation (Ullrich 1980; Yang and Tobias 1980). The literature on interactions between chemical carcinogens and UV light has presented conflicting results, with UV light showing both enhancement and inhibitory effects, or no effects at all (Ullrich 1980). The lack of effect is probably due to the very low levels of UV used in some studies

(Yang and Tobias 1980), but the stimulatory and inhibitory effects are primarily due to the photodynamic activity of most chemical carcinogens (Epstein 1966; Davies and Dodge 1972). The photodynamic activity acts in diametrically opposite ways, namely, development of photosensitisation which enhances the carcinogenic response (Epstein et al 1964), and photochemical degradation which reduces carcinogenicity of the chemical carcinogens (Davies and Dodge 1972). The enhancement effect acts both at the level of initiation (Stenback et al 1973) and promotion (Epstein and Epstein 1962). Experiments designed to examine true carcinogenic interactions must ensure that neither photodegradation nor photosensitisation take place.

Ionising radiation also enhances chemical carcinogenesis (DiPaolo et al 1971). The enhancement of transformation by X-rays is influenced by the X-ray dose, with enhancement ratio increasing logarithmically to a peak at about 250 rads and declining as the dose increases (Yang and Tobias 1980); the decline at high doses is due to cell killing (Ullrich 1980). Argus et al (1962) have reported that X-ray treatment of mouse skin, whether preceding or following DMBA treatment, decreased tumour yield from that induced by DMBA alone. This report is hard to interpret since the radiation doses used (between 0 to 480 rads) were not sufficient to cause severe tissue damage and were comparable to doses used by other investigators who have reported positive interactions in mouse skin.

(b) Cocarcinogenesis With Viruses

An enhancement of chemical carcinogenesis by DNA and RNA viruses has been noted (Casto and DiPaolo 1973; Tennant and Rascati 1980). The enhancement effect is influenced by many factors, including the order in which the cells are exposed to the agents, the type of virus or chemical, and the relative size or proliferative state of the target cell population (Tennant and Rascati 1980). The effect of the viruses is in sensitisation of the cells to the chemical carcinogens but the exact mechanism is not known.

E. Transformation of Cells by Other Oncogenic Agents

A wide variety of mammalian cells can be transformed in vitro and in vivo by agents other than chemical carcinogens. Several recent reviews have provided information on the protocol, mechanisms, effectiveness and usefulness of transformation of cells by viruses (Moyer and Moyer 1981; Sack 1981; Rapp 1981), radiation (Borek 1980; Yang and Tobias 1980), and purified "transforming genes" or oncogenes (Chang et al 1982; Cooper 1982; Robins et al 1981). In vitro transformation of cells by oncogenes is a relatively new method of transforming cells. Because the oncogenes have limited interaction and effects on cellular DNA, it promises to contribute greatly to an understanding of the cause(s) and mechanisms of carcinogenesis.

3. ASSAYS OF CELL TRANSFORMATION

Despite many advances, there is as yet no single marker that defines a transformed cell. This is due partly to the fact that the cell transformation process is complex and multifaceted, and partly due to the inability to give a simple and adequate definition of the normal cell. A commonly used definition of a transformed cell is that it is a cell that has lost control of its growth. This means that the net gain of cells (that is, the balance between the generation of new cells and the rate of cell loss) is faster for transformed cells than for normal cells. It implies that transformed cells either divide faster, live longer, or have a longer proliferative life span than their normal counterparts.

Assays for cell transformation are based on the hypothesis that normal and transformed cells in culture or in vivo differ routinely in one or more characteristics that can be accurately measured and reliably employed as indicators of normalcy or tumorigenicity. These measurements may involve both in vivo and in vitro methods. Normal and transformed cells exhibit phenotypic instability which may lead to spontaneous transformation of normal cells and to the loss or reduction of tumorigenicity of neoplastic cells. Slight differences in culture methods such as the type and concentration of serum, the frequency of subculture, and the density of both plating and harvesting, create different selective pressures which lead to rapid speciation of cells in culture (Sanford et al 1970). Hence, valid attempts to correlate an in vitro trait with in vivo tumorigenicity require that both be experimentally determined at the same time with the same cultures.

A. In Vivo Methods

The most reliable test of cell transformation is to demonstrate the ability of the transformed cells to form tumour when transplated into a suitable host (Cameron and Pool 1981). However, sometimes, in vivo methods may be inadequate, and in such cases in vitro correlates are essential to confirm the in vivo results.

In vivo tests involve transplantation or injection of an inoculum of the transformed cells into animal hosts whose immunological status does not hinder the growth of the transplanted cells. The major variables which affect the results include the manner of tissue preparation, the route of inoculation, inoculum size, the period of observation, the number of host animals involved and tumour immunogenicity (Skehan and Friedman 1981).

(a) Animal Hosts for In Vivo Assays

(i) Isogenic Transplants. Immune rejection of transplated transformed cells constitutes a major artifact of in vivo tests of tumorigenicity. In principle, immune rejection of transplanted transformed cells can be avoided by transplanting the cells into a perfectly isogenic host of the same histocompatibility strain as the animal from which the test tissue is derived. Spontaneously transformed cells transplanted shortly after their original isolation generally display high "take" rates when transplanted into isogenic hosts, but chemically - and virally-transformed cells are frequently subject to host rejection and tend to exhibit low "take rates" (Hewitt 1978). The main reason for the rejection of experimentally transformed cells is because of their considerable immunogenicity, which appears to be a

secondary effect of the transforming agent (Foley 1953; Prehn 1975; Klein and Klein 1977). Also, the speciation of animals of the same nominal histocompatibility strain into distinct substrains as a result of separate propagation in different laboratories also accounts for rejection of experimental tumours (Hewitt 1978).

Hence, since most of the experimental tumours in common use are virally- or chemically-induced rather than spontaneous, or have generally been maintained in culture for extended periods of time, and they are usually tested in animal hosts other than those of their origin, it follows that the use of isogenic transplants as in vivo models of transformation is very limited.

(ii) Immunologically-Privileged Sites. Improvements in tumour transplantation have been achieved largely by the prevention of host immune rejection rather than a manipulation of tumour cell immunogenicity. A significant advance in this regard is the recognition that certain anatomical sites possess an impaired immune capacity and are therefore immunologically privileged. Examples of such sites are the anterior eye chamber, the brain, peritoneal cavity, and hamster cheek pouch (Skehan and Friedman 1981). Some of the sites are more superior in accepting transplants than others, but unfortunately transplantation to the superior sites often involve difficult surgical methods and the results obtained tend to be quantitatively erratic (Giovanella and Fogh 1978).

(iii) Immune-Deficient Hosts. Three types of naturally occurring immune-deficient hosts have been employed in transformation assays: the chick embryo chorioallantoic membrane, fetal and newborn mammals, and

athymic or nude mice (Skehan and Friedman 1981). Further improvements in "take" rates can be achieved by further immunosuppression with chemicals or radiation (Adams et al 1970).

The nude mouse has become a popular immune-deficient model for the analysis of tumorigenicity. Sharkey and Fogh (1984), have reviewed the major considerations in the use of nude mice for cancer research. The homozygous mouse (nu/nu) lacks a thymus gland and is effectively devoid of cytotoxic and suppressor T-cell activity (Collins et al 1980; Skehan and Friedman 1981). However, nude mice have helper T-cell activity (Collins et al 1980), which plays a role in humoral immune response; as a result, serum levels of IgM are normal or elevated, and those of other immunoglobulin classes are up to 25% of normal levels (Rygaard 1973). Other evidence suggests that there exists a "fossil" thymus that appears during ontogeny and is detected when the "present day" thymus is eliminated by the nu mutation (Holub et al 1975). The existence of this cryptic thymus in nu/nu mice has had supportive evidence (Collins et al 1980). Because of the presence of these immunological factors in nu/nu mice, some transplant rejections by nu/nu mice have been noted (Sharkey et al 1978). The immune status of nude mice has been summarized (Kindred 1979; Collins et al 1980).

The major advantage in the use of nude mice in tumorigenicity assay derives from its tolerance to a wide variety of both normal and malignant xenografts (Giovannella and Fogh 1978). Their disadvantages are cost, maintenance and susceptibility to pathogen infection. This latter factor is important since there is evidence that both virus infection and chronic pneumonias produce visible lesions in the lungs

that can be mistaken for tumour metastases (Sharkey and Fogh 1984).

(iv) Immune-Suppressed Hosts. Host animals can be immuno-suppressed by whole body irradiation, treatment with corticoids and anti-lymphocyte serum (ALS), and by thymectomy. All these methods increase the tumour "take rate", particularly when used in concert with immune-deprived recipients (Skehan and Friedman 1981), but in general, the success of the procedure depends on a competition between the establishment of the tumour and the recovery of the immune system. Any rejection of the tumour involves a balance between the growth rate of the tumour, the rate of regeneration of the immune system, and the rate of killing by the effector activity (Collins et al 1980). At present, the most successful method of tumour transplantation is the use of hosts that are thymectomised at birth, lethally irradiated, provided with bone marrow transplants from syngenic animals, and ALS-treated (Skehan and Friedman 1981).

(b) Cell Preparation and Route of Inoculation

In addition to immune rejection, other factors such as the method of cell preparation and the route of inoculation into the host animal also affect the success of in vivo transformation assays.

Cells for injection are administered as single cell suspensions prepared by protease or EDTA dissociation. Both agents are toxic and they lyse or impair the viability of cells (McKeehan 1977), thus reducing the potential of the injected cells to grow as tumours.

The route of inoculation also has an effect on the subsequent results, at least from the point of view of the immunological status of the inoculation site. The most common routes of cell inoculation in

tumorigenicity tests are intraperitoneal (ip), intravascular (iv), intradermal (id), intramuscular (im) and subcutaneous (sc). Generally, iv and ip inoculation sites tend to be inferior routes of inoculation (Skehan and Friedman 1981). In addition, certain types of neoplastic cells display high "take" rates only when inoculated at specific anatomical sites or by specific routes (Outzen and Custer 1975; Epstein et al 1976).

(c) Progressive Growth by Normal Cell Transplants

A useful in vivo tumorigenicity assay must be able to show some degree of progressive growth of neoplastic tissue and at the same time must be unable to support progressive growth of normal tissues. This is often the case, but in some circumstances it is difficult to distinguish between a slow-growing tumour and a normal tissue transplant which has enlarged due to local connective tissue formation, inflammation and trauma (reviewed by Skehan and Friedman 1981). Histological analysis is usually required in order to determine whether the growing tissue is normal or neoplastic.

Two of the cell lines frequently used as normal controls in tumorigenicity assays are the mouse 3T3 and C3H/10T $\frac{1}{2}$ /C18 lines. Both fail to form tumours when inoculated as single cell suspensions but form rapidly and progressively growing malignant tumours when implanted attached to solid substrate (Boone et al 1976; 1979). The hamster BHK 21/C13 line, which has also been used as a normal control in tumorigenicity studies has in fact been found to be neoplastic (reviewed by Skehan and Friedman 1981). These factors should not be ignored when interpreting results of tumorigenicity assays.

B. In Vitro Methods

A variety of in vitro methods can be used to assay for cell transformation. These methods are based on differences between normal and transformed cells in cellular morphology, growth characteristics, membrane-related properties, and biochemical features (reviewed by Skehan and Friedman 1981).

(a) Cellular Morphology

(i) Cytology. Morphological changes are among the first cellular effects observed to occur in cell cultures upon exposure to oncogenic agents (Earle and Nettleship 1943). Changes in several cytological traits such as cell size and shape, karyotype, nuclear number and morphology, the nuclear/cytoplasmic ratio, and cytoplasmic staining characteristics have been noted following cell transformation (reviewed by Sanford 1965; Skehan and Friedman 1981). In most cases the differences between normal and transformed cells are based only on qualitative rather than quantitative or statistical differences in these cytological traits (Skehan and Friedman 1981). In one of the quantitative studies based on statistical evaluations, Montesano et al (1977) found that cytoplasmic basophilia and the nuclear/cytoplasmic ratio both correlated individually with the tumorigenicity of rat liver epithelial lines. Thirteen other traits examined did not individually show a significant correlation with tumorigenicity. However, when classification was based on the joint consideration of all 15 cytological traits rather than on single characteristics, normal and tumorigenic lines could be distinguished from one another with 94% accuracy (Montesano et al 1977). Other investigators have also shown

that by using multiple criteria it is possible to accurately distinguish between normal and neoplastic fibroblast line even if no individual cytological trait correlates significantly with tumorigenicity (Barker and Sanford 1970).

(ii) Cytoskeleton. Reports of a decrease in the number of visible microfilaments or a disorganization and loss of parallel microfilament bundles following exposure of cells to oncogenic agents, have been noted (McNutt et al 1973; Pollack and Rifkin 1975; reviewed by Skehan and Friedman 1981). In most of the studies, however, the status of the cell lines before and after exposure to the oncogenic agent was unknown, thus making it impossible to make any conclusions about the relationship between microfilaments and tumorigenicity. In addition, there are other serious problems with the experimental methods used in the studies, which have been discussed by Skehan and Friedman (1981). Hence, the available data fails to establish a correlation between tumorigenicity and either the number or organization of microfilaments and microtubules.

(b) Growth Regulation

(i) Life Span in Culture. Limited lifespan of normal diploid mammalian cells in culture is well documented (Swim and Parker 1957; Hayflick and Moorhead 1961), and there is evidence that it is also a property of normal tissues serially transplanted in vivo (Hellman et al 1978). In contrast, under favourable growth conditions, cells derived from tumours or premalignant tissue often exhibit unlimited proliferative potential (immortality) both in vitro and in vivo (reviewed by Ponten 1976; Newbold et al 1982). The induction of

immortality does not seem to be an all-or-none process; rather it appears to involve different degrees of reduction in the commitment of cells to a non-proliferative state (Newbold et al 1982). The acquisition of immortality is an early event in the transformation process, preceding the onset of anchorage-independence. Hence, carcinogen-induced immortal cell lines are not necessarily tumorigenic immediately they exceed the normal lifespan but must progress to malignancy after a further period of culture (Newbold et al 1982). This period is highly variable and is a characteristic of the individual cell line. In studies by Newbold et al (1982) some lines remained non-tumorigenic even after 300 population doublings in vitro.

It is possible to induce the anchorage independent phenotype independently of immortality, and such lines (that is, anchorage-independent, senescent cells) do not form tumours in mice in vivo (Newbold et al 1982). This suggests that immortality may be necessary for tumorigenicity. On the other hand, other lines of evidence suggest that the acquisition of immortality is not an obligatory step on the pathway to transformation. First, the assays for senescence seem to be inadequate. By using carefully selected serum (Poiley et al 1978), or by supplementing standard culture media (Parker and Smith 1974), normal human fibroblasts can be maintained over 100 generations, more than twice the number originally reported by Hayflick and Moorhead (1961). This means that even if senescence of a cell population were due to a genetic program, the culture conditions affect the program. Second, many lethal tumours apparently senesce in vitro, supporting the claim that cellular senescence may be an in vitro

phenomenon with little relevance in vivo (Collins et al 1980). Even if senescence occurs in vivo, it need not be a limiting factor for tumorigenicity since a mammalian cell has the potential for at least 50 divisions, which generates enough cells (2^{50}) to give rise to a tumour. Third, the distinction between cells which are senescent and cells which do not divide because they lack appropriate stimulation is not clear (Collins et al 1980).

(ii) Serum Requirement for Growth. Transformed cells generally have lower serum requirements for growth than do their normal counterparts (Temin 1966; Jainchill and Todaro 1970; Dulbecco 1970). Transformed cells are capable of proliferating well in medium containing serum concentrations as low as 1% (Borek et al 1977). This feature holds true for rodent cells but not necessarily for human fibroblasts, where some normal cells as well as the transformants grow at low serum concentrations (Borek 1980b). The absolute levels of serum required may also vary with the source of serum.

(iii) Contact Inhibition and Saturation Density. A loss of contact inhibition of movement (Abercrombie 1966) and replication (Stoker and Rubin 1967), and ability to grow in multilayers (Todaro et al 1964; Terzaghi and Little 1976) are all features that differentiate transformed cells from their normal counterparts grown under the same culture conditions. There are indications that the primary defect that results in loss of contact inhibition of transformed cells is associated with the membrane rather than a result of a general deregulation of growth (Borek and Fenoglio 1976).

(iv) Anchorage Dependence. Anchorage-dependence is the requirement of cells to attach to a substratum or make some other form of contact in order to divide (Stiker et al 1968). Normal cells in culture generally exhibit a pronounced anchorage-dependence, whereas established or transformed cell lines are frequently either anchorage-independent or display a much reduced anchorage requirement (Freedman and Shin 1974). This has led to the hypothesis that the loss of anchorage-dependence in culture accompanies and can be used as an index of oncogenic transformation (Freedman and Shin 1974; Jones et al 1976). It has been proposed that anchorage-independence is the most reliable in vitro correlate of tumorigenicity (Shin et al 1975; Patek et al 1978).

In 1964, two groups independently described a gel culture technique that claimed to assay for anchorage-independence (Burford and Sanders 1964; MacPherson and Montagnier 1964). Many investigators have used these techniques or modified versions of them to measure the colony-forming efficiency (CFE), also known as plating efficiency (PE). CFE is defined as the percentage of cells in an inoculum which give rise to multicellular colonies in the agar suspension.

Skehan and Friedman (1981) have compiled the CFE literature data for rat, mouse, hamster and human cell lines. Four categories of cells are recognised: normal cell lines which are derived from normal tissues, have never been exposed to oncogenic agents, and do not form tumours in vivo; transformed lines which form tumours in vivo; and two categories of uncertain cell lines which are derived from normal tissues, may have been exposed to oncogenic agents, and exhibit varying

degrees of transformation characteristics but were really not tested for in vivo tumorigenicity. Data for crude agar, agarose and methyl cellulose are pooled in the compilation. Hamster cells exhibit a large number of tumorigenic lines with very low CFEs ($<0.01\%$) and of normal lines with moderately high CFEs. Hence, with hamster cells it seems that a low CFE is not indicative of normalcy, nor is a moderate CFE indicative of transformation. However, the compiled data (Skehan and Friedman 1981) show that a high CFE can probably be considered an indicator of tumorigenicity. The situation with mouse and human cells is less clear. Whether tumorigenicity does or does not correlate with CFE depends on whether the lines of uncertain status are actually tumorigenic or normal. If the uncertain cell lines are ignored, then it becomes difficult to interpret the results because of the low number of data for normal mouse and human cell lines.

There are evidence in favour of the argument that a loss of anchorage dependence is a trait selected for by tissue culture conditions, that the apparent correlation between CFE and tumorigenicity which is sometimes observed is coincidental, and that the phenotypic traits which characterise tumorigenic cell lines in culture are not the same as those of tumour cells in vivo. First, cell lines can acquire the capacity for anchorage independent growth in suspension culture or as intraperitoneal ascites only after prolonged adaptation or selection (Owens et al 1953; Earle et al 1954). This effect has been extended also to gel cultures (Giard et al 1973). Second, CFEs of tissue minces and cell suspensions from tumours prepared directly from in vivo tissues without prior growth in culture indicate that tumor cells in vivo are

considerably more likely to be anchorage dependent than transformed cells obtained from cultures.

The CFE of a cell line can vary up to several orders of magnitude depending on the criteria used to assess colony formation, the composition of the suspension gel or liquid, its concentration, the time required for gelation, the inoculum size, the frequency of culture feeding, and the type of water used for preparing assay solutions (Skehan and Friedman 1981). These variables affect different cell lines to different degrees, so that each cell line must be individually optimised. Skehan and Friedman (1981) have reviewed the methodological difficulties associated with the assay.

(c) Cell Membrane-Related Properties

(i) Lectin Agglutinability. Normal cells acquire an increase in cell agglutinability by lectins following in vitro transformation (Inbar and Sachs 1969). On the basis of numerous studies, the hypothesis that increased lectin agglutinability is a correlate of tumorigenicity has become popular (reviewed by Skehan and Friedman 1981). On the other hand, other evidence have contradicted the existence of a correlation between lectin agglutinability and tumorigenicity. First, normal cells are highly variable in their agglutinability; while some are not agglutinable, others are highly so (Sivak and Wolman 1972). Second, tumorigenic lines are also highly variable in their agglutinability (reviewed by Skehan and Friedman 1981). Hence, poor agglutinability by lectins does not necessarily seem to be indicative of normalcy, nor does high agglutinability indicate tumorigenicity.

(ii) Metabolite Uptake. It has been proposed that one of the changes that occur in cell transformation involves alterations in the cell membrane that lead to an increase in transport of metabolites and thus to an increased supply of key nutrients. The increased intracellular concentration of these key nutrients enables malignant cells to proliferate in an uncontrolled manner or at a faster rate than their untransformed counterparts.

A correlation between the uptake rates of glucose, amino acids, and nucleotides and the rate of growth and state of transformation of mammalian cells in culture, has been reviewed (Koren 1980). However, the correlation seems to be related more to the state of growth of the cells than to transformation per se (Dubrow et al 1978; Bush and Shodell 1978).

(d) Biochemical Characteristics

Transformed cells differ from normal cells in several biochemical and biophysical parameters (reviewed in Cameron and Pool 1981). In general, these differences include changes in the cell cycle kinetics, cyclic nucleotides, polyamine metabolism, the state of water of the cell, viscosity, pH, element content, methionine metabolism and DNA methylation, protease induction and secretion, glutamine metabolism and changes in energy metabolism. The expression of these traits may involve induction and/or repression of gene activities. There is extensive interrelationship among these features of the transformed cell. Although many of these differences between transformed and nontransformed cells are not routinely tested in transformation assays, they do form the focus of investigations regarding the

mechanisms of transformation and attempts at cancer therapeutic modalities.

4. GLUTAMINE METABOLISM BY NEOPLASTIC CELLS

Glutamine is required by mammalian cells in several metabolic pathways. It is involved in synthesis of all major classes of macromolecules of the cell, as well as in amidotransfer reactions for synthesis of other amino acids and acetyl CoA. Glutamine is also involved in anabolic, energy-yielding reactions of the cell. The various roles of glutamine in cellular metabolism has been reviewed (Rabinovitz et al 1956; Kitos et al 1962; Patterson 1972; Coleman and Lavietes 1981; McKeehan 1982). Because of the central involvement of glutamine in metabolic activities of the cell, its depletion from the growth medium of mammalian cells results in a rapid response by the cells to the starvation condition (Zetterberg and Engstrom 1981).

(a) Glutamine Requirements of Normal and Neoplastic Cells

The enormous requirement for glutamine by mammalian cells is reflected in the fact that, of all the amino acids supplied in the growth medium, glutamine is the one that is supplied in the highest amount (Eagle 1955a; 1955b; Ham and McKeehan 1979). In general, neoplastic cells have a higher demand for glutamine than their normal counterparts. The lines of evidence which link malignancy with high glutamine utilization are three-fold : First, plasma glutamine levels are found to be lower than normal in patients afflicted with various neoplasia (Roberts and Simonsen 1960); this is interpreted as indicating that malignant cells take up glutamine faster than normal cells. Second, glutaminase activities showed a good correlation with the growth rates and degree of malignancy of several rat tumour cell

lines (Knox et al 1967; 1969; Horowitz et al 1969). Third, there is a direct correlation between the degree of malignancy and the level of oxidative metabolism of glutamine (Horowitz et al 1969; Knox et al 1969; Morris et al 1970).

Initial explanations for the higher demand for glutamine by neoplastic cells relative to normal cells was mainly in connection with its requirement in protein and nucleotide biosyntheses. This was based on the assumption that tumour cells divide faster, and hence, they synthesise greater quantities of proteins and nucleic acids, than normal cells. Later experiments, however, showed that much of the glutamine taken up by malignant cells is utilized in oxidative reactions for energy-yielding purposes. It is now widely thought that the involvement of glutamine in respiratory pathways largely accounts for its differential utilization by transformed and normal cells (reviewed by Coleman and Lavietes 1981; McKeehan 1982).

(b) Role of Glutamine in Macromolecular Biosynthesis

It is well known that glutamine is a constituent of proteins; it is also directly involved in the biosynthesis of nucleotides by providing amido groups. If growth rate correlates with malignancy, then based on these metabolic processes, there should be a greater requirement for glutamine by malignant cells than normal cells. However, evidence suggests that neoplastic cells don't necessarily divide faster than normal cells (Skehan and Friedman 1981).

In recent years, work done by LeJohn and co-workers have given some indication that the differential requirements for glutamine between normal and neoplastic cells could be explained by the role of the amino

acid in nucleic acid biosynthesis (Goh and LeJohn 1977; Goh et al 1977). When transformed cells were deprived of glutamine, they rapidly accumulated a polyphosphorylated compound (HS3); this phenomenon could be prevented or reversed by the addition of glutamine any time during the starvation period (Goh et al 1977). The glutamine deprivation effect could be mimicked by treating cells with inhibitors of nucleotide biosynthesis. Also, the starvation-induced HS3 accumulation could be overcome by supplying the cells with various purines and pyrimidines. Using purine salvage pathway mutants of CHO cells, it was shown that nucleotide starvation, rather than glutamine starvation per se, was responsible for HS3 accumulation (Goh and LeJohn 1977; Goh et al 1977; Goh 1979). These effects were not observed for primary, non-transformed cells. All of these data suggest that the requirement for glutamine is different for transformed cells as compared to primary cell lines, and that the primary role of glutamine in these instances is for nucleic acid biosynthesis.

(c) Role of Glutamine in Respiratory Processes

The role of glutamine in oxidative processes has emerged as the potential pathway in glutamine metabolism for explaining the difference in glutamine requirements between normal and neoplastic cells.

For many decades after the original observation that a high rate of aerobic glycolysis is a hallmark of malignant cells (Warburg et al 1924; Warburg 1926), it has been assumed that glucose provides the major source of energy for cells (Levintow and Eagle 1961; Morell and Froesch 1973). This was based on the fact that glucose is rapidly metabolised by cells and that glucose is present at high concentrations (5 to 10mM)

in blood plasma and in usual tissue culture media. In addition, glucose uptake is higher than normal for neoplastic cells (Koren 1980). In theory, such energy could be derived from glycolytic oxidation to pyruvate and subsequent aerobic oxidation of the latter in the tricarboxylic acid (TCA) cycle. However, various observations suggest that glucose may not be the predominant oxidative respiratory substrate. First, glucose limitation experiments in vitro suggest that the quantitative requirement for glucose for growth of cells that exhibit high rates of glycolysis is much lower than expected (Eagle et al 1958; Graff et al 1965; Renner et al 1972). Much of the glucose taken up by neoplastic cells are not involved in aerobic respiration, which suggests that another respiratory substrate acts as the predominant source of energy. Second, it has been known that tumour cells can grow in much lower concentrations of glucose than is provided in culture media (Eagle et al 1958; McKeehan 1982). Under these conditions, there is a marked reduction in lactate formation without a proportional decrease in the rate of proliferation (McKeehan 1982). Third, in some cultured tumour cells, an external pentose source replaces the hexose requirement for proliferation (McKeehan 1982), thus suggesting that the essential function of glucose in tumour cells may be to provide anabolic substrates rather than fuel for cell proliferation.

The above observations suggest that other molecules instead of glucose (or other hexoses) serve as the source of energy for cell proliferation in neoplastic cells. Correlations between glutamine- and glutamate-dependent respiration in isolated mitochondria from tumour cells, and between the activity of mitochondrial glutaminases and

malignancies suggested that glutamine may be an important respiratory fuel for tumours (Kovacevic and Morris 1972; Knox et al 1969; Morris et al 1970). Stoner and Merchant (1972) noted that cultures of mouse L-M cells utilised glutamine far in excess of the requirement for synthesis of cellular components. About 55% of glutamine carbons utilized by the cells appeared as CO_2 . It was estimated that the quantity of glutamine oxidised provided at least 35% of the energy requirement for cell proliferation, even though glucose was present at high levels in the culture medium. Other data have confirmed this, and some suggest that glutamine can supply essentially all of the cellular requirements for energy when the supply of glucose is extremely low (McKeehan 1982). These reports clearly implicate glutamine as a potentially important respiratory fuel for tumour cells in vivo and in vitro, even when glucose is present.

Although glutamine can replace glucose as the main respiratory molecule of neoplastic cells, evidence shows that glycolysis is not dispensable in neoplastic cells (Reitzer et al 1979; McKeehan 1982). Coleman and Lavietes (1981) have reviewed the evidence that in neoplastic cells glucose carbons are diverted from aerobic respiration towards the synthesis of cholesterol and other lipids, and that the deregulation of lipid biosynthesis is an early and important process in neoplasia. According to the scheme that they present, tumour respiration becomes supported effectively by glutamate which enters the pathway directly into the TCA cycle. Glutamine serves as the source of glutamate. Glutamine also serves as the principal amido donor in the synthesis of purines, pyrimidines and asparagine; in each of these

reactions, glutamate is the by-product which goes to support tumour cell respiration (Coleman and Laviates 1981). Glutamate can substitute for glutamine in isolated mitochondria but not in intact cells (Hems et al 1968). One reason why glutamate fails to replace glutamine in intact cells is, of course, because of the diverse roles of glutamine in cellular metabolism, but it is also because the cell membrane is poorly permeable to glutamate (Kovacevic and Morris 1972). A similar inability of glutamate to substitute for glutamine has been observed in the HS3 response (LeJohn, personal communication).

Several aspects of tumour cell cholesterol and respiratory metabolism support the model that in neoplastic cells glucose is diverted from respiration to support deregulated lipid synthesis, and that glutamine substitutes as the respiratory substrate (for review, see Coleman and Laviates 1981). Also, many metabolic characteristics of neoplastic cells can be explained as resulting from the involvement of glutamine in tumour respiration (reviewed by Coleman and Laviates 1981). It should, however, be emphasised that metabolic shifts, although principally involving events at the mitochondrial/cytoplasmic interface, does not exclude more distal and probably equally important metabolic events at other cellular sites, such as the nucleus, cell membrane and other organelles of the cell.

5. PHOSPHORYLATED DINUCLEOSIDES (HS) IN CELLULAR METABOLISM

Cells have evolved certain intrinsic regulatory mechanisms with which they rapidly adjust their cellular metabolism according to their growth environment. Various types of polyphosphates and unique phosphorylated nucleosides produced during altered growth conditions have been described as mediators of cellular regulation of growth in prokaryotes and eukaryotes (reviewed by Goh 1979; Koch and Richter 1979; Varshavsky 1983). In particular, three phosphorylated dinucleotides, designated HS compounds, have been described in a variety of fungi, and one of them has also been described in a variety of cultured mammalian cells, (LeJohn et al 1975; Goh 1979)

(a) HS In Fungi and Mammalian Cells

The HS compounds (HS1, HS2 and HS3) were first isolated from whole-cell acid extracts of a variety of fungi (LeJohn et al 1975). These compounds were also isolated by osmotic shock treatment of whole cells, thus suggesting that they may be located on the cell membrane (LeJohn et al 1978). One of the three compounds, HS3, was subsequently shown to consist of a glutamyl-ADP-sugar moiety which is covalently linked to a UDP-sugar-tetraphosphate (McNaughton et al 1978). The other two, HS1 and HS2, have been partially characterised and appear to have two uridines each with twelve and ten phosphates respectively in contrast to the eight present in HS3 (McNaughton et al 1978).

One of the fungal HS compounds, HS3, was also detected in numerous cultured mammalian cell lines (Goh and LeJohn 1977). Physiological studies demonstrated that HS3 accumulated only when glutamine was

removed from the growth medium, since isoleucine deprivation (Goh and LeJohn 1977) and glutamate deprivation (LeJohn, unpublished results) were without effect. In a limited survey of various mammalian cell types, it was shown that permanent cell lines could only accumulate HS3 when glutamine was withdrawn from the growth medium; by contrast, primary cell lines failed to alter an apparently high level of HS3 they produced whether or not glutamine was present (LeJohn et al 1979). More recently, Deffie and LeJohn (1984) have provided more direct evidence of the specific patterns of HS3 metabolism with respect to the onset of immortality in cultured cells. Also, using pairs of isogenic transformed and untransformed cell lines, they showed that the specific patterns of HS3 accumulation was intimately connected with the state of transformation of the cell, regardless of the mode of transformation (chemical, viral, oncogene, spontaneous or X-irradiation).

A comparison of the physical, chemical and physiological properties of HS3 from Achlya and CHO cells suggests that HS3 from fungal and mammalian sources may be identical (LeJohn et al 1979; Goh 1979). Fungal and mammalian HS3, however, show differences in their activities against heterologous substrates (LeJohn et al 1979), thus indicating that some as yet undetected difference(s) in HS3 exists between the two sources.

(b) Possible Metabolic Roles of HS Compounds

(i) HS Compounds in Fungi. The three HS compounds synthesised by fungi seem to play a role in fungal sporulation and nucleic acid metabolism (LeJohn et al 1978; 1979). The fact that HS1 and HS2 are absent in mammalian cells (Goh 1979) suggests that these two compounds

are specifically involved in fungal growth regulatory processes not present in mammalian cells, such as fungal sporulation. The relationship between HS compounds and fungal sporulation is further suggested by the observation that a rapid intracellular accumulation of all three HS compounds occurred just prior to sporulation (LeJohn et al 1978). This accumulation of HS by Achlya could be triggered by the depletion of phosphate or some other growth medium components; this nutritional stress invariably also led to sporulation. Supplementation of the starvation medium with glutamine or various purine and pyrimidine bases and nucleosides concomitantly inhibited HS accumulation as well as fungal sporulation. Hence, it was suggested (LeJohn et al 1978) that the intracellular accumulation of all HS compounds may be a prerequisite for sporulation.

HS compounds seem also to be involved in nucleic acid metabolism during the growth cycle of Achlya. An inverse relationship was observed between rates of DNA and RNA synthesis and HS synthesis (LeJohn et al 1975; LeJohn et al 1978). Furthermore, in vitro experiments demonstrated that HS3 and HS2, at physiological concentrations, were potent inhibitors of isolated Achlya DNA-dependent RNA polymerases (McNaughton et al 1975; LeJohn et al 1978) and of fungal ribonucleotide reductase (Lewis et al 1977). The reaction catalysed by ribonucleotide reductase is a major control point in the synthesis of deoxyribonucleoside diphosphates (Larsson and Reichard 1966; Lowdon and Vitols 1973). These observations all suggest that HS compounds may be acting as negative effectors of nucleic acid synthesis in vivo.

The possible involvement of HS compounds in regulating fungal

energy-linked transport systems has also been postulated (Stevenson and LeJohn 1978; Goh and LeJohn 1978).

(ii) HS3 in Mammalian Cells. As mentioned earlier, glutamine deprivation results in the accumulation of HS3 in cultured mammalian cells. Owing to the importance of glutamine as a precursor of de novo nucleotide biosynthesis (Mahler and Cordes 1971), and because of the following observations, it has been suggested that HS3 accumulation may be an important regulatory element that co-ordinates growth and nucleic acid metabolism. First, an inverse relationship exists between RNA, DNA synthesis and HS3 accumulation in glutamine-starved CHO cells (Goh et al 1977). By contrast, HS3 accumulation is independent of protein synthesis. In vitro studies showed that both Achlya and mammalian cell HS3 were potent inhibitors of mammalian ribonucleotide reductase (Lewis et al 1977), the enzyme that catalyses the rate-limiting step in the synthesis of deoxyribonucleotides. Furthermore, HS3 inhibited DNA-dependent RNA polymerases (Goh 1979). Second, azaserine and methotrexate, which are analogues of glutamine and folic acid respectively, and which block de novo purine biosynthesis, were able to induce accumulation of HS3 (Goh and LeJohn 1977). Third, various purine and pyrimidine compounds added to glutamine-starved cells prevented the build-up of HS3 (Goh and LeJohn 1977). However, purine salvage mutants of CHO cells could not respond to the addition of purines even when fed with as much as 2 mM hypoxanthine (Goh et al 1977), suggesting further that HS3 accumulation is linked to defects in purine biosynthesis or utilisation. Fourth, a CHO GAT⁻ mutant, which is auxotrophic for glycine, adenosine and thymidine owing to its defective folate

metabolism (McBurney and Whitemore 1974), accumulated HS3 only when it was deprived of adenosine (Goh and LeJohn 1977). Fifth, glutamine starvation of mammalian cells leads simultaneously to HS3 accumulation, growth restriction, and restriction of nucleic acid biosynthesis (Goh, 1979). These observations demonstrate that HS3 appears to signal perturbations in nucleic acid metabolism, especially purine nucleotide biosynthesis.

Other data (LeJohn, unpublished) have suggested that (a) HS3 may act as a high-energy polyphosphate storage compound during nutritional stress; (b) the so-called inhibition of HS3 biosynthesis by various exogenously supplied purines is in fact due to the consumption of phosphates from HS3 for nucleotide biosynthesis; and (c) the constituent ADP and UDP moieties could be further used for the replenishment of all the nucleotide pools.

6. PROTEOLYTIC ENZYMES IN CELL TRANSFORMATION AND MALIGNANCY

Proteases are enzymes that catalyse the hydrolysis of peptide bonds. Numerous studies show that many tumorous and transformed cells in culture secrete enhanced levels of proteolytic activity when compared with their normal counterparts. It has been proposed that this increased proteolytic activity may be involved in the process of cancer metastasis and in the altered phenotypic characteristics of transformed and tumour cells (Roblin et al 1975; Rossman and Troll 1980). It is likely that several proteases are involved since many proteolytic enzymes have been shown to alter the growth and surface properties of untransformed cells (Reich et al 1975; Sloane and Honn 1984)

(a) Proteases Associated With Transformation and Malignancy

The proteases that are known to play a role in cell transformation and malignancy are divided into four classes: serine, metallo, aspartic and cysteine proteases (Sloane and Honn 1984).

(i) Plasminogen Activator/Plasmin System. Plasminogen activator/Plasmin system belongs to the serine protease group, which are a class of proteolytic enzymes that require an essential serine at their active site. Several serine proteases are involved with the transformation process (Rossman and Troll 1980) but the best studied is the plasminogen/plasmin system. In this system, plasminogen activator(s) (PA), produced and secreted by many transformed cells and a few normal cell lines and tissues (Rifkin et al 1974; Unkeless et al 1974; Christman et al 1975), converts an inactive serum protein, plasminogen, to the active enzyme plasmin (Duffy and O'Grady 1984). Plasmin, in contrast to PA, is a broad specificity enzyme that breaks

down lysine-lysine bonds in fibrin and a variety of other proteins, including all major plasma protein effector systems (Christman et al 1977). Thus the PA/plasmin system has the capacity of activating (or inactivating) other protease systems in serum.

One of the earliest studies on the production of PA by cell lines was reported by Barnett and Baron (1959). They detected PA activity in serum-free culture medium of KB cells (a human epidermoid carcinoma line) or to primary cultures of Rhesus monkey kidney cells. Their results were confirmed by Painter and Charles (1962). Since then, several types of PA have been detected, purified and characterised from a variety of cell types (Christman et al 1977; Duffy and O'Grady 1984).

The concept that fibrinolysis might be related to tumorigenesis and cell transformation is not new (O'Meara 1958; Cliffton and Grossi 1955; Peterson et al 1973). However, it is the work of Reich and co-workers that has provided a conceptually coherent and experimentally validated foundation for further work. Their results, and those of other workers, have defined the potential importance of the expression of PA to the understanding of malignancy and cell transformation by the following observations. First, most malignantly transformed lines, regardless of the transforming agent, demonstrate a significantly increased level of release of PA when compared to their normal counterparts (Unkeless et al 1973; Rifkin et al 1974). Second, when chick embryo fibroblasts are infected with a temperature-sensitive mutant of Rous sarcoma virus, PA is produced by the cells within 2 to 3 hours upon shifting the cells to permissive temperature (Ossowski et al 1973; Unkeless et al 1974), which makes the expression of PA one of the earliest observable events

associated with transformation. Third, infection of cells with a variety of oncogenic viruses stimulates PA secretion from 5- to 45-fold, while infection with either non-transforming strains or with nononcogenic cytotoxic RNA and DNA viruses has little or no effect on PA secretion (Ossowski et al 1973; Unkeless et al 1973). Fourth, when a mouse melanoma line B16 clone B₅59, is grown in the presence of 5-bromodeoxyuridine (BrdU), the cells lose their tumorigenicity concomitant with a decrease in the production of PA; these effects are reversible upon removal of BrdU (Christman et al 1975). Fifth, plasmin is a potent protease capable of cleaving a wide variety of proteins; other proteases have been shown to alter the functional characteristics of animal cells in several ways (Christman et al 1975; Roblin et al 1975). Thus one may conclude from these reports that PA activity correlates with cell transformation or malignancy. The presence of PA within the cell may serve a regulatory function while the extracellular form may enable the transformed cell to alter its environment.

In contrast to the above reports, other studies have found little or no basis for a correlation between the level of PA secreted and the transformed or tumorigenic state. First, there is no consistent relationship between tumorigenicity and PA production by tissue fragments and primary cultures prepared from in vivo tissues. In an investigation of more than 200 human tumours and normal tissues, Clifton and Grossi (1955) observed that normal tissues generally produced high levels of fibrinolytic activity, while production by tumours was variable. This has been confirmed by several other investigators (reviewed by Skehan and Friedman 1981). Second, the

normalcy or tumorigenicity of the cell lines used in some of the early experiments were assumed rather than established by in vivo or in vitro assays. The mere exposure of cells to oncogenic agents does not imply that the cells are transformed since oncogenic agents have effects that are unrelated to their oncogenicity (Skehan and Friedman 1981). Third, clonal variability and replicate variance are ignored in many comparative studies. The difference between replicate samples or between subpopulations within a single culture often equal or exceed the difference in PA between lines (Albrechtsen 1957; Barrett et al 1979). Fourth, density-dependence is also often ignored in comparative studies of PA secretion. Like many cellular processes, fibrinolysis and PA production are density-dependent (Jaken and Black 1979; Laug 1979). In one study, hamster B4 melanoma cells did not start production of PA until after maximum population density was attained (Ramsdahl et al 1961), whereas in another study with calf aortic endothelial cells there was a progressive increase in production of PA with increasing density (Laug 1979). Hence in comparative studies, any differences in the levels of PA may be due to a differential sensitivity to regulation by density-dependent cell interactions. Fifth, many normal cells secrete high levels of PA. Among such cell types are activated macrophages (Unkeless et al 1974) and human WI-26 cells (Mott et al 1974). In addition, not all tumorigenic lines secrete PA, especially those that have been kept in culture for long periods of time (Rossman and Troll 1980; Skehan and Friedman 1981). It follows, therefore, from these discrepancies that there is no consistent pattern of relationship between oncogenic transformation and increase in either fibrinolytic

activity or PA production.

Another serine protease system that has been studied is the prothrombin/thrombin. Although relatively little work has been done with this enzyme system, initial studies indicate that it might play a role in the altered phenotypic properties of virus-transformed cells. Purified thrombin has growth-stimulatory properties but whether or not cells secrete a "thromboplastin" activity which converts serum prothrombin to thrombin is not known (Chen and Buchanan 1975). Thrombin can also activate plasminogen conversion to plasmin (Engel et al 1966). Roblin et al (1975) have reviewed the data on the prothrombin/thrombin system as it relates to cell transformation.

(ii) Collagenolytic Enzymes. These are proteolytic enzymes whose major substrates are the collagens. Collagen is a family of closely-related but chemically and genetically distinct macromolecules (Piez 1984; Miller 1984). The various collagen types have different primary structure and function, and exhibit some specificity with regard to tissue distribution and composition. The molecular architecture of the different collagen fibers requires highly specific proteases for degradation (Gross et al 1980) and for this a variety of serine and metallo-proteases are involved (reviewed by Wooley 1984a).

The importance of collagenolysis in malignancy and cell transformation derives from the fact that, in vitro, collagen represents the major structural protein of all tissues and provides the chief obstacle to passive migration of tumour cells (Wooley 1984a); collagen may also be part of the protein matrix that anchors cells to their substratum in vitro. Since tumour and transformed cells may need to

degrade collagen for various reasons, it follows that collagenolysis is important in these processes. Based on biochemical studies of in vitro cell cultures, it has been shown that transformed cells generally produce increased amounts of collagenolytic activity when compared to normal cells (Murphy et al 1981; Oyomada et al 1983). Immunolocalisation studies have provided direct evidence that the enzymatic activity has a functional role in vivo (Wooley et al 1980a; Barsky et al 1983); this is supported by studies using in vitro model systems of tumour cell invasion where specific inhibitors are used to negate collagenolytic activity and arrest tumour cell invasion (Liotta et al 1982; Thorgiersson et al 1982). Hence, in vivo and in vitro evidence suggest that collagen degradation may play a role in the process of malignancy and cell transformation.

Collagen degradation occurs both extracellularly and intracellularly, but the extracellular pathway seems to be more important for malignancy since it is by this pathway that the cells exert greater influence on their environment. Also, the intracellular enzymes rely on extracellular degradation to produce small collagen fragments suitable for phagocytosis (Wooley 1984a). Some of the extracellular enzymes are membrane-associated while others are secreted freely into the environment.

Among the extracellular enzymes secreted by cells, the collagenases are the most important since they have the ability to degrade highly cross-linked collagen fibres at physiological pH (Wooley 1984a). Collagenases are metallo-proteases (metal-dependent enzymes) that require Ca^{2+} and Zn^{2+} for activity. The properties of collagenases have

been reviewed (Liotta et al 1982; Wooley 1984b) as well as their involvement in malignancy (Wooley et al 1980a; Wooley 1985). Reports from inhibition studies indicate that the physicochemical properties of collagenases from specific tumour cells might differ from that produced by the normal cells from which the tumour is derived (Wooley et al 1980b); this might be related to their functioning.

The collagenases degrade collagen types I, II and III, but have no effect on collagen types IV, V and VI (Wooley 1984a). These latter collagen types are degraded by other extracellular enzymes secreted by cells. Liotta and co-workers have purified and characterised a type IV collagen-degrading metallo-protease from cultured metastatic murine tumour cells (Liotta et al 1981; Salo et al 1983); this enzyme is specific for type IV collagen. Other metallo-proteases and serine proteases that degrade type IV collagen have been reported (Wooley 1984a). Type V collagen-degrading enzymes have also been reported in tumour cells, macrophages and endothelial cells (Wooley 1984a).

Other enzyme systems may indirectly play a role in collagenolysis. For example, plasmin can activate latent forms of collagenase (Paranjpe et al 1980). The interaction between collagenases and plasmin is of importance considering the role of plasmin in tumorigenesis. Another enzyme that has the ability to activate latent collagenases is a membrane-bound enzyme described as a trypsin-like neutral protease (TLNP). TLNP has been found on the surface of Ehrlich ascites cells (Steven et al 1980) but it has not been fully characterised yet and no activity in normal cells is detected.

The lysosomal cysteine proteases, cathepsins B, H, L and N, all

have collagen-degrading properties (Etherington 1979; Sloane and Honn 1984; Wooley 1984a). Since these enzymes are localised intracellularly in the lysosomes in normal cells, their secretion by tumour cells suggests some role in the collagenolytic mechanism and hence in the process of malignancy and cell transformation (Sloane and Honn 1984). Cathepsin B is the most studied among this group of cysteine proteases. It is a broad-specificity endopeptidase that acts against several components of the extracellular matrix such as proteoglycans, fibronectin and the non-helical portion of collagens I - IV (reviewed by Sloane and Honn 1984). Cathepsin B can also regulate collagen degradation through the activation of latent collagenase (Eeckhout and Vaes 1977).

Cathepsin B has been reported in the interstitial fluid of patients with neoplastic disease and in the media of cultured tumour cells (Sloane and Honn 1984; Wooley 1984a). Sloane et al (1981; 1982) have demonstrated a close correlation between cathepsin B activity of variants of murine B16 melanoma and their metastatic potential. In addition, the observations that tumour cathepsin B, in contrast to cathepsin B from normal tissues, may have properties which enable it to possess proteolytic activity extracellularly (Sloane et al 1984) indicate that the secretion of this enzyme by tumour cells is associated with the tumorigenic state. The biological role of cathepsin B in tumorigenesis is, however, complicated by the following observations: First, there have been several reports of negative correlations between cathepsin B activity and transformations (Dolbeare et al 1980; Morgan et al 1981). Second, cathepsin B activity correlates with only certain

malignancies and not others (Sloane and Honn 1984). Third, cathepsin B can be inhibited substantially (up to 50% by serum protease inhibitors such as α_2 -macroglobulin and α_1 and α_2 -thiol proteinase inhibitors (Starkey and Barrett 1973), hence its role in metastasis becomes doubtful. Fourth, cathepsin B activity reduces with passage number of transformed and tumour cells in culture (Recklies et al 1982), yet malignancy or transformation characteristics increase with passage time. Fifth, Giraldi et al (1977) has reported that leupeptin, a serine protease inhibitor, has no effect on spontaneous metastasis of Lewis lung carcinoma. In contrast, Yamamoto et al (1975) had earlier reported that leupeptin decreases the metastatic capacity of injected rat ascites hepatoma cells. These contrasting results might be due to differences in the metastatic capacities of the tumour systems studied. Also, since the investigators did not use the same concentration of leupeptin in their investigations, the discrepancy might be due to the known fact that leupeptin inhibits cysteine proteinases at low concentration (Umezawa 1972).

The role of cathepsins H, L and N in collagenolysis is less clear. Under certain conditions, cathepsin L can have higher collagenolytic activity than cathepsin B (Krischke et al 1982). Cathepsin H, unlike the others, has aminopeptidase activity in addition to endopeptidase activity (Schwartz and Barrett 1980). The role of cathepsins H, L and N in malignancy has been reviewed (Mullins and Rohrllich 1983; Jones and DeClerck 1982; Pauli et al 1983).

It is evident that several collagenolytic pathways might be functional in facilitating metastasis. Because each of the

collagenolytic enzymes has specific requirements for extracellular activity it seems unlikely that they all function simultaneously within the same microenvironment. Extracellular ionic conditions have been implicated in the regulation of protease activity (Sylvén 1968). Furthermore, in vivo regulation of collagenolytic activity of tumour cells is effected by macrophages and mast cells, presumably by secreting collagenase activating proteases (Birkedale-Hansen et al 1976; Henry et al 1983); mast cells could also indirectly modulate collagenolytic activity of tumour cells by stimulating macrophages to secrete further collagenase stimulating factors (Yoffe et al 1984). It is likely that such in vivo regulatory factors may apply to cultured cell lines in vitro. In vitro culture conditions can, however, induce transformed cells to express novel protease activity. For example, an uncharacterised protease activity is induced in transformed cells, but not untransformed cells, when the cells are "heat-shocked" (Deffie and LeJohn 1984); the protease(s) exerts a profound effect on the environment of the tumour cells by breaking down most proteins secreted into the medium. Similar observations have been made for tumour systems in frog (McKinnell and Tarin 1984).

(b) Physiological Role of Proteases in Malignancy and Transformation

The role of proteases in transformation and malignancy has been studied using three general approaches. (1) Correlation of the state of transformation and malignancy with levels of protease secretion by cells; (2) changes in the phenotypic characteristics of untransformed cells after exposure to proteases; and (3) effects of protease inhibitors on properties of transformed cells. Studies utilising these

three approaches have mostly supported the view that proteases play a role in tumour cell metastasis, unrestrained growth of transformed and tumour cells, modulation of external environment by tumour cells for nutritional purposes and the expression of a number of phenotypic properties of transformed cells in culture (see reviews by Roblin et al 1975; Reich et al 1975; Rossman and Troll 1980; Mullins and Rohrllich 1983; Duffy and O'Grady 1984; Sloane and Honn 1984; Wooley 1984a). Several different proteases are involved in the manifestation of these properties, although the proteases may interact at different points for regulation of their activities (Roblin et al 1975; Sloane and Honn 1984; Wooley 1984a).

(i) Relationship Between Proteases and Cell Phenotype. Tumour cells and cells transformed in vitro by a variety of oncogenic agents often exhibit phenotypic characteristics that are qualitatively or quantitatively different from those of their normal counterparts (Roblin et al 1975; Skehan and Friedman 1981). Many studies utilising proteases and protease inhibitors and correlation studies have suggested that proteases are involved in these phenotypic differences between normal and transformed cells. Some of these studies show that the transformed phenotypes are expressed independently of one another, while other studies suggest that proteases may have only a few primary effects on cells and these primary effects are responsible for many of the other phenotypic changes associated with protease treatment (Burger 1970; Roblin et al 1975; Rossman and Troll 1980). It may be of interest at this point to note that protease-activated phospholipase has been reported in transformed cells, in contrast to untransformed cells (Shier

et al 1982). This suggests that in transformed cell cultures proteases help to degrade both the protein and lipid components of cell membrane and of serum components. This may have profound implications for nutrient availability, nutrient uptake and other activities of the cell that are regulated by the cell membrane. However, at least in principle, this could also have implications for tumour cell death and therapy.

In spite of all the evidence that support the view that proteases play a role in transformation, the results of most studies done with proteolytic enzymes and protease inhibitors are often difficult to interpret because of the following observations: (1) Different levels of proteolytic activity may be required to produce different physiological effects (Blumberg and Robbins 1975). (2) The protease inhibitors used in cell culture studies have different mechanisms and they inhibit different proteases to different extents (Roblin et al 1975). (3) Many of the protease inhibitors have undesirable side effects such as inhibition of protein synthesis; hence it is difficult to be sure that the observed effects on cellular phenotype are due to inhibition of proteolytic enzymes. This is particularly true of the low molecular weight inhibitors such as TAME, TPCK and TLCK (Roblin et al 1975; Chou et al 1975). Also, these inhibitors are often very unstable which makes them very unsuitable for long experiments. (4) Many studies with protease inhibitors do not measure the residual level of proteolytic activity in the inhibitor-treated cultures. Results of protease inhibition studies are only significant if the level of inhibition is known. (5) Many studies with protease inhibitors have

shown similar effects on the growth of tranformed and untransformed cells (Roblin et al 1975). A possible reason for this is that the observed effects of the inhibitors on cell growth are not due to protease inhibition (Chou et al 1975), or untransformed cells may produce low levels of protease which are important to their growth (Goldberg 1974). These limitations against the use of proteases and protease inhibitors suggests that their effects on the properties of cells in culture depend upon a number of experimental variables such as the concentration used, cell density and other culture conditions.

(ii) Protease in Tumour Invasion and Metastasis. A requirement for tumour metastasis is that the cells must be able to overcome the natural tissue barriers. Connective tissue is the commonest barrier. Connective tissue contains proteins such as glycoproteins, collagen and proteoglycans. Both the PA/plasmin system and the collagenolytic enzymes are capable of degrading connective tissue (Rossman and Troll 1980; Wooley 1984a; Duffy and O'Grady 1984). The role of the collagenolytic enzyme in cancer metastasis has already been discussed. The involvement of the PA/plasmin system is demonstrated directly by the observation that administration of urokinase (a plasminogen activator) to rabbits with V2 carcinomas enhanced growth and metastasis (Kodama and Tanaka 1978), while tranexamic acid, an inhibitor of plasmin, prevented pulmonary metastasis (Tanaka et al 1981). Also, the PA/plasmin system and the collagenolytic pathway interact with each other resulting in their activation (Paranjpe et al 1980).

(iii) Proteases in Tumour Cell Nutrition. The role of proteases secreted by tumour cells in modulating the environment of the cell for

nutritional purposes is a relatively unexplored area of research. Although there is no report to show that transformed cells, under normal growth conditions, secrete protease(s) that result in complete degradation of proteins for use as an amino acid source, a recent report shows that this can be achieved when transformed cells are "heat-shocked" at 42°C (Deffie and LeJohn 1984). At 42°C for 4 hours a variety of transformed cells, in contrast to their normal isogenic counterparts, were able to produce glutamine from serum protein through the induction of protease(s) (Deffie and LeJohn 1984); this effect was abolished by a variety of protease inhibitors. Both actinomycin D and cycloheximide also abolished the protease and glutamine production, indicating that the heat treatment led to an actual induction of the protease(s) or its activator (Deffie and LeJohn, unpublished results). That the heat-induced proteolytic activity is associated with tumour cell nutrition is suggested by the observation that heat treatment enhanced uptake of glutamine as evidenced by the fact that the level of glutamine found inside the cell was similar to the extracellular level (Deffie and LeJohn 1984). Although these observations may not be critical in the normal physiology of the tumour cell, it may have some value in cancer therapy using combined hyperthermia and chemotherapy.

Proteases are also indirectly involved in transformed and tumour cell nutrition by their effects on the cell membrane which may help to increase nutrient uptake (Roblin et al 1975). There is also evidence that, in vivo, proteases secreted by tumours promote tumour angiogenesis and the greater blood supply may be of nutritional value (Rossman and Troll 1980).

(c) Site of Action of Proteases

Cells which secrete protease also have intracellular pools of the enzyme; in fact, many cells which secrete PA also possess intracellular activity which is often comparable to, and in some cases exceeds, the extracellularly secreted levels (Rifkin and Pollock 1977; Jaken and Black 1979; Laug 1979). Occasionally cells may possess high intracellular levels of PA without secreting any (Jaken and Black 1979). Hence, it becomes unclear whether it is the intra- or extra-cellular level, or both, that is biologically important. Also, qualitative differences between the two protease components may be important.

Accordingly, the site of action of proteases associated with transformation and malignancy may be intracellular or extracellular. Obviously, the extracellular materials such as serum components and connective tissue are targets of the extracellular enzyme. The plasma membrane of cells has been proposed as another extracellular site of action of many proteases. Various hypothesis have been proposed that proteases affect growth control via the cell surface. Cell surface changes resulting from protease action (measured as increased agglutinability by lectin) may lead to reduced adhesiveness, loss of contact inhibition and increased uptake of nutrients or growth factors (Burger 1970; Roblin et al 1975; Rossman and Troll 1980). However, cell surface changes do not account for other transformed phenotypes. Proteolytic action on the cell surface may also influence growth via an alteration in cyclic nucleotide levels (Rossman and Troll 1980).

Evidence has accumulated that at least some of the action of proteases might be intracellular (Rossman and Troll 1980). In

experiments where proteases are added to cells, this would mean that the exogenous proteases must enter cells. This has been shown for a number of proteases (Hodges et al 1973; Martin and Quigley 1977). The intracellular site of action of proteases appear to be the nucleus where the proteases presumably cleave repressors and thereby derepress particular genes whose product initiate DNA synthesis and mitosis (Roberts and Roberts 1975; Katz et al 1977). This mechanism of action is likely to be important only for long-term regulation of growth by proteases and in situations where free intracellular levels of protease are available, like the case of transformed or tumour cells. It may not apply in short-term experiments with added proteases.

7. HYPERTHERMIC RESPONSE OF TRANSFORMED AND NORMAL CELLS

Hyperthermia refers to temperatures that are above the normal body temperature of the animal from which the cells are derived. Most studies involving hyperthermia of mammalian cells are done within a temperature range of 40°C to 43°C, but higher temperatures have been used in some studies. Such studies are done primarily to understand the characteristics and mechanisms of cell killing by heat, and in laboratory animals and humans as a possible method for cancer therapy (Giovannella et al 1980; Raaphorst et al 1983). Equally important for purposes of cancer therapy are studies of the effects of sublethal hyperthermia on cell metabolism and the induction of thermotolerance.

Heat is a physical entity whose destructive effect upon cells depends on the temperature of heating and the duration of application. These two factors are expressed together in the form of the "Thermal Death Time", which is defined as the minimum time of heating that produces irreversible damage to the cells at a given temperature (Dickson and Calderwood 1980). These authors have compiled a list of the thermal death times for various normal and tumour tissues of rodents and rabbits.

(a) Effects of Hyperthermia on Cells

(i) Biologic Effects of Heat. The biologic effects of heat on cultured cells are varied. Studies have shown that various cell lines differ greatly in their heat sensitivity. There is considerable evidence that those aspects of metabolism that control cell replication (DNA, RNA and protein synthesis) represent an important target for

hyperthermic damage (Streffer 1978; Dickson and Calderwood 1980); membrane effects have also been documented (Yatvin 1977; Hahn et al 1977; Coss et al 1979). In discussing the effects of hyperthermia on cell metabolism, the effects on the cell cycle must not be ignored since several metabolic activities of the cell vary according to the phase in the cell cycle. Heat induces resting cells to enter into the cell cycle (Dickson and Calderwood 1976); this has important implications in cancer thermotherapy in view of the observations that cells in S phase are more heat-sensitive than cells in other phases of the cell cycle (Gerweck et al 1974; Raaphorst and Azzam 1983). Also, the cell cycle-dependence of hyperthermia has implications for cellular events which are also cell cycle-dependent.

Perhaps the most dramatic biological effect of hyperthermia on cells is the induction of synthesis of a small number of polypeptides ("heat shock or stress proteins") with a concomitant decrease in the production of normal cellular proteins (reviewed by Adams and Rinne 1982; Schlesinger et al 1982). The heat shock response has been observed in species of bacteria, plants and animals in response to hyperthermia and an enormous variety of other stresses. Using various biochemical and immunological criteria, it has been shown that most of the heat shock proteins (HSPs) show a high degree of conservation among different species (Schlesinger et al 1982). HSPs are expressed at low levels by cells in unstressed, normal tissue culture conditions. The major HSPs have been purified and characterised and several of them are known to be phosphorylated, glycosylated and/or methylated (Wang et al 1981; Welch and Feramisco 1982; Welch et al 1983). The role of these

protein modifications in the functioning of HSPs is unknown. The function(s) of HSPs is still a matter of intense investigation, but a good amount of data indicate that the HSPs may play a protective role during the stress period (Schlesinger et al 1982; Velazquez and Lindquist 1984). One major stress protein, HSP70, has been implicated in the induction of thermotolerance in cultured Drosophila cells, rodent normal cells, and murine cells (Velazquez and Lindquist 1984; Li 1984). HSP70 interacts with DNA material and it is also an RNA-binding protein (Velazquez and Lindquist 1984). HSPs have also been found in association with the mitochondria, which has led to the suggestion that mitochondria are a primary target of the induced stress proteins (Ashburner and Bonner 1979). This suggests a role in respiration for the stress proteins. An indirect support of this is the fact that hypoxia and glucose deprivation are both inducers of HSPs. Also, it has been shown that stress proteins from root anaerobiosis studies possess alcohol dehydrogenase activity which could be useful in maintaining respiration under conditions where oxygen was limiting (Sachs et al 1980; Tanksley and Jones 1981). In this case the stress proteins are executing a sparing action on intermediary metabolism rather than a protective function for the genome.

The effects of hyperthermia on nucleic acid synthesis is less well studied. Sublethal hyperthermia (in terms of dose and/or duration of heat application) enhances DNA synthesis in some transformed cells (Deffie and LeJohn 1984) perhaps due to the ability of heat to induce resting cells to enter into S phase (Dickson and Calderwood 1976). However, potentially lethal hyperthermia may damage DNA synthesis by its

effects on chromatin-protein association, chromatin structure, beta polymerase activity, chromosomal aberrations and sister chromatid exchanges (Dewey et al 1980; Warters and Roti Roti 1981). RNA synthesis follows a similar pattern as protein synthesis in hyperthermic cells (Schlesinger et al 1982), although enhanced synthesis has been recorded for some transformed cells under sublethal hyperthermia (Deffie and LeJohn 1984).

At the membrane level, thermal cell killing has been correlated with changes in fluidity, cholesterol content and membrane active agents (Yatvin 1977; Hahn et al 1977). After hyperthermia, membrane effects such as blebbing, ultrastructural changes and inhibition of membrane transport have been noted (Lin et al 1973; Kwock et al 1978; Coss et al 1979). Cyclic AMP levels are also affected (Lin et al 1978) as well as membrane-associated polyamine leakage from cells (Gerner and Russell 1977). It has been suggested that generalised changes in membrane permeability is an integral aspect of the mechanism by which hyperthermia kills cells (Gerner et al 1980a).

Other biochemical, cytological and morphological changes associated with hyperthermia include the following: (1) inhibition of activity of the polyamine biosynthetic enzymes (Fuller et al 1977); (2) increased number of intracellular actin bundles as well as a rearrangement of the intermediate filament network (Thomas et al 1982); (3) a more flattened and spread morphology (Thomas et al 1982). The direction of these changes is from transformed towards untransformed phenotypic characteristics, in contrast to other effects of heat discussed earlier. Hence the effects of heat in the reversion of transformed

characteristics is an area that should be further investigated.

(ii) Factors That Affect Heat Sensitivity of Cells. Different environmental factors may influence the response of cells to hyperthermia (reviewed by Overgaard and Nielsen 1980; Kim 1984). Factors known to modify the thermosensitivity of mammalian cells are pH, oxygen concentration, cell cycle phases, growth rate, and the nutritional status of the cell.

pH is a potent modifier of hyperthermic susceptibility in a variety of cellular systems. At 37°C, cell proliferation occurs over a wide range of pH in many normal and transformed cells in culture; however, cells under hyperthermia are sensitive to pH changes (Kim 1984). Several quantitative in vitro studies have shown that an extracellular pH below 7.0 during heating results in a markedly reduced viability of the cells (Overgaard and Nielsen 1980). This observation is important in view of the fact that many transformed and tumour cells lower their environmental pH due to increased aerobic glycolysis and impairment of exchange of metabolic products. By comparing different data it seems that the effect of reduced pH is direct; but indirect effects such as a reduced capacity to accumulate sublethal hyperthermic damage and/or a lowering of the level at which thermal resistance occurs have also been attributed to an effect of reduced pH (Overgaard and Nielsen 1980). Based on ultrastructural observations it has been shown that reduced pH primarily accelerates a lysosomal destruction of malignant cells (Overgaard 1977); this may involve proteases. Reduced pH may also involve other mechanisms that have targets in the nucleus or cell membrane (Gerweck 1977). It is not clear whether it is the

extracellular or intracellular pH that is important in the pH effect. At least one study shows that intracellular pH is important for heat sensitivity (Haveman 1978), while another study suggests that the intracellular pH is unrelated to the hyperthermic damage (Dickson and Oswald 1976). Finally, several studies have indicated that, in addition to the effects of pH on the hyperthermic response, hyperthermia also lowers pH of tumour cells (Bicher et al 1980; Wike-Hooley et al 1984). This may be due to increased lactic acid content of tumours that occur immediately after hyperthermia (Song et al 1982). However, hyperthermia-induced changes in tumour pH occur only at temperatures of 43°C or higher (Wike-Hooley et al 1984).

The effect of hypoxia on the hyperthermic response has also been studied in several systems (Overgaard and Nielsen 1980). This is of interest primarily because hypoxia is a typical condition of solid tumours and because hypoxic cells are radioresistant and show some resistance to certain cytotoxic agents. Acute hypoxia does not significantly influence heat sensitivity (Gerweck et al 1974), but chronic hypoxia does confer heat sensitivity on cells (Gerweck and Burlett 1978). Unfortunately, the validity of these observations is limited by the fact that the technique by which chronic hypoxia was attained may alter other parameters such as pH (Gerweck et al 1981).

The sensitivity of plateau-phase cells to hyperthermia may be both more resistant than similar exponentially growing cells, depending on the cell line and the technique used to obtain the plateau-phase culture (Overgaard and Nielsen 1980). At least some of the heat sensitivity observed in cases of unfed plateau-phase cells may be due to an

increased acidity and to lack of sufficient nutrition (Hahn 1974).

Heat sensitivity of mammalian cells is cell cycle-dependent (Gerweck et al 1974). Several reports show that cells in G_1 phase are more heat resistant than cells in S phase (Raaphorst and Azzam 1983). The accumulation of cells in G_1 probably accounts for the increase in heat resistance of plateau phase cells (Raaphorst et al 1978). Heat induces resting cells to enter S phase, but progression through the cycle is much slower, resulting in a partial accumulation of cells in G_2 (Sapareto et al 1978; Kal et al 1975).

(iii) Thermotolerance. Mammalian cells exposed to non lethal doses of heat subsequently show a transient, non-heritable resistance to further heat treatment. This phenomenon, termed thermotolerance, has been reviewed by Henle and Dethlefsen (1978). Two types of thermal tolerance can be induced in mammalian cells: low temperature thermotolerance which involves exposure of cells at temperatures below 43°C for long, continuous periods; and high temperature thermotolerance which is induced by fractionated heat treatments for short periods at temperatures of 43°C or higher (Kim 1984). Low temperature thermotolerance develops during the heat treatment whereas high temperature thermotolerance requires several hours of incubation at 37°C (after hyperthermic treatment) to develop. Hence, the interpretation of experimental results must take into account the kind of thermotolerance that is likely to develop.

There is some evidence that normal tissues possess higher capacity for low temperature thermotolerance than transformed cells (Overgaard and Nielsen 1980). At least part of this differential thermotolerance

between normal and transformed cells is due to environmental acidity, hypoxia and insufficient nutrition, all of which are conditions that reduce the capacity to resist hyperthermia (Overgaard and Nielsen 1980). It is likely that transformed or tumour tissue have greater ability to generate such conditions in their environment than normal tissues. The decay of thermotolerance occurs faster in cells that are cycling than in those that are not (Hall and Roizin-Towle 1984). All of these factors might form a rationale for supposing that a given heat treatment may produce more thermotolerance in normal tissues than in tumour or transformed cells.

Heat shock proteins have been implicated in the development of thermotolerance (Schlesinger et al 1982). Several lines of evidence, including observations of a temporal relationship between HSP synthesis and thermotolerance development, and protein synthesis inhibition studies, have supported this role for HSPs. Whether or not HSPs are involved in the differential effects of heat-sensitivity or thermotolerance between normal and transformed cells is unknown. No qualitative or quantitative difference in HSPs between normal and transformed cells have been reported. Such studies should also correlate specific HSP patterns with the thermosensitivity that is associated with the transformed state. The investigators who have noted quantitative differences in HSP70 that can be correlated with thermotolerance (Li 1984; Velazquez and Lindquist 1984) did not do comparative studies between normal and transformed cells. Hence the role of HSPs in thermotolerance remains speculative.

Thermotolerance can be prevented, or it can be overcome after it

develops by exposure of cells to 0°C, low pH, or addition of polyamines (Gerner et al 1980a). The effect of polyamines is especially interesting since polyamine levels frequently increase in transformed and tumour cells (Scalabrino and Ferioli 1982a; 1982b). Addition of polyamines to cells increases the permeability of the cells resulting in increased uptake of the polyamines, which then stimulates cells into cycling phase (Gerner and Russell 1977; Fuller et al 1977); cycling cells are less thermotolerant than non-cycling cells (Hall and Roizin-Towle 1984). Polyamines also interact with cholesterol with an overall effect of weakening the cell membrane (Gerner et al 1980a). Other membrane components may also be involved.

(b) Selective Sensitivity of Transformed and Tumour Cells to Hyperthermia

A differential effect on tumour cells relative to normal cells is the basis for the use of any antineoplastic agent. In the case of hyperthermia, this differential may be widened by manipulation of the factors known to affect thermal sensitivity and/or thermotolerance. Studies show that transformed cells fall into two zones of thermal sensitivity, 42°C - 43°C, and 45°C - 50°C (Dickson and Calderwood 1980). The lower temperature zone affects thermal sensitivity by the effect of heat on cell metabolism: there is evidence for stimulatory effect of heat, and tumour cells are affected more than normal cells (Dickson and Calderwood 1980; Deffie and LeJohn 1984). The higher temperature range, on the other hand, sensitises cells by direct heat-induced damages to cellular structure and function: there is irreversible inhibition of metabolic processes; although tumour cells are inhibited

more rapidly than normal cells, considerable damage is done to normal cells too (Dickson and Calderwood 1980). Above 44°C, the time/temperature relationships for normal and tumour cells progressively converge and at 46°C they fuse.

(i) Correlation Between Thermosensitivity and Malignancy.

Differences in heat sensitivity between normal and tumour cells have been recorded for several decades now. Giovanella et al (1980) have critically reviewed the studies that correlate thermosensitivity and malignancy, pointing out artifacts and other limitations in some of the studies. Most of the early studies were not designed to allow quantitative estimation of the effects of heating. Some studies used unreliable methods for determining cell viability after hyperthermia, while in some studies there were inaccurate or unreliable definitions of transformation and tumorigenicity. In spite of these limitations, Giovanella et al (1980) conclude that "the acquisition of increased thermosensitivity by a normal cell is linked and probably caused by one of the early steps on the way to malignant transformation".

(ii) Rationale for Use of Hyperthermia in Cancer Therapy. Because of the strong correlation between thermosensitivity and the malignant state, and because the factors which increase thermosensitivity are more likely to be present in malignant tissues than in normal tissue, it is reasonable to consider hyperthermia as a cancer treatment modality. Clinical trials of thermotherapy have been reported (Jain and Gullino 1980; Lowenthal 1984; Kim 1984).

Heat has certain basic biological effects which make it favourable for its use in cancer treatment (Hall and Roizin-Towle 1984). One of

these effects is that heat acts synergistically with other cancer therapeutic agents such as radiation therapy and chemotherapy (Kim 1984; Bull 1984). Data from cell culture studies have provided quantitative information that may be useful in the therapeutic approach to combination hyperthermia and radiation. Heat treatment at temperatures above 41°C enhances radiosensitisation of cultured mammalian cells, and the degree of radiosensitisation is proportional to the temperature change (Kim 1984). Heat-induced radiosensitisation is not due to enhanced radiation damage by heat but to repair inhibition (Warters and Roti 1979). One important observation relevant to combination hyperthermia and radiation is that cells which are radioresistant are the ones which are most thermosensitive: these are nutritionally deficient, hypoxic, and low-pH cells (Emami et al 1984). Hence cells that escape radiotherapy are more likely to be killed by heat.

Gray et al (1953) first showed that hypoxic cells are usually three times more resistant to X-rays than are oxygenated cells. The ratio between the radioresistance of hypoxic cells and oxic cells is known as oxygen enhancement ratio (OER); hyperthermia acts to reduce OER (Robinson et al 1974; Kim et al 1983). Since heat also sensitises many normal tissues when combined with X-irradiation (Stewart and Denekamp 1978), it, therefore, becomes essential to be able to assess the thermal sensitisation in both the tumour and normal tissue in order to evaluate the therapeutic gain. To evaluate the therapeutic gain, two parameters are employed (Kim 1984):

$$\text{Thermal Enhancement Ratio (TER)} = \frac{\text{Dose required to give a specified level of damage without heat}}{\text{Dose to give the same level of damage with heat}}$$

$$\text{Therapeutic Gain Factor (TGF)} = \frac{\text{TER of tumour}}{\text{TER of normal tissue}}$$

A high value of TER is an indication of the effectiveness of hyperthermia in sensitising cells to radiation damage. Similarly, the higher the value of TGF the greater the therapeutic value of the hyperthermic treatment, that is, the greater the differential sensitivity between transformed and untransformed cells to hyperthermia. The available literature on TER of tumours shows that TER values vary widely. The magnitudes of TER may depend on the temperature, sequence of combined therapy, and type of tumour treated. Sometimes the TER of normal tissue is equally enhanced as the tumour, so that the TGF is either very low or marginal (Kim 1984).

Heat also increases the effectiveness of many chemotherapeutic agents. Heat-drug time sequence may partially dictate how the chemical compound affects the cell due to evocation of thermal tolerance, changes in cellular metabolism, cell membrane permeability or membrane transport activities (Bull 1984). Some of the most sophisticated work on the interaction between heat and chemotherapy are in vitro experiments, yet an important limitation or working with drugs in vitro is that some drugs interact with glass or plastic thereby changing their effect (Hahn 1980). Additional limitations of heat-drug studies in general are that the solvents of certain drugs such as ethanol may be cytotoxic with heat, or they may elicit thermal tolerance by inducing synthesis of heat shock proteins; drugs may also be unstable and decay rapidly with heat (Bull 1984). Hahn (1982) describes four classifications of chemical agents by their interaction with heat: (1) Chemotherapeutic agents that

undergo a linear increase in cytotoxicity with increasing temperature. Examples are the alkylating agents. (2) Drugs that do not exhibit a linear increase in cytotoxicity with heat but show a threshold temperature effect, e.g. adriamycin, bleomycin and actinomycin D. In vivo these agents exhibit little change in cytotoxicity above 37°C until a threshold of 42°C to 43°C when a synergistic interaction occurs. (3) Drugs that have no cytotoxicity at 37°C but that become cytotoxic at higher temperatures, e.g. cysteamine and amphotericin B. These drugs are referred to as hyperthermic sensitisers (Kim 1984). This group of drugs is particularly interesting since, in principle, it poses no danger to surrounding normal tissue if the heat treatment is localised strictly to the tumour tissue. (4) Drugs that have no change in cytotoxic effect between 37°C and 45°C, e.g. 5-fluorouracil, methotrexate, vincristine. Each of these drug categories may have their own special usefulness depending on the tumour system and the method of heat delivery to the tumour tissue.

In discussing the interaction of heat and chemotherapy the concept of a therapeutic index is of basic importance. Another concern is whether the hyperthermic treatment has any carcinogenic effect. This is important in view of the fact that other modes of cancer therapy (principally chemotherapy and radiotherapy) are often carcinogenic. The limited number of studies show that hyperthermia by itself is not carcinogenic for temperatures between 40°C and 45°C (Raaphorst et al 1981; Hall and Roizin-Towle 1984). This does not exclude the possibility that heat may act as a cocarcinogen. Indeed there is controversy concerning the carcinogenic potential of combined heat and

X-ray treatments: Harisiadis et al (1980) reported that hyperthermia reduced the incidence of transformation by X-rays, whereas Raaphorst and Azzam (1982) reported that heat increased the incidence of X-ray induced transformation. This difference may be related to the different transformed cells used or to the different protocols of hyperthermia and X-ray applications used in these two studies.

The therapeutic value of hyperthermia in the treatment of cancers certainly need more investigation. An area that is relatively unexplored is studies of the effects of heat on cellular metabolic activities that control the transformed phenotype. The question to answer is this: does heat promote or reduce the tumorigenicity of cells?

8. GENETIC ASPECTS OF ONCOGENESIS

Studies on the genetic aspects of tumorigenesis and cell transformation have been done largely by employing in vitro cell hybridisation techniques, retroviral and cellular DNA transfection, and analysis of the hereditary cancers. The studies have shown that a genetic basis exists with cancers induced by viruses, chemicals, and physical agents as well as the poorly-defined spontaneous tumours. The nature of the genetic change is not clearly understood but current evidence indicates that oncogenesis is a result of either the activation/addition of new functional genes or the inactivation/loss of previously functional genes or gene products. It has been possible to show that more than one genetic locus is involved in the process. Also, it has been shown that the transformed or tumour cell phenotype can be expressed in a dominant or recessive fashion, depending on the etiological agent and the genetic mechanism involved in the genesis of the neoplasia.

A. Analysis of Transformation and Tumorigenesis by Cell Hybridisation

Cell hybridisation techniques have proven to be powerful techniques in the genetic analysis of neoplasia. Barski et al (1961) showed for the first time that somatic cells can hybridise spontaneously in vitro. Following that, investigators showed that UV light-inactivated Sendai virus increased the hybridisation frequency and that the fused cells were viable and capable of cell division (Okada and Tadokoro 1962; Harris and Watkins 1965). Subsequent studies have led to the discovery of new fusogenic agents such as polyethylene glycol (Pontecorvo 1975),

development of numerous selective systems for hybrid cells (Kusano et al 1971), and production and characterisation of a large collection of different mutant somatic cells (Siminovitch 1976).

In order to understand the molecular events that result in the change of normal cells into transformed or tumorigenic cells, it is essential to determine whether the neoplastic phenotype is expressed as a dominant or recessive trait in animal cells. A dominant expression of the neoplastic state in cell hybrids implies that the neoplastic phenotype results from an acquisition or activation of new genetic material, whereas a recessive expression implies loss or inactivation of genetic material. Cell hybridisation studies have also provided information regarding the role of specific chromosomes, the minimum number of genetic loci involved, species-specificity, and the role of the cytoplasm in the regulation of the neoplastic phenotype.

(a) Dominance and Recessivity of Transformation and Tumorigenicity

(i) Intraspecies and Interspecies Cell Hybrids. The use of intra- and interspecies hybrids between normal and neoplastic cells has contributed valuable information about the genetic control of cell transformation and tumorigenicity (reviewed by Ozer and Jha 1977; Croce 1980; Sabin 1981; Stanbridge et al 1982; Miller and Miller 1983).

The studies by Harris and coworkers (Harris et al 1969; Bregula et al 1971) provided the initial evidence for suppression of malignancy by cell fusion. Their studies involved a series of intraspecies hybrids derived from the A9 mouse fibroblast line and each of three highly malignant ascites tumours of mice. A similar suppression of tumorigenicity has been observed for interspecies hybrids between cells

of different mammalian species such as mouse X human (Jonasson and Harris 1977), mouse X rat (Lyons and Thompson 1977), or mouse X hamster (Howell and Sager 1979). Miller and Miller (1983) have reviewed the evidence that the tumorigenicity of many types of tumour cells is suppressed in cell hybrids formed with non-tumorigenic cells; this includes tumours that originate from various tissues (sarcoma, carcinoma, melanoma, lymphoma, teratocarcinoma) and that are initiated by different carcinogens (spontaneous, chemical, viral, physical, and chromosome- or DNA-mediated).

There is considerable evidence also that tumorigenicity can be expressed as a dominant trait in cell hybrids (Ozer and Jha 1977; Croce 1980). Initial reports for this came from studies with intraspecies hybrids (Barski et al 1961; Scarletta and Ephrussi 1965). A major criticism of these early studies, however, is that the "non-tumorigenic" parental lines used were not proven to be normal. More recent data that suggest dominance of tumorigenicity comes from intraspecies mouse hybridomas (Giacomoni 1979). All hybridomas tested in this cross were found to be tumorigenic in syngeneic and/or nude mice, even hybrids that involved two normal B cells and one myeloma cell. Intraspecies hybrids involving human cells have also been found to express tumorigenicity dominantly (Croce et al 1979). Dominance is also expressed in some interspecies hybrids (Yoshida 1972), particularly those involving SV40-transformed cells (Croce 1980; Stanbridge et al 1982).

The expression of in vitro transformation phenotypes in intra- and interspecies hybrids have shown both dominance and recessivity depending on the hybrid system. As a general rule, the transformation phenotypes

of spontaneously transformed cells behave recessively in hybrids formed with normal partners (Marshall and Dave 1978; Szpirer and Szpirer 1980), whereas the transformed properties of DNA virus-transformed cells are often expressed in hybrid cells (Ozer and Jha 1977; Howell and Sager 1979). The situation with chemically and physically-transformed cells is less clear (Jha et al 1978). In some hybrid systems tumorigenicity behaves recessively while transformation is expressed dominantly (Stanbridge and Wilkinson 1978), thus suggesting that regulation of transformation and tumorigenicity are under different controls.

The use of cell hybrids in the genetic analysis of tumorigenicity and transformation depends on several conditions. An important condition is that the outcome of many studies is limited by the chromosomal instability of hybrid cells. In general, intraspecies hybrids, especially those with human cells, are more karyotypically stable than interspecies hybrids (Ephrussi 1972; Stanbridge et al 1982). However, the use of intraspecies hybrids is limited by the fact that it is difficult to determine the parental origin of the chromosomes. In interspecies hybrids, on the other hand, there are several methods of distinguishing between chromosomes of the two parents. In this hybrid system, preferential loss of chromosomes from one of the parents is commonly observed. Generally, hybrids between human cells and rodent cells lose human chromosomes unidirectionally (Weiss and Green 1967); one exception to this rule is found in the hybrids of HT1080 human fibrosarcoma cells and primary rodent cells which segregate mouse chromosomes (Croce 1976; D'Ancona and Croce

1979). Hybrids between cells of different rodent species may segregate chromosomes from both parental genomes, or they may preferentially lose chromosomes of either of the two parents, depending on the cell types used and their differentiated states (Croce 1980). The rate of loss of chromosomes may not be constant throughout the history of the cultivation of the cell hybrids (Croce 1980). These reports of the chromosomal instability of hybrid cells indicate the need for caution in assessing results based on only a limited number of hybrid clones at a single time in their passage history.

(ii) Karyotypic Analysis of Cell Hybrids. The usefulness of hybrids depends on how fast and to what extent the hybrids segregate their chromosomes. For most purposes, the most useful hybrids are those that undergo a gradual loss of chromosomes in a fairly predictable manner (Miller and Miller 1983). Harris et al (1969) first noted an association between chromosome loss and the re-emergence of neoplastic phenotypic characteristics in hybrids in which tumorigenicity was initially suppressed. This has been confirmed with several other hybrid systems (Miller and Miller 1983). A major area of interest in the genetic analysis of tumorigenicity and transformation is to be able to show whether or not specific chromosomal loss is associated with the re-emergence of neoplastic segregants in hybrid cells. For this purpose, interspecific hybrids are of greater practical value than intraspecific hybrids since in the latter case it is difficult to ascertain the parental origin of specific chromosomes.

In hybrid systems with mouse cells as the nontumorigenic parent, re-emergence of tumorigenicity requires the specific loss of chromosome

4 of the normal parent, although both copies of normal 4 need not be lost (Evans et al 1982). Furthermore, the number of copies of chromosome 4 from the neoplastic parent has a reciprocal effect, enhancing tumorigenicity (Evans et al 1982). The effect of normal chromosome 4 in suppressing tumorigenicity has been observed for hybrids involving many kinds of tumors (carcinoma, teratocarcinoma, sarcoma, lymphoma and melanoma) of mice (Schaefer et al 1981; Evans et al 1982). Hence a common genetic lesion of tumors of mice appears to be associated with gene(s) on chromosome 4. Similar studies with Chinese hamster cells indicate that the Chinese hamster chromosome 3 carried gene(s) that support malignancy (Bloch-Shtacher and Sachs 1977).

The situation with human cells seems to be slightly different. Klinger and his associates (Klinger et al 1978; Klinger and Shows 1979) have indicated that no single human chromosome appears to greatly affect tumorigenicity in cell hybrids, but certain combinations of chromosomes from the normal human cell are capable of suppressing tumorigenicity of hybrids. The most effective suppressor combinations are normal human chromosomes 11 and 13, or 11 and 17; also, chromosomes 9 and 17 in combination were a good suppressor. Other minor suppressor combinations have also been reported (Stanbridge et al 1982). These observations suggest that at least two mutations on separate chromosomes are required for a human cell to undergo malignant transformation, and probably accounts for observations that human cells are difficult to transform in vitro (DiPaolo 1983). This is also an indirect support for multistage carcinogenesis.

(b) Complementation Analysis of the Neoplastic Phenotype

Hybrids between two neoplastic cell lines have been used to examine the question of whether a single genetic locus or multiple loci are involved in the expression of tumorigenicity. If a single, common genetic locus is responsible for all malignancies, then the hybrid will be tumorigenic no matter which combinations of cells are used. But if more than one locus is involved, then complementation between different malignant cells will result in a nontumorigenic hybrid. In principle, this kind of complementation analysis should define, from the number of complementation groups, the number of separate genetic defects which can lead to the expression of tumorigenicity.

Experiments by Harris and coworkers (Harris 1971; Weiner et al 1973; 1974) using a number of intraspecific mouse hybrids, showed that malignant x malignant crosses resulted in hybrids that retained their tumorigenicity, suggesting that a single genetic locus is responsible for the tumorigenic phenotype of mouse cells. However, in these studies, some of the tumorigenic cell lines used were virus-transformed and the hybrid clones exhibited a high level of chromosomal instability, both of which are conditions that may influence the regulatory control of the tumorigenic phenotype (Stanbridge et al 1982).

Complementation has been observed in stable hybrids of human tumours of different somatic cell origins (reviewed by Sabin 1981; Stanbridge et al 1982), and in some rodent systems (Miller and Miller 1983). Complementation did not occur in carcinoma-carcinoma and carcinoma-B cell lymphoma hybrids; in the latter case, the fact that the lymphoma cells contained Epstein-Barr viral genomes may have influenced the expression of tumorigenicity in the hybrids. In

contrast, complementation was observed in stable carcinoma-sarcoma and carcinoma-melanoma hybrids. These data are interpreted to mean that in human cells there appears to be a family of genes controlling the expression of tumorigenicity, possibly a distinct gene for each somatic cell type. This situation is analogous to the observation that different oncogenes are more or less effective at transforming different cell types (Bishop 1983; Cooper and Lane 1984).

(c) Role of Cytoplasm in the Regulation of Tumorigenicity

By fusing cytoplasms derived from normal or tumorigenic cells with whole tumorigenic or normal cells, Howell and Sager (1978) found evidence for a role of cytoplasmic factors in the partial suppression of Chinese hamster tumour but not in an experimentally produced SV40 mouse tumour. Whether the differences in response are due to species specificity or to differences in causative agents of tumorigenicity was not reported. The results are difficult to assess since several of the hybrids whose tumorigenic potential was suppressed were nevertheless found to form tumours after variable periods of time (Howell and Sager 1978). Furthermore, Ziegler (1978) found no evidence for a role of cytoplasm in the expression of malignancy when he used a spontaneous mouse mammary carcinoma cell line. Thus, the role of the cytoplasm in regulation of tumorigenicity is still unresolved.

B. Genetic Mechanisms in Oncogenesis

The nature and function of the genetic change in neoplasms have been sought. The genetic study of oncogenesis involves two aspects: genetic abnormality in cancer cells, and genetic predisposition to

cancer. These two aspects may converge to give a more precise understanding of the basic genetic defects that underlie transformation of normal cells into cancer cells.

Studies on the genetic abnormalities of cancer cells has focussed both on gross chromosomal aberrations and on more subtle mutational events in single genes. This has led to the suggestion of the existence of a set of "cancer genes" that may be similar to, or identical with, the oncogenes of cells and certain tumour viruses. It is postulated that cancer may result from the unregulated expression of "cancer genes".

From a study of the hereditary cancers, a second class of "cancer genes" has been identified. The normal form of these genes may suppress the activity of oncogenes and thus in effect constitute a class of "antioncogenes" (Knudson 1983; 1985). Very little is known about "antioncogenes" since a knowledge of their existence derives from their defectiveness rather than from their activity.

(a) Chromosomal Abnormalities in Transformed and Tumour Cells

Most neoplastic cells show chromosomal abnormalities which tend to progress as the cancer progresses (Nowell 1974; Ponder 1980). These have practical value in cancer diagnosis, classification and prognosis, but whether or not they play any role in the origin and development of the cancer is a matter of controversy. The abnormalities are not entirely random, at least in haematological malignancies, since certain chromosomes in human cells (reviewed by Ponder 1980) or mouse cells (reviewed by Miller and Miller 1983) seem to be involved in rearrangements, duplications or deletions more often than would be

expected by chance. Also, particular abnormalities have been found more or less consistently in association with tumours of a particular type.

A particular form of chromosomal abnormality which has attracted interest is sister chromatid exchange (SCE) (reviewed by Wolff 1977). SCE provides a mechanism for mutation or for gene duplication (Wolff et al 1977), and perhaps a mechanism for mitotic recombination between chromatids of homologous chromosomes (Wolff 1977). However, there are inconsistencies about the role of SCEs in chromosomal aberrations as a mechanism of oncogenesis. First, SCEs and chromosomal aberrations do not arise in parallel in response to exposure to chemicals or radiation (Ponder 1980). Second, SCEs do not correlate consistently with the increased chromosomal abnormalities seen in Fanconi's anemia, Bloom's syndrome and ataxia telangiectasia (Wolff 1977). These are rare inherited syndromes in which chromosomal instability, leading to an increased incidence of chromosomal rearrangements, is associated with an increased risk of malignancy.

In the mouse, specific chromosomal abnormalities have been observed for certain neoplasms. In mouse T-cell and B-cell leukemias as well as plasmacytomas, a duplication of chromosome 15, resulting in cells with three copies of that chromosome (trisomy 15), has been associated with tumorigenicity (Miller and Miller 1983). Extra copies of chromosome 15 have also been observed in transformed cell lines in vitro (Miller and Miller 1983). In one study, it was found that the most striking difference between the A9 cell line and a highly tumorigenic derivative of it, A9HT, was an increase from four to six copies per cell of chromosome 15 (Allderdice et al 1973). Hybrids between melanoma cells

and diploid fibroblasts or lymphocytes show suppression of their tumorigenicity, but tumours arise with those hybrids which show trisomy 15 (Jonasson et al 1977). This is not simply a matter of gene dosage since it is not the total number of chromosome 15 but the number derived from the malignant parent that is important. In these studies, tumorigenicity was associated with trisomy 15 only when chromosome 4 of the normal parent was simultaneously absent from the hybrid (Miller and Miller 1983). It seems, therefore, that in the mouse, gene(s) on chromosome 15, when appropriately matched, enhances tumorigenicity, whereas gene(s) on chromosome 4 suppress tumorigenicity. Other less common, specific chromosomal trisomies have been reported in a variety of tumours of mice (Miller and Miller 1980) and humans (Ponder 1980). These multiple types of trisomies may reflect the effect of tissue specificity in malignancies. Some tumours appear to have no chromosomal aberrations (reviewed by Miller and Miller 1983).

Chromosomal imbalances enhance tumorigenicity by allowing the expression of mutant recessive genes (Ohno 1974) or they alter the balance between genes concerned with the expression and suppression of the malignant phenotype (Bloch-Shtacher and Sachs 1977; Sachs 1979). There is evidence that polyploidisation and segregation of chromosomes facilitates the transformation of fibroblasts by virus (McPherson 1970). The role of specific chromosomal aberrations will become clearer when complete identification of genes carried by the specific chromosomes has been carried out.

(b) Gene Amplification Model of Oncogenesis

Gene amplification is the process whereby an increase in the number of copies of a gene or other DNA sequence occurs under a variety of circumstances. The concept that gene amplification relates to the initiation and/or progression of cancer has been supported by evidence from oncogene and cytogenetic studies as well as from observations that tumour promoters stimulate SCE (reviewed by Pall 1981; Shimke 1984). The mechanism whereby gene amplification occurs is believed to involve large tandem duplications and unequal sister chromatid exchange. Both events have been demonstrated in various genetic studies of oncogenesis (Pall 1981). Since unequal crossing over is a rare event and each must occur in a different cell cycle (Pall 1981), the process requires considerable time, thus providing an explanation for the long latency period between exposure of cells to carcinogen and the appearance of the transformed cell.

The oncogene evidence for gene amplification has been obtained mainly by using DNA cloning, restriction mapping and Southern blot techniques. Transformed and tumour cell lines contain amplified oncogenes, including c-myc, c-K-ras and c-abl (Schimke 1984).

Cytogenetic studies have shown that two types of chromosomal abnormalities are found that represent amplified genes: homogeneously staining regions (HSRs) and double minute chromosomes (DMs) (Pall 1981; Miller and Miller 1983). HSRs and DMs occur in a wide variety of neoplastic cells but are very rare in non-neoplastic cells (Levan et al 1977). Presumably, HSRs are genetically homogeneous, representing multiple tandem copies of the same DNA sequence.

The gene amplification model predicts that carcinogens and promoters act by stimulating both cell division and SCE. There are data which show that this might indeed be the case (Kinsella and Radman 1978; Pall 1981). Furthermore, although the correlation between SCEs and chromosomal abnormalities is inconsistent (Wolff 1977), there is at least one report that Bloom's syndrome individuals, who are predisposed to cancer, have high frequencies of SCE (Chaganti et al 1974).

Not all elongated marker chromosomes arise by gene amplification, nor is gene amplification always associated with a visible HSR or DMS (Miller and Miller 1983). Similarly, not every malignancy is likely to be the result of gene amplification, especially those which do not show HSRs or DMS. Three specific categories of malignancies are unlikely to be generated by this mechanism, unless additional features are assumed: the malignancies characterised by specific deletions or translocations (Klein 1981); virus-induced transformation (Temin 1980); and tumours that are considered to be of nongenetic origin such as the teratocarcinomas (Mintz and Illmensee 1975).

(c) DNA Rearrangement Model of Oncogenesis

DNA rearrangement may be defined as a change in the linear order of the DNA sequence and may be accomplished through deletion or translocation of genetic material (Coffey 1983; Klein 1983). The possible role of DNA rearrangements in cancer was initially noted by Temin (1980) who suggested that known proviruses act similarly to transposable elements and that their location at specific sites in the host genome might modify cellular expression and lead to cancer. Also, DNA repair disorders that predispose to cancer manifest chromosomal

rearrangements (Coffey 1983).

Specific chromosomal translocations have been reported for certain kinds of cancers (Klein 1981). The most common are translocations associated with chronic granulocytic leukemia in humans, Burkitt's lymphoma, and murine plasmacytomas. Some of the rearrangements involve specific oncogene sequences, such as the translocations that result in increased expression of c-myc genes (Rowley 1982; Klein 1983), or overexpression of other cellular oncogenes by juxtaposition to a retrovirus promoter with high expression efficiency (Hayward et al 1982).

The DNA rearrangement model of oncogenesis may be used to explain several aspects of the carcinogenic process. First, DNA rearrangements may be a continuing process and could be responsible for tumour progression and tumour cell heterogeneity (Feinberg and Coffey 1982). Second, the high frequency of transformation of some mammalian cells on exposure to carcinogens might be explained by DNA rearrangement rather than by point mutation (Cairns 1981). Third, reversal of DNA translocation in the tumour cell may underlie the apparent reversibility of cancer in several systems such as the teratocarcinomas, neuroblastomas, Lucke's tumours, and plant galls; in these systems, DNA rearrangements could account for the high frequency of reversal from the malignant state (Coffey 1983).

(d) DNA Methylation as a Mechanism of Gene Control

(i) Gene Regulation by DNA Methylation. Mammalian DNA is modified after replication by the enzymatic conversion of a few cytosines to 5-methylcytosine. A methyltransferase enzyme transfers a methyl group

from S-adenosylmethionine (SAM) to the 5-carbon of cytosine. SAM serves as the predominant biological methyl group donor (Cantoni 1953). Methionine is the biosynthetic precursor of SAM (Bennett 1950). Homocysteine, a key intermediate in the pathways of methionine synthesis, can promote growth in place of methionine (Met^1Hcy^+ medium) in cultured normal cells (Mangum and North 1968); these cells are termed methionine-independent. On the other hand, cancer cell lines of very different origins are methionine-dependent since they are unable, or show reduced ability with respect to normal cells, to proliferate when methionine is replaced by homocysteine (reviewed by Hoffman 1982; 1984). The prevalence of methionine dependence in cancer cell lines suggests an important relationship between methionine and oncogenic transformation. This relationship is further suggested by the fact that cancer cells which revert from methionine dependence to methionine independence also revert toward normalcy in some of their transformed properties (Hoffman et al 1979), and by the observations that methionine can serve as a chemoprotectant and prevent cancer in animals (Hoffman 1984). The biochemical basis of methionine dependence is not completely understood, but the level of methionine synthetase is one factor that determines the ability of cells to grow in Met^-Hcy^+ medium (Tautt et al 1982).

Few pieces of evidence are especially relevant at demonstrating the relationship between DNA methylation and gene expression. First, there is a correlation between undermethylation and the high level of expression of a gene (Riggs and Jones 1983). Similarly, cells with hypermethylated DNA express their genes at a lower rate than those with

hypomethylated DNA (Hoffman 1984). The correlation between levels of DNA methylation and gene expression is strongest for the 5' flanking regions, which contain the promoter or other control elements of DNA. But in most cases the coding regions and introns are also less methylated when the gene is being expressed. However, as proposed by Razin and Riggs (1980), undermethylation of DNA may only be a necessary, but not sufficient, condition for gene expression since other control levels are also needed. Second, in vitro methylation of a gene reduces the efficiency of activity of the gene both in vitro and in vivo (Pollack et al 1980). Third, inhibition of methylation activates genes. This has been achieved with several methylation inhibitors, using cells or purified DNA from several mammalian species (Rigg and Jones 1983). Fourth, 5-methylcytosine affects protein-DNA interactions that control transcription of genes (Razin and Riggs 1980). The proteins involved may be structural chromosomal proteins or transcriptional enzymes.

(ii) Altered DNA Methylation in Neoplastic Cells. Cancer cells are often disrupted in their DNA methylation or in methionine metabolism. A measure of the methylation levels in neoplastic cell lines shows that low levels of DNA methylation are seen in spontaneously transformed or tumour cells (Riggs and Jones 1983; Hoffman 1984). The situation with chemically - or virally - transformed cell lines is more complicated. There have been reports of both a decrease and an increase in DNA methylation of cells transformed by a virus or chemical (reviewed by Riggs and Jones 1983; Hoffman 1984). In these instances, as in others described earlier, the increased levels of DNA methylation were accompanied by decreased gene activity. However, there is at least one

report wherein increased methylation resulted in increased gene activity (Tanaka et al 1983). Hence, not only is it possible to have increased methylation upon oncogenic transformation, but in some instances an increased methylation of a gene may lead to increased expression.

In a number of studies, the extent of DNA methylation of specific genes have been examined (reviewed by Hoffman 1984). The cellular oncogenes c-myc, c-K-ras, and c-H-ras have all been found to be hypomethylated in tumours as compared to normal cells (Feinberg and Vogelstein 1983; Hoffman 1984). It may be of interest to note that c-myc and the c-ras oncogenes have almost all their CCGG and GCGC sequences (the methylatable sequences) in the 5' regions of the gene (Capon et al 1983). The fact that the sequences appear predominantly in the regions associated with regulation of the gene indicates a specific role for methylation in silencing those oncogenes. It has been suggested that the non-random overrepresentation of CG doublets in the 5'-regions of genes may be a property of many genes (McClelland and Ivarie 1982). Other genes found to be undermethylated in cancer cells compared to their normal counterparts are the α -feto-protein gene (Vedel et al 1983) and the ribosomal RNA genes (Kunnath and Locker 1982). The former gene is not normally expressed in adult tissues but it is frequently expressed in neoplastic cells (Lawrence and Neville 1972; Alexander 1972).

A variety of chemical carcinogens can directly reduce methylation of DNA segments (Riggs and Jones 1983). These include 5-azacytidine, various alkylating agents and polycyclic aromatic hydrocarbons. Carcinogens which are reagents for sulphydryl groups can act directly on

DNA methyltransferases and inactivate them (Drahovsky and Wacker 1975). The ability of carcinogens to inhibit methylation might also be due to steric hindrance, especially in the case of structurally bulky chemicals (Pfohl-Leszkowicz et al 1982).

Two other potentially important properties influencing DNA methylation have been described, one a presumptive demethylase (Gjerset and Martin 1982) and the other a protein which preferentially binds heavily methylated DNA (Huang et al 1984). It is not known whether these proteins change in cancer.

There are two important predictions of the DNA methylation model of oncogenesis. First, it predicts that oncogenic transformation may be controlled by methionine. Second, if demethylation of DNA is an important carcinogenic event, the possibility arises of remethylating DNA and reversing oncogenesis. The compound 1- β -D-arabino-furanosylcytosine (AraC), which is often used in treatment of cancer (Hoffman 1984), has been shown to increase the overall methylation of P815 mouse mastocytoma cells in culture from 3% to 15% of cytosines methylated (Boehn and Drahovsky 1982). Another approach may be to treat cancer cells with 5-methylcytosine, but initial reports in this direction indicate that 5-methylcytosine is not incorporated into DNA of human leukemia cells (Jekunen et al 1983).

(e) Oncogene Activation

One of the most fruitful investigations of the nature of the genetic changes that underlie carcinogenesis have involved certain critical genes in retroviruses and cells, called oncogenes.

(i) Viral and Cellular Oncogenes. In 1911, Peyton Rous isolated

Rous Sarcoma virus (RSV) as a filterable agent that could produce sarcomas in inoculated chickens (Rous 1911). Much later, the viral gene responsible for tumour induction by RSV was identified in the viral genome (Martin 1970); subsequently a cellular homologue of the retroviral transforming gene was found in normal uninfected cells (Hill and Hillova 1972; Stehelin et al 1976) and it was observed that the cellular gene is conserved in all vertebrates examined (Spector 1978). Prior to the description of the viral and cellular oncogenes, Huebner and Todaro (1969) had proposed the virogene-oncogene hypothesis, which postulated that normal cells contain sequences (oncogenes) homologous to the replicative genes of retroviruses (virogenes).

Viral oncogenes are designated v-oncs, the cellular counterparts as c-oncs, and each of the viral or cellular genes by terms derived from the names of the viruses in question. Oncogenes identified by transfection have been known variously as "tumour genes", "cellular oncogenes", "transforming genes" or merely "oncogenes" (Bishop 1983). Proto-oncogene is used to denote the cellular progenitors of v-oncs (Bishop 1981).

Retroviruses can be classified into two groups: those that contain oncogenes and those that do not (for reviews see Graf and Beug 1978; Hayward et al 1982). The common feature of oncogenesis by both groups of viruses, however, is the activation of a c-onc gene. Although the mechanisms are different, in both cases a cellular gene is placed under the control of viral regulatory sequences and thus expressed constitutively at high levels. Considerable evidence now exists that shows that v-oncs have arisen by transduction of cellular oncogenes

(Bishop 1983).

Three experimental strategies have been used to enlarge the repertoire of c-oncs and to implicate the genes in carcinogenesis (Bishop 1983): transduction by retroviruses; insertional mutagenesis by retroviruses; and DNA-mediated gene transfer. A description of the cellular oncogenes and their viral counterparts that have been identified by these methods is provided in several recent reviews (Bishop 1983; Astrin and Rothberg 1983; Cooper and Lane 1984; Shih and Weeks 1984).

Bishop (1983) and Astrin and Rothberg (1983) have reviewed the studies reporting the chromosomal locations of oncogenes in humans, mice and chicken. Four main conclusions emerge from the studies. First, c-oncs are widely dispersed among the chromosomal complement of human cells. This is not peculiar with oncogenes since there are previous examples of related genes, or of genes whose functions are coordinately induced, that are located on different chromosomes (Hughes et al 1979). Second, in mice there is some evidence of apparent synteny of three genes encoding tyrosine-specific protein kinases (c-src, c-fps/fes, and c-abl) on chromosome 2. However, there is no evidence of functional linkage between the genes. Third, the human chromosomes carrying c-abl, c-fps/fes and c/src each have at least one other locus with the mouse chromosome carrying the same oncogene: adenylate kinase in the case of c-abl (chromosome 2 in mice 20 in human); sorbitol dehydrogenase and β -2 microglobulin in the case of c-fps/fes (2 in mice and 15 in humans); and inosine triphosphatase and adenosine deaminase in the case of c-src (2 in mice, 20 in humans). The importance of these

relationships will depend on whether these loci play any role(s) in the oncogenic process. Fourth, the *myc* gene in humans has been localised on chromosome 8 to band q24, the breakpoint in Burkitt lymphoma-type translocatins (Dalla-Favera et al 1982). Based on present evidence, there is no indication that oncogenes are necessarily located on chromosomes that are identified in cell hybrid studies to play regulatory roles in tumorigenesis.

(ii) Evidence that c-oncs are Involved in Neoplasms. Evidence for the direct involvement of c-oncs in cell transformation and tumorigenesis has come from in vitro and in vivo studies, and from biochemical analysis of cellular oncogenes of normal and neoplastic cells.

Most of the in vitro evidence comes from studies involving DNA-mediated gene transfer (transfection) (Bishop 1983). Using this method, c-onc DNAs from several types of tumours have been shown to be effective at transforming several lines of cells, including cells that are stably diploid and display the multistep pattern of chemically-induced transformation. In contrast, DNAs from normal cells do not transform cells in transfection studies unless certain manipulations are done with them (Cooper 1982).

Another kind of evidence that implicates c-oncs in neoplasia comes from observations of oncogene expression in tumour tissues (Astrin and Rothberg 1983; Bishop 1983; Cooper and Lane 1984). The augmentation of c-*myc* expression in Burkitt's lymphoma is the best studied (Erikson et al 1983), but the activation of other oncogenes have also been reported for other specific tumours (Cooper and Lane 1984). A different

kind of in vivo evidence that implicates c-oncs in tumorigenesis comes from work with avian leukosis viruses (ALV). ALVs are slowly transforming retroviruses that do not contain oncogenes (Weiss et al 1982), yet they induce B-cell lymphomas in chicken at high frequencies (Cooper et al 1978). Tumorigenesis is affected when viral DNA integrates in the vicinity of a c-onc (Hayward et al 1981). The insertions of viral DNA can occur in different orientations and at different positions with respect to the c-onc gene that is activated (Bishop 1983).

The multistep nature of oncogenesis has been viewed from several perspectives, such as the independent roles of initiating and promoting agents (Collins et al 1980), the need for multiple mutations in a somatic cell before a tumour arises (Moolgavkar et al 1981), and the incremental progression of a cell towards a malignant phenotype (Farber and Cameron 1980). Multistep oncogenesis can now also be explained in terms of c-oncs (Bishop 1983; Cooper and Lane 1984). In several types of neoplasias at least two different c-oncs are reproducibly activated; this has also been shown by transfection assays (Cooper and Lane 1984). Experimental data suggest that c-myc activation is probably required at the initiation stage (Cooper and Lane 1984; Macara 1985); a few other c-oncs also seem to be required during the early stage. On the other hand, other c-oncs such as ras, Blym 1, etc. seem to act at later stages (Cooper and Lane 1984; Macara 1985). NIH 3T3 cells, an immortalised cell line, is frequently used in transfection assays. These cells are sensitive to transformation by some oncogenes while they remain insensitive to others. It has been suggested that those c-oncs which do

not induce transformation of NIH 3T3 cells may be involved in early stages of neoplasia and those c-oncs that transform NIH 3T3 cells are involved in the progression stages (Cooper and Lane 1984). The implication of two distinct genes in oncogenesis suggests that the dosage and qualitative theories might be reconciled: enhanced expression of an otherwise normal cellular oncogene (for example, c-myc) initiates tumorigenesis, whereas a subsequent qualitative change in a second oncogene (for example, c-ras) would provide a maintenance function, as appears to be the case with many neoplasia (reviewed by Bishop 1983; Cooper and Lane 1984).

(iii) Modes of Activation of Cellular Oncogenes. Activation of c-oncs follows two major mechanisms (Bishop 1983). The first involves a qualitative change (mutation) to the c-onc gene(s) such that the function(s) of the gene product are altered. The mutation can be in the form of point mutations, recombinations, deletions, or insertions (Shih and Weeks 1984). The second mechanism involves quantitative (or dosage) changes. Excessive expression of a normal c-onc might convert cells to the neoplastic state. The two mechanisms probably operate simultaneously in a single neoplasm, and a single molecular event can result in changes involving both mechanisms. There are experimental evidence and counterevidence for both types of mechanisms (Bishop 1983; Astrin and Rothberg 1983).

Point mutations induced in oncogenesis could be cis- or trans-acting. Mutations within or adjacent to an oncogene might alter the function of the c-onc or make it resistant to negative regulators. This kind of qualitative mechanism of activation has been demonstrated

for the c-ras genes (Shih and Weeks 1984; Cooper and Lane 1984). DNA sequencing of c-H-ras gene has shown that the whole DNA sequence is identical for normal and tumour genes except for a single nucleotide change from G to T at the codon specifying the 12th amino acid of the p21 protein. This results in the substitution of valine for glycine at the 12th amino acid residue (Taparowsky et al 1982; Tabin et al 1982). Other mutations have been reported in the c-ras gene from different tumours, and in other c-onc genes (Shih and Weeks 1984; Cooper and Lane 1984). Point mutations could also occur in regulatory genes thereby destroying their activity and releasing c-oncs from their control. These regulatory loci could be the "antioncogenes" discussed by Knudson (1983; 1985). This would result in a quantitative overexpression of the oncogene. This kind of genetic lesion would be expressed in a recessive manner in cell hybrid systems and it will not be detected by transfection studies. The prevalence of point mutations in neoplasms reinforces the speculation that cancer might be a molecular disease (Wierenga and Hol 1983) akin to other molecular diseases such as sickle cell anemia.

Rearrangements of DNA have also gained popularity as a potential driving force in carcinogenesis (Cairns 1981). The resulting damage to DNA could affect oncogene expression in a manner similar to that outlined for point mutation. Alternatively, rearrangements of DNA could bring silent oncogenes under the influence of active promoters of transcription (Klein 1981). This is what occurs in the myc or mos gene activation in Burkitt's lymphoma (Rowley 1982; Rechavi et al 1982). Another transcriptional promoter that can enhance the expression of

c-oncs is the long terminal repeats (LTRs) of retrovirus proviruses (Varmus 1982). This activity accounts for the induction of c-myc expression by up to 100-fold during the genesis of B-cell tumours by infection with ALV. Similarly, various c-onc genes which do not transform cells in their native states can be made active by in vitro attachment of LTRs (reviewed by Astrin and Rothberg 1983).

(iv) Modes of Action of Oncogenes. Almost all c-oncs act via their protein products (Bishop 1983). Transcription and translation of c-oncs are similar to those for other cellular genes. Protein products for c-oncs act in three different biochemical roles: tyrosine kinase activity; GDP/GTP binding; and nuclear effects (Bishop 1983; Shih and Weeks 1984; Macara 1985). In addition, there is evidence that different oncogenes act at different points on the pathway to malignancy; some are active during the early, initiation stage, while others are active during the progression phase (Heldin and Westermark 1984; Macara 1985).

The c-src gene product typifies the tyrosine kinase class of oncogene products. The product of vertebrate c-src gene is a 60,000 dalton phosphoprotein (pp60^{c-src}) (Brugge and Erikson 1977; Collett et al 1979). pp60^{c-src} phosphorylates protein substrates on tyrosine residues rather than the more commonly phosphorylated sites (serine and threonine) of proteins (Hunter and Sefton 1980). Other c-onc proteins that have tyrosine kinase activity include fes/fps, abl, yes, and ros (Shih and Weeks 1984). The properties and physiological regulation of tyrosine protein kinases have been reviewed (Hunter 1982; Hunter and Cooper 1983; Cobb and Rosen 1984; Nelson and Branton 1984).

The proposition that tyrosine kinase activity plays a key role in the regulation of cell proliferation is based largely on the observation that many c-onc (and v-onc) proteins have tyrosine kinase activity (Shih and Weeks 1984), and that neoplastic cells have several-fold increase in tyrosine residue phosphorylation relative to normal cells (reviewed by Hunter and Cooper 1984). In addition, retroviral mutants lacking tyrosine kinase activity are transformation defective (Anderson et al 1981). This apparent role of tyrosine kinase activity in oncogenesis is, however, difficult to reconcile with the following observations. First, the phosphorylation stoichiometries of all known in vivo substrates are so low that a regulatory role is unlikely (Macara 1985). Second, non-proliferating cells can possess tyrosine kinase activity as high as those of rapidly proliferating cells (Tuy et al 1983). Third, many of the in vivo substrates of the kinases that have been identified are not of obvious regulatory significance (Macara 1985). Sometimes the functions of key growth regulatory proteins are independent of phosphorylation on tyrosine. Fourth, the major sites of tyrosine autophosphorylation of pp60^{C-src} are apparently dispensable for transforming activity in cell culture assays (Snyder et al 1983). Fifth, a temperature-sensitive mutant of RSV has been characterised; it produces a pp60^{V-src} that possess normal tyrosine kinase activity even at the non-permissive temperature (41°C) at which the infected cells are not morphologically transformed (Stoker et al 1984).

One approach for determining the relevance of tyrosine phosphorylation in oncogenesis is to identify the substrates of the kinases and the effects of their phosphorylation in regulation of cell

growth. Several proteins, ranging in size from about 28,000 to 81,000 daltons, have reportedly been identified to act as direct substrates of c-onc protein kinases (reviewed by Houslay 1981; Hunter 1982). But the physiological roles of most of these protein substrates are not known, hence their role in oncogenesis is uncertain. So far vinculin seems to be the only protein substrate whose state of tyrosine phosphorylation appears to be connected with transformation (reviewed by Hynes 1982; Hunter and Cooper 1983).

A different mode of action of oncogene protein kinases has been advanced by Macara and coworkers (Macara et al 1984; Macara 1985). They postulate that oncogene protein kinases regulate the turnover of phosphatidylinositol (PI), either directly (as PI kinase) or indirectly (via tyrosine phosphorylation). PI metabolism, in association with the protein kinase C system, activate several aspects of cellular functions (reviewed by Nishizuka 1984b; Macara 1985).

The c-ras genes each encode a protein with molecular weight of about 21,000 ($p21^{c-ras}$) (Langbeheim et al 1980). $p21^{c-ras}$ proteins are not tyrosine kinases. They specifically bind to guanine nucleotides (GTP and GDP) with high affinity (Bishop 1983; Shih and Weeks 1984). Like the tyrosine kinases, the p21 proteins are also cytosolic proteins or sometimes plasma membrane-associated. p21 protein also shows autophosphorylating activity on serine residues (Shih et al 1980) but no kinase activity for other protein substrates has been reported. The function of the ras proteins remains largely enigmatic. Macara (1985) has presented a model wherein he proposes that the ras proteins act in the progression stage of the oncogenic process.

c-myc, c-myb, and c-fos are nuclear proteins (Shih and Weeks 1984). They seem to play a direct role in the regulation of gene expression (Heldin and Westermark 1984) since they are DNA binding proteins (Macara 1985). It seems that this set of c-oncs represent the ultimate point of action of other oncogene proteins and growth factors (Macara 1985), and this may be the explanation for the wide variety of neoplasias with an abnormal expression of the c-myc gene (Heldin and Westermark 1984).

Oncogenes probably regulate cell growth in a manner similar to the action of growth factors. Heldin and Westermark (1984) have reviewed the data that link different oncogenes to each of the levels of action of growth factors. The c-sis protein in particular shows striking similarity to one of the polypeptide chains of the PDGF molecule (Heldin and Westermark 1984).

The functions of oncogenes in normal cellular processes is not precisely known, but it is believed that they play a role in differentiation (reviewed by Bishop 1983; Astrin and Rothberg 1983).

MATERIALS AND METHODS

MATERIALS

Mammalian Cell Lines

CHO WT cells were obtained from S. H. Goh. and the CHO CR7(ts) and CHO R_C cell lines from Thor Borgford, both of whom were graduate students at the Dept. of Microbiology, U. of Manitoba. The CHO CR7(ts) is a temperature-sensitive mutant of the CHO WT cell line and CHO R_C is a revertant clone of the CHO CR7 line (Ceri and Wright 1977; Wright et al 1978). Mouse B82 (TK⁻) and human SK4476 (HGPRT⁻) cell lines were kindly donated by Dr. L. Donald, Human Genetics Division, Faculty of Medicine, U. of Manitoba. Mouse 10T $\frac{1}{2}$ cell line, its H-ras oncogene transformed counterpart 10T $\frac{1}{2}$ clras-2, and X-ray transformed counterpart 10T $\frac{1}{2}$ MDS-R2, were supplied by Dr. J. Borsa, Atomic Energy Research Station, Pinawa, Manitoba, Canada. The El San (normal human skin diploid fibroblasts), On Ser (human HGPRT⁻ skin diploid fibroblasts), and WI-38 (human lung diploid fibroblasts) and its SV40 transformed counterpart WI-38 VA13 cell lines were purchased from the American Type Culture Collection, Rockville, Md., USA. On Ser-DMBA is a cell line isolated when On Ser was chemically transformed by us (Deffie and LeJohn, 1984) with 9,10-dimethyl-1,2-benzanthracene (5 μ g/ml) according to the procedure of Chen and Heidelberger (1969). The mouse-human hybrid cell clones were obtained by fusing B82 and SK4476 cells with polyethylene glycol according to the method originally developed by Pontecorvo (1975) and optimised by Davidson and Gerald (1976).

Growth Media, Sera, and Culture-wares

Alpha-minimal essential medium (α -MEM) was obtained from GIBCO, Canada. α -MEM lacking glutamine, methionine, or phosphate were prepared by me according to the method of Stanners et al (1971). Other media requiring specific supplements were prepared by adding the appropriate amount of the supplements to standard α -MEM.

Mycoplasma tested and virus screened calf serum, and dialysed fetal bovine serum were purchased from GIBCO, Canada.

Culture plates and tubes and other plastic-ware were obtained from either Lux Scientific Corporation, Ca., USA, or Falcon, Ca., USA. Brockway bottles were purchased from Brockway Glass Co.

Radioisotopes

Radioisotopes were obtained from the radiochemical companies Amersham and New England Nuclear. ^{32}P -orthophosphate (carrier-free) was obtained in dilute HCl (pH 2-3). The specific activities of the radioisotopes used are reported in the appropriate "Methods" and "Results" sections.

Miscellaneous Materials

(a) All chemicals and enzymes used were of analytical grade and were purchased from one of the following commercial sources: Sigma Chemical Co., Difco Laboratories, Calbiochem, J. T. Baker Chemical Co., Fisher Scientific, and Bio-rad Co.

(b) Trypsin used for cell detachment was obtained from Sigma Chemical Co., St. Louis, Mo., USA, or from Difco Laboratories, Detroit, Michigan, USA.

(c) Polyethyleneimine (PEI) cellulose plastic-backed, thin-layer plates were from Brinkman Instruments (Canada).

(d) Kodak X-Omat RP4 films were used for autoradiography.

(e) Gelman glass fibre filters, types A-E, from Gelman Instrument Co., Michigan, USA.

(f) Observation of growth and morphology of the cells was done with ordinary light and phase-contrast microscopes. Photographs of cells were taken with an ordinary light microscope fitted with a camera.

METHODS

1. Growth of Cell Lines

All cell lines were routinely cultured in Brockway bottles at 37°C in a humidified 5% CO₂ atmosphere incubator. Cells were subcultured on reaching confluency, using 0.1% trypsin in phosphate buffered saline to detach them from the glass surface. The split ratios for the permanent cell lines varied while a 1:4 ratio was rigorously followed for all primary cell lines. Primary cell lines were used between passage number 5-35 for all experiments.

The growth medium used for culturing cells was alpha minimal essential medium (α -MEM) (Stanners et al 1971) supplemented with 10% calf serum; fetal bovine serum was used to culture human cell lines, and occasionally, other cell lines too. Penicillin and streptomycin were added at 60.6 mg/liter and 68.6 mg/liter respectively.

2. Growth, ³²P-Orthophosphate Labelling, and Formic Acid Extraction of Cells for Analysis of HS3 During Various Growth Conditions

Labelling of cells with ³²P-orthophosphate, identification and quantitation of acid soluble nucleotides and polyphosphate by one-dimensional chromatography on PEI cellulose plates, followed by autoradiography, have been described (Lewis et al 1976; Goh and LeJohn 1977; Goh 1979).

Cells were grown to near monolayer stage in Brockway bottles as described in section 1 of "Methods", and then trypsinised with 0.1% trypsin for 3-5 minutes. After trypsinisation, the cells were transferred into a pre-warmed growth medium. The cells were then plated

into 60 x 15 mm or 35 x 15 mm plates and allowed to grow for 24 h before use. For experiments involving the protease inhibitors TLCK and TAME, the cells were allowed to grow for 48 h before use, in accordance with suggestions by Schnebli (1975) that cells seeded for less than this period of time are very sensitive to the toxic effects of these protease inhibitors. Permanent cell lines were used at densities of $0.5 - 1.0 \times 10^6$ cells/60 mm plate and $0.2 - 0.5 \times 10^6$ cells/35 mm plate, whereas for primary cell lines the cell densities used were $0.2 - 0.5 \times 10^6$ cells/60 mm plate and $0.1 - 0.2 \times 10^6$ cells/35 mm plate. For ^{32}P labelling studies, the cells were first washed twice with 5 ml of the appropriate pre-warmed medium. Pre-warmed incubation medium containing 10% dialysed fetal bovine serum (DFBS) was next added to each culture plate (3 ml for 60 mm plate, and 1.5 ml for 35 mm plate) together with ^{32}P -orthophosphate (250 $\mu\text{Ci/ml}$). The cells were then incubated for various times at 37°C or 42°C . The ^{32}P labelling was terminated by aspirating the incubation medium, followed by a 5 ml wash with ice-cold 0.9% NaCl. Cells were then rapidly frozen for 24 - 48 h after which they were extracted for at least 30 min at 4°C with 1 M formic acid (200 μl for 60 mm plates, 100 μl for 35 mm plates). The formic acid extract was centrifuged to spin down cell debris and the clear liquid was either kept frozen at -20°C or applied immediately to PEI plates.

3. Chromatography of ^{32}P -Labelled Formic Acid Extract From Mammalian Cells

Ten microliters of the formic acid extract was spotted on lithium chloride-washed and methanol-dried PEI cellulose plates and developed one-dimensionally with 1.5 M KH_2PO_4 at pH 3.65 by ascending

chromatography until the solvent front reached 15 cm from the origin.

After chromatography, the PEI cellulose plates were dried and then exposed to films for 24 - 48 h before development. The appropriate labelled areas were identified, cut out and placed in 10 ml Bray's (1960) scintillation fluid. The radioactivity was determined with a Beckman LS-230 liquid scintillation spectrometer.

4. DNA and RNA Analysis

Cells for these experiments were cultured as described in section 1 of "Methods", and in Goh et al (1977). The plating of cells, washing procedure, and cell densities used were exactly as described earlier.

For DNA labelling, the cells were supplied with 2 $\mu\text{Ci/ml}$ of ^3H -thymidine (methyl- ^3H -TdR, 27 Ci/m mol) for the entire 4 h incubation period. After the labelling period, the medium was aspirated from the culture dish with the cells still attached, the cells were washed once with ice-cold 0.9% NaCl, then extracted with 5 ml ice-cold 10% trichloroacetic acid (TCA) for 1 h. The cells were detached from the culture dish with a rubber policeman, and the entire cell suspension was filtered through a glass fibre filter (Gelman) followed by three successive washes with 5 ml of ice-cold 5% TCA and dried with 10 ml of 70% ethanol. The dried filter was dissolved in 1 ml of Protosol cell and tissue solubiliser for 1 h at 45°C, and then diluted with 9 ml of a toluene-based scintillant. The radioactivity was determined by a Beckman LS-230 liquid scintillation spectrometer and the results expressed on a per cell basis.

For RNA analysis, the cells were supplied with 4 $\mu\text{Ci/ml}$ of ^3H -uracil (45 Ci/m mol) for 4 h, after which the labelling was

terminated by aspiration of the growth medium. The washing procedure, TCA extraction, recovery of RNA-incorporated radioactivity, and determination of radioactive counts were exactly as described above for DNA analysis.

5. Determination of Glutamine Accumulated in the Growth Medium or in Cells

The growth medium and cell extracts from cultures kept under various experimental conditions were used to determine the amount of glutamine generated by the cell cultures. The growth of cells, washing procedures, cell densities used and other experimental conditions have been described in sections 1 and 2 of "Methods", and are further described in the appropriate "Results" section.

Samples for glutamine determination were prepared as follows. The growth medium in the culture dish was decanted and collected for analysis of glutamine in the medium. The cells were washed with cold 0.9% NaCl, covered with 10 mM Tris-Cl, pH 8, and scraped from the culture dish with a rubber policeman. They were then homogenised by hand in a Dounce homogeniser, cell debris removed by centrifugation and the supernatant used as cell extract.

Glutamine was determined as glutamate by the coupled glutaminase/glutamic dehydrogenase assay procedure of Lund (1974). An aliquot of each sample was incubated for 15 min with the protease inhibitor N α -p-tosyl-L-lysine chloromethylketone (100 μ g/ml), and then for 12 h at 37°C with glutaminase (Escherichia coli) in sodium acetate buffer, pH 5. Next, glutamate was quantitated with glutamate dehydrogenase (bovine liver) in a reaction mixture that contained an

aliquot of the glutaminase-treated sample, NADP^+ (1 mM final concentration), tris-acetate, pH 7.5 buffer (100 mM final concentration) and glutamic dehydrogenase enzyme. The reaction was followed in a Gilford recording spectrophotometer at a wavelength setting of 340 nm, using visible light and a blue filter.

6. Measurement of Secreted Protease in the Growth Medium of Cell Cultures

Protease activity in the growth medium of various mammalian cell cultures grown under a variety of experimental conditions was measured according to the method of Rick (1974). Medium samples for the determination of protease activity were obtained by simply collecting the growth medium from cell cultures at the end of the experimental period. The medium was centrifuged to remove any suspended cells before it was used for protease determination.

Protease assay was done by a method (Rick 1974) that uses TAME as substrate of the protease(s). A sample of the growth medium believed to contain protease(s) was mixed with TAME (10 mM final concentration) and tris buffer (46 mM final concentration) and the change in optical density (absorbance) at 247 nm was immediately and directly recorded on a spectrophotometer.

7. Analysis of Proteins from Cells and Medium by Gel Electrophoresis

Cells were cultured and washed as described under sections 1 and 2 of "Methods". $\text{L-}^{35}\text{S}$ -methionine at 100 $\mu\text{Ci/ml}$ was added to each culture plate and incubated for 4 h.

Proteins in the growth medium were precipitated with 10% TCA. The

precipitate was pelleted by centrifugation and then washed twice with 5% TCA and once with ether. The TCA precipitate was resuspended in a buffer composed of 10 mM tris-HCl (pH 7.4), 2 mM phenylmethylsulfonyl fluoride (PMSF) and 1 mM TLCK. An equal volume of a detergent solution consisting of 9.5 M urea, 2% sodium dodecyl sulfate (SDS) and 5% 2-mercaptoethanol was added to the extract together with a few crystals of urea. The mixture was thoroughly stirred to dissolve the proteins and was then analysed by one-dimensional gel electrophoresis immediately or stored at -76°C overnight before analysis.

Cellular proteins were extracted by directly treating cells (collected by trypsinisation and centrifugation at the end of the experimental period) with the detergent solution (9.5 M urea, 2% SDS and 5% 2-mercaptoethanol) and then adding PMSF (2 mM final concentration) TLCK (1 mM final concentration) and 75 $\mu\text{g/ml}$ each of ribonuclease (RNase) and deoxyribonuclease (DNase). The lysed cells were incubated on ice for 15 min, warmed to 30°C for 15 min and then used for one-dimensional gel electrophoresis.

Protein samples (prepared from either the growth medium or from cells) were mixed with an equal volume of a dye solution (50% sucrose, 2% SDS and 0.01% bromophenol blue) and heated for 10 min at 70°C before they were applied onto the gel slab. The gel slabs (0.7 mm thick), prepared according to Dewald et al (1974) with modifications by LeJohn (LeJohn, personal communication), contained 12.8% polyacrylamide (Bio-rad) and 0.1% SDS. The slabs were prepared overnight before use. Aliquots of the protein preparation were applied onto the sample slots of the gel slab. Electrophoresis in one dimension was done in a running

buffer that consisted of glycine (14.40 g/l), tris (3 g/l) and SDS (0.1%), pH 8.3. Samples were run for 1 h at 150 volts, then for 3 h at 200 volts. After electrophoresis, gels were fixed and stained by a procedure (LeJohn, personal communication) that is modified from Neville and Glossman (1974). Gels were washed for 1 h in a solution composed of 40% methanol/7% acetic acid, stained for 1 h in a dye solution consisting of 0.25% Coomassie brilliant blue R/45% methanol/9% acetic acid, and destained in 35% methanol/10% acetic acid solution. Destained gels were dried for 75 min in a Bio-rad slab gel drier before exposure to film. Kodak X-Omat RP4 films were used for autoradiography. Exposure time was 3 days.

8. Transformation of Cells by Chemical Carcinogen, Isolation of Transformed Cells, and Assays for Transformation

Cells were cultured, trypsinised and replated onto 100 mm plates for carcinogen treatment, according to the procedure already described in section 1 of "Methods".

(a) Carcinogen Treatment and Isolation of Transformed Cells

Carcinogen treatment protocol was based on the method of Chen and Heidelberger (1969). Cells (10^3) were inoculated per 100 mm culture dish and grown for 24 h at 37°C in normal growth medium before commencement of carcinogen treatment.

A stock carcinogen solution (1 µg/µl) was prepared by dissolving 9,10-dimethyl-1,2-benzanthracene (DMBA) in dimethylsulfoxide (DMSO) after they were both individually sterilised by autoclaving. Carcinogen was added to the cell cultures at a final concentration of 5 µg/ml (the

concentration of DMSO in contact with the cells was 0.5%). A zero-dose control plate containing DMSO and cells but no carcinogen was also prepared. Carcinogen treatment was carried out for 6 days, with medium changes every 2 days. At the end of the carcinogen treatment period, the cells were cultured for a further period of 2 - 3 weeks in normal growth medium, with medium changes every 3 days. No attempt was made to wash off the carcinogen, so that residual amounts of carcinogen may have been present in the cultures several days after completion of the carcinogen treatment. Transformed clones were recognised by phase contrast microscopy as piled-up colonies in a background of untransformed, monolayer of cells. The transformed colonies were isolated by the ring isolation technique of Ham and Puck (1962). A glass cylinder (8 mm outside diameter, 10 mm high, with a 2 mm wall) was attached to the culture dish surface, around the cell colony, with silicone grease, and the cells encircled in the well were trypsinised and transferred to 35 mm culture dish containing α -MEM medium plus 10% fetal bovine serum. Nontransformed cells from portions of the same carcinogen-treated dishes were also isolated for further studies.

(b) Methylcellulose Assay for Transformation

Methylcellulose assay for transformation was based on a modified version (Peehl and Stanbridge 1981) of a method originally described by MacPherson and Montagnier (1964). The assay solution was prepared by dispersing 2.6 g methylcellulose (Fisher Scientific) in 30 ml of hot (approx. 90°C) glass distilled water. The solution was stirred overnight at 4°C and then autoclaved for 40 min. The solution was stored for at least 24 h at 4°C before it was mixed 1:1 with doubly

supplemented growth medium (α -MEM + serum).

Cells used for the assay were prepared from subconfluent cultures grown under normal culture conditions. The cells were detached from their culture dish by trypsinisation followed by thorough dispersal into single cells in a small volume of growth medium. This cell suspension was then added to the 1.3% methylcellulose assay solution at a cell density of 150 cells/ml of assay solution. Seven millilitres of this assay solution (containing cells) was then layered on top of 100 mm tissue culture dishes containing 5 ml of sterile agar (0.9%) in α -MEM plus serum. The plates were incubated at 37°C for 15 days with addition of 5 ml fresh methylcellulose assay solution every 4 days. At the end of the assay period, colony counts were made with the aid of a light microscope.

9. Cell Fusion and Isolation of Hybrid Cells

Cells were cultured as described in section 1 of "Methods". Cell fusion was achieved in monolayer cultures based on the methods described by Davidson and Gerald (1976).

Parental cells were cultured together in unequal numbers (1.5×10^6 B82, and 0.5×10^6 SK4476 cells/60 mm plate) in order to increase the frequency of hybridisation (Davidson and Ephrussi 1970). The mixed cell culture was kept for 24 h at 37°C before fusion. The medium was aspirated, the cell layer was gently washed twice with α -MEM (without serum), and 1 ml of 50% polyethylene glycol (PEG) 6000 (molecular weight 6000-7500) from Sigma Chemical Co., was added for 1 min with gentle rocking. PEG (50% weight/weight) was prepared by mixing 5 g of warm, autoclaved PEG with 5 ml of warm α -MEM (without serum) (Pontecorvo

1975). The PEG solution was aspirated, the cell layers washed carefully three times with α -MEM (without serum), and 3 ml α -MEM supplemented with 10% calf serum was added to the culture dish. Following incubation for 24 h, the cells were trypsinised, counted, and plated in 100 mm tissue culture dishes at 10^3 cells per dish.

After allowing cell attachment for 24 h the medium was replaced with selective medium which consisted of normal culture medium, plus hypoxanthine (10^{-5} M), aminopterin (3.2×10^{-6} M) and thymidine (10^{-5} M), otherwise known as "HAT" medium (Szybalski and Szybalski 1962; Littlefield 1964). The selective medium was changed every 3 days, and after 10 - 14 days surviving colonies were isolated by the ring isolation technique and transferred to 35 mm tissue culture plates containing HAT medium. Only one hybrid clone was isolated from each dish.

RESULTS

RESULTS

HS3 Synthesis Patterns at 37°C by Cultured Mammalian Cells

Following the discovery that fungal cells accumulated significant quantities of HS compounds when starved of nutrients, especially glutamine (LeJohn et al 1975; 1978), studies showed that glutamine-starved mammalian cells also accumulated one of the HS compounds, HS3. The CHO WT cell line is the first mammalian cell line in which HS3 was identified (Goh and LeJohn 1977; Goh et al 1977; Goh 1979). Fig. 1 illustrates the HS3 production pattern of CHO WT cells. HS3 was found in appreciable amounts only in cells exposed to glutamine-free growth medium, but not in cells exposed to medium containing 2 mM glutamine (compare panels IA and IB, Fig. 1).

A variety of other cultured mammalian cell lines were also tested by Goh (1979) to determine their HS3 production patterns in the presence and absence of glutamine. As illustrated in Table 1, all permanent cell lines tested, except the CHO GAT⁻ cell line, produced HS3 in appreciable amounts only when glutamine was absent in the growth medium; the failure of CHO GAT⁻ cells to produce HS3 in the absence of glutamine is probably due to the presence in its growth medium of adenosine (Goh 1979) a necessary nutrient for the growth of this cell line in culture (McBurney and Whitmore 1974). By contrast, primary cell lines produced HS3 to high levels irrespective of whether or not glutamine was present in the growth medium. Hence it appeared that there are two patterns of HS3 production: a permanent cell line pattern, and a primary cell line pattern. That observation was confirmed in the present study with the

Fig. 1

Effect of L-glutamine deprivation on HS3 levels in CHO WT cells. The growth of cells was as described in 'Methods'. The cells were washed once with 5 ml of prewarmed α -MEM supplemented with or without glutamine plus 10% DFCS. Three millilitres of the appropriate medium containing 50 μ l of ^{32}P i was then added to each plate, followed by a 2 hr incubation. The labelling was terminated by removal of the medium by aspiration and washing of the cells with 5 ml of ice-cold 0.9% NaCl. The cells were then extracted for 30 min at 4°C by the addition of 0.2 ml of 1 M formic acid to each plate. For chromatography, 10 μ l of the formic acid extract was applied on PEI-cellulose thin-layer plates and developed by ascending chromatography. The procedures for chromatography and the determination of the levels of ^{32}P -labelled compounds have been described in the 'Methods'.

(Reproduced, with permission, from Goh, 1979).

FIG. 1

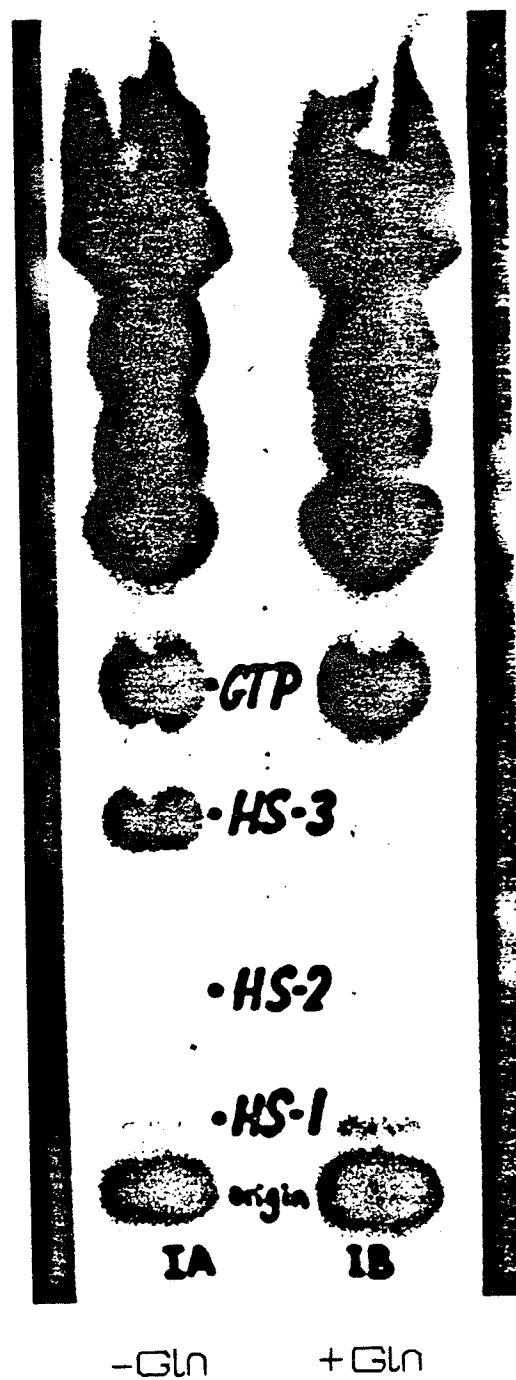


Table 1

Determination of ^{32}P -labelled HS3 in various mammalian cell lines
cultured in the presence or absence of 2 mM L-glutamine.

	HS3 ¹	
	+ glutamine	- glutamine
<u>Permanent cell lines</u>		
CHO WT	12.1 (0.045)*	100 (0.685)
CHO GAT ⁻	96.0 (0.049)	" (0.090)
CHO HGPRT ⁻ (YH 21)	16.7	"
CHO HGPRT ⁻ /APRT (YHD 13)	14.8	"
Balb 3T3 (mouse)	15.8	"
Balb SV 3T3 (mouse)	10.5	"
BHK 21/C1 13	15.1	"
HeLa	24.7	"
L5178Y	41.0	"
<u>Primary cell lines</u>		
WI-38	90.5 (0.183) ²	" (0.214)
Lesch-Nyhan human fibroblast	94.3	"
Normal human fibroblast	99.0	"
Normal human foreskin fibroblast	95.0 (0.68)	" (1.07)
African green monkey kidney cells	106.0	

¹ ^{32}P -labelled HS3 level, after 2 hr in the absence of glutamine is expressed as 100%.

²An average of values after 2-5 hrs in the absence or presence of glutamine (WI-38 only).

*Values in parentheses are ratios of $\frac{\text{HS3}}{\text{GTP}}$ (cpm).

The experimental procedures and the quantitation of HS3 were exactly as described in the legend for Fig. 1 and 'Methods' (Reproduced, with permission, from Goh, 1979)

CHO WT and additional cell types (Table 2).

HS3 Synthesis Patterns in Isogenic Transformed and Untransformed Cell Lines at 37°C

As mentioned above, the study by Goh (1979) revealed that transformed cells accumulated HS3 in the absence but not in the presence of L-glutamine in the growth medium, whereas untransformed cells accumulated HS3 whether or not glutamine was present. The study by Goh (1979), however, was done with a melange of nonisogenic transformed and untransformed cell lines. Also, the transformed cell lines used were established lines which had been kept in culture for long periods of time and, therefore, may have undergone other changes unrelated to transformation. It follows that a direct, causal relationship cannot be established between cell transformation and a change in HS3 production pattern from the studies by Goh (1979). A more thorough investigation into the relationship between transformation and HS3 production pattern required the use of freshly transformed cells and of isogenic transformed and untransformed cell pairs.

In the first line of approach used to study a direct relationship between cell transformation and HS3 response pattern, a human diploid fibroblast cell line, On Ser, was chemically transformed with 9,10-dimethyl-1,2-benzanthracene (DMBA) (5 µg/ml). The transformed cell line was designated On Ser-DMBA and was identified as piled-up focus of cells (Fig. 2a) in a background of fibroblastic, unlayered On Ser cells (Fig. 2b). The cells were isolated and tested for transformation by in vitro methods based on the ability to grow in medium at relatively low serum concentrations (Fig. 3), in semisolid methylcellulose (Table 3),

Table 2

³²P-labelled HS3 in isogenic and non-isogenic transformed and untransformed mammalian cell lines at 37°C and 42°C grown in the presence and absence of 2 mM L-glutamine (gln) in the growth medium.

Cell Line	HS3 (% of control) ¹			
	37°C		42°C	
	-gln	+gln	-gln	+gln
<u>Untransformed</u>				
On Ser	76.5	73.5	74.6	79.5
WI-38	58.8	55.1	63.1	57.1
10T½	61.7	54.9	60.7	58.0
El San	58.2	68.1	61.3	56.4
<u>Transformed</u>				
On Ser-DMBA	116.1	2.1	2.0	2.2
WI-38 VA13	107.1	4.4	3.3	3.1
10T½ ciras-2	88.4	2.9	3.1	2.6
10T½ MDS-R2	89.2	12.3	100.0	11.8
CHO WT	100.0 ²	3.1	2.5	2.5
CHO CR7 (ts)	96.0	3.5	2.4	2.6
CHO Rc	65.1	2.7	1.8	1.5
On Ser-DMBA + TLCK*/TAME**	96.8	4.8	94.8	2.6

¹³²P-labelled HS3 counts were first standardised with respect to the total counts in the formic acid extract, and then expressed as a percentage of the 100% control value. The experimental procedures and quantitation of HS3 were exactly as described in the legend for Fig. 4 and in "Methods".

²Control (100%) is the amount of ³²P-labelled HS3 accumulated in CHO WT cells for 4 h at 37°C in the absence of glutamine. CHO WT is the first mammalian cell line in which the polyphosphate was identified (Goh and LeJohn 1977; Goh et al 1977).

*Na-p-tosyl-L-lysine-chloromethylketone (100 µg/ml)

**Na-p-tosyl-L-arginine methyl ester (100 µg/ml)

Fig. 2

Photographs of cells taken from culture dish of On Ser cells treated with the polycyclic hydrocarbon 9,10-dimethyl-1,2-benzanthracene (DMBA). A sparse culture of On Ser cells (grown in α -MEM + 10% serum) was continuously exposed to 5 μ g/ml of DMBA for 6 days. After the 6-day DMBA treatment period, the cells were allowed to grow for a further period of 14 days during which period the cells attained full confluency. The culture was observed microscopically for rounded, piled-up foci of transformed cells (Fig. 2a) against a background of confluent, monolayer of fibroblastic normal cells (Fig. 2b). Photographs were taken with an ordinary light microscope with a camera.

(magnification x 150)

Fig. 2a

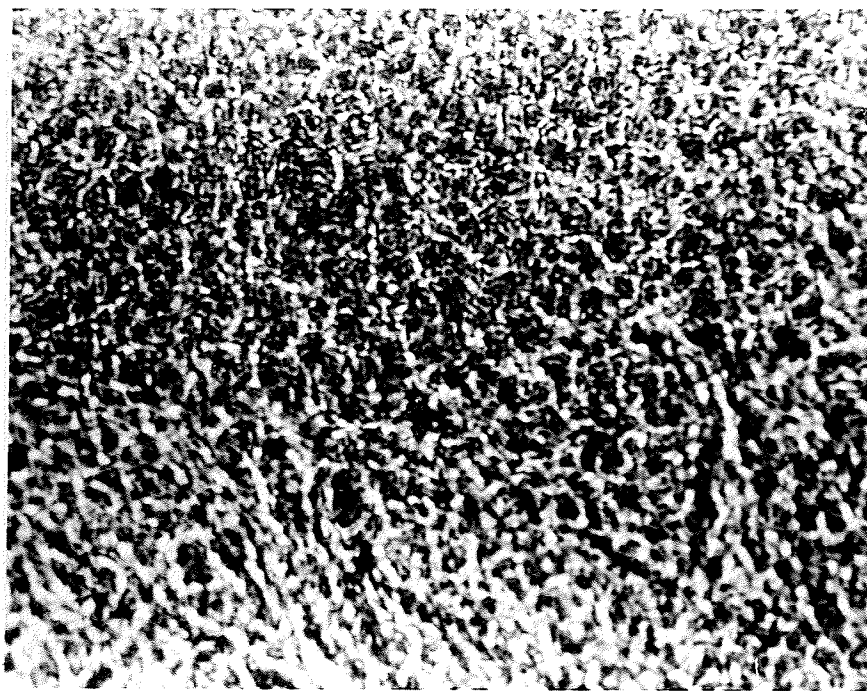


Fig. 2b

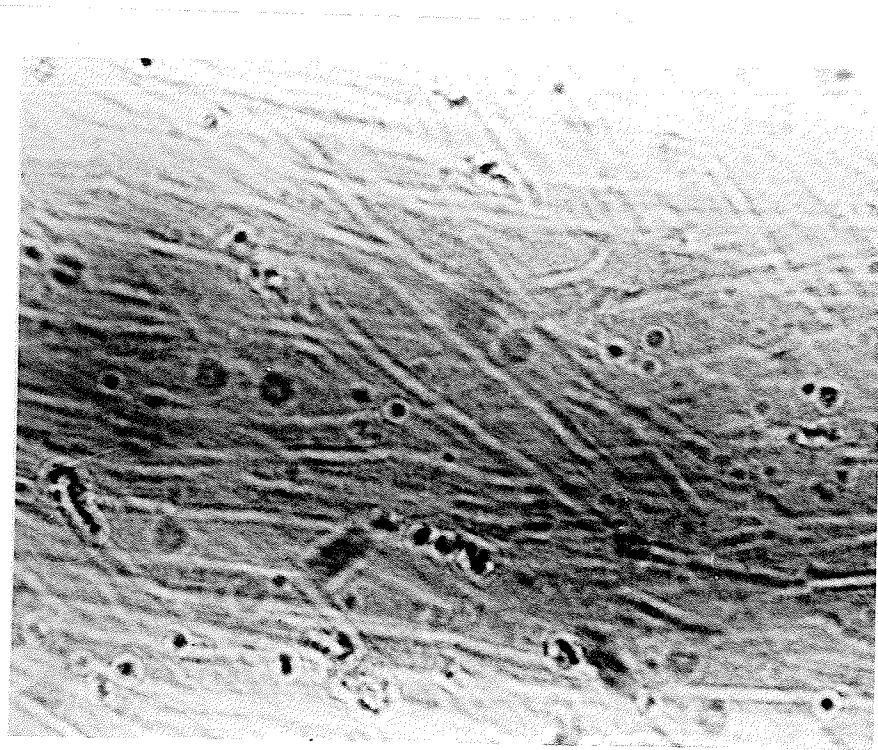


Fig. 3

Growth rates of (a) On Ser, and (b) On Ser-DMBA cells at various concentrations of serum. Cells were plated at low densities in α -MEM + 10% calf serum and grown for 24 h, after which they were transferred to growth medium with the appropriate serum concentration. The cultures were maintained in medium with the appropriate serum concentration for 14 days, with medium change every 2 days. Cell counts were made every 24 h. The 0-h cell count was made at the time the cells were first exposed to medium containing the appropriate serum concentration. All cell counts were made using a Coulter counter after dispersal of cells by trypsin. The results are expressed as $\log_2$ cell count against time, where $\log_2$ is calculated as $\log_{10}$ cell count $\times 3.3219$. (Patterson 1979).

Growth of On Ser (P_{18}) cells in different concentrations of serum

174

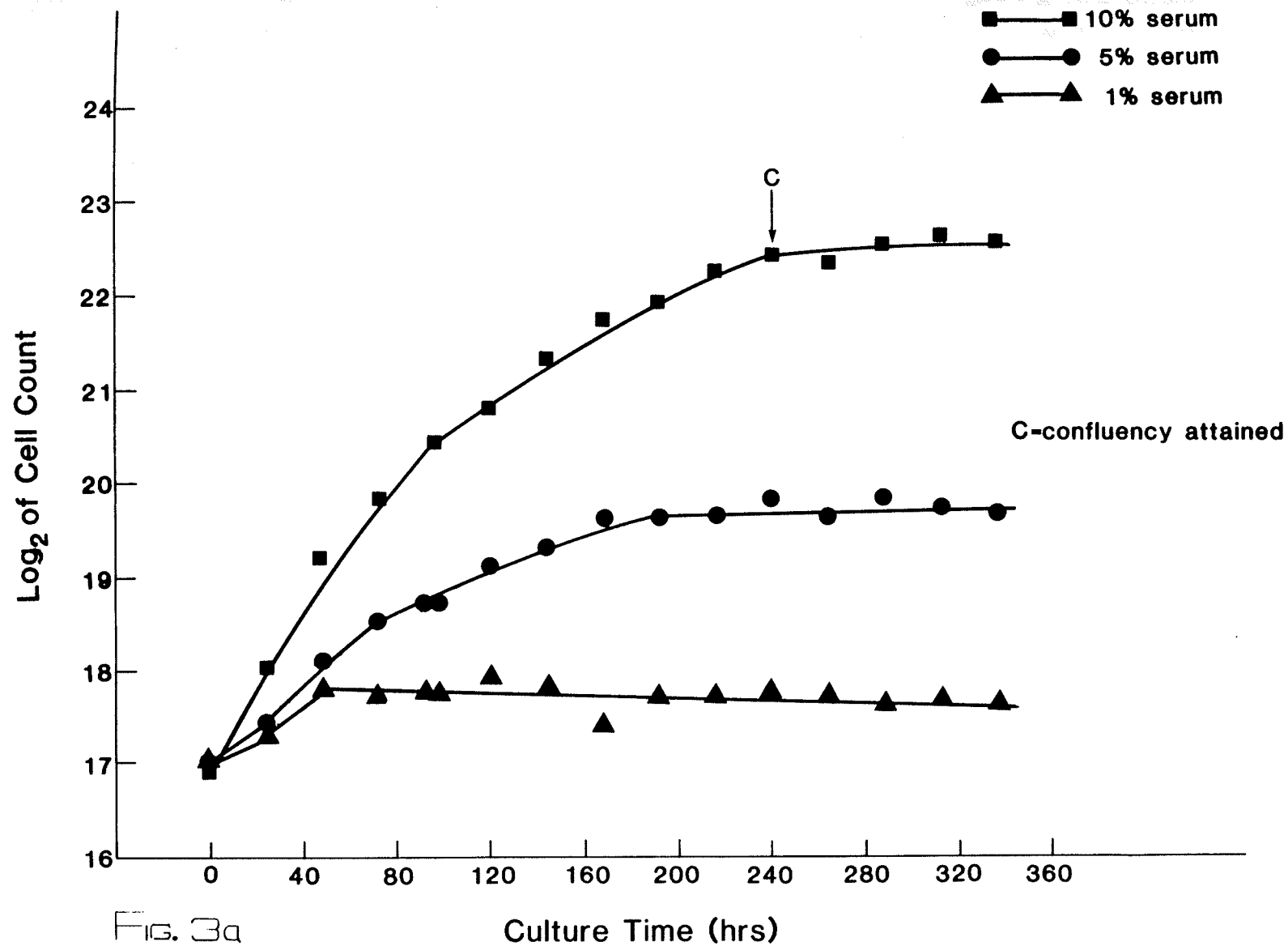


FIG. 3a

Growth of On Ser-DMBA cells in different concentrations of serum

175

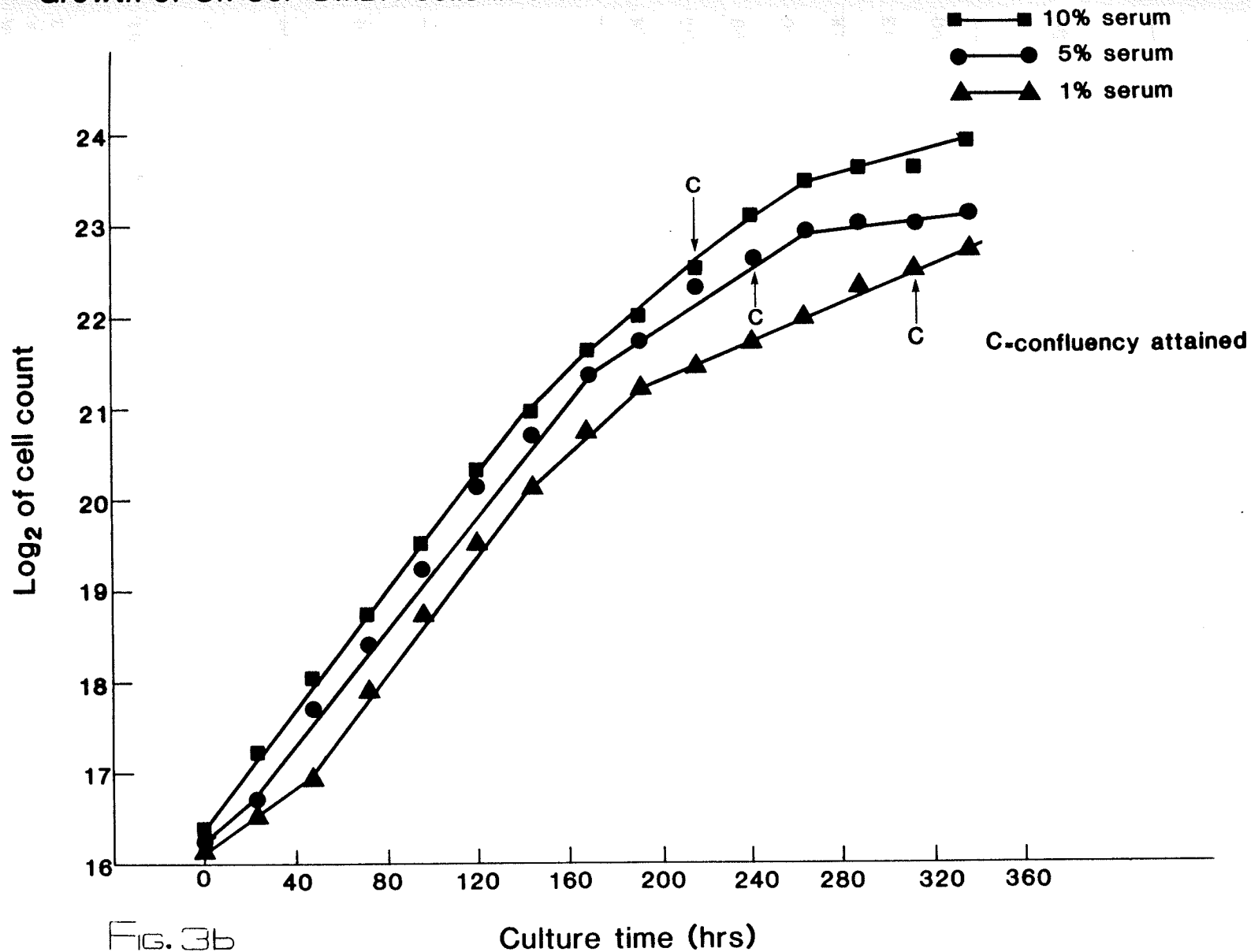


Table 3

Growth characteristics of various mammalian cell lines

Cell Line	Species	Growth Surface ¹		Serum concentration needed for growth to confluency ²			HS3 Response Pattern
		Plastic Surface	Methyl Cellulose	10%	5%	1%	
On Ser	Human	+	-	+	-	-	Primary
El San	Human	+	-	+	-	-	Primary
WI-38	Human	+	-	+	-	-	Primary
On Ser-DMBA	Human	+	+	+	+	+	Permanent
B82	Mouse	+	+	+	+	+	Permanent
CHO WT	Hamster	+	+	+	-	-	Permanent
WI-38 VA13	Human	+	+	+	+	+	Permanent

¹(+) means that cells were able to grow to colonies large enough to be observed by staining or microscopic techniques.

(-) means that there were no identifiable colonies or no observable evidence that cell growth had occurred in the cultures.

²(+) means that, after 14 days of culture, the cells grew to confluency.

(-) means that cell numbers did not significantly increase or there was a net loss of cells over the number originally plated.

Cell culture and counting procedures for cells grown on normal plastic surface and in methyl cellulose have been described in "Methods". Cells were grown in 100 mm tissue culture dishes.

and infinite lifespan in culture. The transformation frequency was about 10^{-7} , which is not unexpected for human cells (Di Paolo 1983). As illustrated in Fig. 4, the HS3 production pattern changed from the primary to the permanent pattern only a few cell generations after transformation of the cells.

In the second line of approach, four pairs of isogenic transformed and untransformed cells (On Ser and its chemically transformed derivative On Ser-DMBA; WI-38 and its virally transformed derivative WI-38 VA13; 10T $\frac{1}{2}$ and its H-ras oncogene transformed and X-ray transformed counterparts 10T $\frac{1}{2}$ ciras-2 and 10T $\frac{1}{2}$ MDS-R2 respectively) were used to show that at 37°C, the untransformed cells accumulated uniformly high levels of HS3 whether or not glutamine was present in the growth medium, whereas their transformed counterparts accumulated high levels of HS3 only when they were starved of glutamine (Table 2). Significantly, this pattern was observed no matter what the agent of transformation was - chemical, viral, oncogene, X-ray and undeciphered (spontaneous).

Hence at 37°C, cell transformation was causally and directly related to a change in HS3 production pattern from the primary to the permanent pattern.

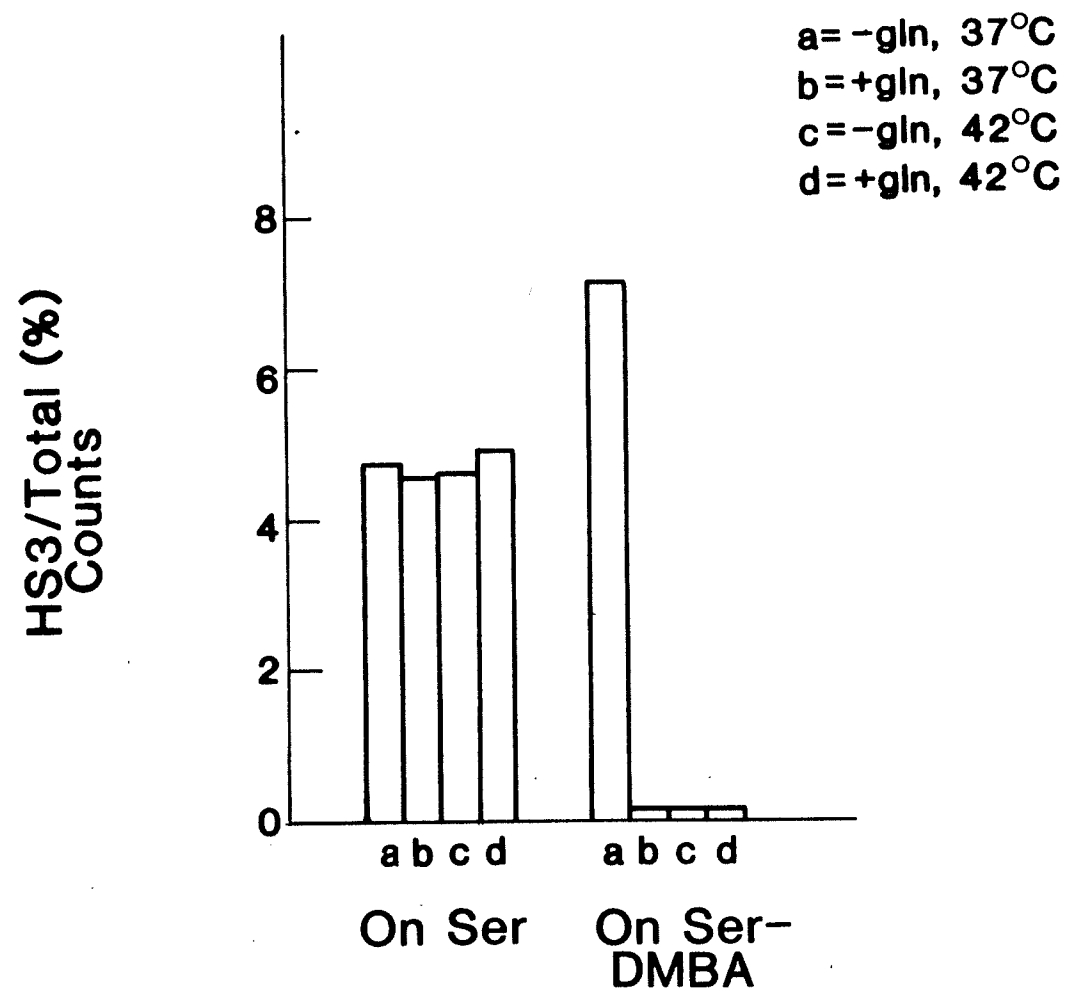
Correlation Between Specific Growth Characteristics and HS3 Response Pattern

Transformed cells differ from untransformed cells in many aspects of their growth characteristics in culture (Skehan and Friedman 1981). The most commonly studied relate to their lifespan in culture, serum requirements for growth, contact inhibition and saturation density, and

Fig. 4

^{32}P -labelled HS3 in On Ser and On Ser-DMBA cells at 37°C and 42°C in α -MEM with or without glutamine and supplemented with 10% DFBS. The growth of cells and plating and washing procedures were as described in sections 1 and 2 of 'Methods'. Cells were plated in 35 mm tissue culture plates. One and a half millilitres of the appropriate medium containing $250\text{ }\mu\text{Ci/ml}$ of ^{32}Pi was added to each plate, followed by a 4-h incubation at the appropriate temperature. The labelling was terminated by aspiration of the medium and washing of the cells three times with 2.5 ml of ice-cold 0.9% NaCl. The cells were extracted for 30 min at 4°C by the addition of $100\text{ }\mu\text{l}$ of 1 M formic acid to each plate. For chromatography, $10\text{ }\mu\text{l}$ of the formic acid extract was applied onto PEI-cellulose thin-layer plates and developed by ascending chromatography. The procedures for chromatography and the determination of the levels of ^{32}P -labelled compounds have been described in 'Methods'.

Fig. 4



anchorage dependence for growth. It was, therefore, of interest to determine whether any single growth characteristic correlates with the pattern of HS3 response in a variety of transformed and untransformed cell lines.

The results of Table 3 show that all cell lines which exhibited the primary pattern of HS3 production did not grow in methylcellulose or in medium with low serum concentration and they had limited life in culture. On the other hand, cell lines that exhibited the permanent pattern of HS3 production have infinite life in culture, formed colonies in methylcellulose and most of them grew well in media containing 1% serum; the CHO WT cell line is an exception in that it failed to grow in medium supplemented with 5% or 1% serum.

Effects of Varying Concentrations of L-Glutamine and Phosphate on the HS3 Response in On Ser and On Ser-DMBA Cells at 37°C

Experiments were performed to determine the effects of varying concentrations of glutamine on HS3 metabolism in On Ser and On Ser-DMBA cells grown at 37°C. The purpose of these experiments were two-fold: firstly, to measure the threshold level of glutamine necessary to suppress HS3 production in transformed cells; and secondly, to determine whether non-transformed cells were responsive in their HS3 response to pharmacological levels of glutamine in the medium.

It can be observed from Fig. 5a that On Ser cells did not significantly alter their levels of HS3 in any particular direction with increasing concentrations of glutamine (0 to 10 mM) in their growth medium. This was true for all three levels of phosphate at which the glutamine concentration studies were done. There were somewhat low

Fig. 5 ^{32}P -labelled HS3 in On Ser cells in the presence of different concentrations of glutamine and three different levels of phosphate in the growth medium. Similar specific activities of $^{32}\text{P}_i$ were maintained for the three levels of phosphate used in the growth medium. Experiments were conducted at (a) 37°C , and (b) 42°C . Procedures for cell culture and washing, and quantitation of HS3 are described in the legend for Fig. 4 and in "Methods".

FIG. 5a

On Ser, 37°C

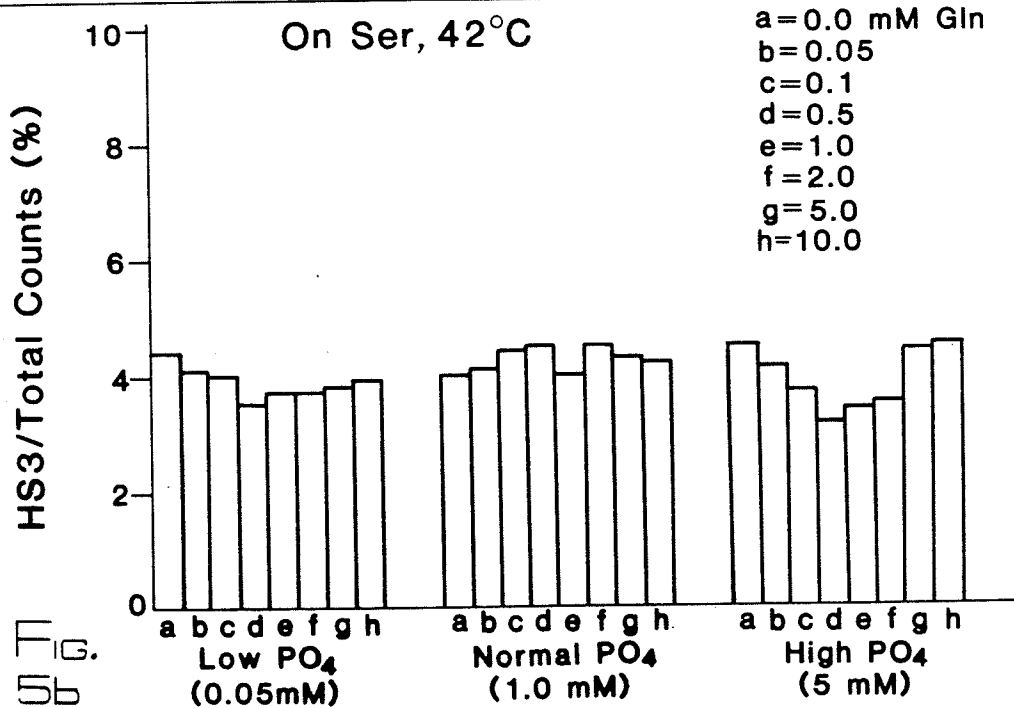
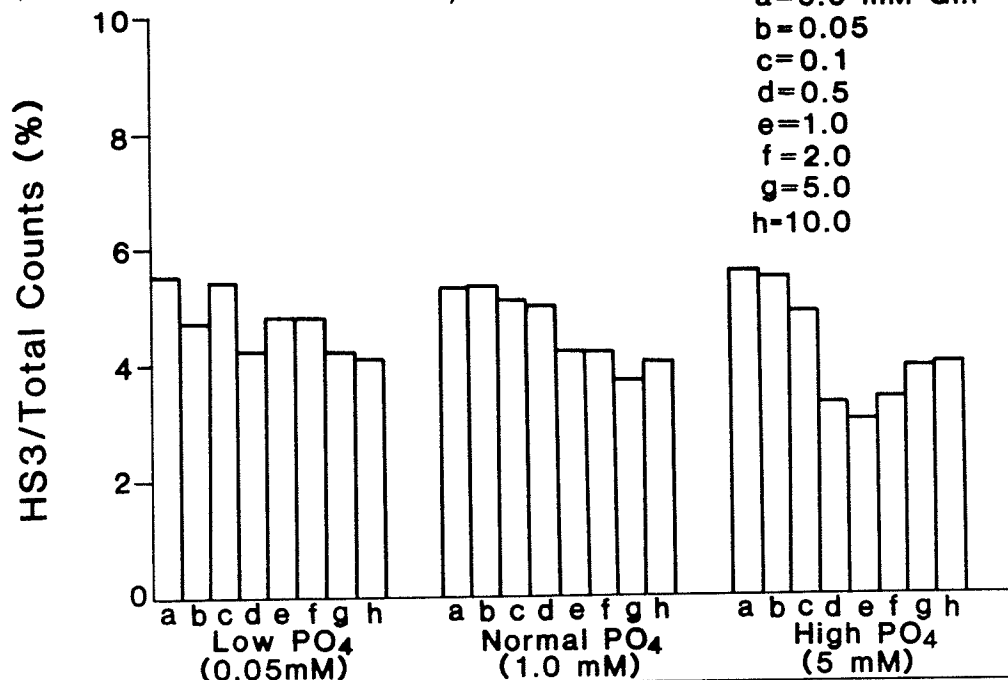


FIG. 5b

levels of HS3 at glutamine concentrations of 0.5, 1.0 and 2.0 mM in high phosphate medium, but this may not have any significance since the HS3 values increased again at higher glutamine concentrations. The normal level of glutamine in α -MEM medium is 2 mM (Stanners et al 1971), so that the highest concentration of glutamine used (10 mM) could be considered pharmacological. The pattern of HS3 synthesis typical for primary cell lines was maintained with On Ser cells at all glutamine and phosphate levels used. By contrast, with On Ser-DMBA cells at 37°C (Fig. 6a) there was an inverse relationship between the levels of HS3 accumulated and the concentration of glutamine in the growth medium, and the slope of the response curve varied with the amount of phosphate in the growth medium. The threshold level of glutamine needed to deplete HS3 levels to near zero also varied with the amount of phosphate in the medium; at low (0.05 mM) and normal (1.0 mM) phosphate levels, HS3 levels dropped to zero at a glutamine concentration of 0.5 mM or higher, whereas at high phosphate concentration (5.0 mM) the cells required up to 5 mM or more glutamine to deplete HS3 levels to zero. Similar findings were reported for a watermold by LeJohn (1983). Another feature of the glutamine response curve for On Ser-DMBA cells is that at high phosphate, the HS3 levels decreased as a smooth function of glutamine concentration but, on the other hand, in the lower phosphate medium the HS3 response showed a sharp demarcation between 0.1 and 0.5 mM glutamine.

Experiments were also performed to determine the effects of varying amounts of phosphate (0.1 to 4 mM) on HS3 levels in On Ser and On Ser-DMBA cells grown at 37°C in the presence or absence of glutamine.

Fig. 6 ^{32}P -labelled HS3 in On Ser-DMBA cells in the presence of different concentrations of glutamine and three different levels of phosphate in the growth medium. Similar specific activities of $^{32}\text{P}_i$ were maintained for the three levels of phosphate used in the growth medium. Experiments were conducted at (a) 37°C , and (b) 42°C . Procedures for cell culture and washing, and quantitation of HS3 are described in the legend for Fig. 4 and in "Methods".

FIG. 6a

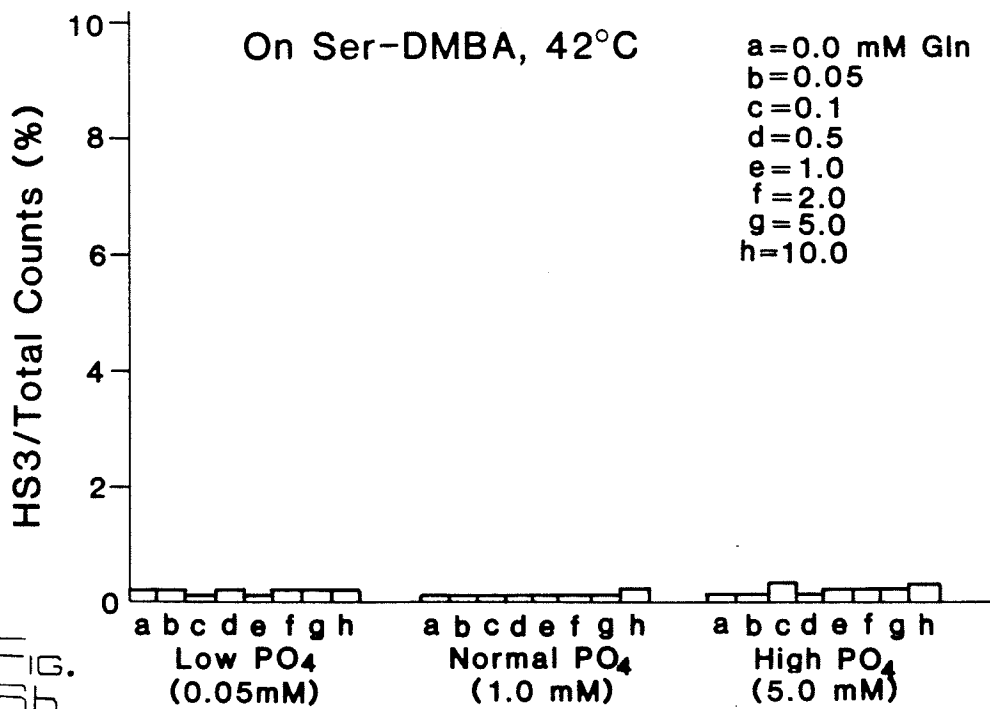
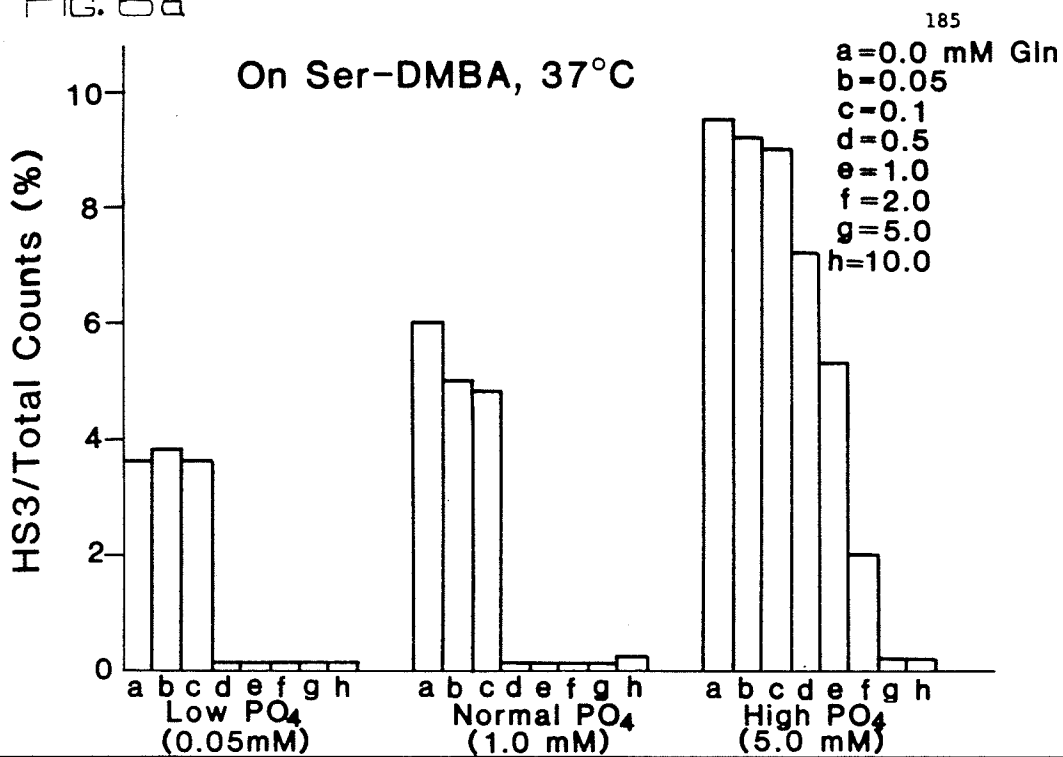


FIG. 6b

These experiments were meant to determine whether phosphate limitation redirects the available phosphate into molecules other than HS3, measured as a reduced amount of incorporation of ^{32}P -orthophosphate into HS3. Also, whether excess phosphate levels lead to more HS3 accumulation or even induce HS3 accumulation under conditions where it is normally not produced. The results presented in Fig. 7 show that with On Ser cells the levels of HS3 accumulated were approximately uniformly high (ranging from 3 to 5% of total ^{32}P counts) at all phosphate concentrations used, whether or not glutamine was present in the growth medium. With On Ser-DMBA cells (Fig. 8) the amount of HS3 produced increased in direct proportion with the increase of phosphate in the medium when the cells were grown at 37°C in glutamine-free medium. Very little HS3 was synthesised by On Ser-DMBA at all phosphate concentrations used when 2 mM glutamine was present in the medium (Fig. 8). Less than 0.5% HS3/total ^{32}P counts was detected at 4 mM phosphate in the presence of glutamine, compared to 9.3% HS3/total ^{32}P in the absence of glutamine at the same phosphate level.

HS3 Metabolism at 42°C : The Temperature Effect

The effect of moderate thermal stress (treatment of cells at 42°C for 4 h) on HS3 metabolism was studied in On Ser and On Ser-DMBA cells and a variety of other transformed and untransformed cell lines. All cell lines used grew optimally at 37°C but were thermally stressed at 42°C although they survived and resumed normal growth if returned to 37°C after 4 h. Prolonged incubation (12 h) at 42°C was lethal. On Ser cells maintained their individual levels of HS3 whether or not they were glutamine-deficient or thermally stressed (Fig. 4) even in the presence

Fig. 7 ^{32}P -labelled HS3 at 37°C and 42°C in On Ser cells grown in media with varying phosphate concentrations in the presence or absence of 2 mM glutamine. The same specific activity of $^{32}\text{P}_i$ was maintained for all levels of phosphate used in the growth medium. Cell culture and washing procedures and the method of quantitation of HS3 are described in the legend for Fig. 4 and in "Methods".

Fig. 8 ^{32}P -labelled HS3 at 37°C and 42°C in On Ser-DMBA cells grown in media with varying phosphate concentrations in the presence or absence of 2 mM glutamine. The same specific activity of $^{32}\text{P}_i$ was maintained for all levels of phosphate used in the growth medium. Cell culture and washing procedures and the method of quantitation of HS3 are described in the legend for Fig. 4 and in "Methods".

FIG. 7

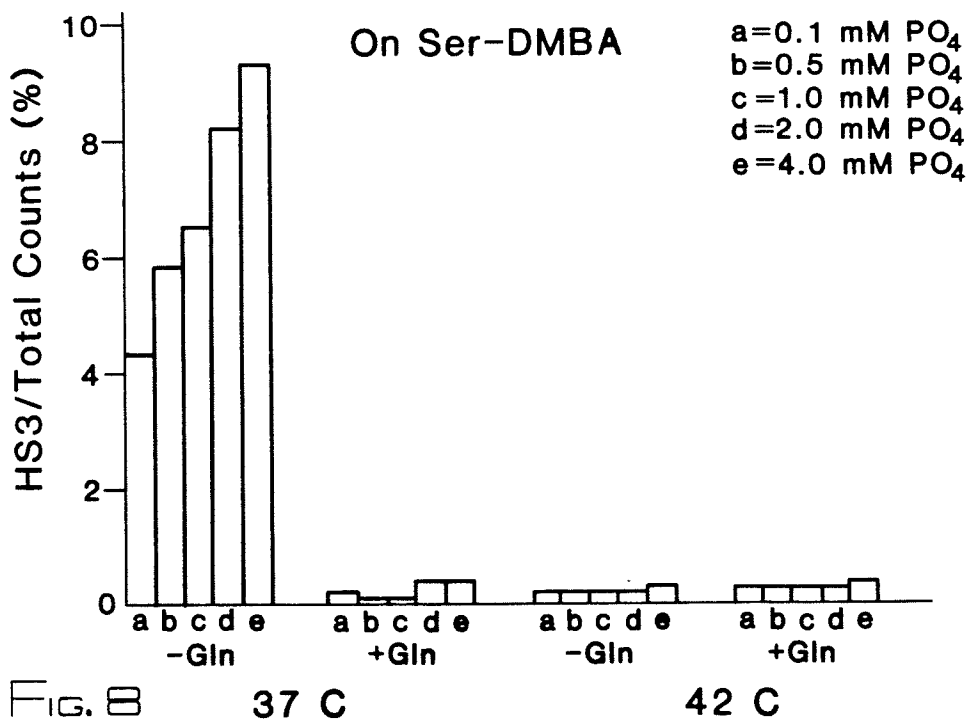
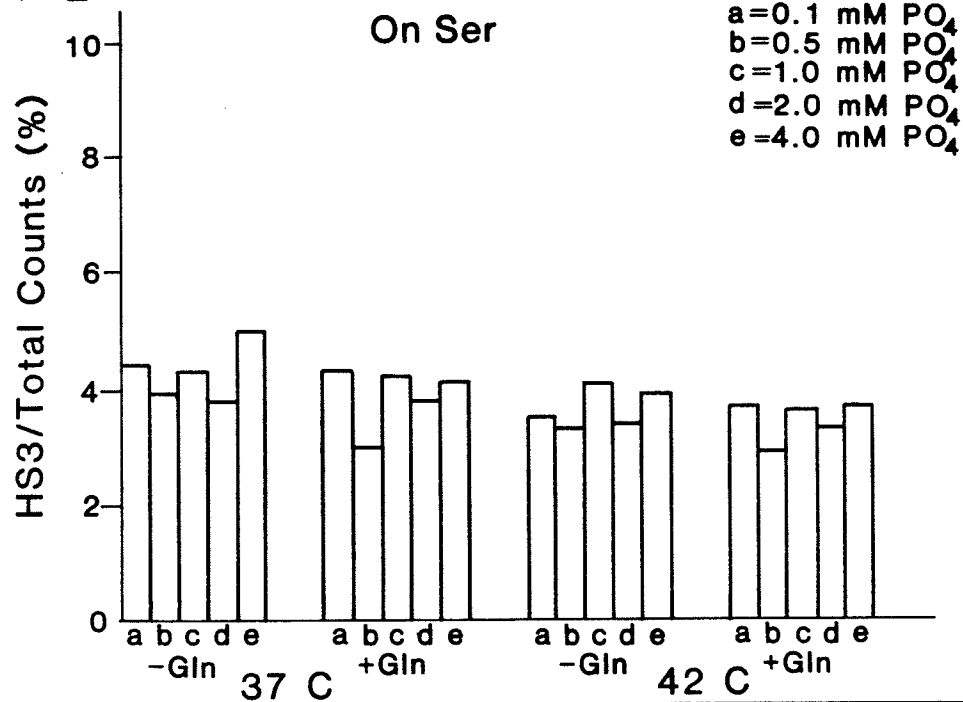


FIG. 8

of high or low levels of glutamine and phosphate (Fig. 5a, 5b, 7). By contrast, On Ser-DMBA cells accumulated negligible amounts of HS3 at 42°C even in glutamine-free medium, irrespective of the level of phosphate in the medium (Fig. 4, 6a, 6b, 8). Studies on HS3 metabolism in a variety of other transformed and untransformed cells under thermal stress (Table 2) show that thermal stress did not affect HS3 levels in all untransformed cells, but with transformed cells (except the 10T½ MDS-R2 cell line) thermal stress prevented the build-up of HS3 by the cells (compare columns 1 and 3, Table 2). Since some of the transformed and untransformed cell lines are in isogenic pairs, the results imply that untransformed and transformed cells have different responses to thermal stress with respect to their HS3 response. Thermal stress appears to condition transformed cells into behaving as if they were not starving for glutamine. The failure of 10T½ MDS-R2 cells to respond to thermal stress in a similar manner as other transformed cells will be contemplated in the "Discussions" section.

DNA and RNA Synthesis at 37°C and 42°C by On Ser and On Ser-DMBA Cells

Previous work by Goh and LeJohn (1977) and Goh (1979) showed that there is an inverse relationship between nucleic acid synthesis and HS3 levels in transformed cells. The current results support that conclusion and show (Table 4) that at 37°C DNA and RNA synthesis was affected by glutamine withdrawal in both transformed and untransformed cells. DNA was labelled twice as effectively and RNA three times as effectively in cells when glutamine was supplied than in its absence (see columns 2, 4, 6 and 8, Table 4). On the other hand, at 42°C transformed cells synthesised DNA and RNA optimally and independently of

Table 4

DNA and RNA synthesis by On Ser and On Ser-DMBA cells in the presence or absence of L-glutamine and in the presence or absence of the protease inhibitors TLCK and TAME at 37°C and 42°C.

Addition	³ H-thymidine incorp. (% of control)				³ H-uracil incorp. (% of control)			
	On Ser		On Ser-DMBA		On Ser		On Ser-DMBA	
	37°C	42°C	37°C	42°C	37°C	42°C	37°C	42°C
-gln	50	43	64	121	35	33	17	74
+gln (2 mM)	100*	89	119	132	100*	118	48	73
-gln + TLCK**	47	42	55	53	30	ND	13	15
+gln + TLCK	82	90	109	100	78	ND	71	62
-gln + TAME***	49	46	54	53	ND	36	17	13
+gln + TAME	95	77	113	114	ND	80	65	59

*Control (100%) is the incorporation of ³H-thymidine or ³H-uracil into DNA or RNA respectively by On Ser grown in complete medium for 4 h at 37°C.

**N α -p-tosyl-L-lysine-chloromethylketone (100 μ g/ml)

***N α -p-tosyl-L-arginine methyl ester (100 μ g/ml)

ND = not determined

Procedures for cell culture and washing, radioactive labelling, recovery of labelled nucleic acids and quantitation of radioactivity were described in "Methods".

glutamine (see columns 5 and 9, Table 4) while untransformed cells maintained their reliance on glutamine for optimal DNA and RNA synthesis (columns 3 and 7, Table 4). Therefore, as in the case of HS3 metabolism, thermal stress altered nucleic acid metabolism in transformed cells in a way that caused them to become independent of supplied glutamine for optimal synthesis of DNA and RNA.

Influence of the Protease Inhibitors TLCK and TAME on Nucleic Acid and HS3 Metabolism

While several hypotheses can be proposed to explain the thermal stress phenomenon of transformed cells, one hypothesis in particular was experimentally tested; that is, the "apparent" non-requirement for glutamine at 42°C was due to protease(s) degradation of serum proteins thereby providing the cells with glutamine.

An early indication that protease(s) might be involved in providing glutamine for transformed cells at 42°C came from studies involving the protease inhibitors TLCK and TAME. At 42°C, On Ser-DMBA cells did not accumulate HS3 and synthesised nucleic acid optimally when cultured in glutamine-free medium, but in the presence of TLCK and/or TAME they accumulated HS3 (Table 2) and nucleic acid synthesis became dependent on exogenous supply of glutamine (Table 4). Both TLCK and TAME did not have any effect on either HS3 or nucleic acid synthesis by the transformed cells at 37°C, indicating that the effect of the protease inhibitors noted at 42°C was primarily to control the action of the proposed protease(s) in transformed cells at 42°C.

Evidence that Glutamine is Generated in Medium of Thermally-Stressed Transformed Cultures

As mentioned earlier, heat shock seemed to condition transformed cells into behaving as if they were not starving for glutamine. The simplest mechanism, in view of the results of experiments with protease inhibitors discussed in the preceding section, is that thermally-stressed transformed cells produced and secreted protease(s) which degraded proteins to generate glutamine in the medium. It is an established fact that under normal growth conditions, transformed cells secrete abundant amounts of proteases compared to untransformed cells (Bosmann, 1969; Roblin et al 1975; Rossman and Troll 1980), but the effects of heat stress on protease secretion by transformed and untransformed cells had not previously been studied.

That protease(s) might be breaking down proteins to provide glutamine for transformed cells under thermal stress was suggested by the observations that upon shifting the temperature from 37°C to 42°C, significant amounts of glutamine accumulated in the growth medium (which was initially glutamine-free) in On Ser-DMBA and other transformed cell cultures, but not in On Ser and other untransformed cultures (Table 5). The amount of released glutamine was estimated from a glutamine concentration standard curve (Fig. 9). (The level of released glutamine measured in CHO WT cell cultures at 42°C was somewhat lower than that of other transformed cells.) Protease inhibitors added prior to the temperature shift prevented the build-up of glutamine in glutamine-free cultures of transformed cells (Table 5). Since protease inhibitors had parallel effects on HS3 and nucleic acid metabolism (Tables 2 and 4), it

Table 5

Accumulation of L-glutamine and protease(s) in the medium of various transformed and untransformed mammalian cell cultures at 37°C and 42°C in the presence or absence of 2 mM L-glutamine and in the presence or absence of protease inhibitors in the growth medium. The growth of cells and determination of glutamine and protease were done according to the procedures described in "Methods". All experiments were done using near-confluent cultures in Brockway bottles containing 10 ml of incubation medium.

<u>Cell Line and Treatment Condition¹</u>	<u>L-glutamine in medium² (mM)</u>	<u>Protease activity in medium³ (% of control)</u>
<u>On Ser</u>		
-gln, 37°C	0	11
+gln, 37°C	0.8	11
-gln, 37°C + TLCK/TAME	0	ND
+gln, 37°C + TLCK/TAME	0.8	ND
-gln, 42°C	0	11
+gln, 42°C	1.0	22
-gln, 42°C + TLCK/TAME	0	ND
+gln, 42°C + TLCK/TAME	0.8	ND
<u>On Ser-DMBA</u>		
-gln, 37°C	0	100*
+gln, 37°C	0.6	122
-gln, 37°C + TLCK/TAME	0	ND
+gln, 37°C + TLCK/TAME	0.6	ND
-gln, 42°C	0.25	256
+gln, 42°C	0.4	200
-gln, 42°C + TLCK/TAME	0.0025	ND
+gln, 42°C + TLCK/TAME	0.6	ND

... cont'd

Table 5 (cont'd)

<u>Cell Line and Treatment Condition¹</u>	<u>L-glutamine in medium² (mM)</u>	<u>Protease activity in medium³ (% of control)</u>
<u>10T$\frac{1}{2}$</u>		
-gln, 37°C	0	22
+gln, 37°C	0.8	11
-gln, 42°C	0	11
+gln, 42°C	1.0	11
<u>10T$\frac{1}{2}$ ciras-2</u>		
-gln, 37°C	0	67
+gln, 37°C	0.8	44
-gln, 42°C	0.2	178
+gln, 42°C	0.6	111
<u>CHO WT</u>		
-gln, 37°C	0	11
+gln, 37°C	1.0	11
-gln, 42°C	0.05	22
+gln, 42°C	0.9	22

¹All experiments were performed with cells grown in Brockway bottles in α -MEM supplemented with or without glutamine, plus 10% DFBS and, where applicable, 100 μ g/ml each of TLCK and TAME.

²The values for L-glutamine concentration in growth medium were extrapolated from the glutamine standard curve (Fig. 9). Corrections were made for L-glutamate in the medium.

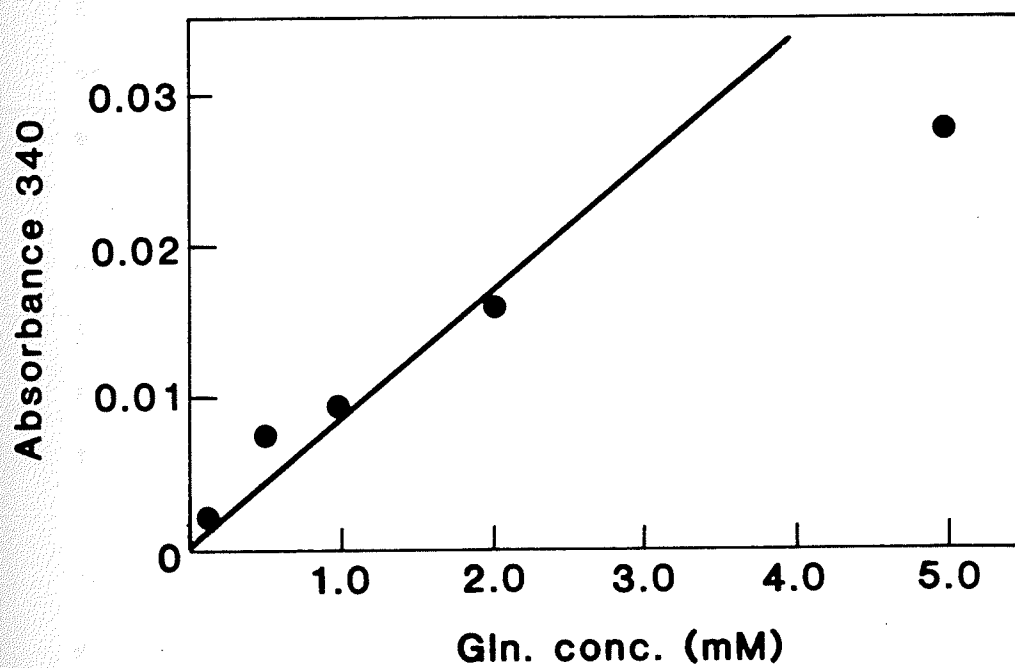
³Protease activity is expressed as a percentage of a 100% control value.

*Protease control (100%) is the absorbance reading for On Ser-DMBA cells grown for 4 h at 37°C in medium without glutamine.

ND = not determined

Fig. 9 Standard curve that correlates optical density (absorbance) with L-glutamine concentration. Glutamine (dissolved in 100 mM tris-acetate buffer, pH 7.5) was determined by the coupled assay procedure of Lund (1974) as described in "Methods". The reaction mixtures and spectrophotometer specifications are described in "Methods".

FIG. 9



suggests a direct relationship between the thermal effects on these two metabolic events and the glutamine build-up. On Ser-DMBA cells, but not On Ser cells, also accumulated glutamine within cells at 42°C but not at 37°C (Table 6).

Two other, less direct lines of evidence implicate protease generated in the thermal effect. First, Table 7 shows that methionine sulphoximine, an analogue and competitive inhibitor of glutamine (LeJohn, personal communication) allowed On Ser-DMBA cells to accumulate HS3 in glutamine-free medium at 42°C in a concentration-dependent manner; the cells did not accumulate HS3 at 42°C in the absence of methionine sulphoximine. Methionine sulphoximine did not affect HS3 levels at 37°C suggesting that its effect was primarily as an inhibitor of glutamine. Second, glutaminase [an enzyme that breaks down glutamine to glutamate (Hartman 1979)] allowed heat-stressed On Ser-DMBA cells to accumulate HS3 to control levels in glutamine-free medium (Table 8); there was no noticeable effect of glutaminase on HS3 levels at 37°C.

Studies were done to determine what the target proteins might be for the heat-induced protease(s) of transformed cells. Theoretically, glutamine could be derived by breakdown of either cellular proteins or serum proteins, but a reasonable conjecture in these studies was that glutamine was derived from proteins in the medium since it would be unproductive for the cell to derive glutamine from its own proteins. The experimental approach was to determine whether the removal of serum (the main source of proteins in the medium) from the culture medium circumvents the thermal effect on HS3 metabolism. As shown in Table 9 (and other results presented earlier) heat-stressed On Ser-DMBA cells in

Table 6

Accumulation of L-glutamine in cells of On Ser and On Ser-DMBA cultures at 37°C and 42°C in the presence or absence of glutamine in the growth medium.

<u>Cell Line and treatment condition</u>	<u>L-glutamine in cell extract</u> (mM)
<u>On Ser</u>	
-gln, 37°C	0
+gln, 37°C	0
-gln, 42°C	0
+gln, 42°C	0
<u>On Ser-DMBA</u>	
-gln, 37°C	0
+gln, 37°C	0.05
-gln, 42°C	0.2
+gln, 42°C	0.25

The growth of cells, preparation of cell extracts, and determination of L-glutamine were according to the procedures described in "Methods". L-glutamine concentrations were extrapolated from the standard curve. Corrections were made for L-glutamate in the medium.

Table 7

³²P-labelled HS3 in On Ser-DMBA cells at 37°C and 42°C in growth medium without glutamine but containing varying concentrations of methionine sulphoximine.

Treatment condition (On Ser-DMBA cells incubated in medium without gln)	HS3/Total counts ¹ (%)	HS3 ² (% of control)
Cells at 37°C, without MS	5.26	85.0
+ 0.1 mM MS*	4.96	80.1
+ 0.5 mM MS	5.87	94.8
+ 1.0 mM MS	5.92	95.6
Cells at 42°C, without MS	0.67	10.8
+ 0.1 mM MS	2.40	38.8
+ 0.5 mM MS	4.02	64.9
+ 1.0 mM MS	4.26	68.8

¹³²P-labelled HS3 counts expressed as a percentage of the total radioactive counts in the formic acid extract.

²³²P-labelled HS3 (relative to total counts) expressed as a percentage of control value. Control (100%) is the amount of labelled HS3 accumulated in CHO WT cells for 4 h at 37°C in the absence of glutamine.

*Methionine sulphoximine. A 10 mM stock solution was prepared in α -MEM without glutamine.

The growth of cells and quantitation of HS3 were carried out as described in the legend for Fig. 4 and "Methods". All experiments were carried out in α -MEM without glutamine, plus 10% DFBS. Cells were incubated for 4 h.

Table 8

³²P-labelled HS3 in On Ser-DMBA cells at 37°C and 42°C in the presence or absence of glutaminase (Glnase) in growth medium without glutamine.

<u>Treatment conditon</u>	<u>HS3/Total counts</u> (%)	<u>HS3</u> (% of control)
<u>37°C for 4 h</u>		
-gln med.	5.42	87.6
-gln med. + Glnase*	5.68	91.8
+gln med. + Glnase	0.97	15.7
<u>42°C for 4 h</u>		
-gln med.	0.88	14.2
-gln med. + Glnase	5.21	84.2
+gln med. + Glnase	1.03	16.6

*10 units/ml of glutaminase was used for each sample. Glutaminase was added at the start of the 4-h incubation period.

Cell culture method and quantitation of HS3 were described in the legend for Fig. 4 and in "Methods". HS3 is expressed relative to total radioactive counts in the formic acid extract, and as a percentage of a control value, as described in the legend for Table 7.

Table 9

³²P-labelled HS3 in On Ser-DMBA cells at 37°C and 42°C in the presence or absence of serum, and with bovine serum albumin substituting for serum.

<u>Treatment conditon</u>	<u>HS3/Total counts</u> (%)	<u>HS3</u> (% of control)
<u>37°C</u>		
without serum	4.24	68.5
without serum + 10% BSA*	4.79	77.4
with serum + 10% BSA	5.01	80.9
<u>42°C</u>		
without serum	4.69	75.8
without serum + 10% BSA	4.19	67.7
with serum + 10% BSA	0.92	14.9

*Bovine serum albumin. 10% (weight/volume) BSA was prepared in α -MEM without glutamine.

Cell culture procedures and quantitation of HS3 were described in the legend for Fig. 4 and in "Methods". All experiments were carried out using α -MEM without glutamine.

HS3 is expressed relative to total radioactive counts in the formic acid extract, and as a percentage of a control value, as described in the legend for Table 7.

serum-supplemented medium without glutamine accumulated very small amounts of HS3 (only about 15% of control). However, when serum was removed from the medium, appreciable amounts of HS3 (up to 75% of control) accumulated at 42°C (Table 9). Presumably, bovine serum albumin (BSA) could not substitute for general calf serum. The removal of serum from the medium did not have any dramatic effect at 37°C. The presence of 10% BSA in the medium did not adversely affect the viability of the cells within the experimental period.

Gel electrophoresis patterns of ^{35}S -methionine-labelled proteins derived from cellular material of On Ser and On Ser-DMBA cells at 37°C and 42°C (Fig. 10) showed slight differences in the labelling patterns. In general, the protein labelling intensity was lower in On Ser-DMBA than in On Ser cells at both 37°C and 42°C (compare panels 1 - 4 to panels 6 - 9, Fig. 10). But the labelling intensity of On Ser-DMBA cells was similar for 37°C and 42°C samples. A few protein spots are unique to the On Ser-DMBA samples, but none could be related to a temperature effect. There is no evidence of a "heat shock response" at 42°C normally expressed as a synthesis of a set of highly conserved proteins with a concomittant reduction or absence of other cellular proteins (Schlesinger et al 1982). The labelling period at 42°C was probably too long to elicit the "heat shock response". Heat shock proteins may not appear if the cells have already adapted to the high temperature (see Appendix).

Fig. 10 Autoradiogram of ^{35}S -methionine-labelled cellular proteins from On Ser-DMBA and On Ser cells grown at 37°C and 42°C in the presence or absence of glutamine in the incubation medium. Experiments were performed in medium lacking methionine, except for the radioactively-labelled methionine (100 $\mu\text{Ci}/\text{ml}$). Proteins were analysed by one-dimensional polyacrylamide gel electrophoresis. Cell culture methods, protein sample preparation, electrophoresis procedures, and preparation of autoradiogram were described in 'Methods'. Cells for protein sample preparations were collected following a 4-h incubation period at the appropriate temperature after addition of the radioactive label.

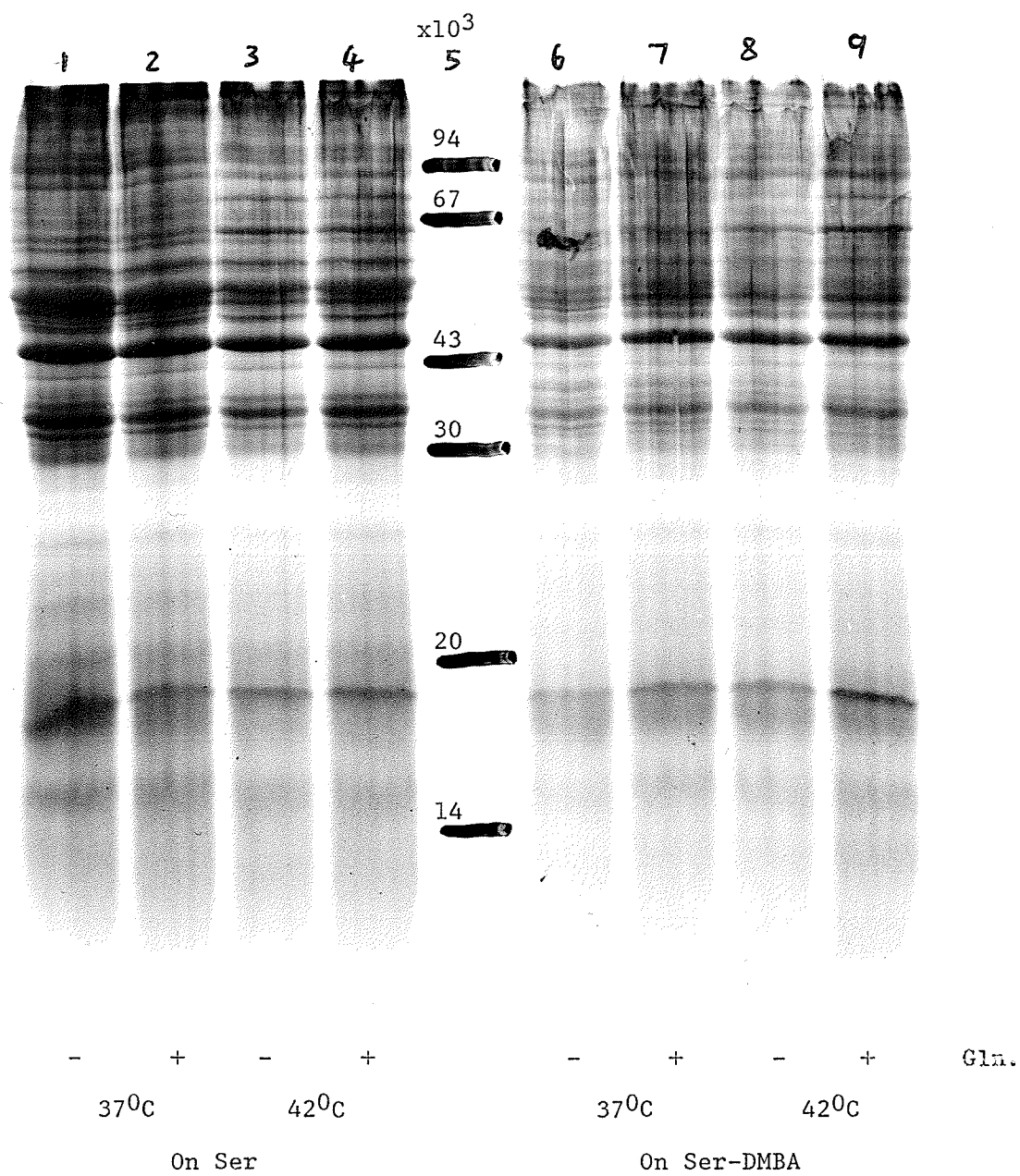
Gln = glutamine

(-) means glutamine absent in the growth medium

(+) means glutamine present in the growth medium.

The molecular weight standards were marked according to the positions of those proteins on a similarly run gel with the standards.

Fig. 10



Protease Activity in Media of Transformed and Untransformed Cells at 37°C and 42°C

A direct proof that protease(s) are primarily involved in the heat-induced generation of glutamine in cultures of transformed cells is best provided by measurement of protease activity in the medium, and by isolation of the culprit enzyme(s) under the appropriate conditions. As shown in Table 5, analysis of the medium for proteolytic activity revealed that the medium from transformed cell cultures that were incubated at 42°C for 4 h had at least twice as much protease activity as medium from cultures incubated at 37°C. Only about 10% of protease activity detectable in transformed cultures was demonstrated in the medium of untransformed cells. CHO WT cultures showed very low levels of protease activity, similar to the levels measured in untransformed cultures. This corresponds with the low levels of glutamine generated in the medium of CHO WT cultures.

Experiments were performed in an effort to partially characterise the protease(s) induced by heat in transformed cultures. First, both cycloheximide and actinomycin D reduced the level of the heat-induced protease activity in transformed cultures by nearly 60% (Table 10). However, the level of protease activity in the presence of these inhibitors was still much higher than that found in untransformed cultures (compare Tables 5 and 7). In spite of the fact that up to 40% of protease activity remained in cultures treated with cycloheximide and actinomycin D, virtually no glutamine was released in those cultures (Table 10). Another result revealed in Table 10 concerns the question of whether cells are required for the heat-induced protease activity or

Table 10

Influence of cycloheximide and actinomycin D, and the role of cells in the heat-induced accumulation of L-glutamine and protease activity in the medium of On Ser-DMBA cultures.

<u>Treatment condition</u>	<u>L-glutamine generated in medium¹ (μM)</u>	<u>Protease activity in medium¹ (% of control)</u>
<u>2 h at 37°C, 4 h at 42°C</u>	250	189
+ CHX*	2.5	78
+ ACT.D*	0	78
<u>2 h at 37°C, med. at 42°C</u>		
0 h**	0	89
1 h	0	89
2 h	0	67
3 h	0	89
4 h	0	67

¹Quantitation of L-glutamine and protease activity in the growth medium were by procedures described in the legend for Table 5 and in "Methods".

*CHX refers to cycloheximide
ACT.D refers to actinomycin D

Cycloheximide or actinomycin D were added at the time that the cultures were shifted from 37°C to 42°C. 10 μ g/ml of cycloheximide, and 1 μ g/ml of actinomycin D were used.

**Cultures were initially incubated for 4 h at 37°C, after which the medium was collected and centrifuged to remove any suspended cells. The cell-free medium was divided into five parts for incubation at 42°C for the various time periods specified.

All experiments were conducted with cells kept for the specified amounts of time and temperature in α -MEM without glutamine, plus 10% DFBS.

whether the enzyme is already present in the medium at 37°C but activated by heat. On Ser-DMBA cells were incubated at 37°C for 4 h in medium lacking glutamine, the medium was removed and heated at 42°C for 1 to 4 h and then assayed for glutamine and protease activity. The results in Table 10 (rows 4 to 8) show that when cell-free medium (which was pre-incubated with cells at 37°C for 4 h) was heated for 1 to 4 h, there was little or no change in the basal level of protease activity, but the heat-induced portion of proteolytic activity was absent; under these conditions, it seemed that the protease activity measured represents protease(s) normally secreted by these cells at 37°C. The results clearly show that cells are required for the component of protease activity induced at 42°C which results in generation of glutamine in transformed cell cultures. There was no effect of the duration of heating period on the level of protease induced, further indicating that all the measured proteolytic activity is protease(s) normally induced or secreted by cells at 37°C.

In a series of experiments, it was found that the protease(s) induced by heat in transformed cells were inhibited, either in their induction or action, by a variety of protease inhibitors (Table 11). (The inhibition of protease activity was measured as the degree of inhibition of glutamine production in the transformed cell cultures at 42°C in the presence of the protease inhibitor.) Four protease inhibitors were used in the investigations: benzamidine and nitrophenyl-p-guanidobenzoate (NPGb), which inhibit plasminogen activator but not plasmin; and tranexamic acid and ε-aminocaproic acid (ε-ACA) which inhibit plasmin but not plasminogen activator (Quigley

Table 11

Influence of various protease inhibitors on the heat-induced accumulation of glutamine in the growth medium of On Ser-DMBA cultures.

Treatment condition ¹	% Inhibition of glutamine production ²
<u>2 h 37°C no inhibitor, 4 h 42°C</u>	
+ benzam	100
+ NPGB	50
+ benzam. + NPGB	50
<u>2 h 37°C, 4 h 42°C</u>	
+ benzam	100
+ NPGB	100
+ benzam + NPGB	50
<u>2 h 37°C no inhibitor, 4 h 42°C</u>	
+ tranexamic acid	50
+ ε-ACA	50
+ tranexamic + δ-ACA	50
<u>2 h 37°C no inhibitor, 4 h 42°C no inhibitor*</u>	0

¹ Near confluent cultures containing glutamine-deficient medium were incubated for 2 h at 37°C and then transferred to 42°C for 4 h. Protease inhibitors were added either at the start of the 37°C incubation or at the time of transfer to 42°C, as specified in the table above. Protease inhibitors used are as follows (Quigley, 1979):

Benzamidine	(benzam)	10 ⁻³ M
Nitrophenyl-p-guanidinobenzoate (NPGB)		10 ⁻⁵ M
Tranexamic acid		10 ⁻² M
ε-amino-n-caprioc acid	(ε-ACA)	10 ⁻² M

Combinations of protease inhibitors were used at half dosages.

² Results are expressed as % inhibition of glutamine production by the protease inhibitor(s), where 0% inhibition is the amount of glutamine generated in cultures kept for 2 h at 37°C and 4 h at 42°C without any added protease inhibitors.

*0% inhibition control (explained above)

Glutamine quantitation were by procedures described in the legend for Table 5 and in "Methods".

1979). The plasminogen activator/plasmin system is an important proteolytic enzyme system in transformed cells (Roblin et al 1975). The results of Table 11 show that all four inhibitors added individually or in combinations inhibited the heat-induced protease activity by at least 50%. Benzamidine was the most effective since it resulted in 100% inhibition of glutamine production when it was added to the cultures either at the beginning of the 37°C pre-incubation or at the time of transfer to 42°C. NPGb was also 100% effective when it was added at the start of the 37°C pre-incubation but only 50% effective when it was added at the time of transfer to 42°C. Surprisingly, a combination of benzamidine and NPGb at half dosages was only 50% effective at inhibiting glutamine production, irrespective of the time they were added to the cultures. This suggests that the two inhibitors are not additive in their actions, which also suggests that the two inhibitors may be acting on different protease entities. There is also the possibility that the protease inhibitors affect either glutaminase or glutamic dehydrogenase or both, the two enzymes used in the assay system for glutamine produced as a result of protease(s) action. A further possibility is that the inhibition of glutamine production by the protease inhibitors is spurious and only about 50% inhibition actually occurs. If this is so, it might mean that the protease(s) responsible for glutamine production is hardly affected by the inhibitors, suggesting that only the plasminogen activator/plasmin system is affected by these inhibitors. Tranexamic acid and ϵ -ACA, whether added individually or in half-dose combination, were only 50% effective. None of the protease inhibitors used appeared to affect the viability of the

cells.

HS3 Response Patterns of B82 and SK4476 Cell Lines and of B82 x SK4476 Hybrid Clones at 37°C in the Presence and Absence of L-Glutamine

The HS3 synthesis patterns of a transformed mouse cell line, B82, and an untransformed human cell line, SK4476, was studied. The B82 cell line showed several transformation characteristics such as ability to grow in agar and in medium containing low serum, and infinite lifespan in culture (Table 3). As shown in Table 12, B82 cells had HS3 synthesis patterns that are similar to those of other transformed cells (compare Table 2) when they are cultured at 37°C in the presence or absence of L-glutamine in the medium. On the other hand, the SK4476 cell line, like other untransformed cell lines, showed the untransformed pattern of HS3 synthesis. An important parameter that was measured in these studies is the "HS3 Ratio", which is defined as the ratio of HS3 synthesised by cells in the absence of glutamine to that synthesised in the presence of glutamine in the growth medium. At 37°C, the HS3 ratio of untransformed cells (Tables 2 and 12) is approximately 1, whereas the HS3 ratio of transformed cells (Tables 2 and 12) varies between 7 and 50. In particular, the HS3 ratio of the SK4476 cells was 1.01 and for B82 cells it was 17.25.

The HS3 ratios of hybrid cells constructed by fusing B82 and SK4476 cells were also determined. These interspecies hybrids between a transformed and an untransformed cell lines were meant to study the regulation of the HS3 response. The hybrids were selected by their ability to grow in HAT medium (see "Discussions"). Occasionally throughout the experimental period, and for at least three cell

Table 12

³²P-labelled HS3 in B82 and SK4476 cells at 37°C in the presence or absence of 2 mM glutamine in the growth medium.

<u>Cell line</u> ¹	<u>Treatment condition</u> ¹	<u>HS3/Total</u> ² (%)	<u>HS3</u> ² (% of control)	<u>HS3 Ratio</u> ³ (-/+ gln)
B82	-gln (1)	6.08	98.2	17.25
	(2)	6.17	99.7	
	+gln (1)	0.38	6.1	
	(2)	0.33	5.3	
SK4476 P ₁₁	-gln	3.65	59.0	1.01
	+gln	3.63	58.6	
SK4476 P ₁₃	-gln	4.73	76.4	1.01
	+gln	4.69	75.8	

¹Experiments for the B82 cell line were done in duplicate whereas two experiments were conducted separately for the SK4476 cell line at two passage levels (P₁₁ and P₁₃).

²HS3 is expressed relative to total radioactive counts in the formic acid extract, and as a percentage of a control value, as described in the legend for Table 7.

³HS3 Ratio is calculated as ³²P-HS3 in cells in glutamine-deficient medium divided by ³²P-HS3 in cells in complete medium.

Cell culture procedures and quantitation of HS3 were described in the legend for Fig. 4 and in "Methods".

generations at the end of the experimental period, all hybrid clones were tested and shown to grow in HAT medium. At no time were the B82 and SK4476 cells able to grow in HAT medium. An examination of Table 13 shows that the HS3 ratios of all hybrid clones were initially similar to that of the untransformed SK4476 cell line. However, as the cells underwent repeated divisions the HS3 ratios gradually approached or exceeded the value for the transformed B82 cell line. The rate and point at which the HS3 ratios changed from the untransformed to the transformed values during passage of the hybrid cells varied from clone to clone. Hybrid clones 3, 5, 6 and 14 attained values of HS3 ratios that were very close to that of the B82 parental cell line. Hybrid clone 16 had HS3 ratio that nearly doubled the value for B82. Clones 20 and 21 maintained HS3 ratios that were closer to the untransformed cell pattern than to the transformed pattern. The rest of the hybrid clones (clones 7, 8, 12, 13 and 19) had HS3 ratios that were intermediate between those of B82 and SK4476; but they all nevertheless attained HS3 values within the range for transformed cells. In general, the hybrids required 12 to 18 cell divisions (population doublings) to change their HS3 ratios from the untransformed to the transformed patterns, and the change was in several steps.

Growth Rates of B82, SK4476 and B82 x SK4476 Hybrid Cells at 37°C in α -MEM Supplemented with 10% or 1% Serum

A study was done to determine the growth rates of B82, SK4476 and the hybrid clones at 37°C in α -MEM supplemented with 10% or 1% calf serum (Figs. 11 to 22). It is established that transformed and untransformed cells differ in their ability to grow in medium containing

Table 13

HS3 Ratios for hybrid cells (obtained by fusion of B82 and SK4476 cells) at different cell passages.

		HS3 Ratio										
Hybrid Clones ²	Pop Doublings ¹											
		6	8	10	12	14	16	18	20	22	24	
3			1.2	1.3	1.0	1.0	1.1	12.4	17.0			
5			1.1	1.1	1.3		16.5	10.9				
6		1.6	3.2	3.3	6.4	12.1						
7			2.5	1.5		1.5	4.9	6.7				
8		1.0	1.0		1.2	5.4	8.7	7.2				
12			1.0	5.7	5.8	6.6	10.4	9.9				
13		2.7	3.2	3.6	3.4	5.5	9.4					
14		2.4	2.3		2.5	2.5			7.3	16.8	6.1	
16		1.2	0.9	1.4	1.5		3.3	30.6				
19		1.1	1.4	1.1	1.7	3.3	9.2					
20			2.9		3.6	3.3		4.1	3.2			
21			1.2	0.8	1.1		1.6	3.1	2.9			

¹Number of population doublings represent the number of times the cells in the culture underwent cell division, starting from the time the culture first attained confluency in Brockway bottles after isolation of the hybrid clone. Example, for a 2:1 subculture split ratio, the cultures undergo one population doubling in order to attain another confluency.

²Hybrid clones were assigned numbers according to the order in which they were isolated from culture plates. The hybrid clone numbers that are not listed were either lost due to bacterial/fungal contamination, or they failed to grow or grew too poorly for practical purposes.

Cell culture procedures, cell hybridisation and isolation of hybrid clones, and quantitation of HS3 and of HS3 ratios were described in the legends for Fig. 4 and Table 12, and in "Methods".

low serum (Temin 1966; Jainchill and Todaro 1970; Dulbecco 1970). A correlation between changes in HS3 ratios and patterns of growth in 10% and 1% serum-supplemented medium for the hybrid cells could give valuable information regarding the regulation of HS3 metabolism.

The B82 parental cell line grew beyond confluency in both 10% and 1% serum-supplemented medium, with growth rate and final cell density higher in medium with 10% serum. On the other hand, the SK4476 cell line grew to confluency in medium with 10% serum, but failed to grow in medium with 1% serum; in addition these cells were unable to grow post-confluent. B82 cells grew faster and attained higher final cell densities than SK4476 cells (Figs. 11 to 22).

The hybrid clones showed variations in their abilities to grow in medium containing 10% or 1% serum. In medium with 10% serum, most hybrid clones grew with rates similar to that of the transformed B82 parental cells (see Figs. 11a to 22a). Hybrid clone 3 (Fig. 11a) had a somewhat irregular growth pattern, with at least two periods of alternating rapid and slow growth phases. Another hybrid clone with unusual growth pattern is clone 21 (Fig. 22a), which initially grew at a similar rate as the untransformed SK4476 cell line, but later on in the course of its culture history, the hybrid grew much faster than the SK4476 cells to take the growth pattern of the B82 cell line.

In medium containing 1% serum, there was also some variation in the growth patterns among the hybrid clones. Clones 5 (Fig. 12b), 20 (Fig. 21b) and 21 (Fig. 22b) failed to grow in 1% serum (like the SK4476 parental line). The growth rate of clone 3 (Fig. 11b) was similar to that of SK4476 cells during the first 250 hrs of culture, after which

Fig. 11
to 22

Growth rates of B82, SK4476 cell lines, and hybrid clones cultured at 37°C in medium supplemented with (a) 10%, and (b) 1% calf serum.

The growth rate studies are similar to the procedures described in the legend for Fig. 3.

The growth curves for B82 and SK4476 cell lines were obtained in single studies and have been reproduced for each hybrid clone for comparison.

"c" indicates when the cultures attained confluency.

FIG. 11a

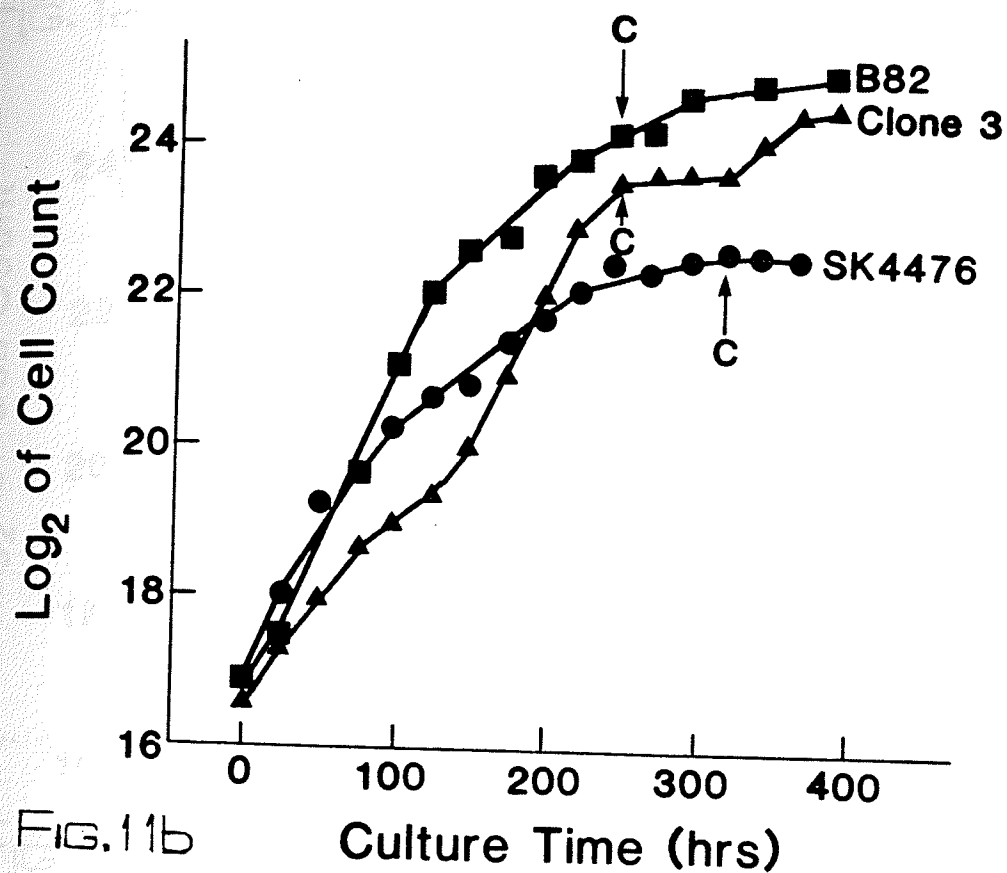


FIG. 11b

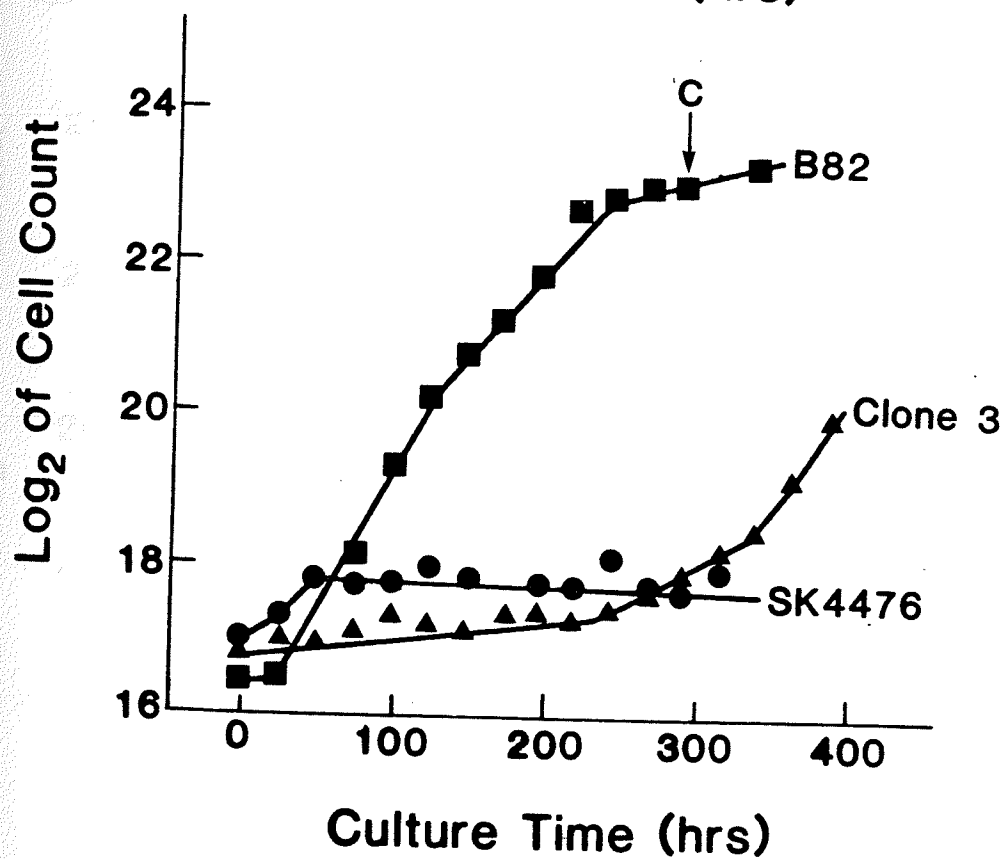


FIG. 12a

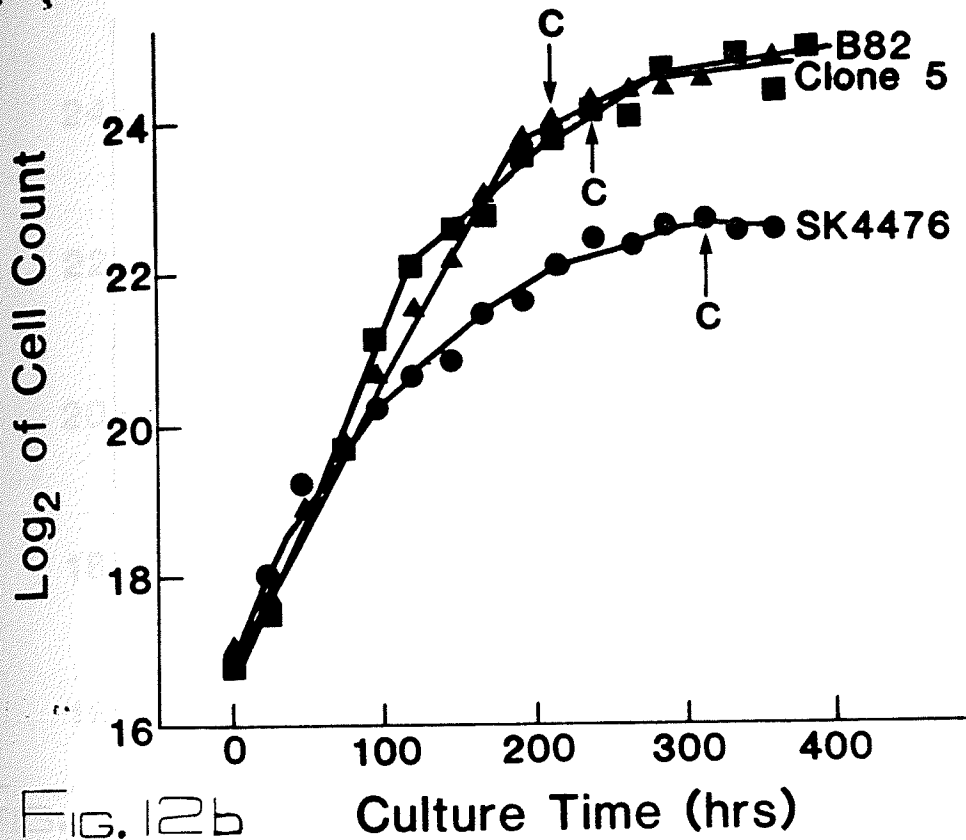


FIG. 12b

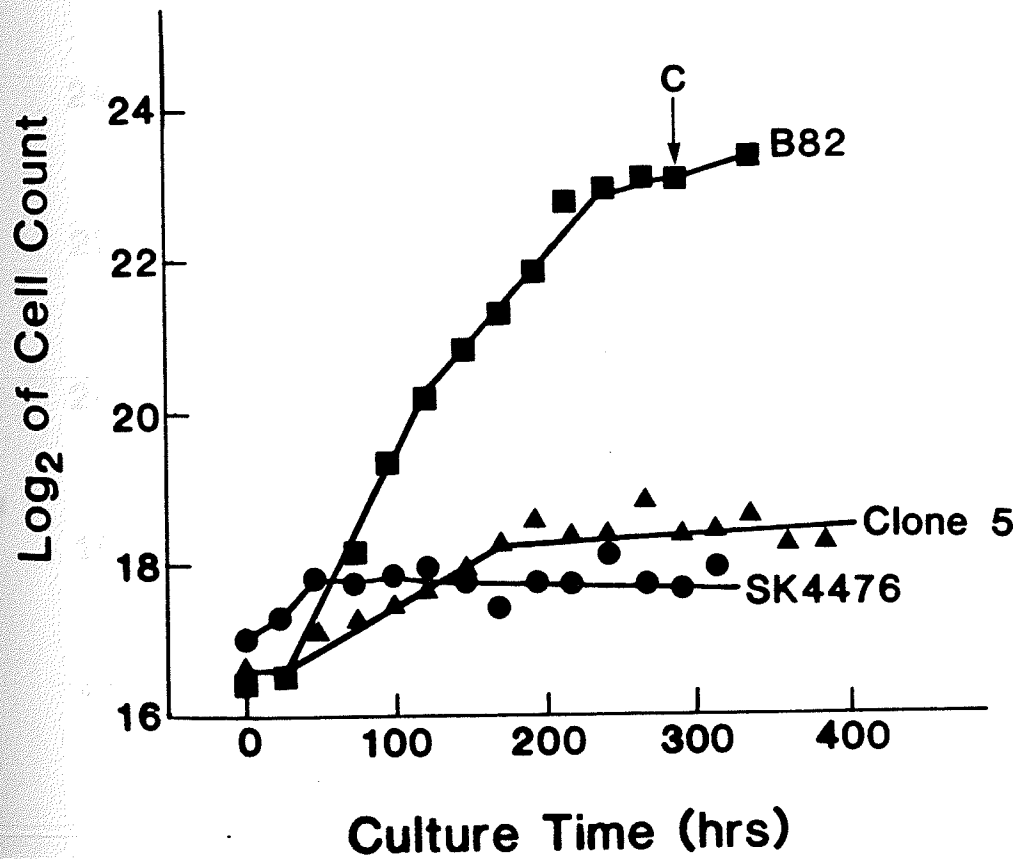


Fig. 13a

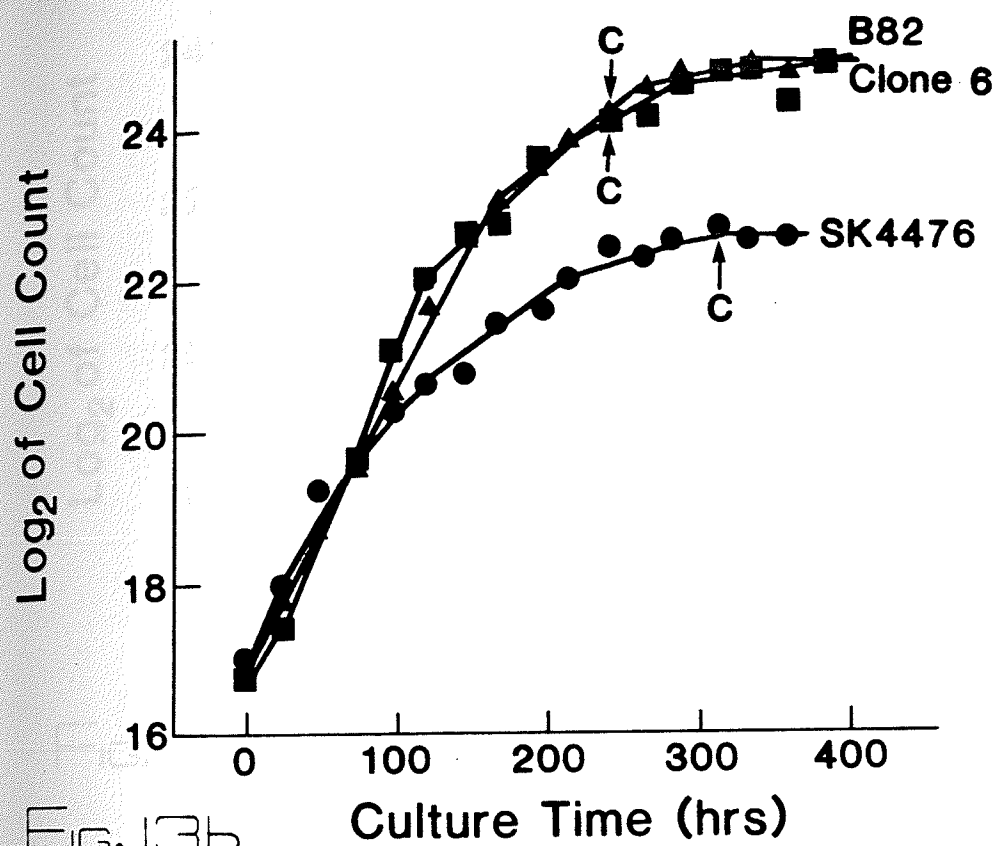


Fig. 13b

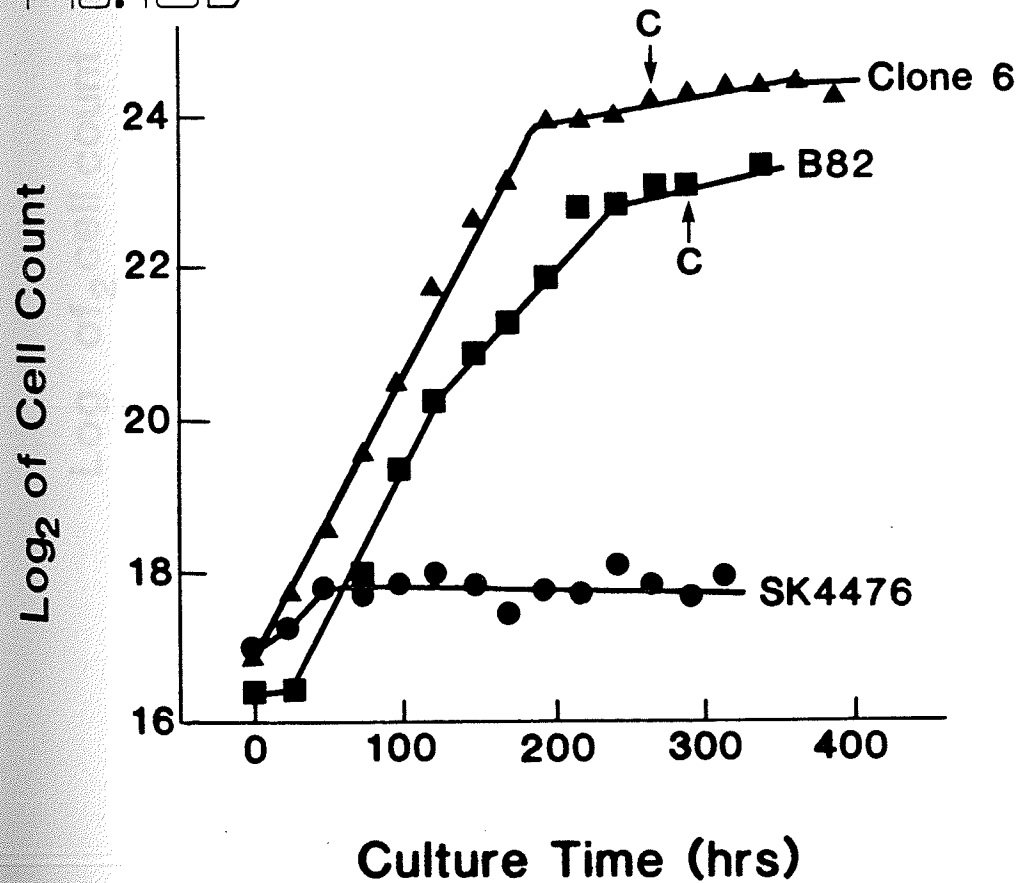


FIG. 14a

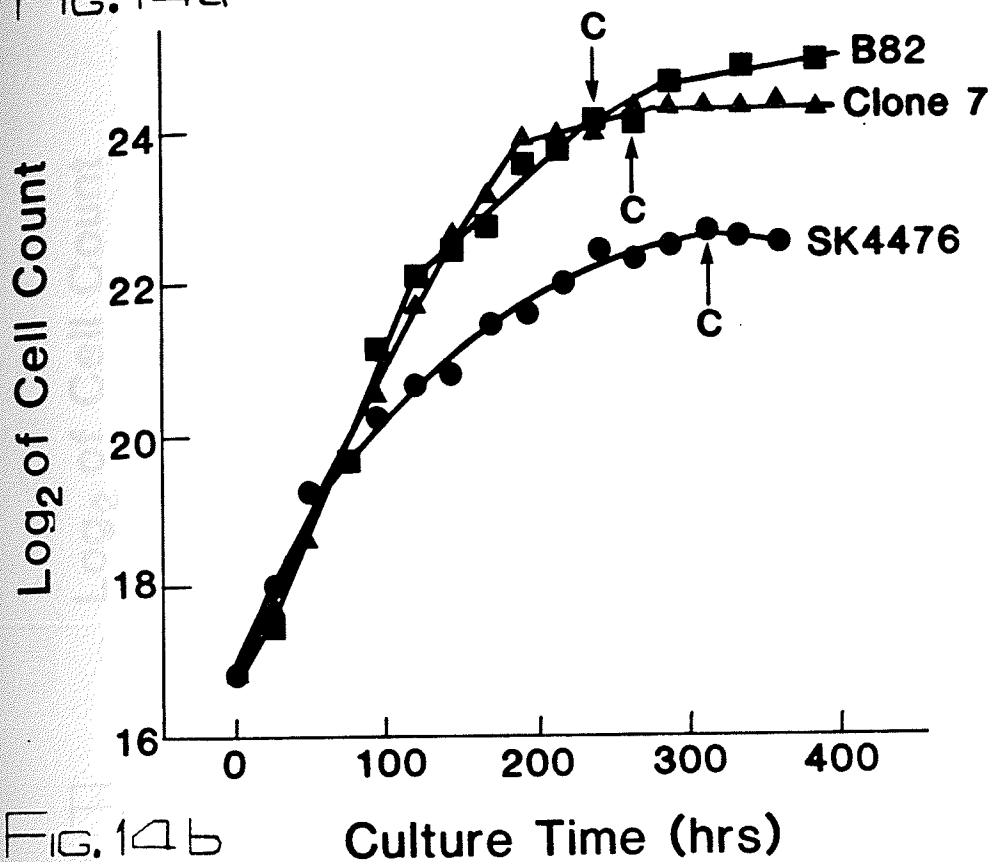


FIG. 14b

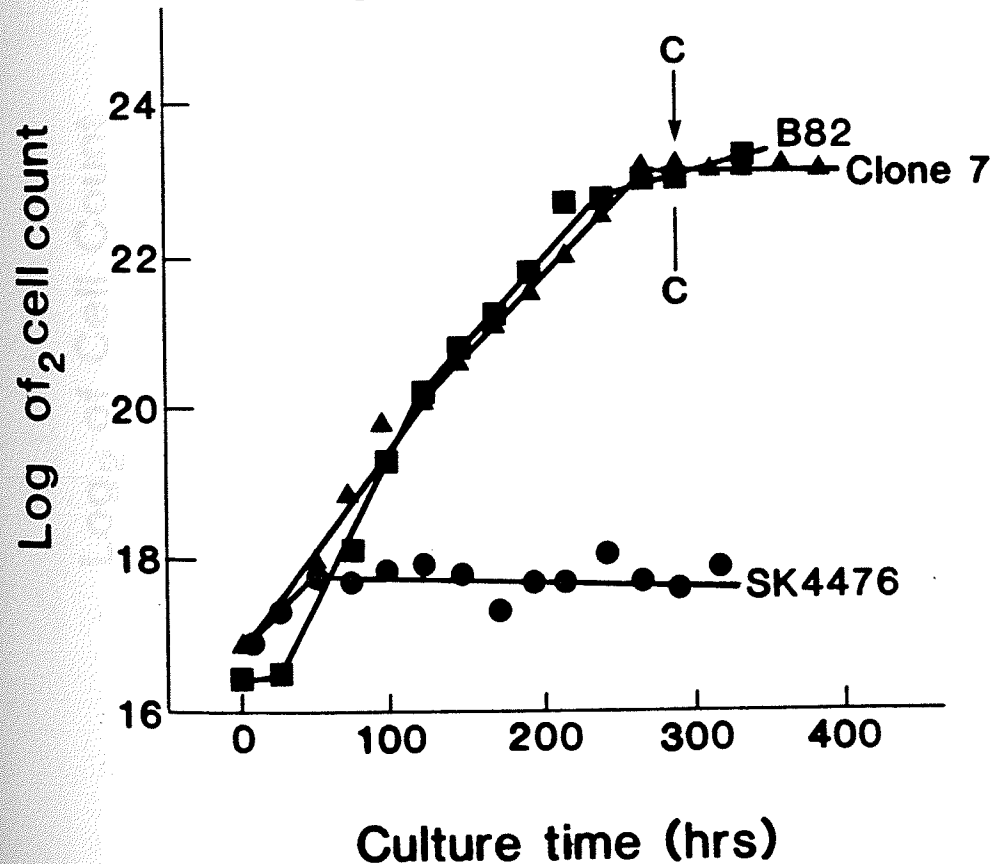


Fig. 15a

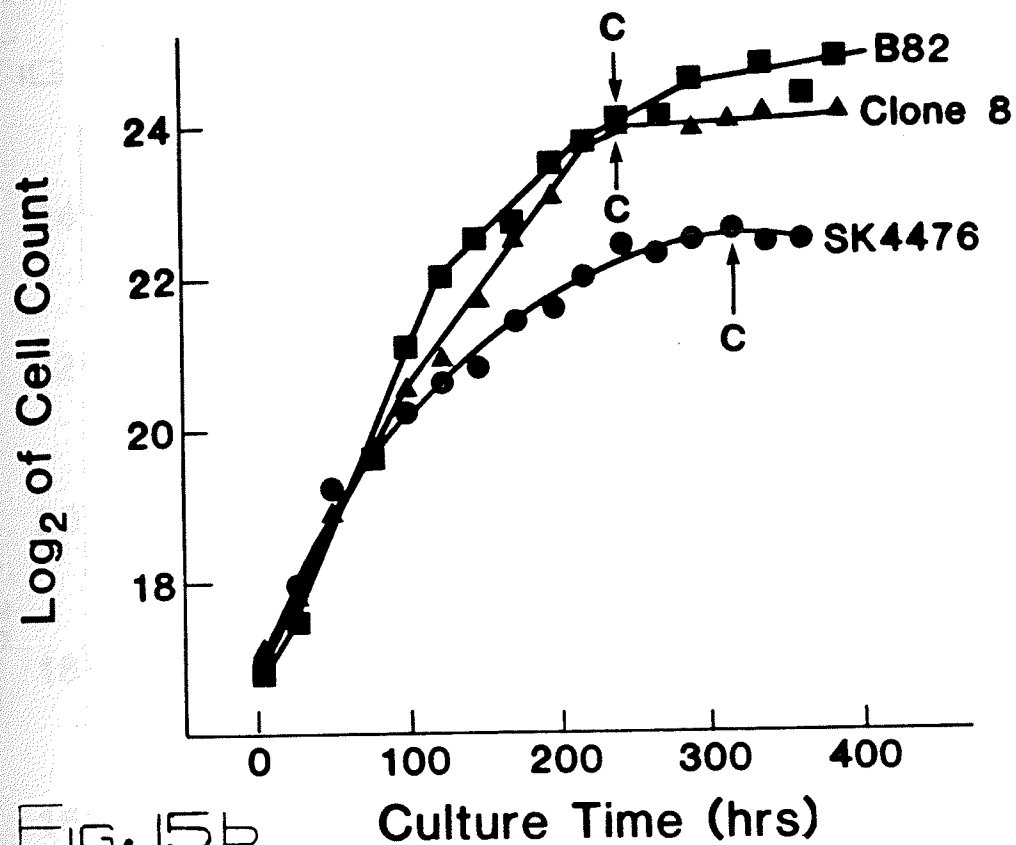


Fig. 15b

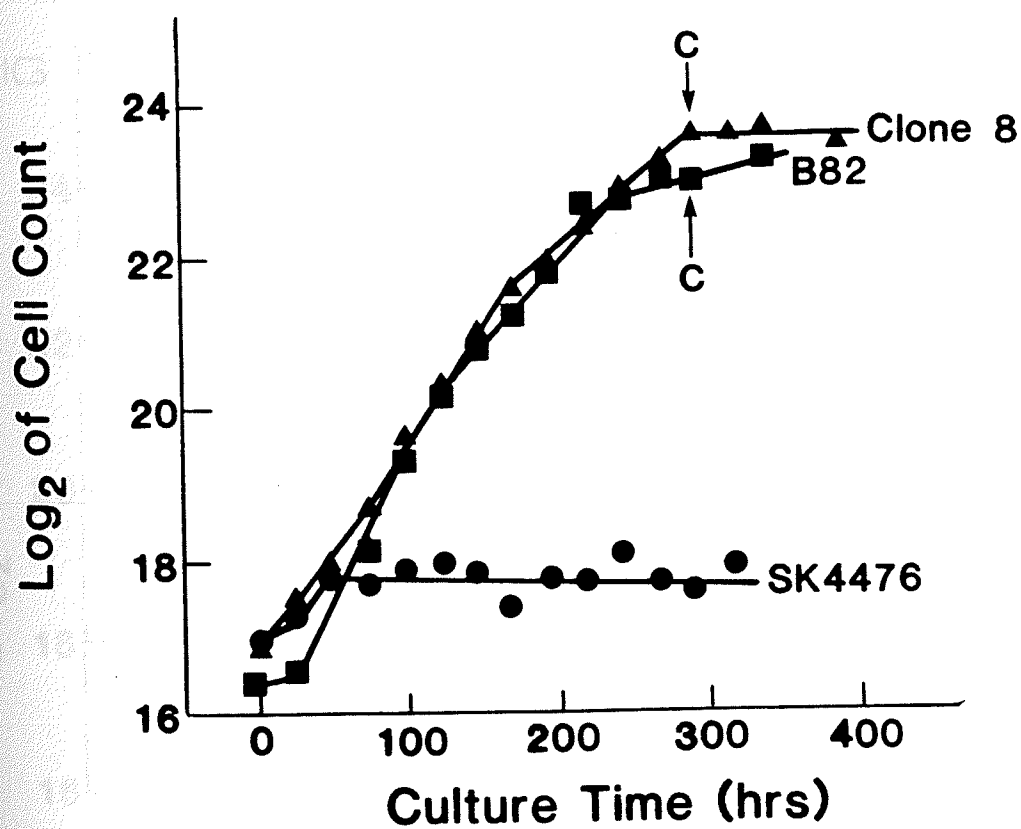


FIG 16a

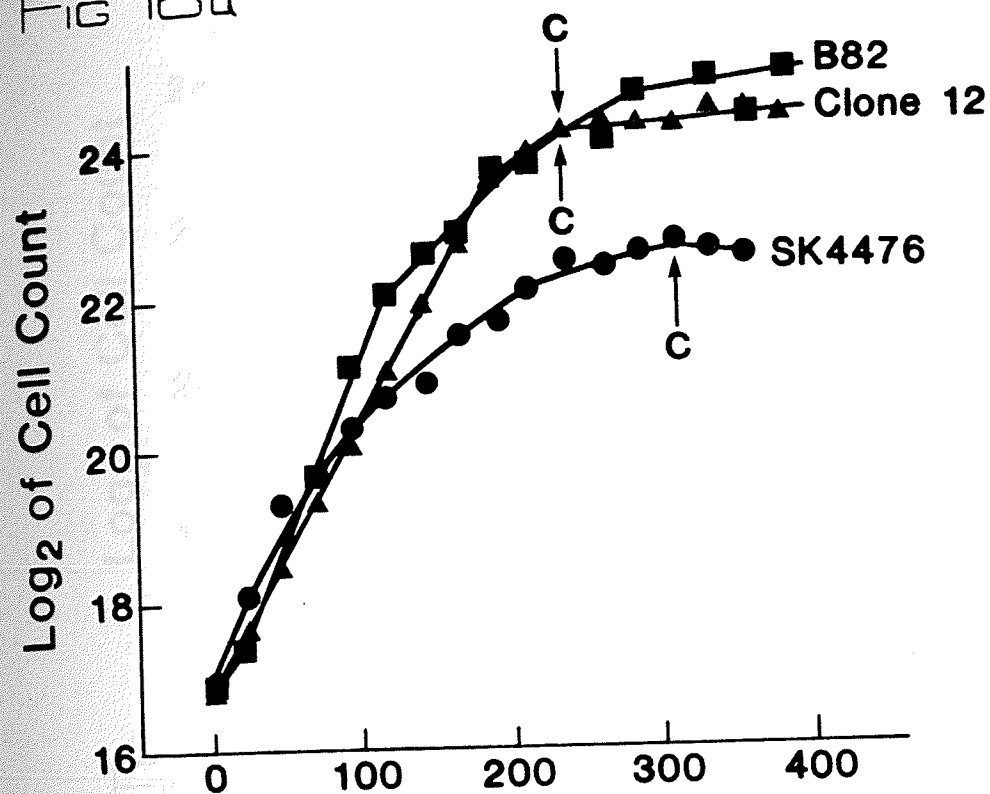


FIG 16b

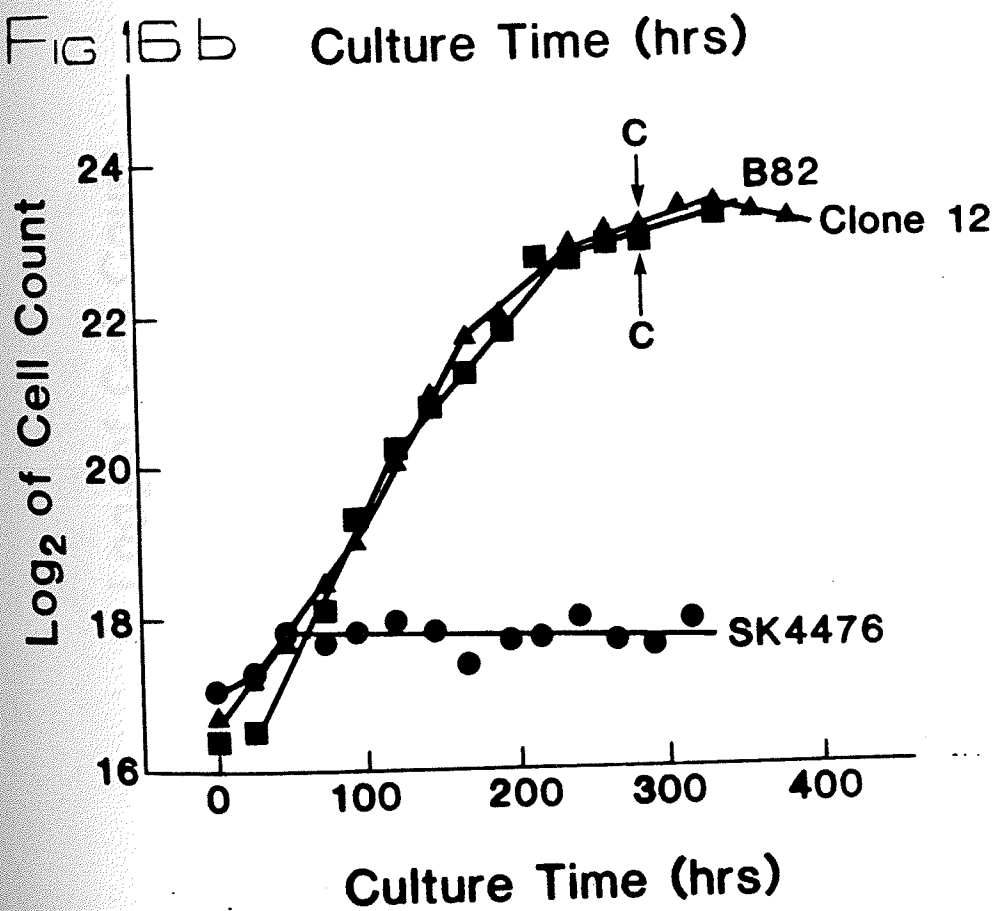


FIG. 17a

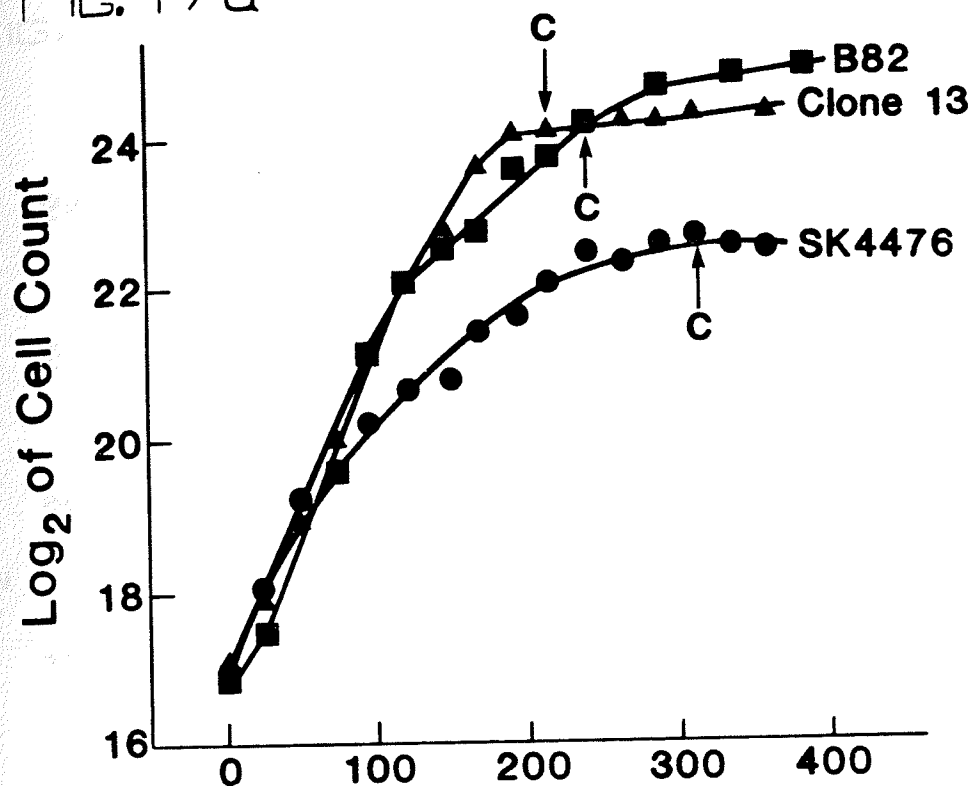


FIG. 17b

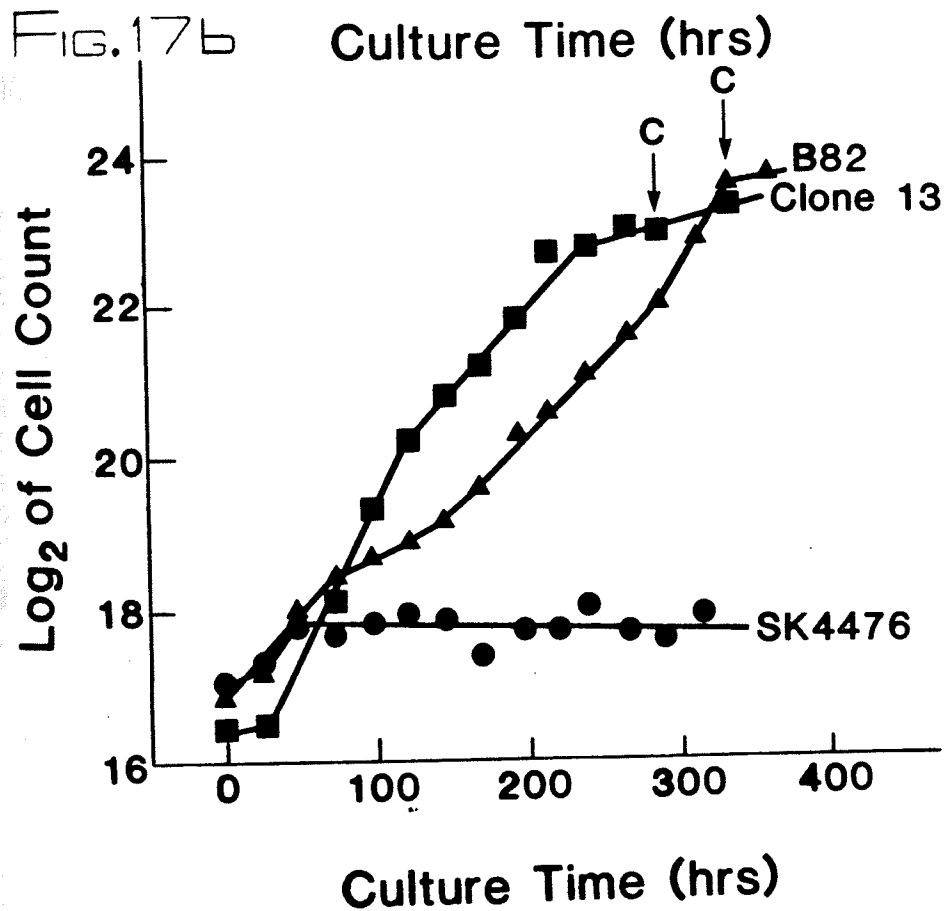


FIG. 18a

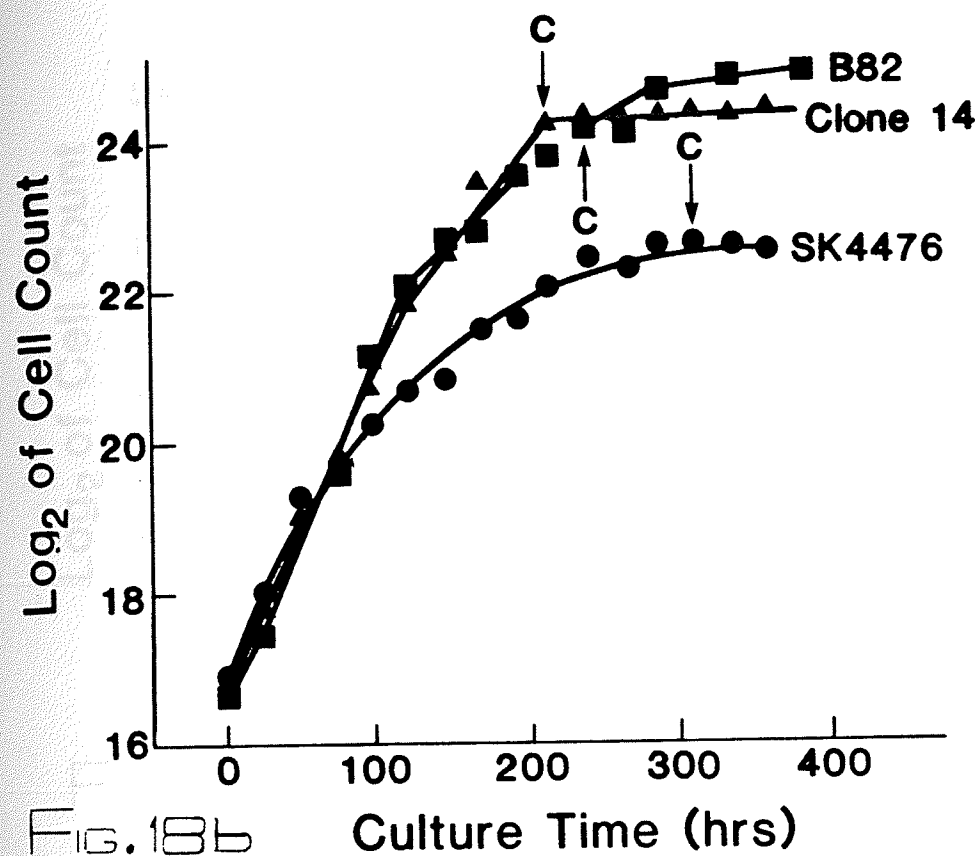


FIG. 18b

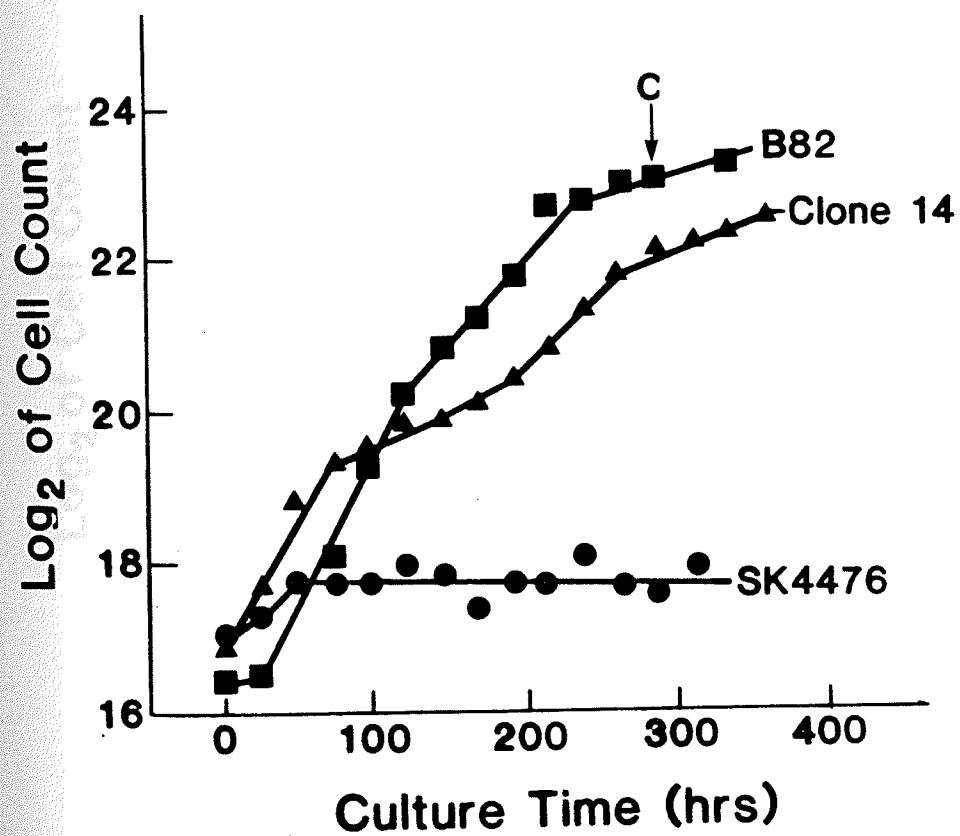


Fig. 19a

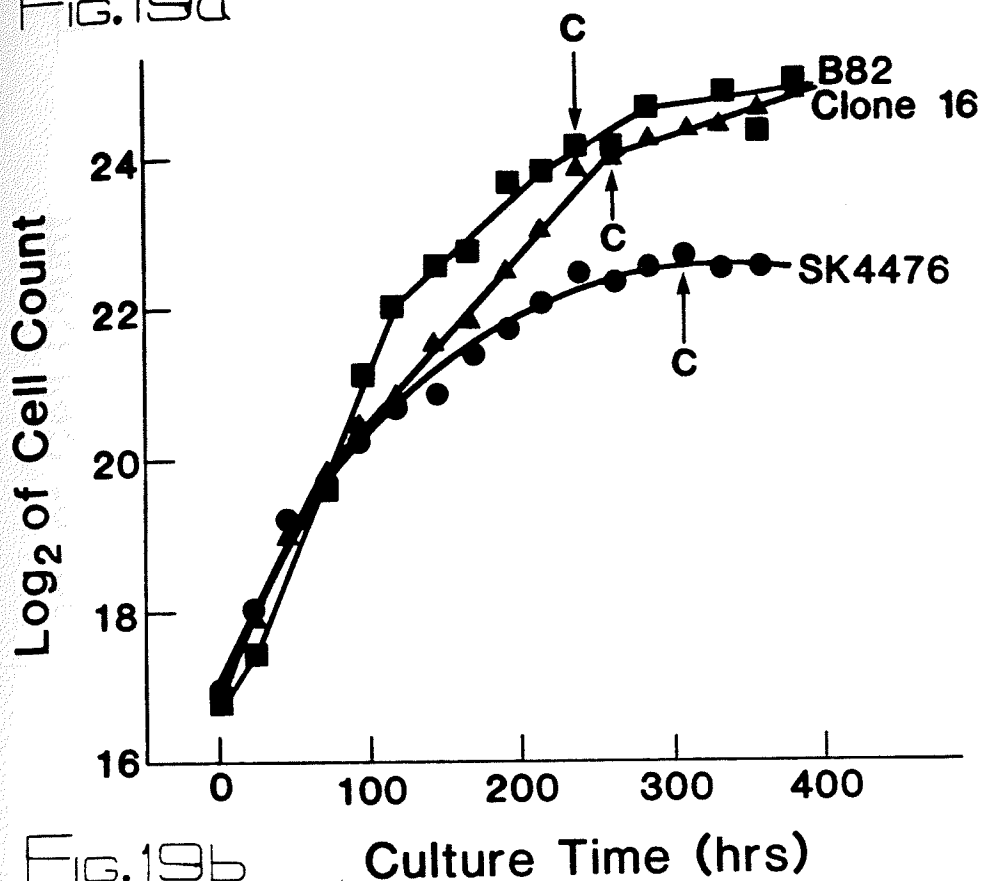


Fig. 19b

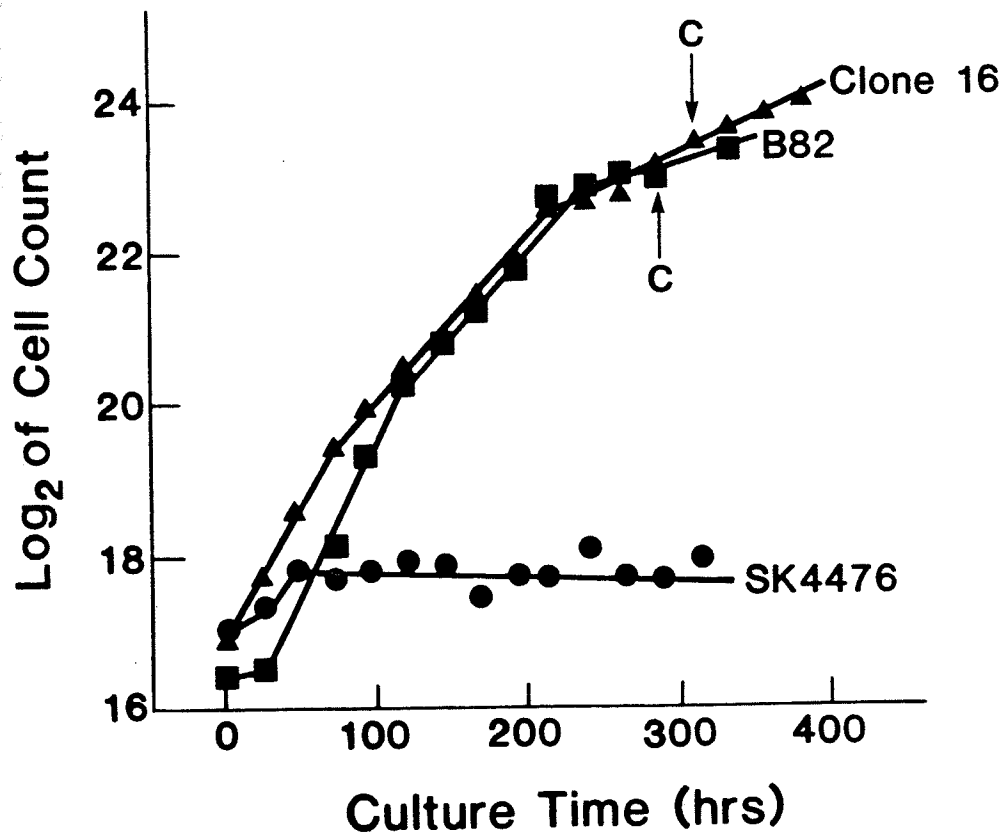


FIG. 20a

225

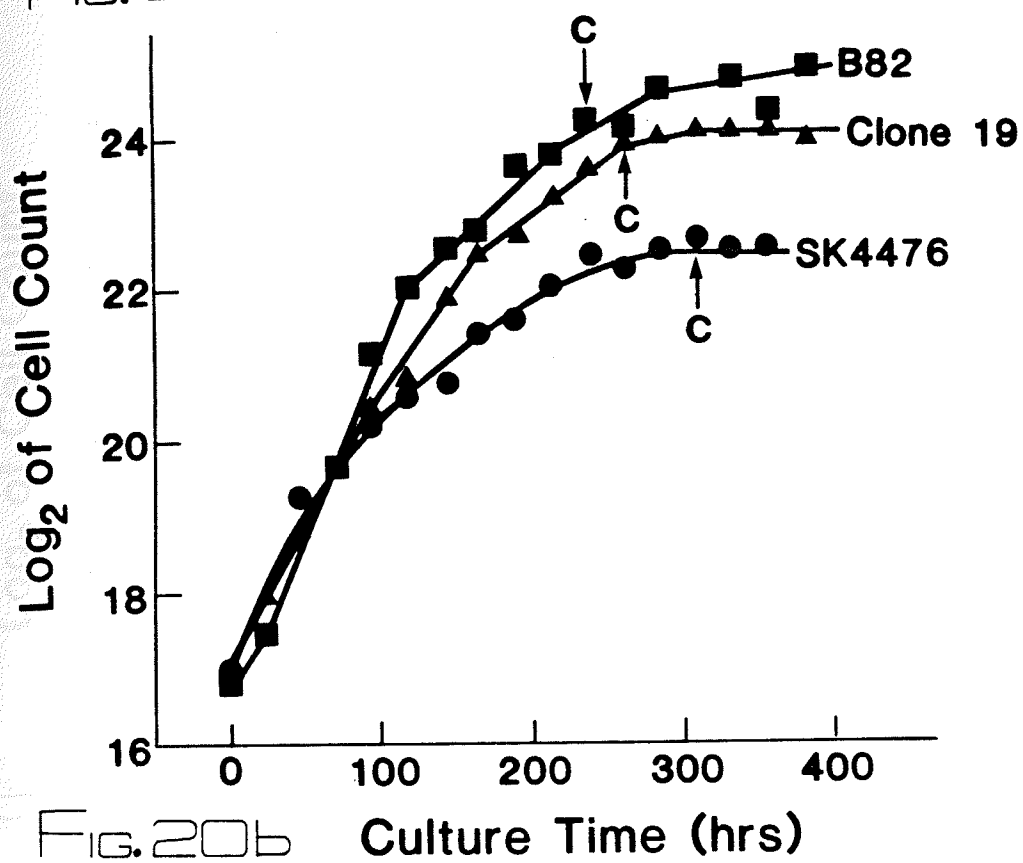


FIG. 20b

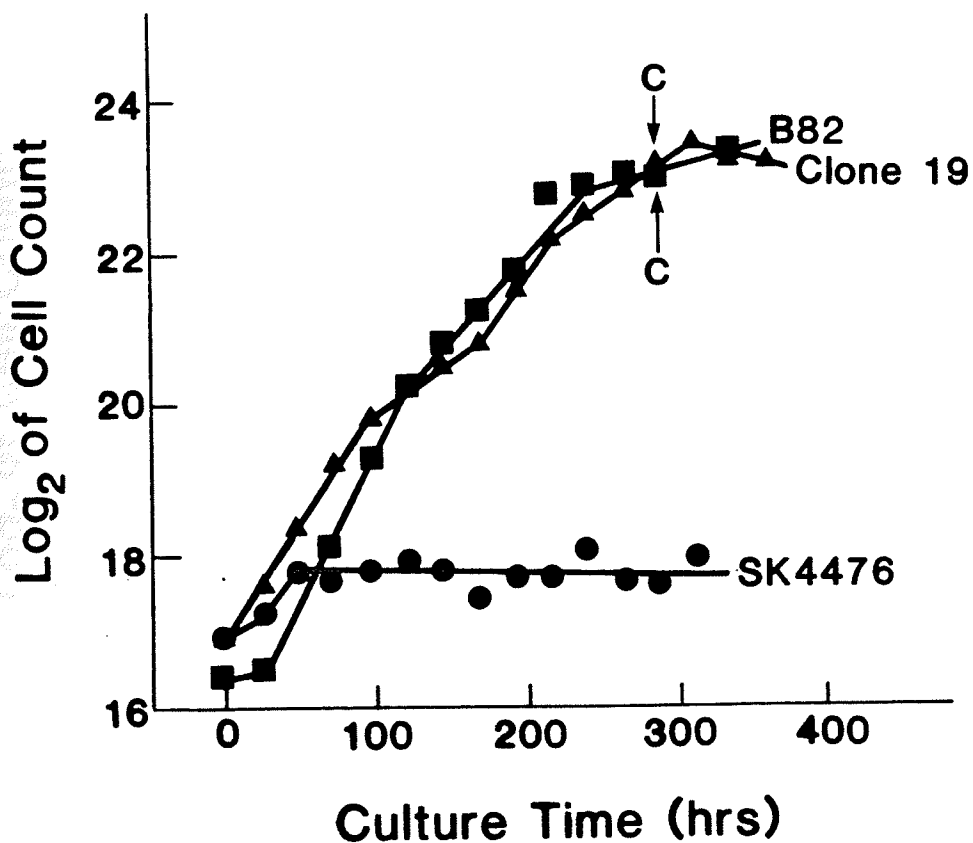


FIG. 21a

226

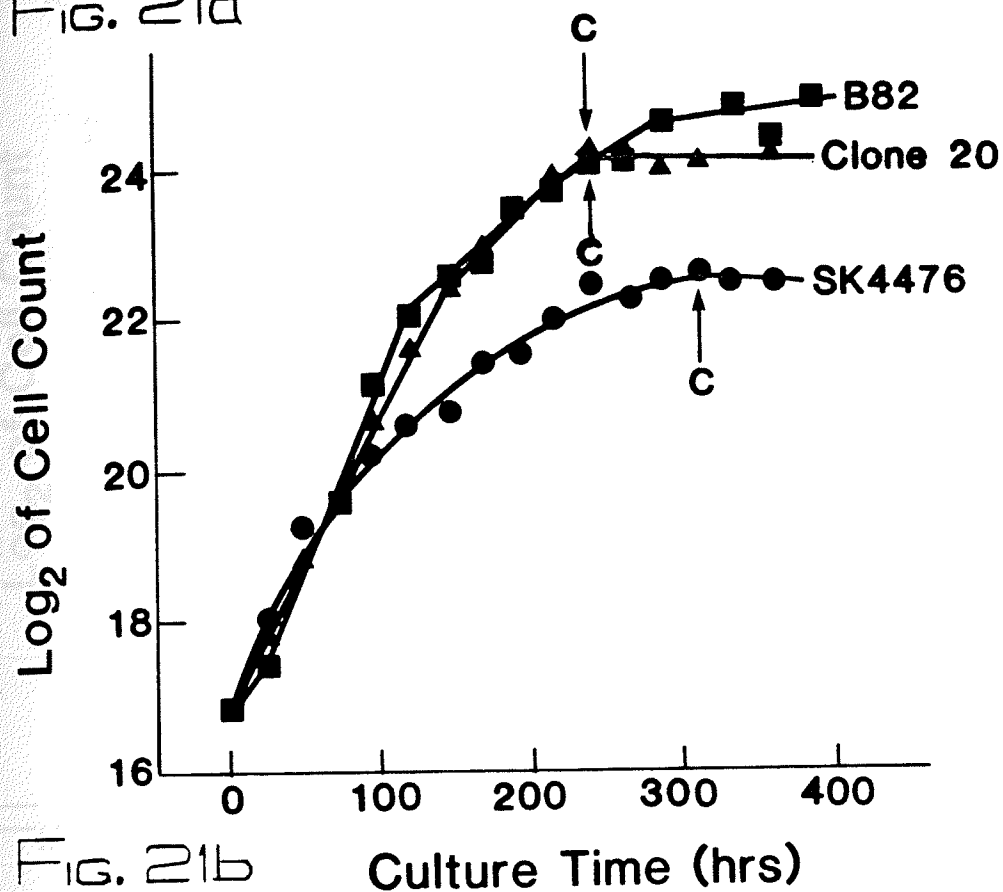


FIG. 21b

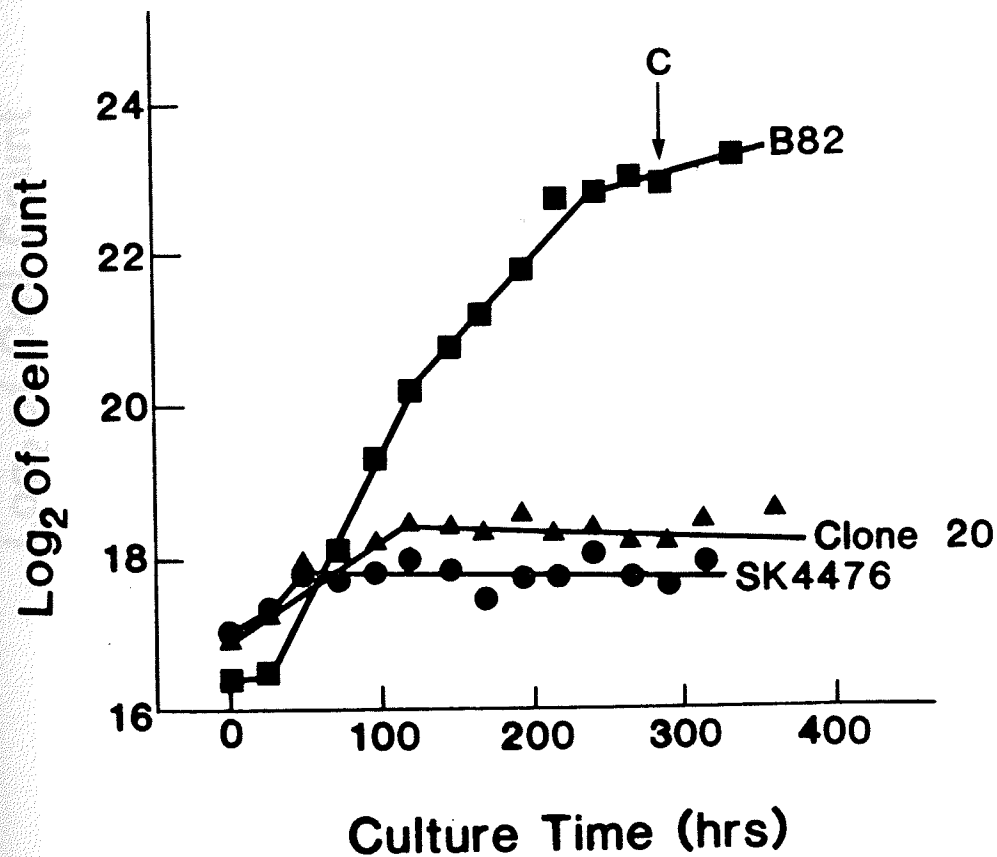


FIG. 22a

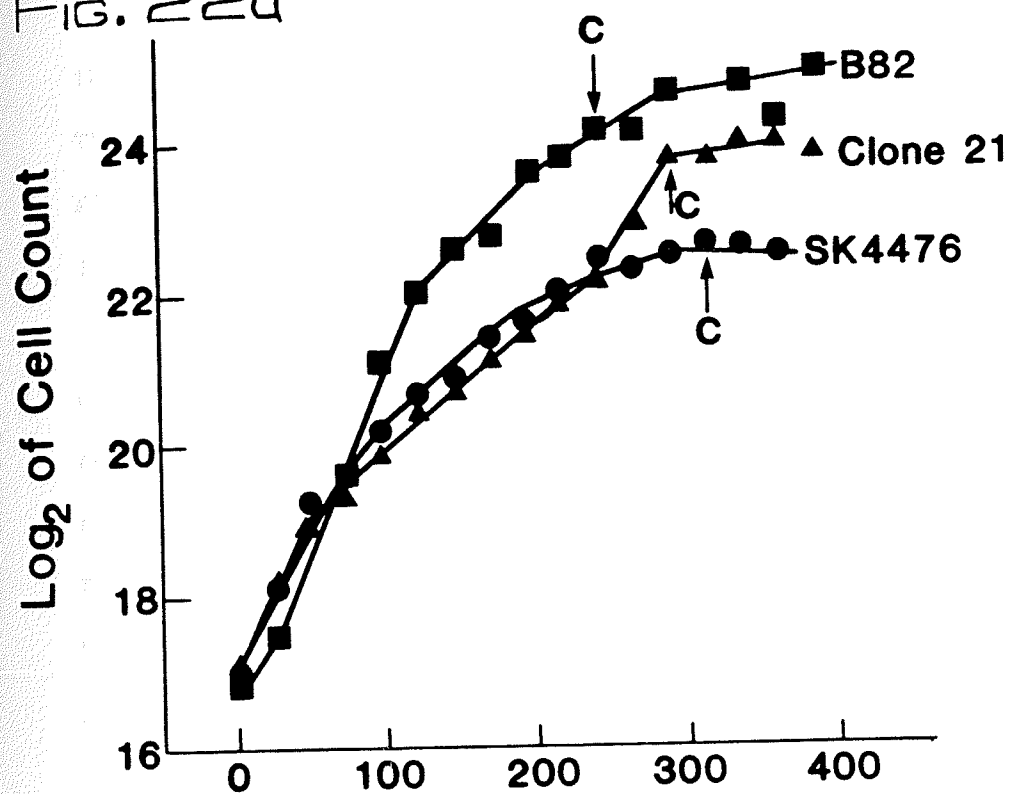
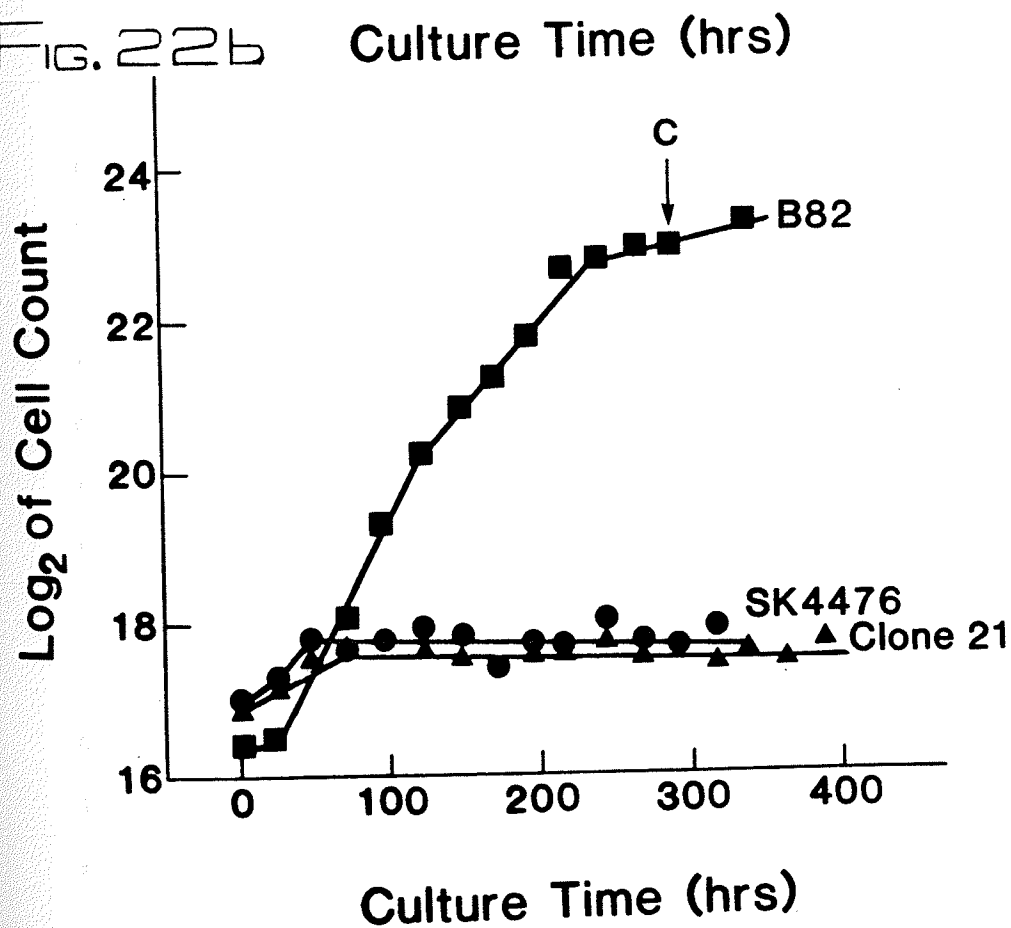


FIG. 22b



the cells started growing at a rate similar to the untransformed culture. The rest of the hybrid clones (clones 6, 7, 8, 12, 13, 14, 16 and 19; Figs. 14b to 20b) grew well in 1% serum at rates similar to the B82 cell line.

The hybrid cells also differed in their ability to grow post-confluent. It is known that, in general, transformed cells in culture continue to grow even after they attain confluency, whereas non-transformed cells stop growing at that point (Todaro and Green 1963; Todaro et al 1964; Tarzaghi and Little 1976). The phenomenon whereby high cell density inhibits growth in non-transformed cultures is referred to as density-dependent inhibition of growth (Stoker and Rubin 1967); transformed cells do not exhibit density-dependent inhibition of growth. In this study, B82 clearly continued to grow after attainment of confluency when cultured in medium with 10% or 1% serum (see Fig. 11 to 22). By contrast, SK4476 cells did not grow post-confluent. With the hybrid cells, only clone 16 (Fig. 19) grew post-confluent in both 10% and 1% serum-containing medium. On the other hand, several clones (clones 7, 8, 12, 19, 20 and 21) exhibited density-dependent inhibition of growth when they were grown in medium containing either 10% or 1% serum. The other hybrid clones (clones 5 and 6) grew post-confluent in 10% serum but not in 1% serum.

Table 14 is a summary of the results presented in Fig. 11 to 22. It presents the growth characteristics of B82, SK4476 and hybrid clones at 37°C in 10% and 1% serum-containing medium. An examination of Table 14 shows that only hybrid clone 16 has identical growth characteristics as the transformed B82 parental cells. Two hybrid clones (clones 20 and

21) have identical growth characteristics as the untransformed SK4476 parental cell line. The rest of the hybrid clones had growth characteristics intermediate between the B82 and SK4476 cell lines. A comparison of the data in Tables 13 and 14 show that some relationships exist between the growth characteristics and HS3 ratios (see "Discussions").

Membrane Characteristics of B82, SK4476 and Hybrid Cells

In Achlya it has been shown that HS3 is associated with a proteoglycan located on the cell wall - membrane complex (Cameron and LeJohn 1978). Similarly, it has been suggested that in mammalian cells the HS3 molecule is probably synthesised at the cell membrane (LeJohn, unpublished data). For these reasons, studies were done to determine some membrane characteristics of the hybrids and the parental cells and to correlate these with HS3 synthesis patterns. The membrane characteristic that was chosen for study is the ability of the cells to grow in the presence of varying concentrations of ouabain in the growth medium. Ouabain is a cardiac glycoside which kills cells by inhibiting the Na^+/K^+ -ATPase at the K^+ selective site (Post et al 1960; Bakkeren and Bonting 1968; Hokin 1976). Ouabain has been shown to inhibit cell growth in a wide variety of cell culture systems (reviewed by Cameron et al 1982); human cells are much more sensitive to the drug than mouse cells (Willis and Ellory 1983). Hybrids between mouse and human cells may have different proportions of the Na^+/K^+ -ATPase from the mouse and human sources. Since the mouse and human cell lines used in the cell hybridisation studies reported have differed in their HS3 responses, it follows that the hybrids are potentially useful in assessing the role of

Table 14

Growth characteristics of B82 and SK4476 cell lines and hybrid clones (obtained by fusion of B82 and SK4476 cells) at 37°C in α -MEM supplemented with 10% or 1% calf serum. (This table is a summary of the results presented in Figures 11 to 22).

Cell line or Hybrid clone	Growth in medium with		Ability to grow above confluency in med. with	
	10% serum	1% serum	10% serum	1% serum
B82 (transformed)	+	+	+	+
SK4476 (untransf.)	+	-	-	-
Hybrid clone 3	+	-/+*	+	ND ¹
5	+	-	+	-
6	+	+	+	-
7	+	+	-	-
8	+	+	-	-
12	+	+	-	-
13	+	+	-	ND
14	+	+	-	ND
16	+	+	+	+
19	+	+	-	-
20	+	-	-	-
21	+	-	-	-

¹"ND" refers to "not determinable" since cultures did not grow to the required cell density within the experimental time period.

*-/+ means that cultures were initially unable to grow, but later during the course of the culture period the cells started growing.

Procedures for cell culture, cell hybridisation and isolation of hybrids, and growth studies were described in the legend for Fig. 3 and in "Methods".

the cell membrane in the HS3 production pattern.

Table 15 summarises the effects of ouabain on survival of B82, SK4476 and hybrid cells. A measure of the susceptibility of cells to the drug is the D_{10} , which is defined as the lowest ouabain concentration at which 10% or less of the cells survived relative to cultures not retreated with the drug (column 2, Table 15). Another measure of the effect of the drug on cells is the lowest ouabain concentration at which there is a net loss of cells over the cells originally plated (column 3, Table 16). By both measurements, it is clear that the SK4476 cell line was much more susceptible to the effects of ouabain than the B82 cell line. The hybrid cells had D_{10} values that were very close to that of the B82 cell line, indicating that the cell membrane components of the hybrids were essentially similar to that of the mouse B82 cell line with respect to the Na^+/K^+ -ATPase enzyme.

Table 15

Influence of ouabain on the survival of B82 and SK4476 cell lines, and hybrid cells (obtained by fusion of B82 and SK4476 cells).

<u>Cell line or hybrid clone</u>	<u>D₁₀ value¹</u> (mM ouabain)	<u>Ouabain conc. at which</u> <u>net loss of cells occur²</u> (mM)
SK4476 (human)	0.005	0.005
B82 (mouse)	0.2	0.7
Hybrid clone 3	0.2	0.3
5	0.2	0.6
6	0.2	0.5
7	0.2	0.3
8	0.2	0.2
12	0.1	0.2
13	0.3	
14	0.4	0.3
16	0.2	
19	0.2	
20	0.2	
21	0.3	0.5

¹D₁₀ value is the lowest ouabain concentration at which only 10% or less of cells survive relative to control (see below for explanation of control).

²Refers to the lowest ouabain concentration at which there is a net loss of cells over the cells originally plated.

Procedures for cell structure, cell hybridisation and isolation of hybrids were described in the legend for Fig. 3 and in "Methods". All experiments were done at 37°C in α -MEM supplemented with 10% calf serum. Cells were plated at low densities in 100 mm plates and grown for 14 days in the presence of the appropriate amounts of ouabain. A control plate with cells grown without ouabain was also included. At the end of the culture period, surviving cells were counted.

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DISCUSSION

DISCUSSION

The Pattern of HS3 Synthesis at 37°C Changes as Normal Cells Become Transformed

The initial observations that mammalian cells synthesise HS3 when they are deprived of glutamine was made with CHO WT cells (Goh and LeJohn 1977; Goh et al 1977; see also Fig. 1); CHO WT cells did not synthesise HS3 when normal levels of glutamine were supplied in the growth medium. Subsequent studies with a wide variety of transformed and untransformed cell lines showed that, in general, transformed cells accumulated HS3 only when glutamine was absent from the growth medium, whereas untransformed cells accumulated HS3 irrespective of whether or not glutamine was present in the growth medium (Goh 1979; LeJohn et al 1979; see also Table 1). Hence distinct patterns of HS3 synthesis, with respect to availability of glutamine, appeared to exist for transformed and untransformed cell lines. The question that emerged after the studies by Goh (1979) is whether there is a direct, causal relationship between cell transformation and the different patterns of HS3 synthesis with respect to glutamine availability that are observed for transformed and untransformed cell lines. The results of Goh (1979) could not give an adequate answer to that question since he used nonisogenic transformed and untransformed cells. Also, many of the untransformed cell lines that he used had been kept in culture for long periods of time such that the possibility cannot be ruled out that secondary changes unrelated to transformation may have taken place in the cells and may actually be responsible for the different patterns of

HS3 synthesis.

The present study confirms the original observation by Goh (1979) that transformed and untransformed cells have different patterns of HS3 synthesis in response to glutamine availability. In addition, this study attempts to establish a direct, causal relationship between cell transformation and a change in the pattern of HS3 synthesis from the untransformed to the transformed type. Two approaches were adopted. First, a human diploid fibroblast cell line (On Ser) was transformed by a chemical carcinogen and then tested for its HS3 synthesis pattern. Second, four pairs of isogenic transformed and untransformed cell lines were studied for their patterns of HS3 synthesis. A discussion of results obtained with these two approaches follows.

In the first line of approach, On Ser cells were transformed by the carcinogenic agent, 9,10-dimethyl-1,2-benzanthracene (DMBA), and the transformed cell derivative was designated On Ser-DMBA. On Ser-DMBA cells were tested for transformation by the usual in vitro transformation criteria such as morphology (Fig. 2a and 2b), serum requirement for growth (Fig. 3a and 3b; Table 3), anchorage requirement for growth (Table 3), saturation density and contact inhibition of growth (Fig. 3a and 3b), and unlimited lifespan in culture (see Temin 1966; Skehan and Friedman 1981; Newbold et al 1982). The cells were not tested for in vivo tumorigenicity, but it is generally accepted that anchorage-independence for growth correlates very closely with in vivo tumorigenicity (Shin et al 1975; Skehan and Friedman 1981). The transformation frequency of about 10^{-7} was rather low, but this is not unexpected for diploid human cells in culture (see review by DiPaolo

1983). Several reasons may account for the low transformation frequency observed with this system. DMBA binds with high affinity to RNA and proteins (Kuroki et al 1971; Selkirk 1980) thereby reducing the effective dose of the carcinogen for interaction with DNA which is considered the critical site of action in the carcinogenic process (Selkirk 1980). Furthermore, chemical carcinogenesis is considered a multistage process requiring promoter(s) after initial treatment of cells with a carcinogen (Collins et al 1980; Weinstein et al 1984). Since promoters were not used in this transformation system it is not unexpected that the frequency of transformation was low. It was prudent to avoid the use of promoters since many tumour promoters are chemical compounds that have pleiotropic effects on cells and some of the effects are unnecessary or unconnected to the promotion process (Colburn 1980; Weinstein et al 1984). The long latent period it took for transformed cells to appear after carcinogenic treatment may have been due in part to the fact that tumour promoters were not used and also that DMBA belongs to the class of chemical carcinogens that require metabolic activation by cells (Slaga 1980). Also, it is known that, generally, human cells are difficult to transform with carcinogenic treatment (DiPaolo 1983).

On Ser-DMBA cells had an HS3 synthesis pattern that differed from that of On Ser when the tests were performed only a few cell generations upon isolation of the transformed clone. On Ser-DMBA and On Ser cells showed HS3 responses typical of other transformed and untransformed cell lines respectively (Fig. 4). Since the tests were performed only a few cell generations after the transformed cells were isolated, it suggests

that cell transformation is directly related to the change in the pattern of HS3 synthesis. It can be concluded that the acquisition of the transformed pattern of HS3 synthesis is an early event in the process of cell transformation. The On Ser cells used in the tests of HS3 synthesis and growth characteristics were obtained from the same carcinogen-treated dish as the On Ser-DMBA cells. Since those On Ser cells exhibited the untransformed pattern of HS3 synthesis and they failed to show any transformation characteristic (Table 3), it indicates that the different HS3 patterns observed for On Ser and On Ser-DMBA cells were not simply due to the exposure of the cells to the carcinogen, but rather it represents a true indication of transformation.

The second line of approach used to establish a direct, causal relationship between cell transformation and the change in pattern of HS3 synthesis involved the use of pairs of isogenic transformed and untransformed cell lines. Three untransformed cell lines (On Ser, WI-38 and 10T $\frac{1}{2}$) produced the untransformed pattern of HS3 synthesis at 37°C, whereas their transformed counterparts (On Ser-DMBA, WI-38 VA13, 10T $\frac{1}{2}$ ciras-2 and 10T $\frac{1}{2}$ MDS-R2) produced the transformed pattern (Table 2). Since isogenic cell pairs were used, the only conceivable genetic difference between members of any pair would involve genes that are related to the transformed phenotype or transformation process. It is also clear that regardless of the mode of transformation (chemical, viral, oncogenic and X-irradiation for isogenic pairs; and spontaneous for the CHO cell lines, Table 2), mammalian cells from diverse species, tissues and organs exhibited the same transformed pattern of HS3 synthesis at 37°C. This consistent feature of diverse transformed cells

suggests that the change in the pattern of HS3 synthesis is probably an early and obligatory event in the transformation process. It also suggests a common level of action of all major classes of carcinogenic agents. The implication is that diverse oncogenic agents may have a common mechanism of action, possibly involving common biochemical, genetic or histological site(s).

The Temperature Effect

As presented in the "Results", the patterns of polyphosphate metabolism in transformed and untransformed cells in response to thermal stress were different. With the exception of the 10T $\frac{1}{2}$ MDS-R2 cell line, transformed cells were sensitive to hyperthermia in their HS3 accumulation in that they failed to accumulate HS3 at 42°C even when the cells were deprived of glutamine. This effect was observed regardless of the mode of transformation (chemical, viral, oncogenic or spontaneous). Untransformed cells, on the other hand, were insensitive to the high temperature treatment in that they accumulated HS3 at uniform levels at both 37°C and 42°C whether or not glutamine was present. These observations suggest that the pattern of HS3 synthesis in transformed and untransformed cells under thermal stress may be a valuable indicator of transformation. The failure of the 10T $\frac{1}{2}$ MDS-R2 cell line to show the temperature effect may be due to damages that may have been done to the cells by the X-irradiation used as the agent of transformation. Indirect support for this is that the 10T $\frac{1}{2}$ MDS-R2 cells grew much slower than other transformed cells and even slower than its non-transformed parental 10T $\frac{1}{2}$ cells when cultured under standard tissue culture conditions. Also, they exhibited contact inhibition of growth,

in contrast to the 10T½ cirs-2, and morphologically they appeared much larger than the 10T½ cells or its derivatives. Hence the 10T½ MDS-R2 cell line was either incompletely transformed or it was damaged by the X-irradiation treatment.

Differences in heat sensitivity between normal and transformed cells have been reported. It is believed that the acquisition of increased thermosensitivity by cells is linked and probably caused by one of the early steps in the transformation process (Giovannella et al 1980). This, and other factors discussed in the "Historical", have prompted the possibility that hyperthermia can be used as a cancer treatment modality (Jain and Gullino 1980; Lowenthal 1984; Kim 1984).

In theory, several possible mechanisms may account for the thermal sensitivity of HS3 synthesis observed with transformed cells. Either, (1) HS3 is not synthesised at 42°C, that is, transformed cells are temperature-sensitive with respect to polyphosphate polymerisation (poly-P polymerase ts mutation?); or (2) HS3 is rapidly consumed at 42°C via activation of an HS3 degradative pathway; or (3) transformed cells somehow generate glutamine at 42°C. The fact that HS3 in transformed cells accumulated to high levels at 42°C in the presence of the protease inhibitors TLCK and TAME (Table 2) indicates that transformed cells are not necessarily temperature sensitive in their polyphosphate metabolism. Neither does it support the possibility that HS3 may be rapidly degraded at 42°C by activation of an HS3 degradative enzyme system. Similarly, the effect of heat on nucleic acid biosynthesis does not support any generalised temperature sensitivity of synthetic activity in transformed cells. Hence the evidence suggests

that the first two possible mechanisms of the temperature effect listed above are unlikely in this instance. On the other hand, the same evidence with protease inhibitors implicates a mechanism involving proteases. In the following sections, a discussion of the results presented earlier reveals that the third mechanism, whereby transformed cells at 42°C secrete protease(s) that generate glutamine, operates in the temperature effect; the evidence shows that an induced proteolytic activity breaks down proteins to supply glutamine to the cells.

Transformed Cells Under Thermal Stress Induce Protease(s) Which Degrade Proteins to Generate Glutamine in Their Medium

As mentioned earlier, transformed cells grown at 42°C for 4 h in glutamine-free medium behaved as if they were not starving for glutamine, both in their polyphosphate and nucleic acid metabolism. While several hypothesis can be proposed to explain the phenomenon, the evidence suggests that the "apparent" non-requirement for glutamine was due to protein degradation by protease(s), thereby providing the cells with glutamine. It is an established fact that transformed cells secrete abundant amounts of proteases compared to untransformed cells (Roblin et al 1975; Rossman and Troll 1980). However, there has been no previous reports that further activation of protease(s) or induction of new protease(s) occur in cultured cells under hyperthermic conditions.

Direct as well as indirect evidence support the contention that under heat stress glutamine is generated in the growth medium of transformed cells. The most direct evidence comes from the observation that when transformed cells were grown at 42°C, abundant amounts of

glutamine were detected in the initially glutamine-free growth medium (Table 5). Glutamine was not detected in similar cultures grown at 37°C or in untransformed cultures grown at 37°C or 42°C. The amount of glutamine generated in the growth medium of transformed cells was about 0.2 mM (per 10 ml of medium). This is a minimum value since similar amounts of glutamine were detected within the heat-stressed transformed cells (Table 6) and the source of glutamine may have been from the medium. Although the 0.2 mM glutamine generated in the medium is much lower than the 2 mM glutamine normally supplied in α -MEM medium (Stanners et al 1971), it is apparently sufficient to prevent HS3 accumulation. From the results of the glutamine concentration effects on HS3 accumulation (Fig. 6a), only about 0.1 to 0.5 mM glutamine is sufficient for complete suppression of HS3 accumulation.

There are also less direct evidence that transformed cultures generate glutamine in their growth medium when they are heat-stressed, and that it is the generated glutamine that mediates the temperature effect. First, methionine sulfoximine, an analogue and competitive inhibitor of glutamine (LeJohn, personal communication), allowed transformed cells to accumulate HS3 at 42°C (Table 7). Methionine sulfoximine acted in a concentration-dependent manner, which is expected of a competitive inhibitor. At the highest concentration of methionine sulfoximine used (1.0 mM), the amount of HS3 that accumulated at 42°C was more than 70% of that accumulated under similar conditions at 37°C. Methionine sulfoximine did not have any significant effect on HS3 levels at 37°C probably because no glutamine was generated in the cultures at that temperature. Hence it seems that

the effect of methionine sulfoximine is primarily that of an exogenous antagonist of glutamine. The second indirect evidence that implicates glutamine as a mediator of the temperature effect in transformed cultures involves the use of glutaminase, an enzyme that deaminates glutamine to glutamate (Hartman 1970). When glutaminase was added to heat-stressed transformed cells cultured in glutamine-free medium, HS3 accumulated under the heat stress condition (Table 8). Presumably, glutaminase breaks down any protease-generated glutamine before the latter interacts with the HS3 metabolic pathway(s). Since glutamate is a product of the action of glutaminase (Hartman 1970), the high levels of HS3 accumulation in the presence of glutaminase supports the earlier suggestion (Goh et al 1977; LeJohn and Goh, unpublished) that the effect of glutamine on HS3 metabolism is specific. Glutaminase was unable to induce HS3 accumulation in cultures containing the normal level (2 mM) of glutamine in the medium. This is probably because the effective enzyme concentration was not sufficient to break down glutamine fast enough to make the medium glutamine-deficient for the purpose of HS3 accumulation.

It has been mentioned earlier that the glutamine generated in transformed cultures under heat stress may be a result of the action of induced or activated protease(s) under the hyperthermic condition. An initial indication of this comes from the observation that the protease inhibitors TLCK and TAME permitted the build-up of glutamine in heat-stressed transformed cell cultures (Table 5). Glutamine-dependent metabolic pathways, such as HS3 metabolism (Table 2) and nucleic acid biosynthesis (Table 4), were also affected by TLCK and TAME in a manner

that implicates secreted protease(s). The most direct evidence of the involvement of protease(s) in the temperature effect comes from the fact that high levels of proteolytic activity were detected in the growth medium of transformed cells at 42°C under conditions where glutamine was generated. The appearance of glutamine is directly correlated with proteolytic activity in the medium. This suggests that the source of glutamine is, at least in part, derived from the action of the secreted protease(s), possibly degrading proteins external to the cell, including serum proteins. Evidence whereby serum deprivation led to accumulation of HS3 at 42°C, presumably because the absence of serum does not provide the necessary protein substrates for action of the protease(s) to generate glutamine (Table 9), indicates that authentic serum proteins may be major substrates for the heat-induced protease(s). There is, however, no direct evidence that any serum protein is a substrate for the protease(s) secreted by the heat-stressed transformed cells since attempts to run electrophoretic gels of serum proteins failed to be explicit due to the very high amounts of some serum proteins and very low quantity of others (see Appendix). Bovine serum albumin (BSA) was ineffective as a substitute substrate for normal calf serum on which protease(s) that generate glutamine during heat stress can act (Table 9). Since albumin is the major protein component of serum (Putman 1975), its ineffectiveness at replacing serum suggests that the minor serum proteins, rather than albumin, may be the targets for the protease(s). BSA did not affect the viability of the cells, the effectiveness of serum, or the levels of HS3 synthesised at 37°C.

Other evidence suggest that cellular proteins may also be targets

of proteases induced in transformed cell cultures. The gel electrophoresis patterns of cellular proteins (Fig. 10), however, show that there is no evidence that specific cellular proteins are targets for protease(s) induced by heat stress. In general, proteins from On Ser-DMBA cells were less intensely labelled than those from On Ser at 37°C or 42°C. This could be due to isotopic dilution resulting from unlabelled amino acid derived from protease action on serum proteins. But while there is no difference in the labelling patterns or intensities between the 37°C samples and the heat-stressed samples of On Ser-DMBA cells, it seems that the only proteins of cellular origin that might be substrates for the heat-induced protease(s) are those proteins secreted by the cells into the medium.

The CHO WT cells, although a transformed cell line, did not secrete any measurable amount of protease; neither did they generate glutamine in their medium under hyperthermic conditions. Yet they exhibited the temperature effect in their HS3 accumulation, suggesting that a mechanism, other than protease-mediated generation of glutamine, may also be involved in the temperature effect.

Further Characterisation of Thermally-Induced Proteolytic Activity of Transformed Cultures

Attempts were made to partially characterise the protease(s) secreted by thermally-stressed transformed cells. The strategy included the use of various protease inhibitors, metabolic inhibitors, and identification of possible substrates for the protease(s).

The major proteolytic enzyme systems secreted by transformed cells under normal culture conditions are the plasminogen activator/plasmin

system and the collagenolytic enzyme system (see "Historical" section). These enzyme systems can activate other proteases including all major plasma protein effector systems (Christman et al 1977; Wooley, 1984a). It is likely that the proteolytic activity measured in the medium of transformed cells grown at 37°C is that of the plasminogen activator/plasmin system and/or the collagenolytic enzyme system. These enzyme systems, and others induced or activated in transformed cell cultures at 37°C, have limited or specific action on one or a few serum proteins with little or no release of glutamine (see "Historical" section). There is no report that hyperthermia results in any further activation or induction of these enzyme systems. Hence the phenomenon of heat-induced enhancement of proteolytic activity in transformed cell cultures reported here is novel.

Several lines of evidence support the view that the proteolytic activity induced by heat represents a separate and distinct entity from proteases normally secreted by transformed cells at 37°C. The first evidence comes from the fact that the action of the heat-induced protease(s) resulted in complete degradation of proteins to amino acids. This is measured as heat-induced generation of glutamine in the growth medium of transformed cells. The presence of other amino acids was untested for obvious reasons. It follows, therefore, that at least part of the proteolytic activity induced by heat is an oligopeptidase. None of the protease(s) secreted by transformed cells at 37°C have been shown to bear oligopeptidase activity, either individually or in combinations. If the heat-induced protease(s) is an oligopeptidase, then its substrates are the products of the action of other proteases secreted or

activated by cells. Hence there is the possibility that a coordinated action exists between the various proteases secreted by the cells. It is known that different proteolytic enzyme systems interact in a coordinated fashion (Paranjpe et al 1980; Steven et al 1980).

Studies with protease inhibitors have provided further information in the characterisation of the heat-induced protease(s) secreted by transformed cells. The proteolytic activity, measured as glutamine production in the growth medium, was inhibited to various degrees by TLCK, TAME, benzamidine, NPGB, transexamic acid, or ϵ -ACA. Benzamidine and NPGB inhibit plasminogen activator but not plasmin, thrombin or elastase; on the other hand, tranexamic acid and ϵ -ACA inhibit plasmin but not plasminogen activator (Quigley 1979). TLCK also is not an inhibitor of plasminogen activator (Quigley 1979). The fact that all of these inhibitors reduced or eliminated the activity of the putative heat-induced oligopeptidase(s) suggests that the plasminogen activator/plasmin enzyme system may be involved in the action of the enzyme(s), possibly in an activating role. Alternatively, the enzyme(s) is probably cross-sensitive to the same inhibitors as plasminogen activator and plasmin. It is possible that the heat-induced proteolytic activity consists of several separate enzymes that act at different points in the degradation of proteins to amino acids, so that each component may be sensitive to a different type of inhibitor. This is implied in the results of Table 11.

Studies with actinomycin D and cycloheximide show that both transcription and translation are required for the heat-induced protease activity. Since cycloheximide and actinomycin D were added at the time

of transfer of cultures from 37°C to 42°C, the metabolic inhibitors affected only the portion of protease(s) that are induced or activated at 42°C, but not the proteases that are normally induced and secreted at 37°C. Hence the synthesis of plasminogen activator and subsequent activation of plasmin were not prevented in these experiments. Cycloheximide and actinomycin D treatment each resulted in about 60% reduction in total proteolytic activity and complete inhibition of glutamine production; presumably, the remaining 40% proteolytic activity represents proteases that are synthesised at 37°C, before the metabolic inhibitors were added. The cycloheximide and actinomycin D data clearly show that the plasminogen activator/plasmin system, or other proteases secreted by cells under normal culture conditions, are distinct from the heat-induced proteases.

Another type of evidence that supports the distinctiveness of the heat-induced protease(s) comes from studies whereby cell-free growth medium from transformed cell cultures were heated for up to 4 hr at 42°C, after a prior incubation with cells at 37°C (Table 10). With this experimental protocol, plasminogen activator, plasmin and other proteases secreted by cells at 37°C, are present in the medium at the time of the cell-free incubation at 42°C. No glutamine was detected in the medium, and only about 40% of protease activity remained, representing proteases secreted by cells at 37°C. The implication of this is that cells are required for the heat-induced proteolytic activity. This may mean that heat-stressed cells directly secrete the putative oligopeptidase(s), or alternatively that cells under hyperthermia secrete an "activating factor" that activates a latent enzyme already

present in serum. Heat-stressed cells may also be secreting a zymogen that is activated by enzymes secreted at 37°C. These are all situations that are known to apply to other well-studied proteases secreted by transformed or tumour cells at 37°C. For example, transformed cells secrete plasminogen activator which activates the production of plasmin from a normal serum protein plasminogen (Rifkin et al 1974; Duffy and O'Grady 1984); transformed cells secrete latent zymogen forms of collagenases which are activated by plasmin and other serum proteins (Paranjpe et al 1980; Steven et al 1980).

Possible Effects of the Heat-Induced Protease(s)

Numerous studies have revealed that proteases secreted under physiological conditions by neoplastic cells play a role in tumour metastasis enhancement of growth potential of the neoplastic cell, modulation of the external environment for nutritional purposes, and the expression of certain in vitro phenotypic characteristics of neoplastic cells (reviewed by Roblin et al 1975; Reich et al 1975; Rossman and Troll 1980; Mullins and Rohrllich 1983; Duffy and O'Grady 1984; Sloane and Honn 1984; Wooley 1984a). A physiological role(s) of the heat-induced protease activity cannot easily be established since the heat stress temperature (42°C) is non-physiological.

Cultured mammalian cells synthesise a small number of polypeptides, referred to as "heat shock proteins" (HSPs), when they are exposed to temperatures within the range of 42°C to 45°C (Adams and Rinne 1982; Schlesinger et al 1982). These proteins are believed to play roles in thermotolerance (Velazquez and Lindquist 1984; Li 1984), respiratory processes (Ashburner and Bonner 1979), and various protective functions

(Schlesinger et al 1982). The heat shock proteins of transformed cells are similar to those of untransformed cells; indeed, the heat shock proteins are highly conserved (Schlesinger et al 1982). Also, the heat shock proteins are intracellular proteins, with no evidence that they are secreted by cells into the growth medium. No heat shock protein in mammalian cells has been reported to have proteolytic activity or to act as an activator of latent proteases. For these reasons, the protease(s) secreted by transformed cells under heat stress are not likely to be the same or related proteins as the HSPs.

In spite of the probability that the heat-induced protease(s) may not have a physiological role, evidence gathered in this study indicate that the protease(s) may have effects that may be important in transformed cell nutrition and for cancer therapeutic modalities. First, the proteolytic activity observed in transformed cell cultures under thermal stress resulted in the complete degradation of proteins, thereby providing the cells with amino acids. In addition, heat-stressed transformed cells were found to have much higher intracellular levels of glutamine than cells at 37°C (Table 6), suggesting that the hyperthermic condition facilitates uptake of glutamine. (It is, of course, also possible that the high intracellular levels of glutamine under heat stress conditions was due to breakdown of intracellular proteins.) Nontransformed cells did not have any detectable intracellular glutamine at both 37°C and 42°C, suggesting that perhaps the higher uptake of glutamine observed in heat-stressed transformed cells may be due to action of the protease(s) secreted by transformed cells at 42°C. It has been suggested that one effect of

proteases secreted by transformed cells is to modify the cell membrane in a way that affects nutrient transport properties (Roblin et al 1975). Secreted proteases degrade cell membrane proteins (Burger 1970; Rossman and Troll 1980), as well as activate phospholipases that degrade membrane lipids (Shier et al 1982). None of these effects are observed with nontransformed cells. Hence, the heat-induced protease(s) seem to have profound implications for nutrient availability, nutrient uptake, and other aspects of the cellular metabolism that are regulated by the cell membrane. However, some caution must be exercised at suggesting whether the protease(s) has a role that is meant primarily for nutritional purposes, or whether its possible role in transformed cell nutrition is secondary to some other role. A primary role in nutrition would require that induction or secretion of the protease(s) be regulated by availability of nutrients in the growth medium. However, the evidence shows that the levels of secreted protease(s) is the same whether the nutritional requirements are complete or not. Also, it is difficult to explain why transformed cells would secrete protease(s) at 42°C primarily for the purpose of providing amino acids for growth stimulation, since 42°C is an unphysiological and unfavourable growth temperature.

The heat-induced proteolytic activity secreted by transformed cells has implications for cancer therapeutic modalities. The use of hyperthermia in the treatment of cancer has a biological as well as clinical basis (Jain and Gullino 1980; Lowenthal 1984; Kim 1984; Hall and Roizin-Towle 1984). Heat seems to act synergistically with other cancer therapeutic agents such as radiation therapy and chemotherapy

(Kim 1984; Bull 1984). At the same time, proteases secreted by transformed cells alter the expression of a number of phenotypic properties of the cells in a manner that is growth stimulatory (reviewed by Roblin et al 1975; Reich et al 1975; Rossman and Troll 1980; Duffy and O'Grady 1984; see also "Historical"). In this study, it was observed that hyperthermic treatment of cells resulted in enhanced secretion of protease(s) by transformed cells above the level normally secreted at 37°C. In addition, the heat-induced protease(s) seem to be a different type of protease(s) than those secreted under normal growth conditions. The heat-induced protease(s) seem to have effects that may be growth-stimulatory. Since growing cells are more heat-sensitive than stationary phase cultures (Raaphorst and Azzam 1983), it follows that at least part of the greater heat-sensitivity of neoplastic cells relative to normal cells may be explained by the effects of the heat-induced protease(s) of neoplastic cells. It also points to the possibility that the heat sensitivity of neoplastic cells may be manipulated to increase differential thermosensitivity between normal and neoplastic cells. Another implication for cancer therapy is that the heat-induced proteases may alter cell membrane properties that may increase uptake of cancer chemotherapeutic drugs. As well, there is the possibility that the heat-induced protease(s) may have an intracellular role in transformed cells.

Does HS3 Act as a Phosphate Store?

The linkage between HS3 accumulation and glutamine availability, and the fact that the patterns of HS3 accumulation are different for transformed and non-transformed cells is an indication that transformed

cells differ from untransformed cells in their requirements for glutamine. Glutamine is involved in several biochemical pathways (Rabinovitz et al 1956; Patterson 1972; McKeehan 1982). LeJohn and coworkers (Goh and LeJohn 1977; Goh et al 1977; Goh 1979) have provided biochemical and genetic evidence that the role of glutamine as a precursor for de novo purine biosynthesis mainly accounts for the glutamine-dependent accumulation of HS3 in transformed cells. The implication of their investigations is that, in transformed cells, HS3 might be acting as a "nutritional shock signal", specifically for indicating the level of glutamine in the growth medium. The glutamine concentration experiments (Fig. 6a) support this contention.

HS3 itself has been shown to affect nucleic acid biosynthesis by its inhibitory effects on ribonucleotide reductase (Lewis et al 1976), an enzyme that is involved in the conversion of ribonucleoside diphosphates to their deoxy derivatives (Larsson and Reichard 1966); and on DNA-dependent RNA polymerases (Goh 1979), a set of enzymes implicated in the synthesis of ribosomal RNA, heterogenous nuclear RNA, and 5S and pre-4S RNA (Chambon 1975). Hence with transformed cells, glutamine starvation affects nucleic acid biosynthesis in at least two ways: first the absence of glutamine inhibits purine and pyrimidine biosynthesis since glutamine is a precursor in the de novo synthesis of these nucleotides; and second, the glutamine-deficient condition induces HS3 accumulation which inhibits nucleic acid biosynthesis as outlined above.

The results presented in this study give indirect experimental evidence that HS3 may be acting as a storage compound for phosphate

during nutritional stress, particularly in the case of transformed cells. First, as the results of Fig. 8 show, transformed cells exposed to increasing amounts of phosphate in their medium accumulated proportionately higher levels of HS3 when the cells were cultured in glutamine-free medium. HS3 accumulation did not occur at any phosphate concentration used when 2 mM glutamine was supplied or when the cells were grown at 42°C, a temperature at which significant amounts of glutamine are generated in the growth medium (see earlier discussions).

Presumably, HS3 accumulation occurs when phosphate is not consumed in nucleic acid biosynthesis due to lack of glutamine for nucleotide biosynthesis. This is in line with the observation by Goh et al (1977) and Goh (1979) that exogenously supplied purines lead to rapid depletion of accumulated HS3 due to the consumption of the phosphates of HS3 for nucleic acid biosynthesis. It is also believed that the constituent ADP and UDP moieties of HS3 (McNaughton et al 1978) could be further used for the replenishment of all the nucleotide pools (LeJohn, unpublished). The observed inverse relationship between HS3 levels and nucleic acid biosynthesis (Table 4) is an indication that the phosphates not going into HS3 under conditions of HS3 depletion are probably used in the synthesis of DNA and RNA.

Untransformed cells do not alter their HS3 levels in response to changes in glutamine or phosphate levels in the growth medium. This apparent insensitivity to glutamine and phosphate exhibited by untransformed cells tend to undermine the proposed roles and effects of HS3 discussed earlier for transformed cells. Since HS3 levels in untransformed cells have no correlations with varying glutamine levels

(Fig. 5a, b), or varying phosphate levels (Fig. 7), or with levels of nucleic acid biosynthesis (Table 4), it is difficult to ascribe functions such as regulation of nucleic acid biosynthesis and phosphate storage roles to HS3 in untransformed cells. It is tempting to propose that in untransformed cells, the constantly high levels of HS3 under all growth conditions may be involved in long-term regulation of growth of the cells, rather than a means of quickly responding to specific changes in growth conditions as seems to be the case with transformed cells. Long-term regulation of growth may be related to differences in behaviour between transformed and untransformed cells, particularly those relating to the limited lifespan of untransformed cells in culture.

Recessivity of the Transformed Pattern of HS3 Synthesis

Studies involving the fusion of transformed and untransformed cells have routinely been used to determine if the neoplastic state is expressed as a dominant or recessive trait (Ozer and Jha 1977; Croce 1980; Sabin 1981; Stanbridge et al 1982; Miller and Miller 1983). A dominant trait indicates that neoplasms arise through an acquisition or activation of new genetic material, whereas a recessive trait implies loss or inactivation of genetic material. In the present study, the regulation of HS3 synthesis was studied by fusing a transformed mouse cell line (B82) with a non-transformed human cell line (SK4476). The two cell lines possessed growth characteristics and HS3 synthesis patterns that are typical of other transformed and untransformed cell lines respectively.

In usual growth media, normal cell lines synthesise de novo the

necessary purines and pyrimidines and thus addition of preformed purines and pyrimidines is superfluous (Mahler and Cordes 1971; Chu and Powell 1976). However, if the de novo pathways are blocked by metabolic inhibitors or due to the absence of appropriate precursors, the cells will require an exogenous supply of preformed nucleosides which they can utilise in the salvage pathway for nucleic acid biosynthesis (Mahler and Cordes 1971; Chu and Powell 1976). Cells require two functional enzymes, namely hypoxanthine-guanine phosphoribosyl transferase (HGPRT) and adenine phosphoribosyl transferase (APRT), for utilisation of purines; and one enzyme, namely thymidylate kinase (TK), for utilisation of pyrimidines in the salvage pathways (Mahler and Cordes 1971; Chu and Powell 1976; Adams and Harkness 1976; Wright et al 1980). Cells which do not have functional HGPRT/APRT or TK enzymes cannot survive in conditions wherein the de novo nucleotide biosynthesis pathway is blocked, even if preformed nucleosides are provided.

Interspecific mouse-human cell hybrids were selected among a predominant population of cells that consisted of unfused mouse and human cells, mouse-mouse hybrids, and human-human hybrids by use of a selective medium that allowed growth of only the interspecific hybrids. The selective medium consisted of normal growth medium supplemented with hypoxanthine, aminopterin and thymidine ("HAT"), developed by Szybalski and Szybalski (1962), and used in selecting somatic cell hybrids (Littlefield 1964; Ruddle 1973). Aminopterin is a strong inhibitor of dihydrofolate reductase (EC 1.5.1.3), an enzyme that is involved in the formation of 5,10-methylenetetrahydrofolate which is the active coenzyme required for de novo purine and pyrimidine biosynthesis (Mahler and

Cordes 1971). Hypoxanthine and thymidine serve as sources of purines and pyrimidines respectively for use in the salvage pathways. The HAT medium is doubly selective, that is, the products of two gene loci, HGPRT and TK, must be present in order to survive in the presence of aminopterin. The B82 cell line lacks functional TK enzyme, whereas SK4476 lacks functional HGPRT. Because of these enzyme deficiencies, these two cell lines rely solely on the de novo pathways to meet their requirements for DNA synthesis; they cannot survive under conditions where the de novo pathways are blocked, even if purines and pyrimidines are supplied to the cells. However, when B82 and SK4476 cells are fused, the hybrids formed can synthesise their DNA by the salvage pathways since in the hybrids the two cell lines can complement each other in their individual deficiencies for TK or HGPRT enzymes. Hence hybrids between B82 and SK4476 cells can grow in HAT-supplemented medium. Unfused mouse and human cells as well as intraspecific fusion products cannot grow in HAT-supplemented medium since no complementation of the deficient enzymes occurs in these cell types.

The HS3 ratio (defined earlier in the "Results" section) for B82 cell line is 17 and for SK4476 it is 1. These values are within the range for transformed and untransformed cells respectively (compare Tables 2 and 12). In general, transformed cells exhibit a rather wide range of values of HS3 ratios, which is probably a reflection of the fact that the transformation process is multistep (Collins et al 1980; Weinstein et al 1984). With the hybrids, all clones initially had HS3 ratios that are typical or close to the value for the untransformed (SK4476) parental cell line; however, after a variable number of

population doublings, most hybrid clones acquired HS3 ratios that are typical of transformed cells. In interpreting these observations, several tentative conclusions can be made. First, the transformed pattern of HS3 synthesis seems to be recessive to the untransformed pattern. This is in line with earlier reports that, in general, the transformation phenotypes of spontaneously transformed cells behave recessively in hybrids formed with normal partners (Marshall and Dave 1978; Szpirer and Szpirer 1980). The dominance of the untransformed pattern of HS3 synthesis does not seem to be carried by gene(s) on human chromosome 17, which is the human chromosome that carries the TK gene (Boone et al 1972); this conclusion is derived from the observation that all hybrids, even upon changing from the untransformed to the transformed pattern of HS3 synthesis, were capable of growth in HAT medium and, therefore, presumably contained the human chromosome with the TK gene. It is, of course, possible that small portions of chromosome 17 (containing the TK gene) may have been translocated onto other chromosomes in the hybrid such that it may not have been necessary for retention of the entire chromosome 17. However, it is unlikely that this occurred in all the hybrid clones examined.

Second, the fact that a wide range of values of HS3 ratios were observed in the hybrids may be an indication that probably several regulatory elements may be involved in the determination of the pattern of HS3 synthesis. Further support for this contention comes from the observation that the HS3 ratios of the hybrid cells approached the transformed pattern in several steps, rather than a single step. It is probable that the step-wise change in HS3 ratio in hybrid cells is a

result of the loss of specific human chromosomes from the hybrids. It is known that, as a general rule, hybrids between human and rodent cells segregate human chromosomes unidirectionally (Weiss and Green 1967). Studies have shown that no single human chromosome appears to greatly influence tumorigenicity in cell hybrids; instead certain combinations of chromosomes from the normal human cell suppress tumorigenicity in hybrids (Klinger et al 1978; Klinger and Shows 1979). This is in contrast to the situation with mouse cells as the nontumorigenic partner, whereby reemergence of tumorigenicity requires the specific loss of only one copy of mouse chromosome 4 (Evans et al 1982). Hence it is apparent that the value of HS3 observed in the hybrids is determined by the number of specific human chromosomes in the hybrid cells. The rate of loss of the specific human chromosomes determines the rate of increase of HS3 ratio.

Relationships Between HS3 Response Patterns and Growth Characteristics of Cells

Transformed cells differ from their normal counterparts in many respects, including differences in morphology, growth characteristics, membrane-related properties, and many biochemical features (see "Historical" section). It is probable that the distinctive patterns of HS3 response observed for transformed and untransformed cell lines is a result of the overall properties of transformed cells, rather than the acquisition of any single, specific transformation characteristic by the neoplastic cell. Indirect support for this comes from the studies with cell hybrids (Tables 13 and 14). Hybrid clones 20 and 21 which showed close similarity to the untransformed (SK4476) cell line in their growth

patterns maintained the lowest HS3 ratios. Similarly, hybrid clone 16 which had identical growth characteristics with the transformed (B82) cell line had the highest HS3 ratio.

Since a fundamental feature of neoplastic cells involves growth regulation (Cameron and Pool 1981), many aspects of growth control such as lifespan in culture, serum requirements for growth, anchorage dependence for growth, contact inhibition of growth and saturation density are routinely employed as markers of transformation or normalcy. In this study, an attempt was made to determine which aspect of growth control most consistently correlates with the transformed or untransformed patterns of HS3 synthesis.

A growth feature which showed a distinct relationship with the pattern of HS3 synthesis is anchorage requirement for growth. All cell lines which exhibited the untransformed pattern of HS3 synthesis did not grow in methylcellulose, a medium that does not permit anchorage of cells to their culture dish (MacPherson and Montagnier 1964). On the other hand, cell lines with the transformed pattern of HS3 synthesis were able to grow in methylcellulose (that is, they are anchorage-independent for growth) (see Table 3). This is not surprising, considering that anchorage-independence for growth is believed to be the most reliable and consistent in vitro correlate of tumorigenicity (Freedman and Shin 1974; Shin et al 1975; Patek et al 1978). It should, however, be noted that the cell lines studied by Goh (Table 1) were not tested for their anchorage requirement for growth, so that the correlation between this feature of growth and HS3 synthesis pattern is restricted to the cell lines examined in this study (Table 3).

The correlation between lifespan in culture and pattern of HS3 synthesis is also intriguing. All the untransformed cell lines that had finite lifespan in culture (On Ser, WI-38, and El San) expressed the untransformed pattern of HS3 synthesis (Tables 2 and 3). On the other hand, all transformed cell lines, which invariably have infinite lifespan in culture, expressed the transformed pattern of HS3 synthesis (Tables 1, 2 and 3). An interesting situation is observed with two untransformed, but immortal, cell lines. The BALB/3T3 (Aaronson and Todaro 1968) and 10T $\frac{1}{2}$ (Reznikoff et al 1973a; 1973b) are both untransformed cell lines but they have permanent lifespan in culture. Like other untransformed cell lines, they show a high degree of sensitivity to post-confluence inhibition of division, do not grow in methylcellulose, and they do not form tumours in nude mice; but unlike other untransformed cells, these two cell lines are aneuploid, and are immortal in culture (Reznikoff et al 1973a; Freedman and Shin 1974). While the BALB/3T3 cell line expressed the transformed cell pattern of HS3 synthesis (Table 1), the 10T $\frac{1}{2}$ cell line, by contrast, retained the untransformed cell pattern of HS3 synthesis (Table 2). Whether it is transformation per se or immortality that determines the pattern of HS3 synthesis depends on how the results with 10T $\frac{1}{2}$ and BALB/3T3 cells are interpreted. If the HS3 pattern of 10T $\frac{1}{2}$ cells is considered an anomaly then, based on the results for the other transformed and untransformed cells, it can be concluded that expression of the transformed pattern of HS3 synthesis is correlated with immortality rather than transformation per se. By contrast, if HS3 synthesis pattern of BALB/3T3 cells is anomalous then the critical factor is transformation instead of

immortality. It is not surprising, however, if the HS3 pattern correlates with immortality since the acquisition of immortality is believed to be an early event in the transformation process, often preceding the onset of anchorage-independence (Newbold et al 1982). However, immortal cells are not necessarily transformed since they must progress after a further period of culture (Newbold et al 1982). This is probably why 10T½ cells show the untransformed pattern of HS3 synthesis, whereas the BALB/3T3 cells show the transformed pattern, even though both are immortal. It seems that the BALB/3T3 cells are a step(s) further towards transformation than the 10T½ cells. Indeed, BALB/3T3 cells exhibit a higher level of tumorigenicity than 10T½ cells (Grieg et al 1985).

Another transformation characteristic that shows some correlation with the pattern of HS3 synthesis is the ability of cells to grow in medium with low serum. As Table 3 shows, all untransformed cell lines (On Ser, El San, and WI-38) failed to grow in medium with 1% serum and they all had the untransformed pattern of HS3 synthesis. By contrast, transformed cell lines, except the CHO WT line, grew well in 1% serum and expressed the transformed pattern of HS3 synthesis. Similarly, with the hybrid cell clones, all the hybrid clones, except clone 5, which attained the transformed cell value of HS3 ratio were able to grow in medium with 1% serum, whereas the two hybrid clones (clones 20 and 21) which did not attain high HS3 ratios were unable to grow in medium with 1% serum. Although the CHO WT cells expressed the transformed pattern of HS3 synthesis, yet still they failed to grow in 1% serum. It can be recalled that the CHO WT cell line was also the only transformed cell

line tested that did not have any detectable levels of secreted protease and subsequent generation of glutamine in cultures subjected to hyperthermic conditions (see earlier parts of this "Discussion"). The inability of CHO WT cells and hybrid clone 5 to grow in medium with low serum indicates that the relationship between serum requirement for growth and the HS3 response pattern is not consistent. This may be related to the fact that the feature of serum requirement for growth is not considered a consistent and early indication of transformation (Borek 1980).

Possible Role of the Cell Membrane in the HS3 Response

The cell membrane may determine several properties of neoplastic cells such as increased growth rate, prolonged survival, decreased adhesion, loss of contact inhibition, increased invasiveness, expression of repressed antigens, and escape from immune attack (reviewed in Wallach 1968). In addition, the cell membrane is the organelle that is in direct contact with the external environment of the cell and hence it is in a position to monitor the conditions of the external environment and transmit the information to the cell. The cell membrane also exerts a selective control upon the transport of regulatory molecules, which might be either positive or negative effectors of growth (Pardee 1964). Various small molecules are known to mediate many of the functions of the mammalian cell membrane, the best studied being the cyclic nucleotides and several monovalent and divalent ions (see "Historical" section).

The HS3 molecule has the properties of a small molecular weight compound that may be involved in the membrane-mediated coordination for

macromolecular biosynthesis with availability of specific, key nutrients in the extracellular environment. In the first place, HS3 levels dramatically increased within a short time upon removal of glutamine, a key metabolic nutrient, from the external environment of transformed cells in culture. At the same time, in vitro as well as semi-in vivo (by use of permeabilised cells) studies have indicated that the accumulated HS3 due to glutamine deprivation may be involved in various aspects of nucleic acid biosynthesis in a negative way (Goh 1979). This is a clear example of how HS3 may be involved as a coordinator of external growth conditions with key intracellular metabolic events. It is not known by which mechanism HS3 may act in this capacity. It has been suggested that in mammalian cells the HS3 molecule is probably synthesised at the cell membrane (LeJohn, unpublished data).

The results presented in this study do not give a conclusive insight into the role of the cell membrane in the HS3 response. The cell hybridisation studies, which involved the use of a transformed mouse cell line and an untransformed human cell line as the parental cells for production of cell hybrids, show that, although all the hybrid clones had cell membrane components that seemed similar to the transformed cell membrane (measured as sensitivity of the cells to killing by ouabain), yet still there was no overwhelming correspondence among the hybrids to exhibit the transformed pattern of HS3 response. However, there was a general tendency among the hybrid clones to acquire the transformed pattern of HS3 synthesis with prolonged growth in culture. It may also be significant to note that hybrid clone 16, which exhibited the highest HS3 ratio (Table 13) and the only hybrid clone

that showed identical growth characteristics with the transformed parental line (Table 14), was also the only hybrid clone that showed identical ouabain sensitivity as the transformed parental line (Table 15). This may strengthen the argument for the proposal that HS3 is linked with the cell membrane.

The use of ouabain sensitivity to measure the role of the cell membrane in the HS3 response in cell hybrids has some inherent limitations. First, in the assay procedure, the effect of ouabain is noted only at the end of a 14-day incubation of cells with the drug. Within this period of time, several genetic events may have occurred in the hybrids such that it becomes impossible to tell whether functional mouse and human forms of Na^+/K^+ ATPase (the enzyme that is sensitive to ouabain) were still present in the hybrids. Also, it becomes impossible to correlate changes in HS3 response at different cell passages with sensitivity of the cells to ouabain. Second, there is no conclusive published report that the Na^+/K^+ ATPase involved in the assay is a key enzyme affected in the transformation process. Hence it is possible that the membrane feature that was measured is not strongly linked with transformation.

CONCLUSIONS

CONCLUSIONS

The results which have been presented relates the distinctive patterns of HS3 synthesis reported earlier for transformed and untransformed cell lines (Goh 1979) with other known physiological features of transformed cells. The following conclusions and hypotheses can be drawn from the results that have been presented:

(1) The pattern of HS3 synthesis in response to glutamine availability changes in a direct and causal manner as soon as normal cells in culture become transformed, irrespective of the mode of transformation; the change in HS3 synthesis pattern occurs early in the transformation process. The HS3 synthesis pattern for transformed cells can be related to some specific aspects of transformed cell phenotype, and there are indications that it may be a result of recessive genetic change(s) in the cell.

(2) An integrated approach in the study of the characteristics of transformed cells may be a more reliable way of defining transformation than study of individual characteristics in isolation. In this study, it is clear that the heat-induced modification of HS3 synthesis involving proteases is a reliable method of distinguishing between truly transformed cells from cells that are untransformed but express some transformed characteristics, a distinction which may not be reliable if only individual characteristics were considered in isolation.

(3) Heat-induced protease(s) secretion seems to be a consistent feature of genuinely transformed cells notwithstanding the variety in causative agents of transformation. This suggests a common action of the various oncogenic agents. The importance of the heat-induced protease(s) in transformed cells is not known.

APPENDIX

APPENDIX

Note: The experiments described in this Appendix were done after the final draft of the thesis was already prepared. At that time, I felt it necessary to make further attempts to characterise the putative heat-induced proteolytic system.

In further attempts to identify the putative heat-induced protease(s), and possible substrates for the protease(s), the growth medium as well as cell extracts of heat-treated On Ser-DMBA cells were analysed by polyacrylamide gel electrophoresis and autoradiographic techniques. Based on the observation that a high level of protease activity was present in the medium of heat-treated On Ser-DMBA cells (table 5), it is reasonable to expect degradation of serum proteins when the cell cultures are exposed to hyperthermic conditions. The growth of cells, labelling methods, heat treatment, preparation of serum and cell samples, and electrophoretic and autoradiographic methods were as described in "Methods" and in the legends to the appropriate figures.

Fig. A-1a is a Coomassie blue-stained gel electrophoretic pattern of proteins in the growth medium of On Ser-DMBA cultures treated at 37°C or 43°C. The first lane (fig. A-1a) shows proteins normally present in serum without exposure to cells. A large protein band (MW about 55,000 to 60,000) is easily identified, plus other minor bands in higher and lower molecular weight ranges. No noticeable degradation of serum proteins occurred when the cells were incubated at 37°C (compare lanes 1 and 2, fig. A-1a). However, when the cells were incubated for 2h or 4h at 43°C, (lanes 3 and 4 respectively, fig. A-1a), significant breakdown of serum proteins occurred, especially in the

Fig. A-1a Coomassie blue-stained polyacrylamide gel

electrophoretic patterns of proteins in the growth medium of On Ser-DMBA cultures kept for 4h at 37°C (37→37; lanes 2 and 6), or 2h at 43°C followed by 2h at 37°C (43→37; lanes 3 and 7), or 4h at 43°C (43→43; lanes 4 and 8) in glutamine-deficient medium. Lane 1 (S) represents proteins contained in the growth medium containing 10% serum without prior exposure to cells. Molecular weight markers (lane 5) consisting of a mixture of proteins with known molecular weight are run; the figures represent the molecular weights ($\times 10^3$ daltons) of the proteins. The cells were cultured and treated at the appropriate temperatures as described in the "Methods". At the end of the experimental period, the medium was recovered, mixed in a 1:1 ratio with detergent solution (urea/SDS/ ^{β} -mercapto-ethanol) and an aliquot applied directly onto the gel slab. Procedures for gel slab preparation, electrophoresis, and post-electrophoresis treatment are described in "Methods".

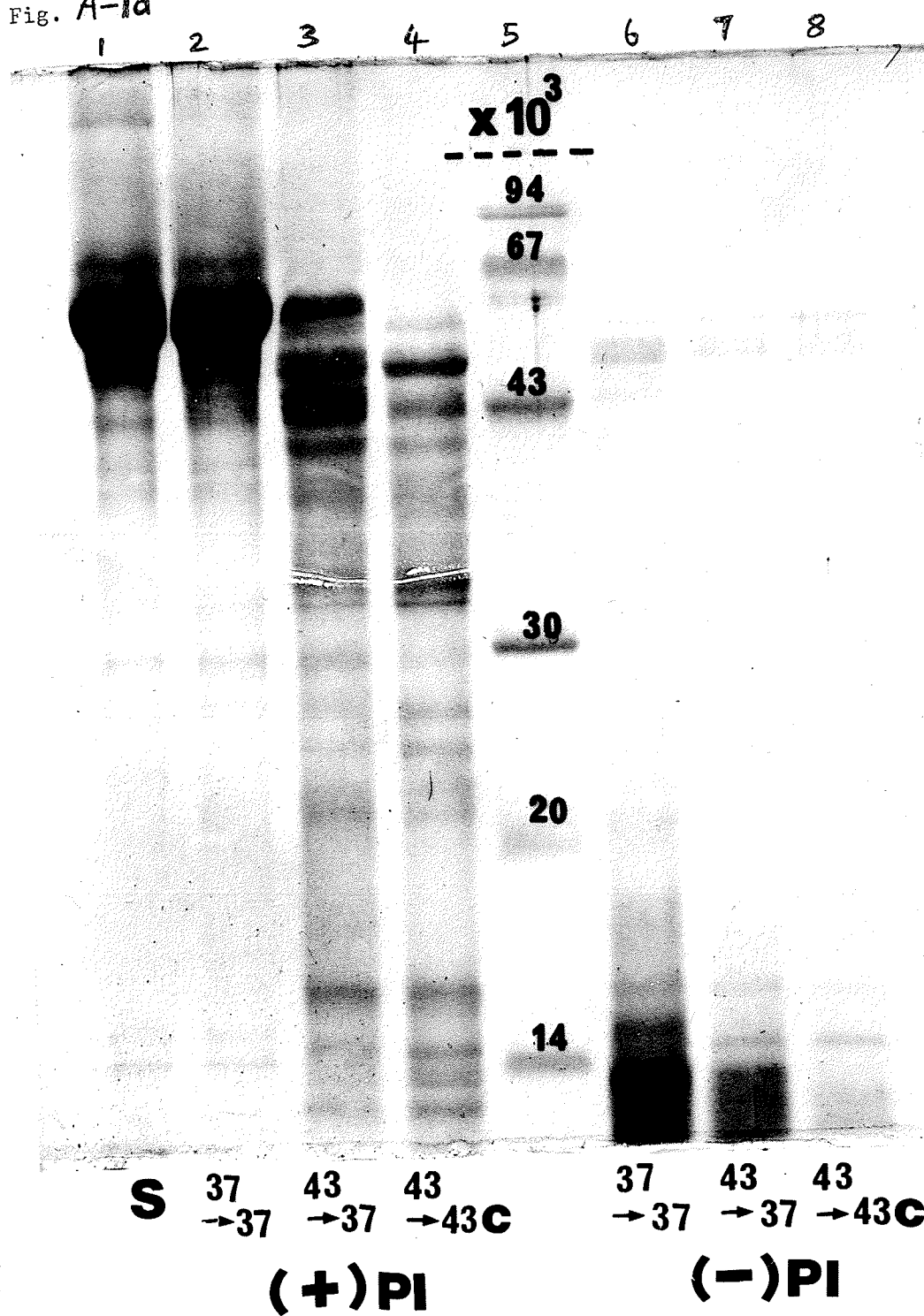
PI = protease inhibitors

(+) means protease inhibitors added to samples at end of experimental period, after cells were separated from the medium.

(-) means no protease inhibitors added.

A mixture of protease inhibitors (TLCK, TAME, PMSF and benzamidine) was added.

Fig. A-1a



major protein band (55,000 to 60,000 daltons). The disappearance of that protein band(s) is accompanied by a concomitant appearance of lower molecular weight protein bands in the heat-treated cells, suggesting that the lower molecular weight proteins may represent degradation products of the major protein species in the growth medium. Based on the intensity of protein bands, it is evident that greater degradation of serum proteins occurred when the cells were incubated at 43°C for 4h than for 2h (compare lane 4 to lane 3, fig. A-1a). The evidence, therefore, shows that authentic serum proteins may be substrates for the heat-induced protease(s). Another interesting feature is that significantly more degradation of serum proteins occurred when protease inhibitors were not added to the growth medium as compared to samples with added protease inhibitors; this was the case when the inhibitors were added either after (fig. A-1a) or prior to (fig. A-1b) thermal stress. These results indicate the strong likelihood that more than one proteinase activity is present in the medium. One activity probably degrades major protein(s) to smaller oligopeptides. The second protease activity may be less specific; this may be a generalised peptidase that is induced during the course of heat treatment.

Fig. A-2 is an autoradiogram of ³⁵S-methionine-labelled proteins in the growth medium of On Ser-DMBA cultures treated for various time periods at 37°C and 43°C. Theoretically, proteins in the medium identified by ³⁵S-methionine incorporation should represent proteins secreted by the cells into the medium. There are more protein bands in the medium of heat-treated cells than in medium of cells kept only at 37°C; these probably represent degradation products of the major high MW band, which tend to decrease in labelling intensity at high temperature with a concomitant appearance of lower MW protein bands. Again, as was

Fig. A-1b Coomassie blue-stained polyacrylamide gel

electrophoretic patterns of proteins in the growth medium of On Ser-DMBA cultures kept for 4h at 37°C (37/37; lanes 2 and 3), or 2h at 43°C followed by 2h at 37°C (43/37; lanes 4 and 5), or 4h at 43°C (43/43; lanes 6 and 7) in glutamine deficient medium. Lane 1 represents proteins in growth medium containing 10% serum without prior exposure to cells. Molecular weight markers (lane 8) consisted of a mixture of proteins with MW given ($\times 10^3$ daltons). Procedures for cell culture, sample preparation, electrophoresis, and post-electrophoresis treatment are described in the legend to fig. A-1a and in "Methods".

PI = protease inhibitors

(+) means PI added prior to temperature treatment

(-) means no PI added

A mixture of protease inhibitors (TLCK, TAME, PMSF and benzamidine) was added.

Fig. A-1b

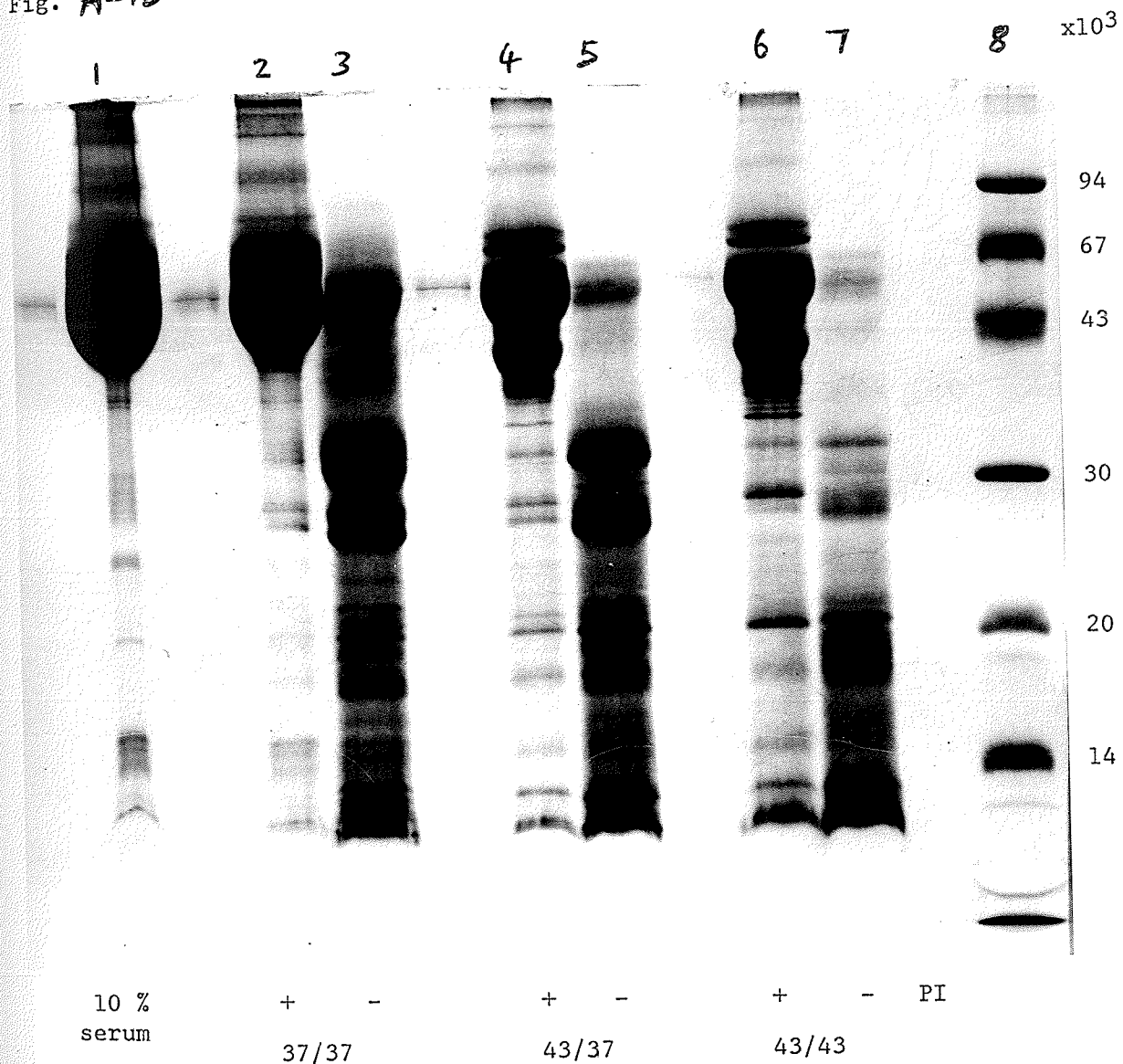


Fig. A-2 Autoradiogram of ^{35}S -methionine-labelled proteins in the incubation medium of On Ser-DMBA cells kept for 4h at 37°C ($37\rightarrow 37$), or for 2h at 43°C followed by 2h at 37°C ($43\rightarrow 37$), or for 4h at 43°C ($43\rightarrow 43$). All experiments were done in glutamine-deficient medium plus 10% DFBS. The growth of cells, labelling procedure, temperature treatments, electrophoresis and autoradiography have all been described in 'Methods' and in the legend to fig. A-1a. The medium was mixed in a 1:1 ratio with detergent buffer (urea/SDS/mercaptoethanol) before the samples were applied onto the gel slab.

PI means protease inhibitors.

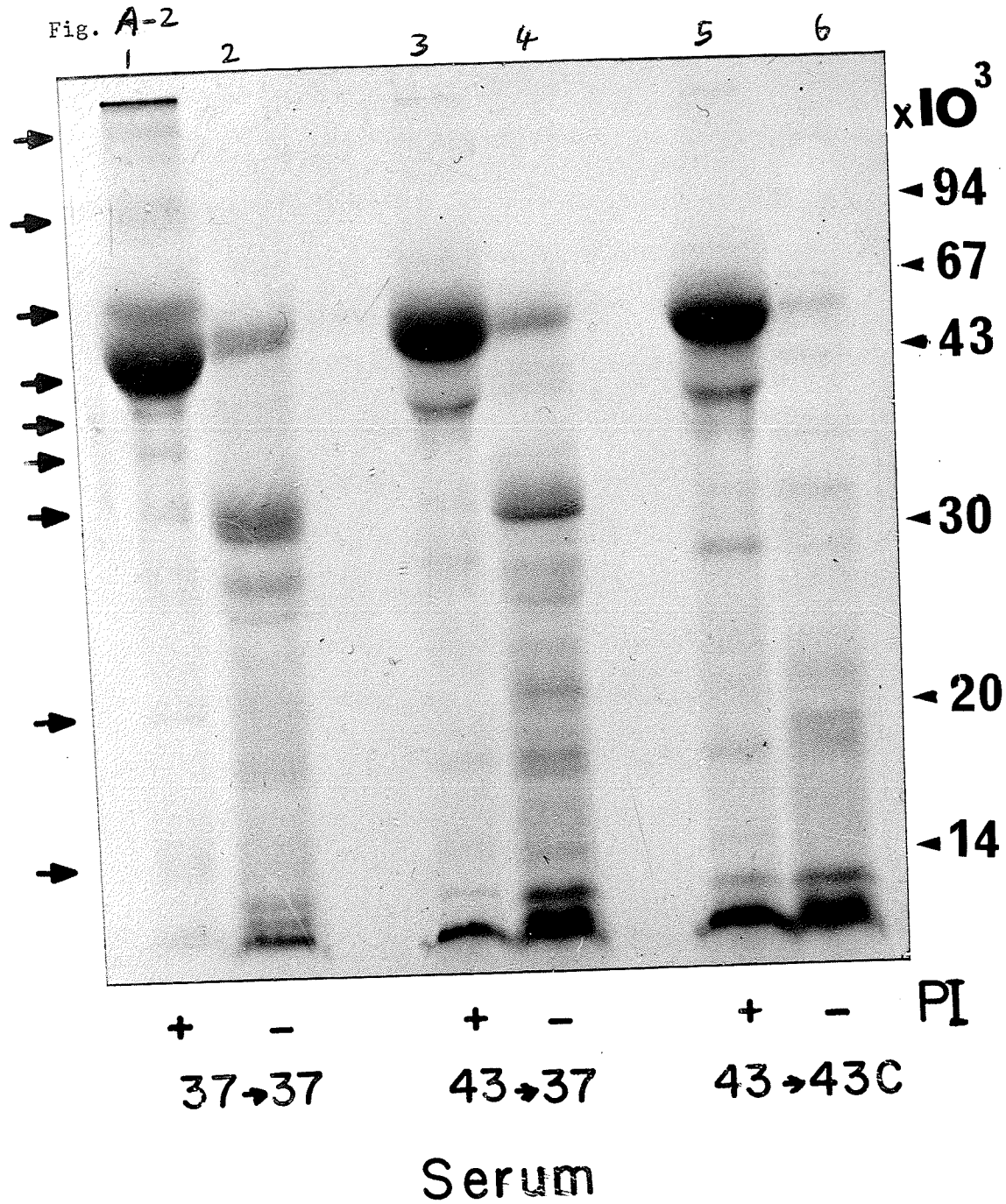
(+) means PI added prior to temperature treatment

(-) means no PI was added to samples.

A mixture of protease inhibitors (TLCK, TAME, PMSF and benzamidine) was added.

Arrows on the left hand side of the fig. represent identifiable bands in lane 1. Numbers on the right hand side of the fig. are MW markers, as described in the legend to fig. A-1a.

Fig. A-2



described, there was more protein degradation when protease inhibitors were not added (compare adjacent lanes in each pair of lanes, fig. A-2). It follows, therefore, that secreted cellular proteins may also be targets for the heat-induced protease(s). It appears that the proteins secreted abundantly by cells are identical to the major serum proteins (compare figs. A-1a and A-1b to fig. A-2). The possibility remains, however, that the radioactive bands seen in the autoradiogram are serum proteins that got contaminated with the radioactive label, especially when the bands on the autoradiogram compare very closely with the Coomassie blue-stained bands (compare figs. A-1a and A-1b to fig. A-2).

The question of whether non-secreted cellular proteins are also targets of the heat-induced protease(s) was investigated. Fig. A-3 shows Coomassie blue-stained proteins from cell extracts of On Ser and On Ser-DMBA cultures treated at 37°C and 42°C in the presence or absence of L-glutamine in the growth medium. In general, the protein patterns of On Ser and On Ser-DMBA cells under the various conditions of treatment were similar. However, proteins from On Ser cells appeared to have a higher intensity of staining than proteins from On Ser-DMBA cells, especially proteins of MW of approximately 35,000, 45,000, and 55,000 daltons (compare lanes 2-5 to lanes 7-10, fig. A-3). The intensely stained protein(s) of about 55,000 daltons in On Ser cells may actually be absent in On Ser-DMBA cells (fig. A-3). It is interesting that the absence or reduced quantity of this 55,000 dalton protein in the On Ser-DMBA cells may correlate with the presence of the ^{35}S -methionine-labelled secreted protein of a similar MW (compare fig. A-2 to fig. A-3). The electrophoregram (fig. A-3) shows no apparent differences in protein patterns or intensity of staining that could be attributed to

Fig. A-3 Coomassie blue-stained polyacrylamide gel electrophoresis of cellular proteins recovered after treatment of On Ser and On Ser-DMBA cells at 37°C and 42°C for 4h. Cells were incubated in medium with or without glutamine. Cell sample preparation and electrophoresis procedures are described in 'Methods' and in the legend to fig. A-1a.

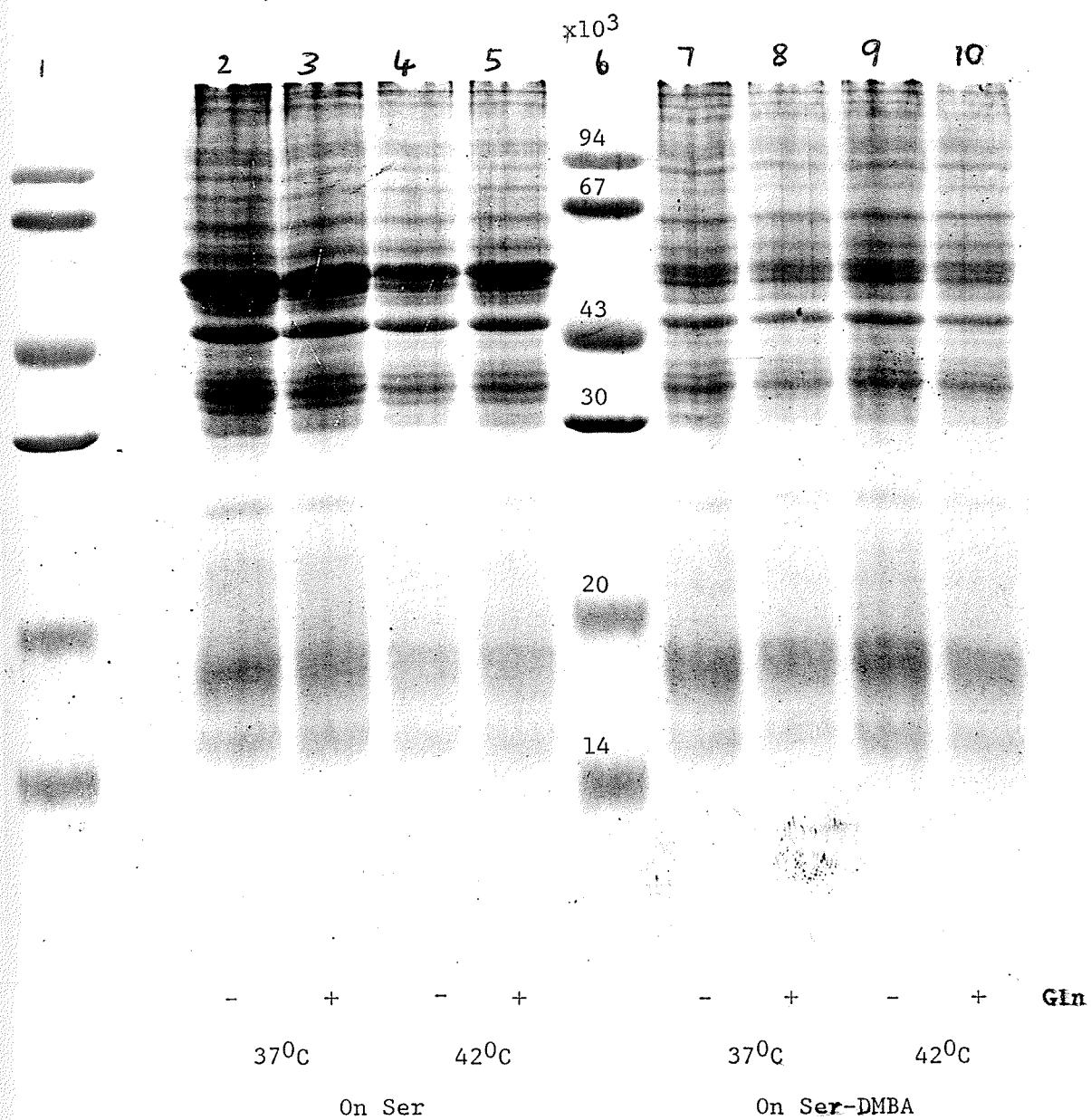
Gln = glutamine

(-) means glutamine absent in the incubation medium

(+) means glutamine present in the incubation medium.

MW markers (lanes 1 and 6) were run as described in the legend to fig. A-1a.

Fig. A-3



heat or nutritional differences. Hence non-secreted cellular proteins may not be substrates for the heat-induced protease(s). Alternatively, the heat-induced protease(s) may not exist in an active form in cells.

In summary, the results presented here suggest that proteins contained in the growth medium, possibly authentic serum proteins (figs. A-1a and A-1b) as well as proteins secreted by cells (fig. A-2), may be the main substrates of the heat-induced protease(s). The evidence shows that non-secreted cellular proteins are not degraded to any significant extent in a heat-related manner (fig A-3).

BIBLIOGRAPHY

- AARONSON, S. A., & TODARO, G. J. (1968). Development of 3T3-like lines from BALB/c mouse embryo cultures. *J. Cell. Physiol.* 72:141-148.
- ABERCROMBIE, M. (1966). Contact inhibition: the phenomenon and its biological implications. *Natl. Cancer Inst. Monogr.* 26:249-277.
- ADAMS, A., & HARKNESS, R. A. (1976). Developmental changes in purine phosphoribosyltransferases in human and rat tissues. *Biochem. J.* 160:565-576.
- ADAMS, C., & RINNE, R. W. (1982). Stress protein formation: gene expression and environmental interaction with evolutionary significance. *Int. Rev. Cytol.* 79:305-315.
- ADAMS, R. A., FOLEY, G. E., FARBER, S., FLOWERS, A., LAZARUS, H., & HELLERSTEIN, E. E. (1970). Serial transplantation of Burkitt's tumor (EB3) cells in newborn Syrian hamsters and its facilitation by antilymphocyte serum. *Cancer Res.* 30:338-345.
- ALBRECHTSEN, O. K. (1957). The fibrinolytic activity of human tissues. *Br. J. Haematol.* 3:284-291.
- ALLDERDICE, P. W., MILLER, O. J., MILLER, D. A., WARBURTON, D., PEARSON, P. L., KLEIN, G., & HARRIS, H. (1973). Chromosome analysis of two related heteroploid mouse cell lines by quinacrine fluorescence. *J. Cell Sci.* 12:263-274.
- ALLFREY, V. G. (1977). Post-synthetic modifications of histone structure: a mechanism for the control of chromosome structure by the modulation of histone-DNA interactions. In: "Chromatin and Chromosome Structure", (H. J. Li and R. A. Eckhardt, eds.), pp. 167-192. Academic Press, New York.
- ALLRED, L. E. (1978). The contact receptor-chalone hypothesis: a model for the control of cell proliferation. *J. Cell Biol.* 79:48a.
- ALPEN, E. L. & JOHNSTON, M. E. (1967). DNA synthetic rate and DNA content of nucleated erythroid cells. *Exp. Cell Res.* 47:177-192.
- AL-SHAIKHALLY, M. H. M., NEDERGAARD, J., & CANNON, B. (1979). Sodium-induced calcium release from mitochondria in brown adipose tissue. *Proc. Natl. Acad. Sci. USA* 76:2350-2353.
- ANDERSON, D. D., BECKMAN, R. P., HARMS, E. H., NAKAMURA, K., & WEBER, M. J. (1981). Biological properties of partial transformation mutants of Rous sarcoma virus and characterisation of their pp60^{v-src} kinase. *J. Virol.* 37:445-458.

- ANTONIADES, H. N. (1981). Human platelet-derived growth factor (PDGF): purification of PDGF-I and PDGF-II and separation of their reduced subunits. *Proc. Natl. Acad. Sci. USA* 78:7314-7317.
- ANTONIADES, H. N., SCHER, C. D., & STILES, C. D. (1979). Purification of human platelet-derived growth factor. *Proc. Natl. Acad. Sci. USA* 76:1809-1813.
- ARGUS, M. F., KANE, J. F., SAKUNTALA, M., & RAY, F. E. (1962). Effects of ionising radiation on 9, 10-dimethyl-1,2-benzanthracene tumorigenesis. *Radiat. Res.* 16:37-43.
- ASHBURNER, M., & BONNER, J. J. (1979). The induction of gene activity in *Drosophila* by heat shock. *Cell* 17:241-254.
- ASTRIN, S. M., & ROTHBERG, P. G. (1983). Oncogenes and cancer. *Cancer Invest.* 1:355-364.
- BABA, T., AOKI, K., & ISHII, M. (1967). Relation between the so-called two-phase theory and summation in carcinogenesis. *Gann.* 58:161-166.
- BACKER, J. M., & WEINSTEIN, I. B. (1983). A phorbol ester tumor promoter accelerates hydrolysis of ATP by isolated rat liver mitochondria. *Proc. Am. Assoc. Cancer Res.* 24:109 (abstr.).
- BACKER, J. M., BOERSIG, M. R., & WEINSTEIN, I. B. (1982). Inhibition of respiration by a phorbol ester tumor promoter in murine cultured cells. *Biochem. Biophys. Res. Commun.* 105:855-866.
- BALK, S. D. (1971). Calcium as a regulator of the proliferation of normal, but not of transformed, chicken fibroblasts in a plasma-containing medium. *Proc. Natl. Acad. Sci. USA* 68:271-275.
- BALK, S. D. (1970). Precipitates and particulates cause proliferative activity of density-inhibited cultured cells by disturbing the distribution boundary layer: an artefact superimposed upon an artefact. *Life Sci.* 27:1917-1920.
- BARKER, B. E., & SANFORD, K. K. (1970). Cytologic manifestations of neoplastic transformation in vitro. *J. Natl. Cancer Inst.* 44:39-63.
- BARNES, D., & SATO, G. (1980). Serum-free cell culture: a unifying approach. *Cell* 22:649-655.
- BARNETT, E. V., & BARON, S. (1959). An activator of plasminogen produced by cell culture. *Proc. Soc. Exp. Biol. Med.* 102:308-311.
- BARRETT, J. C., CRAWFORD, B. D., MIXTER, L. O., SCHECHTMAN, L. M., TS'O, P. O. P., & POLLACK, R. (1979). Correlation of in vitro growth properties and tumorigenicity of Syrian hamster cell lines. *Cancer Res.* 39:1504-1510.

- BARSKI, G., SORIEUL, S., & CORNEFERT, F. (1961). "Hybrid" type cells in combined cultures of two different mammalian cell strains. *J. Natl. Cancer Inst.* 26:1269-1291.
- BASERGA, R. (1976). "Multiplication and Division in Mammalian Cells". Dekker, New York.
- BAUGH, C. L., & TYTELL, A. A. (1967). Propagation of the human diploid cell WI-38 in galactose medium. *Life Sci.* 6:371-380.
- BAUGH, C. L., LECHER, R. W., & TYTELL, A. A. (1967). The effect of pH on the propagation of the diploid cell WI-38 in galactose medium. *J. Cell. Physiol.* 70:225-228.
- BAXTER, G. C., & STANNERS, C. P. (1978). The effect of protein degradation on cellular growth characteristics. *J. Cell. Physiol.* 96:139-146.
- BENSON, R. W. (1978). RNA polymerase activities in heart tissue of aging mice. *Exp. Gerontol.* 13:305-310.
- BERENBLUM, I. (1982). Sequential aspects of chemical carcinogenesis: skin. In: "Cancer: A Comprehensive Treatise, Vol. 1, Edition 2, (F. F. Becker, ed.), pp. 102-149. Plenum Publishing Corp., New York.
- BERTRAM, J. S. (1979). Modulation of cellular interactions between 3CH/10T $\frac{1}{2}$ cells and their transformed counterpart by phosphodiesterase inhibitors. *Cancer Res.* 39:3502-3508.
- BETTGER, W. J., & HAM, R. G. (1982). The critical role of lipids in supporting clonal growth of human diploid fibroblasts in a defined medium. In: "Growth of Cells in Hormonally Defined Media", (G. H. Sato, A. B. Pardee, & D. A. Sirbasku, eds.), pp. 61-64. Cold Spring Harbor Conference on Cell Proliferation, Vol. 9. Cold Spring Harbor Publications, New York.
- BICHER, H. I., HETZEL, F. W., SANDHU, T. S., FRINAK, S., VAUPEL, P., O'HARA, M. D., & O'BRIEN, T. (1980). Effects of hyperthermia on normal and tumour microenvironment. *Radiology* 137:523-530.
- BIRKEDAL-HANSEN, H., COBB, C. M., TAYLOR, R. E., & FULLMER, H. M. (1976). Activation of fibroblast procollagenase by mast cell proteinases. *Biochem. Biophys. Acta* 438:273-286.
- BISHOP, J. M. (1981). Enemies within: the genesis of retroviral oncogenes. *Cell* 23:5-6.
- BISHOP, J. M. (1983). Cellular oncogenes and retroviruses. *Ann. Rev. Biochem.* 52:301-354.
- BISHOP, R., MARTINEZ, R., NAKAMURA, K. D., & WEBER, M. J. (1983). A

- tumor promoter stimulates phosphorylation on tyrosine. *Biochem. Biophys. Res. Commun.* 115:536-543.
- BJORKSTEN, J. (1963). Aging, primary mechanism. *Gerontologia* 8:179-182.
- BLOCK-SHTACHER, N., & SACHS, L. (1977). Identification of a chromosome that controls malignancy in Chinese hamster cells. *J. Cell. Physiol.* 93:205-212.
- BLUMBERG, P. M. (1980). In vitro studies on the mode of action of the phorbol esters, potent tumor promoters: Part 1. *CRC Crit. Rev. Toxicol.* 8:199-234.
- BLUMBERG, P. M. (1981). In vitro studies on the mode of action of the phorbol esters, potent tumor promoters: Part 2. *CRC Crit. Rev. Toxicol.* 9:153-197.
- BLUMBERG, P. M., & ROBBINS, P. W. (1975). Relation of protease action on the cell surface to growth control and adhesion. In: "Proteases and Biological Control", (E. Reich, D. Rifkin, & E. Shaw, eds.), pp. 945-956. Cold Spring Harbor Laboratories, Cold Spring Harbor, New York.
- BOEHM, T., & DRAHOVSKY, D. (1982). Elevated level of enzymatic DNA methylation in cells treated with 1-i-D-arabinofuranosylcytosine. *Cancer Res.* 42:1537-1540.
- BOONE, C. W., TAKEICHI, N., PARANJPE, M., & GILDEN, R. (1976). Vasoformative sarcomas arising from BALB/3T3 cells attached to solid substrates. *Cancer Res.* 36:1626-1633.
- BOONE, C. W., VEMBU, D., WHITE, B. J., TACHEICHI, N., & PARANJPE, M. (1979). Karyotypic, antigenic, and kidney-invasive properties of cell lines from fibrosarcomas arising in C3H/10T½ cells implanted subcutaneously attached to plastic plates. *Cancer Res.* 39:2172-2178.
- BOREK, C. (1980). In: "Advances in Modern Experimental Toxicology. I. Mammalian Cell Transformation by Chemical Carcinogens", (V. Dunkel, N. K. Mishra, & M. Mehlman, eds.), pp. 297-318. Senate Press, Princeton, New Jersey.
- BOREK, C. (1982). Radiation oncogenesis in cell culture. *Adv. Cancer Res.* 37:159-232.
- BOREK, C., & FENOGLIO, C. M. (1976). Scanning electron microscopy of surface features of hamster embryo cells transformed in vitro by X-irradiation. *Cancer Res.* 36:1325-1334.
- BOREK, C., HIGASHINO, S., LOEWENSTEIN, W. R. (1969). Intercellular communication and tissue growth. *J. Memb. Biol.* 1:274-293.

- BOREK, C., PAIN, C., & MASON, H. (1977). Neoplastic transformation of hamster embryo cells irradiated in utero and assayed in vitro. *Nature* (London) 266:452-454.
- BORUN, T. W. (1975). Histones, differentiation, and the cell cycle. In: "Results and Problems in Cell Differentiation, Vol. 7, Cell Cycle and Cell Differentiation", (J. Reinert and H. Holtzer, eds.), pp. 249-290. Springer-Verlag, New York.
- BOSMANN, H. B. (1971). Mitochondrial biochemical events in a synchronised mammalian cell populations. *J. Biol. Chem.* 246:3817-3823.
- BOUTWELL, R. K. (1974). The function and mechanism of promoters of carcinogenesis. *CRC Crit. Rev. Toxicol.* 2:419-443.
- BOUTWELL, R. K. (1977). The role of the induction of ornithine decarboxylase in tumor promotion. In: "Origins of Human Cancer, Book B, (H. H. Hiatt, J. D. Watson, & J. A. Winsten, eds.), pp. 49-58. Cold Spring Harbor Laboratory, New York.
- BOYNTON, A. L., WHITFIELD, J. F., & ISAACS, R. J. (1976). The different roles of serum and calcium in the control of proliferation of BALB/c3T3 mouse cells. *In Vitro* 12:120-123.
- BRAVO, R., FEY, S. J., BELLATIN, J., LARSEN, P. M., AREVALO, J., & CELIS, J. E. (1981). Identification of a nuclear and of a cytoplasmic polypeptide whose relative proportions are sensitive to changes in the rate of cell proliferation. *Exp. Cell Res.* 136:311-319.
- BRAY, G. (1960). A simple efficient liquid scintillator for counting aqueous solutions in a liquid scintillation counter. *Anal. Biochem.* 1:279-285.
- BREGULA, V., KLEIN, G., & HARRIS, H. (1971). The analysis of malignancy by cell fusion. II. Hybrids between Ehrlich cells and normal diploid cells. *J. Cell Sci.* 8:673-680.
- BRUGGE, J. S., & ERIKSON, R. L. (1977). Identification of a transformation-specific antigen induced by an avian sarcoma virus. *Nature* 269:346-348.
- BRUNET, N., GOURDJI, D., TIXIER-VIDAL, A., & RIZZINO, A. (1982). Role of attachment and spreading factors: effect of fetuin on proliferation and prolactin secretion by GH3 cells and primary cultures of normal rat pituitary cells. In: "Growth of Cells in Hormonally Defined Media", (G. H. Sato, A. B. Pardee and D. A. Sirbasku, eds.), Cold Spring Harbor Conferences on Cell Proliferation, Vol. 9, pp. 169-177. Cold Spring Harbor, New York.
- BRUNET, N., RIZZINO, A., GOURDJI, D., & TIXIER-VIDAL, A. (1981).

- Effects of thyroliberin (TRH) on cell proliferation and prolactin secretion by GH3/B6 rat pituitary cells: a comparison between serum-free and serum-supplemented media. *J. Cell. Physiol.* 109:363-372.
- BULL, J. M. C. (1984). An update on the anticancer effects of a combination of chemotherapy and hyperthermia. *Cancer Res. (Suppl.)* 44:4853s-4856s.
- BURFORD, F. K., & SANDERS, B. O. (1964). Ascites tumours from BHK-21 cells transformed in vitro by polyoma virus. *Nature (London)* 201:786-789.
- BURGER, M. M. (1970). Proteolytic enzymes initiating cell division and escape from contact inhibition of growth. *Nature (London)* 227:170-171.
- BURMER, G. C., MOTULSKY, H., ZEIGLER, C. J., & NORWOOD, T. H. (1983). Inhibition of DNA synthesis in young cycling human diploid fibroblast-like cells upon fusion to enucleate cytoplasts from senescent cells. *Exp. Cell Res.* 145:79-84.
- BURNET, F. M. (1977). *Interdiscip. Topics Gerontol.* 11:30-36.
- BUSH, H., & SHODELL, M. (1978). Uptake of low molecular weight substances by SV40-transformed 3T3 cells is invariant with growth rates in the presence and absence of serum. *Exp. Cell Res.* 114:27-30.
- CAIRNS, J. (1981). The origin of human cancers. *Nature* 289:353-357.
- CAMERON, I. L., & POOL, T. B. (1981). The transformed cell: some introductory comments. In: "The Transformed Cell", (I. L. Cameron & T. B. Pool, eds.), pp. 1-6. Academic Press, New York.
- CAMPISI, J., MEDRANO, E. E., MORREO, G., & PARDEE, A. B. (1982). Restriction point control of cell growth by a labile protein: evidence for increased stability in transformed cells. *Proc. Natl. Acad. Sci. USA* 79:436-440.
- CAPON, D., CHEN, E., LEVINSON, A., SEEBURG, P., & GOEDEL, D. (1983). Complete nucleotide sequences of the T24 human bladder carcinoma oncogene and its normal homologue. *Nature* 302:33-37.
- CARAFOLI, E., & CROMPTON, M. (1978). The regulation of intracellular calcium. *Current Top. Membr. Transport* 10:151-216.
- CARREL, A. (1912). On the permanent life of tissues outside of the organism. *J. Exp. Med.* 15:516-528.
- CASTAGNA, M., TAKAI, U., KAIBUCHI, K., SANO, K., KIKKAWA, U., & NISHIZUKA, Y. (1982). Direct activation of calcium-activated, phospholipid-dependent protein kinase by tumor promoting phorbol esters. *J. Biol. Chem.* 257:7847-7851.

- CASTO, B. C., & DiPAOLO, J. A. (1973). Viruses, chemicals, and cancer. *Prog. Med. Virol.* 16:1-47.
- CASTOR, C. W., RITCHIE, J. C., WILLIAMS (Jr.), C. H., SCOTT, M. E., WHITNEY, S. L., MYERS, S. L., SLOAN, T. B., & ANDERSON, B. E. (1979). Connective tissue activation: composition and actions of a human platelet antacid mediator. *Arthritis Rheum.* 22:260-272.
- CECCARINI, C., & EAGLE, H. (1971). pH as a determinant of cell growth and contact inhibition. *Proc. Natl. Acad. Sci. USA* 68:229-233.
- CERUTTI, P. A., EMERIT, I., & AMSTAD, P. (1983). Membrane-mediated chromosomal damage. In: "Genes and Proteins in Oncogenesis", (I. B. Weinstein and H. J. Vogel, eds.), pp. 55-67. Academic Press, New York.
- CHAFOLEAS, J. G., & MEANS, A. R. (1982). Calmodulin is an important regulatory molecule in cell proliferation. In: "Ions, Cell Proliferation, and Cancer", (A. L. Boynton, W. L. McKeehan, & J. F. Whitfield, eds.), pp. 142-150. Academic Press, New York.
- CHAFOLEAS, J. G., BOLTON, W. E., HIDAHA, H., BOYD, A. E., & MEANS, A. R. (1982). Calmodulin and the cell cycle: involvement in regulation of cell cycle progression. *Cell* 28:41-50.
- CHAGANTI, R. S. K., SCHONBERG, S., & GERMAN, J. (1974). A manyfold increase in sister chromatid exchanges in Bloom's syndrome lymphocytes. *Proc. Natl. Acad. Sci. USA* 71:4508-4512.
- CHAMBON, P. (1975). Eucaryotic nuclear RNA polymerases. *Ann. Rev. Biochem.* 44:613-638.
- CHAN, J. Y. H., & BECKER, F. F. (1979). Decreased fidelity of DNA polymerase activity during N-2-fluorenylacetamide hepatocarcinogenesis. *Proc. Natl. Acad. Sci. USA* 76:814-818.
- CHANDRASEKHAR, S., & MILLIS, A. T. J. (1980). Fibronectin from aged fibroblasts is defective in promoting cellular adhesion. *J. Cell. Physiol.* 103:47-54.
- CHANG, E. H., FURTH, M. E., SCOLNICK, E. M., & LOWY, D. R. (1982). Tumorigenic transformation of mammalian cells induced by a normal human gene homologous to the oncogene of Harvey murine sarcoma virus. *Nature* 297:479-483.
- CHEN, L. B., & BUCHANAN, J. M. (1975). Mitogenic activity of blood components. I. Thrombin and prothrombin. *Proc. Natl. Acad. Sci. USA* 72:131-135.
- CHEN, T. T., & HEIDELBERGER, C. (1969). Quantitative studies on the malignant transformation of mouse prostate cells by carcinogenic hydrocarbons in vitro. *Int. J. Cancer* 4:166-178.

- CHILDS, C. B., PROPER, J. A., TUCKER, R. F., & MOSES, H. L. (1982). Serum contains a platelet-derived transforming growth factor. *Proc. Natl. Acad. Sci. USA* 79:5312-5316.
- CHOIE, D. D., & RICHTER, G. W. (1978). G₂ sub-population in mouse liver induced into mitosis by lead acetate. *Cell Tissue Kinet.* 11:235-239.
- CHOU, I-N., BLACK, P. H., & ROBLIN, R. (1975). Effects of protease inhibitors on growth of 3T3 and SV3T3 cells. In: "Control of Proliferation in Animal Cells", (B. Clarkson & R. Baserga, eds.), pp. 339-350. Cold Spring Harbor Laboratories, Cold Spring Harbor, New York.
- CHRISTMAN, J. K., ACS, G., SILAGI, S., & SILVERSTEIN, S. C. (1975). Plasminogen activator: biochemical characterisation and correlation with tumorigenicity. In: "Proteases and Biological Control", (E. Reich, D. B. Rifkin, & E. Shaw, eds.), pp. 827-839. Cold Spring Harbor Laboratories, Cold Spring Harbor, New York.
- CHRISTMAN, J. K., SILVERSTEIN, S. C., & ACS, G. (1977). Plasminogen activators. In: "Proteinases in Mammalian Cells and Tissues", (A. Barrett, ed.), pp. 91-149. Elsevier, Amsterdam.
- CHURCH, K. (1967). Pattern of DNA replication in binucleate cells occurring in mouse embryo cell cultures. *Exp. Cell Res.* 46:639-641.
- CLIFTON, E. E., & Grossi, C. E. (1955). Fibrinolytic activity of human tumors as measured by the fibrin plate method. *Cancer* 8:1146-1154.
- COBB, M. H., & ROSEN, O. M. (1984). The insulin receptor and tyrosine protein kinase activity. *Biochem. Biophys. Acta* 738:1-8.
- COFFEY, D. S. (1983). The concept of DNA rearrangement in carcinogenesis: the nuclear matrix. In: "Genes and Proteins in Oncogenesis", (I. B. Weinstein and H. J. Vogel, eds.), pp. 339-349. Academic Press, New York.
- COLBURN, N. H. (1979). The use of tumour promoter responsive epidermal cell lines to study preneoplastic progression. In: "Neoplastic Transformation in Differentiated Epithelial Cell Systems In Vitro", (L. M. Franks & C. B. Wigley, eds.). Academic Press, New York.
- COLBURN, N. H. (1980). Tumour promotion and preneoplastic progression. In: "Carcinogenesis, Vol. 5: Modifiers of Chemical Carcinogenesis", (T. J. Slaga, ed.), pp. 33-56. Raven Press, New York.
- COLBURN, N. H., LAU, S., & HEAD, R. (1975). Decrease of epidermal histidase activity by tumor-promoting phorbol esters. *Cancer Res.* 35:3154-3159.

- COLEMAN, P. S. & LAVIETES, B. B. (1981). Membrane cholesterol, tumorigenesis, and the biochemical phenotype of neoplasia. *CRC Crit. Rev. Biochem.* 11:341-393.
- COLLETT, M. S., ERIKSON, E., PURCHIO, A. F., BRUGGE, J. S., & ERIKSON, R. L. (1979). A normal cell protein similar in structure and function to the avian sarcoma virus transforming gene product. *Proc. Natl. Acad. Sci. USA* 76:3159-3163.
- COLLINS, J. L., PATEK, P. Q., & COHN, M. (1980). Cancer: a problem in somatic cell evolution. *Contempr. Top. Immunol.* 11:1-79.
- COOPER, G. M. (1982). Cellular transforming genes. *Science* 218:801-806.
- COOPER, G. M., & LANE, M. (1984). Cellular transforming genes and oncogenesis. *Biochem. Biophys. Acta* 738:9-20.
- COOPER, M. D., PAYNE, L. M., DENT, P., BURMESTER, B. R., & GOOD, R. A. (1968). Pathogenesis of avian lymphoid leukosis. I. Histogenesis. *J. Natl. Cancer Inst.* 41:373-389.
- COSS, R. A., DEWEY, W. C., & BAMBURG, J. R. (1979). Effect of hyperthermia (41.5°C) on Chinese hamster ovary cells analysed in mitosis. *Cancer Res.* 39:1911-1918.
- COURTOIS, Y., & HUGHES, R. C. (1976). Surface labelling of senescent chick fibroblasts by lactoperoxidase-catalysed iodination. *Gerontology* 22:371-379.
- CRICHTON, R. R. (ed.) (1975). "Proteins of Iron Storage and Transport in Biochemistry and Medicine". North-Holland, Amsterdam.
- CRISTOFALO, V. J. (1970). Metabolic aspects of aging in diploid human cells. In: "Aging in Cell and Tissue Culture", (E. Holeckova & V. J. Cristofalo, eds.), pp. 83-119. Plenum Press, New York.
- CRISTOFALO, V. J. (1972). Animal cell cultures as a model system for the study of aging. *Adv. Gerontol. Res.* 4:45-79.
- CRISTOFALO, V. J., & KABAKJIAN, J. (1975). Lysosomal enzymes and aging in vitro: subcellular enzyme distribution and effect of hydrocortisone on cell life span. *Mech. Aging Dev.* 4:19-28.
- CRISTOFALO, V. J., & KRITCHEVSKY, D. (1965). Growth and glycolysis in the human cell strain WI-38. *Proc. Soc. Exp. Biol. Med.* 118:1109-1113.
- CRISTOFALO, V. J., & STANULIS-PRAEGER, B. M. (1982). Cellular senescence in vitro. *Adv. Cell Culture* 2:1-68.
- CRISTOFALO, V. J., PALAZZO, R., & CHARPENTIER, R. (1979). Chronologic

- versus replicative life span in human cell cultures: a reexamination. *Gerontologist*, Pt. II, 19:59.
- CROCE, C. M. (1976). Loss of mouse chromosomes in somatic cell hybrids between HT-1080 human fibrosarcoma cells and mouse peritoneal macrophages. *Proc. Natl. Acad. Sci. USA* 73:3248-3252.
- CROCE, C. M. (1980). Cancer cells in cell hybrids. *Biochem. Biophys. Acta* 605:411-430.
- CROCE, C. M., BARRICK, J., LINNENBACH, A., & KAPROWSKI, H. (1979). Expression of malignancy in hybrids between normal and malignant cells. *J. Cell. Physiol.* 99:279-286.
- CROOK, R. B., LOUIE, M., DENEL, T. F., & TOMKINS, G. M. (1978). Regulation of glutamine synthetase by dexamethasone in hepatoma tissue culture cells. *J. Biol. Chem.* 253:6125-6131.
- CROWLEY, C., & CURTIS, H. J. (1963). The development of somatic mutations in mice with age. *Proc. Natl. Acad. Sci. USA* 49:626-628.
- DALLA-FAVERA, R., BREGNI, M., ERIKSON, J., PATTERSON, D., GALLO, R., & CROCE, C. M. (1982). Human c-myc oncogene is located on the region of chromosome 8 that is translocated in Burkitt lymphoma cells. *Proc. Natl. Acad. Sci. USA* 79:7824-7827.
- D'ANCONA, G. G., & CROCE, C. M. (1979). Segregation of rat chromosomes in somatic cell hybrids between rat cells and HT-1080 human fibrosarcoma cells. *Human Genet.* 53:17-21.
- DAVIDSON, R. L., & EPHRUSSI, B. (1970). *Exp. Cell Res.* 61:222-226.
- DAVIDSON, R. L., & GERALD, P. S. (1976). Improved techniques for the induction of mammalian cell hybridisation by polyethylene glycol. *Somat. Cell Genet.* 2:165-176.
- DAVIES, P. F., & ROSS, R. (1978). Mediation of pinocytosis in cultured arterial smooth muscle and endothelial cells by platelet-derived growth factor. *J. Cell Biol.* 79:663-671.
- DAVIES, R. E., & DODGE, H. A. (1972). Modification of chemical carcinogenesis by phototoxicity and photochemical decomposition of carcinogen. *Proc. 1st Ann. Meet. Am. Soc. Photobiol.*, June 1973, p. 138.
- DEFFIE, A. M., & LeJOHN, H. B. (1984). Involvement of protease in L-glutamine control of nucleic acid and polyphosphate metabolism in cells transformed by 9, 10-dimethyl-1,2-benzanthracene, SV40, and H-ras oncogene. *Biochem. Biophys. Res. Commun.* 124:6-13.
- DeLARCO, J. E., & TODARO, G. J. (1978). Growth factors from murine

- sarcoma virus-transformed cells. Proc. Natl. Acad. Sci. USA 75:4001-4005.
- DeLEO, A. B., JAY, G., APPELLA, E., DUBOIS, G. C., LAW, L. W., & OLD, L. J. (1979). Detection of a transformation-related antigen in chemically induced sarcomas and other transformed cells of the mouse. Proc. Natl. Acad. Sci. USA 76:2420-2424.
- DELL'ORCO, R. J., MERTENS, J. G., & KRUSE, P. F. (1973). Doubling potential, calendar time, and senescence of human diploid cells in culture. Exp. Cell Res. 77:356-360.
- DEWALD, B., DULANEY, J. T., & TOUSTER, O. (1974). Solubilisation and polyacrylamide gel electrophoresis of membrane enzymes with detergents. In: "Methods in Enzymology", (S. Fleischer & L. Packer, eds.), Vol. 32, pp. 82-91. Academic Press, New York.
- DEWEY, W. C., FREEMAN, M. L., RAAPHORST, G. P., CLARK, E. P., WONG, R. S. L., HIGHFIELD, D. P., SPIRO, I. J., TOMASOVIC, S. P., DENMAN, D. L., & COSS, R. A. (1980). Cell biology of hyperthermia and radiation. In: "Radiation Biology in Cancer Research", (R. E. Meyn & H. R. Withers, eds.), pp. 589-621. Raven Press, New York.
- DICKSON, J. A., & CALDERWOOD, S. K. (1976). In vivo hyperthermia of Yoshida tumor induces entry of non-proliferating cells into cycle. Nature 263:772-774.
- DICKSON, J. A., & OSWALD, B. E. (1976). The sensitivity of a malignant cell line to hyperthermia (42°C) at low intracellular pH. Br. J. Cancer 34:262-271.
- DICKSON, J. A., & CALDERWOOD, S. K. (1980). Temperature range and selective sensitivity of tumors to hyperthermia: a critical review. Ann. N.Y. Acad. Sci. 335:180-205.
- DiPAOLO, J. A. (1983). Relative difficulties in transforming human and animal cells in vitro. J. Natl. Cancer Inst. 70:3-8.
- DiPAOLO, J. A., DeMARINIS, A. J., & DONIGER, J. (1981). Transformation of Syrian hamster embryo cells by sodium bisulfite. Cancer Letts. 12:203-208.
- DiPAOLO, J. A., DONOVAN, P. J., & NELSON, R. L. (1971). X-irradiation enhancement of transformation by benzo(a)pyrene in hamster embryo cells. Proc. Natl. Acad. Sci. USA 68:1734-1737.
- DIPPOLD, W. G., JAY, G., DeLEO, A. B., KHOURY, G., & OLD, L. J. (1981). p53 transformation-related protein: detection by monoclonal antibody in mouse and human cells. Proc. Natl. Acad. Sci. USA 78:1695-1699.
- DOLBEARE, F., VANDERLAAN, M., & PHARES, M. (1980). Alkaline phosphatase

- and acid arylamidase as marker enzymes for normal and transformed WI-38 cells. *J. Histochem. Cytochem.* 28:419-426.
- DRAHOVSKY, D., & WACKER, A. (1975). Inactivation of mammalian DNA methylase activity by N-methyl-N-nitrosoguanidine. *Eur. J. Cancer* 11:517-519.
- DRESCHER-LINCOLN, C. K., & SMITH, J. R. (1983). Inhibition of DNA synthesis in proliferating human diploid fibroblasts by fusion with senescent cytoplasts. *Exp. Cell Res.* 144:455-462.
- DUBROW, R., PARDEE, A. B., & POLLACK, R. (1978). 2-amino-isobutyric acid and 3-O-methyl-D-glucose transport in 3T3, SV40-transformed 3T3 and revertant cell lines. *J. Cell. Physiol.* 95:203-212.
- DUFFY, M. J., & O'GRADY, P. (1984). Plasminogen activator and cancer. *Eur. J. Cancer Clin. Oncol.* 20:577-582.
- DULAK, N. C., & TEMIN, H. M. (1973). A partially purified polypeptide fraction from rat liver cell conditioned medium with multiplication-stimulating activity for embryo fibroblasts. *J. Cell. Physiol.* 81:153-160.
- DULBECCO, R. (1970). Topoinhibition and serum requirement of transformed and untransformed cells. *Nature* 227:802-806.
- DULBECCO, R., & ELKINGTON, J. (1973). Conditions limiting multiplication of fibroblastic and epithelial cells in dense cultures. *Nature* 246:197-199.
- DULBECCO, R., & ELKINGTON, J. (1975). Induction of growth in resting fibroblastic cell cultures by Ca^{2+} . *Proc. Natl. Acad. Sci. USA* 72:1584-1588.
- EAGLE, H. (1955a). The specific amino acid requirements of a human carcinoma cell (strain HeLa) in tissue culture. *J. Exp. Med.* 102:37-48.
- EAGLE, H. (1955b). The specific amino acid requirements of a mammalian cell (strain L) in tissue culture. *J. Biol. Chem.* 214:839-852.
- EAGLE, H., & PIEZ, K. A. (1962). The population-dependent requirement by cultured mammalian cells for metabolites which they can synthesise. *J. Exp. Med.* 116:29-43.
- EAGLE, H., BARBAN, S., LEVY, M., & SCHULZE, H. O. (1958). The utilisation of carbohydrates by human cell cultures. *J. Biol. Chem.* 233:551-558.
- EAGLE, H., OYAMA, V. I., LEVY, M., & FREEMAN, A. E. (1957). Myo-inositol as an essential growth factor for normal and malignant human cells in tissue culture. *J. Biol. Chem.* 226:191-205.

- EARLE, W. R., & NETTLESHIP, A. (1943). Production of malignancy in vitro. V. Results of injections of cultures into mice. J. Natl. Cancer Inst. 4:213-227.
- EARLE, W. R., SCHILLING, E. L., BRYANT, T. C., & EVANS, V. J. (1954). The growth of pure L strain mouse cells in fluid suspension cultures. J. Natl. Cancer Inst. 14:1159-1171.
- EASTMENT, C. T., & SIRBASKU, D. A. (1980). Human platelet lysate contains growth factor activities for established cell lines derived from various tissues of several species. In Vitro 16:694-705.
- EECKHOUT, Y., & VAES, G. (1977). Further studies on the activation of procollagenase, the latent precursor of bone collagenase. Effects of lysosomal cathepsin B, plasmin and kallikrein, and spontaneous activation. Biochem. J. 166:21-31.
- EMAMI, B., MITTAL, B. M., & SAPARETO, S. (1984). Sequencing of the total course of hyperthermia and irradiation. Cancer Res. (Supplement) 44:4731s-4732s.
- ENGEL, A., ALEXANDER, B., & PECHET, L. (1966). Activation of trypsinogen and plasminogen by thrombin preparations. Biochemistry 5:1543-1551.
- ENGELHARDT, D. L., LEE, G. T-Y., & MOLEY, J. F. (1979). Patterns of peptide synthesis in senescent and presenescent human fibroblasts. J. Cell. Physiol. 98:193-198.
- ENGSTROM, W., & ZETTERBERG, A. (1984). The relationship between purines, pyrimidines, nucleosides and glutamine for fibroblast cell proliferation. J. Cell. Physiol. 120:233-241.
- ENGSTROM, W., ZETTERBERG, A., & AUER, G. (1982). Calcium, phosphate and cell proliferation. In: "Ions, Cell Proliferation, and Cancer", (A. L. Boynton, W. L. McKeehan, and J. F. Whitfield, eds.), pp. 341-357. Academic Press, New York.
- EPHRUSSI, B. (1972). "Hybridisation of Somatic Cells". Princeton University Press, Princeton, New York.
- EPSTEIN, A. L., HERMAN, M. M., KIM, H., DORFMAN, R. F., PATH, M. R. C., & KAPLAN, H. S. (1976). Biology of the human malignant melanomas. III. Intra-cranial heterotransplantation in the nude, athymic mouse. Cancer 37:2158-2176.
- EPSTEIN, J. H. (1966). Ultraviolet light carcinogenesis. In: "Advances in Biology of Skin", Vol. 7, (W. Montagna & R. Dobson, eds.), pp. 215-236. Pergamon, New York.
- EPSTEIN, J. H., & EPSTEIN, W. L. (1962). Cocarcinogenic effect of

- ultraviolet light on DMBA tumor initiation in albino mice. J. Invest. Dermatol. 39:455-460.
- EPSTEIN, S., SMALL, M., FALK, H. L., & MONK, N. (1964). On the association between photodynamic and carcinogenic activities in polycyclic compounds. Cancer Res. 24:855-862.
- ERIKSON, J., AR-RU, A., DRWINGA, H. L. (1983). Transcriptional activation of the translocated c-myc oncogene in Burkitt's lymphoma. Proc. Natl. Acad. Sci. USA 80:820-824.
- ETHERINGTON, D. J. (1979). Proteinases in connective tissue breakdown. Ciba Found. Symp. 75:87-103.
- EVANS, E. P., BURTENSCHAW, M. D., BROWN, B. B., HENNION, R., & HARRIS, H. (1982). The analysis of malignancy by cell fusion. IX. Reexamination and clarification of the cytogenetic problem. J. Cell Sci. 56:113-130.
- FARBER, E., & CAMERON, R. (1980). The sequential analysis of cancer development. Adv. Cancer Res. 31:125-226.
- FEINBERG, A., & VOGELSTEIN, B. (1983). Hypomethylation of ras oncogenes in primary human cancers. Biochem. Biophys. Res. Commun. 111:47-54.
- FEINBERG, A. P., & COFFEY, D. S. (1982). In: "Tumor Cell Heterogeneity", (A. H. Owens, D. S. Coffey, & S. B. Baylin, eds.), pp. 469-494. Academic Press, New York.
- FISCHER, A. (1925). "Tissue Culture. Studies in Experimental Morphology and General Physiology of Tissue Cells in vitro". Levin & Munksgaard, Copenhagen.
- FISHER, H. W., PUCK, T. T., & SATO, G. (1958). Molecular growth requirements of single mammalian cells: the action of fetuin in promoting cell attachment to glass. Proc. Natl. Acad. Sci. USA 44:4-10.
- FISHER, P. B. (1983). Chemical-viral interactions in cell transformation. Cancer Invest. 1:495-509.
- FOLEY, E. J. (1953). Antigenic properties of methylcholanthrene-induced tumours in mice of the strain of origin. Cancer Res. 13:835-837.
- FOLKMAN, J., & GREENSPAN, H. P. (1975). Influence of geometry on control of cell growth. Biochem. Biophys. Acta 417:211-236.
- FOLKMAN, J., & MOSCONA, A. (1978). Role of cell shape in growth control. Nature 273:345-349.
- FOX, T. O., SHEPPARD, J. R., & BURGER, M. M. (1971). Cyclic membrane

changes in animal cells: transformed cells permanently display a surface architecture detected in normal cells only during mitosis. *Proc. Natl. Acad. Sci. USA* 68:244-247.

FREEDMAN, V. H., & SHIN, S. (1974). Cellular tumorigenicity in nude mice: correlation with cell growth in semi-solid medium. *Cell* 3:355-359.

FULLER, D. J. M., GERNER, E. W., & RUSSELL, D. H. (1977). Polyamine biosynthesis and accumulation during G₁ to S phase transition. *J. Cell. Physiol.* 93:81-88.

FURUYA, M., TAKAOKA, T., NAGAI, Y., & KATSUTA, H. (1971). Inositol requirement of mammalian cells in tissue culture. *Jpn. J. Exp. Med.* 41:471-475.

GAMOW, E. I., & PRESCOTT, D. M. (1970). The cell life cycle during early embryogenesis of the mouse. *Exp. Cell Res.* 59:117-123.

GAULDEN, M. E. (1956). DNA synthesis and X-ray effects at different mitotic stages in grasshopper neuroblasts. *Genetics* 41:645-660.

GAVOSTO, F., & PILERI, A. (1971). Cell cycle of cancer cells in man. In: "The Cell Cycle and Cancer", (R. Baserga, ed.), pp. 99-131. Dekker, New York.

GELBOIN, H. V. (1980). Benzo(a)pyrene metabolism, activation, and carcinogenesis: role and regulation of the mixed-function oxidases and related enzymes. *Physiol. Rev.* 60:1107-1166.

GELBOIN, H. V., KINOSHITA, N., & WIEBEL, F. J. (1972). Microsomal hydroxylases: induction and role in polycyclic hydrocarbon carcinogenesis and toxicity. *Fed. Proc.* 31:1298-1309.

GELFANT, S. (1977). A new concept of tissue and tumor cell proliferation. *Cancer Res.* 37:3845-3862.

GELFANT, S. (1981). Cycling-noncycling cell transitions in tissue aging, immunological surveillance, transformation, and tumor growth. *Int. Rev. Cytol.* 70:1-25.

GERNER, E. W., & RUSSELL, D. H. (1977). The relationship between polyamine accumulation and DNA replication kinetics in synchronised CHO cells after heat shock. *Cancer Res.* 37:482-489.

GERNER, E. W., CRESS, A. E., STICKNEY, D. G., HOLMES, D. K., & CULVER, P. S. (1980a). Factors regulating membrane permeability alter thermal resistance. *Annals N.Y. Acad. Sci.* 335:215-233.

GERWECK, L. E. (1977). Modification of cell lethality at elevated temperatures. The pH effect. *Radiat. Res.* 70:224-235.

- GERWECK, L. E., & BURLETT, P. H. (1978). In: "Cancer Therapy by Hyperthermia and Radiation", (C. Streffer, ed.), pp. 178-180. Urban and Schwarzenberg, Baltimore, Md.
- GERWECK, L. E., GILLETTE, E. L., & DEWEY, W. C. (1974a). Effect of heat and radiation on synchronous Chinese hamster cells: killing and repair. *Radiat. Res.* 59:139-140.
- GERWECK, L. E., GILLETTE, E. L., & DEWEY, W. C. (1974b). Killing of Chinese hamster cells in vitro by heating under hypoxic or aerobic conditions. *Eur. J. Cancer* 10:691-693.
- GERWECK, L. E., RICHARDO, B., JENNINGS, M. (1981). The influence of variable oxygen concentration on the response of cells to heat or X-irradiation. *Radiat. Res.* 85:314-320.
- GEY, G. O., COFFMAN, W. D., & KUBICEK, M. T. (1952). Tissue culture studies of the proliferative capacity of cervical carcinoma and normal epithelium. *Cancer Res.* 12:264-265.
- GIACOMONI, D. (1979). Tumorigenicity and intracisternal A-particle expression of hybrids between murine myeloma and lymphocytes. *Cancer Res.* 39:4481-4484.
- GIARD, D. G., AARONSON, S. A., TODARO, G. J., ARNSTEIN, P., KERSEY, J. H., DOSIK, H., & PARKS, W. P. (1973). In vitro cultivation of human tumors: establishment of cell lines derived from a series of solid tumors. *J. Natl. Cancer Inst.* 51:1417-1423.
- GILLIES, R. J. (1981). Intracellular pH and growth control in eukaryotic cells. In: "The Transformed Cell", (I. L. Cameron and T. B. Pool, eds.), pp. 347-395. Academic Press, New York.
- GIOVANELLA, B. C., & FOGH, J. (1978). Present and future trends in investigations with nude mouse as a recipient of human tumor transplants. In: "The Nude Mouse in Experimental and Clinical Research", (J. Fogh and B. C. Giovanella, eds.), pp. 281-312. Academic Press, New York.
- GIOVANELLA, B. C., STEHLIN, J. S., & YIM, S. O. (1980). Correlation of the thermosensitivity of cells to their malignant potential. *Annals N.Y. Acad. Sci.* 335:206-214.
- GIRALDI, T., NISI, C., & SAVA, G. (1977). Lysosomal enzyme inhibitors and antimetastatic activity in the mouse. *Eur. J. Cancer* 13:1321-1323.
- GJERSET, R. A., & MARTIN, D. W. (1982). Presence of a DNA demethylating activity in the nucleus of murine erythroleukemic cells. *J. Biol. Chem.* 257:8581-8583.
- GLASS, G. A., & GERSHON, D. (1981). Enzymatic changes in rat

erythrocytes with increasing cell and donor age: loss of superoxide dismutase activity associated with increases in catalytically defective forms. *Biochem. Biophys. Res. Commun.* 103:1245-1253.

GLAUMANN, H. (1970). Studies on the synthesis and transport of albumin in microsomal subfractions from rat liver. *Biochem. Biophys. Acta* 224:206-218.

GOH, S. H. (1979). A study of the metabolism of a novel dinucleoside polyphosphate (HS3) found in mammalian cells: possible regulation of nucleic acid biosynthesis by HS3 during step-down conditions. Ph.D. Thesis, University of Manitoba, Winnipeg, Manitoba, Canada.

GOH, S. H., & LeJOHN, H. B. (1977). Genetical and biochemical evidence that a novel dinucleoside polyphosphate coordinates salvage and de novo nucleotide biosynthetic pathways in mammalian cells. *Biochem. Biophys. Res. Commun.* 74:256-263.

GOH, S. H., & LeJOHN, H. B. (1978). Glucose transport in Achlya: characterisation and possible regulatory aspects. *Can. J. Biochem.* 56:246-256.

GOH, S. H., WRIGHT, J. A., & LeJOHN, H. B. (1977). Possible regulation of macromolecular biosynthesis in mammalian cells by a novel dinucleoside polyphosphate (HS3) produced during step-down growth regulation. *J. Cell. Physiol.* 93:353-362.

GOLDBERG, A. R. (1974). Involvement of cell and serum proteases in exposing tumor-specific agglutinin sites. *Annals N.Y. Acad. Sci.* 234:348-354.

GOSPODAROWICZ, D. (1975). Purification of a fibroblast growth factor from bovine pituitary. *J. Biol. Chem.* 250:2515-2526.

GOSPODAROWICZ, D., & MORAN, J. S. (1976). Growth factors in mammalian cell culture. *Ann. Rev. Biochem.* 45:531-558.

GOSPODAROWICZ, D., VLODAVSKY, I., GREENBERG, G., & JOHNSON, L. K. (1979). Cellular shape is determined by the extracellular matrix and is responsible for the control of cellular growth and function. In: "Hormones and Cell Culture", (R. Ross and G. Sato, eds.), pp. 561-592. Cold Spring Harbor, New York.

GRAF, T., & BEUG, H. (1978). Avian leukemia virus: interaction with their target cells in vivo and in vitro. *Biochem. Biophys. Acta* 516:269-299.

GRAFF, S., MOSER, H., KASTNER, O., GRAFF, A. M., & TANNENBAUM, M. (1965). The significance of glycolysis. *J. Natl. Cancer Inst.* 34:511-519.

- GRAVES, J. A. M. (1972). DNA synthesis in heterokaryons formed by fusion of mammalian cells from different species. *Exp. Cell Res.* 72:393-403.
- GRAY, L. H., CONGER, A. D., EBERT, M., HORNSEY, S., & SCOTT, C. A. (1953). The concentration of oxygen dissolved in tissues at the time of irradiation as a factor in radiotherapy. *Br. J. Radiol.* 26:636-648.
- GREENBERG, D. B., BARSH, G. S., TSUNG-SHANG, H., & CUNNINGHAM, D. D. (1977). Serum-stimulated phosphate uptake and initiation of fibroblast proliferation. *J. Cell. Physiol.* 90:193-210.
- GRINNELL, F., HAYS, D. G., & MINTER, D. (1977). Cell adhesion and spreading factor: partial purification and properties. *Exp. Cell Res.* 110:175-190.
- GROPPI, V. E., & COFFINO, P. (1980). G₁ and S phase mammalian cells synthesise histones at equivalent rates. *Cell* 21:195-204.
- GROSS, J., HIGHBERGER, J. H., JOHNSON-WINT, B., & BISWAS, C. (1980). Mode of action and regulation of tissue collagenases. In: "Collagenase in Normal and Pathological Connective Tissue", (D. E. Wooley and J. M. Evanson, eds.), pp. 11-35. John Wiley & Sons, Chichester.
- GRUNBERGER, D., & SANTELLA, R. M. (1983). Conformational changes in DNA induced by chemical carcinogens. In: "Genes and Proteins in Oncogenesis", (I. B. Weinstein & H. J. Vogel, eds.), pp. 13-40. Academic Press, New York.
- HAFF, R. J., & SWIM, H. E. (1956). Serial propagation of 3 strains of rabbit fibroblasts; their susceptibility to infection with vaccinia virus. *Proc. Soc. Exp. Biol. Med.* 93:200-204.
- HAHN, E. W., FEINGOLD, S. M., & KIM, J. H. (1977). Repair of normal tissue injury from local hyperthermia. *Radiat. Res.* 70:634 (abstr.).
- HAHN, G. M. (1974). Metabolic aspects of the role of hyperthermia in mammalian cell inactivation and their possible relevance to cancer treatment. *Cancer Res.* 34:3117-3123.
- HAHN, G. M. (1980). Comparison of the malignant potential of 10T½ cell and transformants with their survival responses to hyperthermia and amphotericin B. *Cancer Res.* 40:3763-3767.
- HAHN, G. M. (1982). "Hyperthermia and Cancer". Plenum Publishing Corp., New York.
- HAHN, G. M., LI, G. C., & SHIU, E. (1977). Interaction of amphotericin B and 43°C hyperthermia. *Cancer Res.* 37:761-764.

- HALL, E. J., & ROIZIN-TOWLE, L. (1984). Biological effects of heat. *Cancer Res. (Suppl.)* 44:4708s-4713s.
- HAM, R. G. (1974). Nutritional requirements of primary cultures. A neglected problem of modern biology. *In Vitro* 10:119-129.
- HAM, R. G. (1982). Importance of the basal nutrient medium in the design of hormonally defined media. In: "Growth of Cells in Hormonally Defined Media". Cold Spring Harbor Conference on Cell Proliferation, Vol. 9, (G. H. Sato, A. B. Pardee, & D. A. Sirbasku, eds.), pp. 39-60. Cold Spring Harbor, New York.
- HAM, R. G., & McKEEHAN, W. L. (1979). Media and growth requirements. *Methods in Enzymology* 58:44-93.
- HAM, R. G., & PUCK, T. T. (1962). Quantitative colonial growth of isolated mammalian cells. In: "Methods in Enzymology", (S. P. Colowick and N. O. Kaplan, eds.), Vol. 5, pp. 90-99. Academic Press, New York.
- HAMILTON, P. B. (1945). Glutamine: a major constituent of free amino acids in normal animal tissues and blood plasma. *J. Biol. Chem.* 158:397-409.
- HAMILTON, W. G., & HAM, R. G. (1977). Clonal growth of Chinese hamster cell lines in protein-free media. *In Vitro* 13:537-547.
- HAREL, L., BLAT, C., & CHATELAIN, G. (1983). Density-dependent inhibition of growth: fractionation of inhibitory diffusible factor(s) released by dense cultures of 3T3 cells. *Biol. Cell* 48:11-16.
- HAREL, L., CHATELAIN, G., & GOLDE, A. (1984). Density-dependent inhibition of growth: inhibitory diffusible factors from 3T3 and Rous sarcoma virus (RSV)-transformed 3T3 cells. *J. Cell. Physiol.* 119:101-106.
- HAREL, L., JULLIEN, M., & DeMONTI, M. (1978). Diffusible factor(s) controlling density inhibition of 3T3 cell growth: a new approach. *J. Cell. Physiol.* 96:327-332.
- HARISIADIS, L., MILLER, R. C., HARISIADIS, S., & HALL, E. J. (1980). Oncogenic transformation and hyperthermia. *Br. J. Radiol.* 53:479-482.
- HARLEY, C. B., POLLARD, J. W., CHAMBERLAIN, J. W., STANNERS, C. P., & GOLDSTEIN, S. (1980). Protein synthetic errors do not increase during aging of cultured human fibroblasts. *Proc. Natl. Acad. Sci. USA* 77:1885-1889.
- HARMAN, D. (1956). Aging: a theory based on free radical and radiation chemistry. *J. Gerontol.* 11:298-300.

- HARMAN, D. (1981). The aging process. *Proc. Natl. Acad. Sci. USA* 78:7124-7128.
- HARRIS, H. (1971). Cell fusion and the analysis of malignancy. *Proc. Royal Soc. London, Series B*, 179:1-20.
- HARRIS, H., & WATKINS, J. F. (1965). Hybrid cells derived from mouse and man: artificial heterokaryons of mammalian cells from different species. *Nature* 205:640-646.
- HARRIS, H., MILLER, O. J., KLEIN, G., WORST, P., & TACHIBANA, T. (1969). Suppression of malignancy by cell fusion. *Nature* 223:363-368.
- HART, R. W., & SETLOW, R. B. (1974). Correlation between deoxyribonucleic acid excision-repair and life-span in a number of mammalian species. *Proc. Natl. Acad. Sci. USA* 71:2169-2173.
- HART, R. W., & SETLOW, R. B. (1976). DNA repair in late-passage human cells. *Mech. Ageing Dev.* 5:67-77.
- HARTMAN, S. C. (1972). Glutaminase and gamma-glutamyl transferases. In: "The Enzymes", IV, (P. D. Boyer, ed.), pp. 79-100. Academic Press, New York.
- HARVEY, R. G. (1982). Polycyclic hydrocarbons and cancer. *Amer. Scientist* 70:386-393.
- HAVEMAN, J. (1978). The pH of the cytoplasm as an important factor in the survival of in vitro cultured cells after hyperthermia or irradiation. *Int. J. Radiat. Biol.* 34:557.
- HAYAKAWA, M. (1969). Progressive changes of the growth characteristics of the human diploid cells in serial cultivation in vitro. *Tohoku J. Exp. Med.* 98:171-179.
- HAYASHI, I., & SATO, G. H. (1976). Replacement of serum by hormones permits growth of cells in a defined medium. *Nature* 259:132-134.
- HAYFLICK, L. (1965). The limited in vitro lifetime of human diploid cell strains. *Exp. Cell Res.* 37:614-636.
- HAYFLICK, L. (1974). The longevity of cultured human cells. *J. Am. Geriatr. Soc.* 22:1-12.
- HAYFLICK, L., & MOORHEAD, P. (1961). The serial cultivation of human diploid cell strains. *Exp. Cell Res.* 25:585-621.
- HAYWARD, W. S., NEEL, B. G., & ASTRIN, S. M. (1981). Activation of a cellular onc gene by promoter insertion in ALV-induced lymphoid leukemia. *Nature* 290:475-479.
- HAYWARD, W. S., NEEL, B. G., & ASTRIN, S. M. (1982). Avian leukemia

- viruses: activation of cellular oncogenes. *Adv. in Viral Oncology* 1:207-233.
- HECKER, E., FUSENIG, N. E., KUNZ, W., MARKS, F., & THIELMANN, H. W. (1982). "Carcinogenesis: A Comprehensive Survey", Vol. 7. Raven Press, New York.
- HELDIN, C-H., & WESTERMARK, B. (1984). Growth factors: mechanism of action and relation to oncogenes. *Cell* 37:9-20.
- HELDIN, C-H., WASTESON, A., & WESTERMARK, B. (1977). Partial purification and characterisation of platelet factors stimulating the multiplication of normal human glial cells. *Exp. Cell Res.* 109:429-437.
- HELLMAN, S., BOTNICK, L. E., HANNON, E. C., & VIGNEULLE, R. M. (1978). Proliferative capacity of murine hematopoietic stem cells. *Proc. Natl. Acad. Sci. USA* 75:490-494.
- HEMS, R., STUBBS, M. & KREBS, H. A. (1968). Restricted permeability of rat liver for glutamate and succinate. *Biochem. J.* 107:807-815.
- HENLE, K. J., & DETHLEFSON, L. A. (1978). Heat fractionation and thermotolerance: a review. *Cancer Res.* 38:1843-1851.
- HENNINGS, H., SHORES, R., WENK, M. L., SPANGLER, E. F., TARONE, R., & YUSPA, S. (1983). Malignant conversion of mouse skin tumours is increased by tumour initiators and unaffected by tumour promoters. *Nature* 304:67-69.
- HENRY, N., & van LAMSWEERDE, A. L. (1983). Collagen degradation by metastatic variants of Lewis lung carcinoma: cooperation between tumor cells and macrophages. *Cancer Res.* 43:5321-5327.
- HEWITT, A., KLEINMAN, H. K., PENNYPACKER, J. P., & MARTIN, G. R. (1980). Identification of an adhesion factor for chondrocytes. *Proc. Natl. Acad. Sci. USA* 77:385-388.
- HEWITT, H. B. (1978). The choice of animal tumors for experimental studies of cancer therapy. *Adv. Cancer Res.* 27:149-200.
- HIGGINSON, J. (1979). Environmental carcinogenesis: a global perspective. In: "Environmental Carcinogenesis", (P. Emmelot & E. Kriek, eds.), pp. 9-24. Elsevier, North Holland.
- HILL, M., & HILLOVA, J. (1972). Virus recovery in chicken cells tested with Rous sarcoma cell DNA. *Nature New Biol.* 237:35-39.
- HOCHHAUSER, S. J., STEIN, J. L., & STEIN, G. S. (1981). Gene expression and cell cycle regulation. *Int. Rev. Cytol.* 71:95-243.
- HODGES, G. M., LIVINGSTON, D. C., & FRANKS, L. M. (1973). The

- localisation of trypsin in cultured mammalian cells. *J. Cell Sci.* 12:887-902.
- HOFFMAN, R. M. (1982). Methionine dependence in cancer cells - a review. *In Vitro* 18:421-428.
- HOFFMAN, R. M. (1984). Altered methionine metabolism, DNA methylation and oncogene expression in carcinogenesis. *Biochem. Biophys. Acta* 738:49-87.
- HOFFMAN, R. M., JACOBSEN, S. J., & ERBE, R. W. (1979). Reversion to methionine independence in Simian virus 40-transformed human and malignant rat fibroblasts is associated with altered ploidy and altered properties of transformation. *Proc. Natl. Acad. Sci. USA* 76:1313-1317.
- HOLLEY, R. W. (1975). Control of growth of mammalian cells in cell culture. *Nature* 258:489-490.
- HOLLEY, R. W., & KIERNAN, J. A. (1968). "Contact inhibition" of cell division in 3T3 cells. *Proc. Natl. Acad. Sci. USA* 60:300-304.
- HOLLEY, R. W., & KIERNAN, J. A. (1974). Control of the initiation of DNA synthesis in 3T3 cells: low molecular weight nutrients. *Proc. Natl. Acad. Sci. USA* 71:2942-2945.
- HOLLIDAY, R. (1975). Growth and death of diploid and transformed human fibroblasts. *Fed. Proc. Fed. Am. Soc. Exp. Biol.* 34:51-55.
- HOLLIDAY, R., & TARRANT, G. M. (1972). Altered enzymes in ageing human fibroblasts. *Nature* 238:26-30.
- HOLMES, R., HELMS, J., & MERCER, G. (1969). Cholesterol requirement of primary diploid human fibroblasts. *J. Cell Biol.* 42:262-271.
- HOLUB, M., ROSSMAN, P., TSASKOLOVA, H., & VIDMAROVA, H. (1975). Thymus rudiment of the athymic nude mouse. *Nature* 256:491-493.
- HOROWITZ, A., GREENEBAUM, E., & WEINSTEIN, I. B. (1981). Identification of receptors for phorbol ester tumor promoters in intact mammalian cells and of an inhibitor of receptor binding in biologic fluids. *Proc. Natl. Acad. Sci. USA* 78:2315-2319.
- HOROWITZ, M. L., KNOX, W. E., & MORRIS, H. P. (1969). Glutaminase activities and growth rates of rat hepatomas. *Cancer Res.* 29:1195-1199.
- HOUCK, J. C., & HENNINGS, H. (1973). Chalones: specific endogenous mitotic inhibitors. *FEBS Letts.* 32:1-8.
- HOUSLAY, M. D. (1981). Membrane phosphorylation: a crucial role in the action of insulin, EGF, and pp60^{src}. *Bioscience Rep.* 1:19-34.

- HOWARD, A., & PELC, S. R. (1951). Nuclear incorporation of p^{32} as demonstrated by autoradiographs. *Exp. Cell Res.* 2:178-187.
- HOWARD, B. V., & KRITCHEVSKY, D. (1969). The lipids of normal diploid (WI-38) and SV40-transformed human cells. *Int. J. Cancer* 4:393-402.
- HOWELL, A. N., & SAGER, R. (1978). Tumorigenicity and its suppression in hybrids of mouse and Chinese hamster cell lines. *Proc. Natl. Acad. Sci. USA* 75:2358-2362.
- HOWELL, A. N., & SAGER, R. (1979). Noncoordinate expression of SV40-induced transformation and tumorigenicity in mouse cell hybrids. *Somat. Cell Genet.* 5:129-143.
- HUANG, L-H., WANG, R., GAMA-SOSA, M. A., SHENOY, S., & EHRLICH, M. (1984). A protein from human placental nuclei binds preferentially to 5-methylcytosine-rich DNA. *Nature* 308:293-295.
- HUEBNER, R. J., & TODARO, G. J. (1969). Oncogenes of RNA tumor viruses as determinants of cancer. *Proc. Natl. Acad. Sci. USA* 64:1087-1094.
- HUGGINS, C. B., PATAKI, J., & HARVEY, R. G. (1967). Geometry of carcinogenic polycyclic aromatic hydrocarbons. *Proc. Natl. Acad. Sci. USA* 58:2253-2260.
- HUGHES, S. H., STUBBLEFIELD, E., PAYRAR, F., ENGEL, J. D., DODYSON, J. B., SPECTOR, D., CORDELL, B., SCHIMKE, R. T., & VARMUS, H. E. (1979). Gene localisation by chromosome fractionation: globin genes are on at least two chromosomes and three estrogen-inducible genes are on three chromosomes. *Proc. Natl. Acad. Sci. USA* 76:1348-1352.
- HULSER, D. F., & FRANK, W. (1971). Stimulation of embryonic rat cells in culture by a protein fraction isolated from fetal calf serum. I. Electrophysiological measurements at the cell surface membranes. *Z. Naturforsch* 26B:1045-1056.
- HUNTER, T. (1982). Phosphotyrosine - a new protein modification. *Trends Biochem. Sci.* 7:246-249.
- HUNTER, T., & COOPER, J. A. (1983). Role of tyrosine phosphorylation in malignant transformation by viruses and in cellular growth control. *Prog. Nucleic Acids Res. Mol. Biol.* 29:221-232.
- HUNTER, T., & COOPER, J. A. (1984). Tyrosine protein kinases and their substrates: an overview. *Adv. Cyclic Nucleo. Prot. Phos. Res.* 17:443-455.
- HUNTER, T., & SEFTON, B. M. (1980). Transforming gene product of Rous sarcoma virus phosphorylates tyrosine. *Proc. Natl. Acad. Sci. USA* 77:1311-1315.

- HYNES, R. O. (1976). Cell surface proteins and malignant transformation. *Biochem. Biophys. Acta* 458:73-107.
- HYNES, R. O. (1982). Phosphorylation of vinculin by pp60^{src}: what might it mean? *Cell* 28:437-438.
- INBAR, M., & SACHS, L. (1969). Interactions of the carbohydrates-binding compound concanavalin A with normal and transformed cells. *Proc. Natl. Acad. Sci. USA* 63:1418-1425.
- IQBAL, K., & OTTOWAY, J. H. (1970). Glutamine synthetase in muscle and kidney. *Biochem. J.* 119:145-156.
- IVERSEN, O. H. (1982a). Enhancement of methylnitrosourea skin carcinogenesis by inhibiting cell proliferation with hydroxyurea or skin chalone. *Carcinogenesis* 8:881-889.
- IVERSEN, O. H. (1982b). Hydroxyurea enhances methylnitrosourea skin tumorigenesis when given shortly (especially 30 min.) before, but not after, the carcinogen. *Carcinogenesis* 8:891-894.
- IVERSEN, O. H., & ASTRUP, E. G. (1984). The paradigm of two-stage carcinogenesis: a critical attitude. *Cancer Invest.* 2:51-60.
- IVERSEN, O. H., & IVERSEN, U. (1982). Must initiators come first? Tumorigenic and carcinogenic effects on skin of 3-methylcholanthrene and TPA in various sequences. *Br. J. Cancer* 45:912-920.
- JACKSON, M. J., & SHIN, S. (1982). Inositol as a growth factor for mammalian cells in culture. In: "Growth of Cells in Hormonally Defined Media", (G. H. Sato, A. B. Pardee and D. A. Sirbasku, eds.), pp. 75-86. Cold Spring Harbor Conferences on Cell Proliferation, Vol. 9. Cold Spring Harbor Laboratory, New York.
- JAINCHILL, J., & TODARO, G. J. (1970). Stimulation of cell growth in vitro by serum with and without growth factor: relation to contact and viral transformation. *Exp. Cell Res.* 54:137-146.
- JAKEN, S., & BLACK, P. H. (1979). Differences in intracellular distribution of plasminogen activation in growing, confluent, and transformed 3T3 cells. *Proc. Natl. Acad. Sci. USA* 76:246-250.
- JEKUNEN, A., PUUKA, M., & VILPO, J. (1983). Exclusion of exogenous 5-methyl-2'-deoxycytidine from DNA in human leukemic cells. A study with [2-¹⁴C]- and [methyl-¹⁴C]-5-methyl-2'-deoxycytidine. *Biochem. Pharmacol.* 32:1165-1168.
- JHA, K., CACCIAPUOTI, J., & OZER, H. (1978). Expression of transformation in cell hybrids. II. Non-suppression of the transformed phenotype in hybrids between chemically transformed and nontransformed derivatives of BALB/3T3. *J. Cell. Physiol.* 97:145-152.

- JOHNSON, R. T., & HARRIS, H. (1969). DNA synthesis and mitosis in fused cells. II. HeLa-chick erythrocyte heterokaryons. *J. Cell Sci.* 5:625-644.
- JONASSON, J., & HARRIS, H. (1977). The analysis of malignancy by cell fusion. VIII. Evidence for the intervention of an extrachromosomal element. *J. Cell Sci.* 24:255-263.
- JONASSON, J., POVEY, S., & HARRIS, H. (1977). The analysis of malignancy by cell fusion. VII. Cytogenetic analysis of hybrids between malignant and diploid cells and of tumors derived from them. *J. Cell Sci.* 24:217-254.
- JONES, P. A., & DECLERCK, Y. A. (1982). Extracellular matrix destruction by invasive tumor cells. *Cancer Metastasis Rev.* 1:289-317.
- JONES, P. A., LAUG, W. E., GARDNER, A., NYE, C. A., FINK, L. M., & BENEDICT, W. F. (1976). In vitro correlates of transformation in C3H/10T $\frac{1}{2}$ clone 8 mouse cells. *Cancer Res.* 36:2863-2867.
- JOSEPH, S. K., & MEIJER, A. J. (1981). The inhibitory effects of sulphhydryl reagents on the transport and hydrolysis of glutamine in rat liver mitochondria. *Eur. J. Biochem.* 119:523-529.
- KAJI, K., & MATSUO, M. (1978). Ageing of chicken embryo fibroblasts in vitro. II. Relationship between cell proliferation and increased multinuclear cells. *Mech. Ageing Dev.* 8:233-239.
- KAL, H. B., HATFIELD, M., & HAHN, G. M. (1975). Cell cycle progression of murine sarcoma cells after X-irradiation or heat shock. *Radiology* 117:215-217.
- KATZ, J., TROLL, W., ADLER, S. W., & LEVITZ, M. (1977). Antipain and leupeptin restrict uterine DNA synthesis and function in mice. *Proc. Natl. Acad. Sci. USA* 74:3754-3757.
- KAUFMAN, U., ZAPP, J., & FROESCH, E. R. (1977). Demonstration of specific serum carrier protein of nonsuppressible insulin-like activity in vivo. *J. Clin. Endocrinol. Metabol.* 44:160-165.
- KELLEY, R. O. (1976). Development of the aging cell surface: a freeze-fracture analysis of GAP junctions between human embryo fibroblasts aging in culture. A brief note. *Mech. Ageing Dev.* 5:339-345.
- KIHARA, H., & De LAFOR, S. D. (1968). Arginase in fetal calf serum. *Proc. Soc. Exp. Biol. Med.* 129:303-304.
- KIKKAWA, U., TAKAI, Y., TANAKA, Y., MYAKE, R., & NISHIZUKA, Y. (1983). Protein kinase C as a possible receptor protein of tumor-promoting phorbol esters. *J. Biol. Chem.* 258:11442-11445.

- KILLANDER, D., & ZETTERBERG, A. (1965). A quantitative cytochemical investigation of the relationship between cell mass and initiation of DNA synthesis in mouse fibroblasts in vitro. *Exp. Cell Res.* 40:12-20.
- KIM, J. H. (1984). Combined hyperthermia and radiation therapy in cancer treatment: current status. *Cancer Invest.* 2:69-80.
- KIM, J. H., KIM, S. H., & HAHN, E. W. (1983). Hypoxic cell radiosensitisation by moderate hyperthermia and glucose deprivation. *Radiat. Res.* 93:416-420.
- KINDRED, B. (1979). Nude mice in immunology. *Prog. Allergy* 26:137-238.
- KINSELLA, A. R., & RADMAN, M. (1978). Tumor promoter induces sister chromatid exchanges: relevance to mechanisms of carcinogenesis. *Proc. Natl. Acad. Sci. USA* 75:6149-6153.
- KIRSCHKE, H., KEMBHAVI, A. A., BOHLEY, P., & BARRETT, A. J. (1982). Action of rat liver cathepsin L on collagen and other substrates. *Biochem. J.* 201:367-372.
- KITOS, D. A., SINCLAIR, R., & WAYMOUTH, C. (1962). Glutamine metabolism by animals cells growing in a synthetic medium. *Exp. Cell Res.* 27:307-316.
- KLEIN, G. (1981). The role of gene dosage and genetic transpositions in carcinogenesis. *Nature* 294:313-318.
- KLEIN, G. (1983). Specific chromosomal translocations and the genesis of B-cell-derived tumors in mice and men. *Cell* 32:311-315.
- KLEIN, G., & KLEIN, E. (1977). Rejectionability of virus-induced tumors and nonrejectionability of spontaneous tumors: a lesson in contrasts. *Transplant. Proc.* 9:1095-1104.
- KLEVECZ, R. R., & FORREST, G. L. (1977). In: "Growth, Nutrition and Metabolism of Cells in Culture", (V. Cristofalo, ed.), Vol. III, Chap. 5, pp. 149-196. Academic Press, New York.
- KLINGER, H. P. (1980). Suppression of tumorigenicity in somatic cell hybrids. *Cytogenet. Cell Genet.* 27:254-266.
- KLINGER, H. P., & SHOWS, T. B. (1979). Human chromosomes which in cell hybrids suppress tumorigenicity of heteroploid Chinese hamster (CHO) cells. *Cytogenet. Cell Genet.* 25:172-173.
- KLINGER, H. P., BAIM, A. S., EUN, C. K., SHOWS, T. B., & RUDDLE, F. H. (1978). Human chromosomes which affect tumorigenicity in hybrids of diploid human with heteroploid human or rodent cells. *Cytogenet. Cell Genet.* 22:245-249.

- KNOX, P., & GRIFFITHS, S. (1979). A cell spreading factor in human serum that is not cold-insoluble globulin. *Exp. Cell Res.* 123:421-424.
- KNOX, W. E., HOROWITZ, M. L., & FRIEDEL, G. H. (1969). The proportionality of glutaminase content to growth rate and morphology of rat neoplasms. *Cancer Res.* 29:669-680.
- KNOX, W. E., TREMBLAY, G. C., SPANIER, B. B., & FRIEDEL, G. H. (1967). Glutaminase activities in normal and neoplastic tissues of the rat. *Cancer Res.* 27:1456-1458.
- KNUDSON, A. G. (1983). Hereditary cancers of man. *Cancer Invest.* 1:187-193.
- KNUDSON, A. G. (1985). Hereditary cancer, oncogenes, and antioncogenes. *Cancer Res.* 45:1437-1443.
- KOCH, G., & RICHTER, D. (eds.) (1979). "Regulation of Macromolecular Synthesis by Low Molecular Weight Mediators". Academic Press, New York.
- KODANA, Y., & TANAKA, K. (1978). Effects of urokinase on growth and metastasis of rabbit V2 carcinoma. *Gann.* 69:9-15.
- KOREN, R. (1980). The relevance of the state of growth and transformation of cells to their patterns of metabolic uptake. *Int. Rev. Cytol.* 68:127-172.
- KOVACEVIC, Z., & MORRIS, H. P. (1972). The role of glutamine in the oxidative metabolism of malignant cells. *Cancer Res.* 34:3403-3407.
- KRIEG, L., KUHLMANN, J., & MARKS, F. (1974). Effect of tumor-promoting phorbol esters and of acetic acid on mechanisms controlling DNA synthesis and mitosis (chalones) and on the biosynthesis of histidine-rich protein in mouse epidermis. *Cancer Res.* 34:3135-3146.
- KRIEK, E., & WESTRA, J. G. (1978). In: "Chemical Carcinogens and DNA", (P. L. Grover, ed.), Vol. II, pp. 1-28. CRC Press, Boca Raton, Florida.
- KRUSE, P. F., & MIEDEMA, E. (1965). Glucose uptake related to proliferation of animal cells in vitro. *Proc. Soc. Exp. Biol. Med.* 119:1110-1112.
- KUNNATH, L., & LOCKER, J. (1982). Variable methylation of the ribosomal RNA genes of the rat. *Nucleic Acids Res.* 10:3877-3892.
- KUROKI, T., HUBERMAN, E., HARQUARDT, H., SELKIRK, J. K., HEIDELBERGER, C., GROVER, P. L., & SIMS, P. (1971). Binding of K-region epoxides and other derivatives of benz(a)anthracene and

- dibenz(a,h)anthracene to DNA, RNA and proteins of transformable cells. *Chem. Biol. Interact.* 4:389-397.
- KUSANO, T., LONG, C., & GREEN, H. (1971). A new reduced human-mouse somatic cell hybrid containing the human gene for adenine phosphoribosyltransferase. *Proc. Natl. Acad. Sci. USA* 68:82-86.
- KWOCK, L., LIN, P. S., HEFTER, K., & WALLACH, D. F. H. (1978). Impairment of Na⁺-dependent amino acid transport in a cultured human T-cell line by hyperthermia and irradiation. *Cancer Res.* 38:83-87.
- LAERUM, O. D., & RAJEWSKY, M. F. (1975). Neoplastic transformation of fetal rat brain cells in culture after exposure to ethylnitrosourea in vivo. *J. Natl. Cancer Inst.* 55:1177-1187.
- LAJTHA, L. G. (1963). On the concept of the cell cycle. *J. Cell. Comp. Physiol.* 62:143-145.
- LANGBEHEIM, H., SHIH, T. Y., & SCOLNICK, E. M. (1980). Identification of a normal vertebrate cell protein related to the p21^{src} of Harvey murine sarcoma virus. *Virology* 106:292-300.
- LARSSON, A., & REICHARD, P. (1966). Enzymatic synthesis of deoxyribonucleotides: IX and X. *J. Biol. Chem.* 241:2533-2549.
- LAUG, W. E. (1979). Anchorage independent growth and plasminogen activator production by bovine endothelial cells. *Proc. Am. Assoc. Cancer Res.* 20:96-103.
- LAWRENCE, P. A. (1968). Mitosis and the cell cycle in the metamorphic moult of the milkweed bug Oncopeltus fasciatus. A radioautographic study. *J. Cell Sci.* 3:391-443.
- LEFFERT, H. L. (1982). Monovalent cations, cell proliferation and cancer: an overview. In: "Ions, Cell Proliferation and Cancer", (A. L. Boynton, J. F. Whitfield, & W. L. McKeehan, eds.), pp. 93-102. Academic Press, New York.
- LeJOHN, H. B., CAMERON, L. E., KLASSEN, G. R., & MEUSER, R. U. (1978). Effects of L-glutamine and HS compounds on growth and sporulation metabolism in Achlya. *Can. J. Biochem.* 56:227-236.
- LeJOHN, H. B., CAMERON, L. E., McNAUGHTON, D. R., & KLASSEN, G. R. (1975). Diguanosine nucleotides of fungi that regulate RNA polymerases isolated and partially characterised. *Biochem. Biophys. Res. Commun.* 66:460-467.
- LeJOHN, H. B., KLASSEN, G. R., & GOH, S. H. (1979). HS3 - a bizzare dinucleoside polyphosphate as a possible regulator of eukaryotes. In: "Regulation of Macromolecular Synthesis by Low Molecular Weight Mediators", (G. Koch & D. Richter, eds.), pp. 193-207. Academic Press, New York.

- LEVAN, A., LEVAN, G., & MITELMAN, F. (1977). Chromosomes and cancer. *Hereditas* 86:15-30.
- LEVINTOW, L., & EAGLE, H. (1961). Biochemistry of cultured mammalian cells. *Ann. Rev. Biochem.* 30:605-640.
- LEWIS, W. H., McNAUGHTON, D. R., GOH, S. H., LeJOHN, H. B., & WRIGHT, J. A. (1977). Inhibition of mammalian ribonucleotide reductase by a dinucleotide produced in eukaryotic cells. *J. Cell. Physiol.* 93:345-352.
- LEWIS, W. H., McNAUGHTON, D. R., LeJOHN, H. B., & WRIGHT, J. A. (1976). Regulation of fungal ribonucleotide reductase by unusual dinucleotides. *Biochem. Biophys. Res. Commun.* 71:128-135.
- LI, G. C. (1984). Thermal biology and physiology in clinical hyperthermia: current status and future needs. *Cancer Res. Suppl.* 44:4886s-4893s.
- LICHTI, U., YUSPA, S. H., & HENNINGS, S. (1978). Ornithine and S-adenosylmethionine decarboxylases in mouse epidermal cell cultures treated with tumour promoters. In: "Carcinogenesis, Vol. 2. Mechanisms of Tumor Promotion and Cocarcinogenesis", (T. J. Slaga, A. Sivak, & R. K. Boutwell, eds.), pp. 221-232. Raven Press, New York.
- LIEBERMAN, I., & OVE, P. (1957). Purification of a serum protein required by a mammalian cell in tissue culture. *Biochem. Biophys. Acta* 25:449-450.
- LIMA, L., & MACIEIRO-COELHO, A. (1972). Parameters of aging in chicken embryo fibroblasts cultivated in vitro. *Exp. Cell Res.* 70:279-284.
- LIN, P. S., KWOK, L., HEFTER, K., & WALLACH, D. F. H. (1978). Modification of rat thymocyte membrane properties by hyperthermia and ionising radiation. *Int. J. Radiat. Biol.* 33:371-382.
- LIN, P. S., WALLACH, D. F. H., & TSAI, S. (1973). Temperature-induced variations in the surface topology of cultured lymphocytes are revealed by scanning electron microscopy. *Proc. Natl. Acad. Sci. USA* 70:2492-2496.
- LINN, S., KAIRIS, M., & HOLLIDAY, R. (1976). Decreased fidelity of DNA polymerase activity isolated from aging human fibroblasts. *Proc. Natl. Acad. Sci. USA* 73:2818-2822.
- LINZER, D. I. H., & LEVINE, A. J. (1979). Characterisation of a 45K dalton cellular SV40 tumor antigen present in SV40-transformed cells and uninfected embryonal carcinoma cells. *Cell* 17:43-52.
- LIOTTA, L. A., THORGIERSSON, U. P., & GARBISA, S. (1982). Role of

collagenases in tumor cell invasion. *Cancer Metastasis Rev.* 1:277-288.

- LIOTTA, L. A., TRYGGVASON, K., GARBISA, S., GEHRON-ROBEY, P., & ABE, S. (1981). Partial purification and characterisation of a neutral protease which cleaves type IV collagen. *Biochemistry* 20:100-104.
- LIPETZ, J., & CRISTOFALO, V. J. (1972). Ultrastructural changes accompanying the ageing of human diploid cells in culture. *J. Ultrastruct. Res.* 39:43-56.
- LISKAY, M. R. (1977). Absence of a measurable G₂ phase in two Chinese hamster cell lines. *Proc. Natl. Acad. Sci. USA* 74:1622-1625.
- LISKAY, M. R., KARNFELD, D., FULLERTON, P., & EVANS, R. (1980). Protein synthesis and the presence or absence of a measurable G₁ in cultured Chinese hamster cells. *J. Cell. Physiol.* 104:461-467.
- LITTLEFIELD, J. W. (1964). Selection of hybrids from mating of fibroblasts in vitro and their presumed recombinants. *Science* 145:709-711.
- LOWDON, M., & VITOLS, E. (1973). Ribonucleotide reductase activity during the cell cycle of Saccharomyces cerevisiae. *Arch. Biochem. Biophys.* 158:177-184.
- LUND, P. (1974). Determination (of L-glutamine) with glutaminase and glutamate dehydrogenase. In: "Methods of Enzymatic Analysis, Vol. 4, (H. U. Bergmeyer, ed.), pp. 1719-1721. Verlag Chemie Weinheim/Academic Press, New York.
- LYONS, L. B., & THOMPSON, E. B. (1977). Delayed malignancy and altered growth properties of somatic cell hybrids between rat hepatoma and mouse L cells. *J. Cell. Physiol.* 90:179-192.
- MACARA, I. G. (1985). Oncogenes, ions, and phospholipids. *Am. J. Physiol.* 248:C3-C11.
- MACIEIRO-COELHO, A. (1983). Changes in membrane properties associated with cellular aging. *Int. Rev. Cytol.* 83:183-220.
- MACIEIRO-COELHO, A., PONTEN, A., & PHILIPSON, L. (1966). The division cycle and RNA synthesis in diploid human cells at different passage levels in vitro. *Exp. Cell Res.* 42:673-684.
- MacKENZIE, C. G., MacKENZIE, J. B., & BECK, P. (1961). The effect of pH on growth, protein synthesis and lipid-rich particles in cultured mammalian cells. *J. Biophys. Biochem. Cytol.* 9:141-156.
- MacPHERSON, I. (1970). The characteristics of animal cells transformed in vitro. *Adv. Cancer Res.* 13:169-215.

- MacPHERSON, I., & MONTAGNIER, L. (1964). Agar suspension culture for the selective assay of the cells transformed by polyoma virus. *Virology* 23:291-294.
- MAHLER, H. R. (1961). Interrelationships with enzymes. In: "Mineral Metabolism, Vol. 1, Part B, (C. L. Comar & F. Bronner, eds.), pp. 743-879. Academic Press, New York.
- MAHLER, H. R., & CORDES, E. H. (1971). "Biological Chemistry", 2nd edition, pp. 819-847. Harper and Row, New York.
- MANGUM, J., & NORTH, J. (1968). Vitamin B₁₂-dependent methionine biosynthesis in HEp-2 cells. *Biochem. Biophys. Res. Commun.* 32:105-110.
- MANGUM, J. H., MURRAY, B. K., & NORTH, J. A. (1969). Vitamin B₁₂-dependent methionine biosynthesis in cultured mammalian cells. *Biochemistry* 8:3496-3499.
- MARQUARDT, H., HUNKAPILLER, H. W., HOOD, L. E., TWARDZIK, D. R., DeLARCO, J. E., STEPHENSON, J. R., & TODARO, G. J. (1983). Transforming growth factors produced by retrovirus-transformed fibroblasts and human melanoma cells: amino acid sequence homology with epidermal growth factor. *Proc. Natl. Acad. Sci. USA* 80:4684-4688.
- MARSHALL, C., & DAVE, H. (1978). Suppression of the transformed phenotype in somatic cell hybrids. *J. Cell Sci.* 33:171-190.
- MARTIN, B. M., & QUIGLEY, J. P. (1977). Binding and uptake of thrombin: possible role in the thrombin-induced mitogenesis of chick embryo fibroblasts. In: "Chemistry and Biology of Thrombin", (R. L. Lundblad, J. W. Fenton II, and K. G. Nann, eds.), pp. 531-544. Ann Arbor Science, Ann Arbor, Michigan.
- MARTIN, G. S. (1970). Rous sarcoma virus: a function required for the maintenance of the transformed state. *Nature* 227:1021-1023.
- McBURNEY, M. W., & WHITMORE, G. F. (1974). Isolation and biochemical characterisation of folate deficient mutants of Chinese hamster cells. *Cell* 2:173-182.
- McCLELLAND, M., & IVARIE, R. (1982). Asymmetrical distribution of CpG in an "average" mammalian gene. *Nucleic Acids Res.* 10:7865-7877.
- McHALE, J. S., MOUTON, M. L., & McHALE, J. T. (1971). Limited culture lifespan of human diploid cells as a function of metabolic time instead of division potential. *Exp. Gerontol.* 6:89-93.
- McKEEHAN, W. L. (1977). The effect of temperature during the trypsin treatment on viability and multiplication potential of single normal human and chicken fibroblasts. *Cell Biol. Int. Rep.* 1:335-343.

- McKEEHAN, W. L. (1982a). Glycolysis, glutaminolysis and cell proliferation. *Cell Biol. Int. Rep.* 6:635-650.
- McKEEHAN, W. L. (1982b). Growth factor-nutrient interrelationships in control of normal and transformed cell proliferation. In: "Growth of Cells in Hormonally Defined Media". Cold Spring Harbor Conferences on Cell Proliferation, Vol. 9, Part A, (G. H. Sato, A. B. Pardee and D. A. Sirbasku, eds.), p. 65-74. Cold Spring Harbor Laboratory, New York.
- McKINNELL, R. G., & TARIN, D. (1984). Temperature-dependent metastasis of the Lucke renal carcinoma and its significance for studies on mechanisms of metastasis. *Cancer Metastasis Rev.* 3:373-386.
- McNAUGHTON, D. R., KLASSEN, G. R., & LeJOHN, H. B. (1975). Phosphorylated guanosine derivatives of eukaryotes: regulation of DNA-dependent RNA polymerases I, II, and III in fungal development. *Biochem. Biophys. Res.* 66:468-474.
- McNAUGHTON, D. R., KLASSEN, G. R., LOEWEN, P. C., & LeJOHN, H. B. (1978). Recharacterisation of fungal dinucleoside polyphosphate (HS3). *Can. J. Biochem.* 56:217-226.
- McNUTT, N. S., CULP, L. A., & BLACK, P. G. (1973). Contact-inhibited revertant cell lines isolated from SV40-transformed cells. IV. Microfilament distribution and cell shape in untransformed, transformed, and revertant BALB/c3T3 cells. *J. Cell Biol.* 56:412-428.
- MEANS, A. R., TASH, J. S., & CHAFOULEAS, J. G. (1982). Physiological implications of the presence, distribution, and regulation of calmodulin in eukaryotic cells. *Physiol. Revs.* 62:1-39.
- MELLMAN, W. J. (1971). A biochemical genetic view of human cell culture. *Adv. Hum. Genet.* 2:259-306.
- MEYER, R. R., & RIS, H. (1966). Incorporation of tritiated thymidine and tritiated deoxyadenosine into mitochondrial DNA of chick fibroblasts. *J. Cell Biol.* 31:76A.
- MICHL, J. (1977). *Cell Biol. Int. Rep.* 1:427-433.
- MICHL, J., & SPURNA, V. (1975). Pinocytosis as an essential transport mechanism of metazoan cells in culture. *Exp. Cell Res.* 93:39-46.
- MICHL, J., & SPURNA, V. (1976). Contact inhibition of pleiotropin pinocytosis as a critical factor in metazoan cell aging. *Gerontology* 22:56-78.
- MICHL, J., TOLAR, M., SPURNA, V., & REZACOVA, D. (1975). Growth-promoting alpha-globulin and ageing. In: "Cell Impairment in Aging and Development", (V. J. Cristofalo & E. Holeckova, eds.),

- pp. 137-146. Adv. Exp. Med. Biol., Vol. 53. Plenum Press, New York.
- MILCAREK, C., & ZAHN, K. (1978). The synthesis of ninety proteins including actin throughout the HeLa cell cycle. J. Cell Biol. 79:833-838.
- MILLER, D. A., & MILLER, O. J. (1983). Chromosomes and cancer in the mouse: studies in tumours, established cell lines, and cell hybrids. Adv. Cancer Res. 39:153-182.
- MILLER, E. C. (1978). Some current perspectives on chemical carcinogenesis in human and experimental animals: Presidential address. Cancer Res. 38:1479-1496.
- MILLER, E. C., & MILLER, J. A. (1960). The carcinogenicity of fluoro derivatives of 10-methyl-1,2-benzanthracene. I. 3- and 4-monofluoro derivatives. Cancer Res. 20:133-137.
- MILLER, E. C., & MILLER, J. A. (1976). The metabolism of chemical carcinogenesis to reactive electrophiles and their possible mechanism of action in carcinogenesis. In: "Chemical Carcinogens", (C. E. Searle, ed.), pp. 737-762. American Chemical Society, Washington D.C.
- MILLER, E. J. (1984). Chemistry of the collagens and their distribution. In: "Extracellular Matrix Biochemistry", (K. A. Piez and A. H. Reddi, eds.), pp. 41-82. Elsevier, New York.
- MILLER, J. A. (1970). Carcinogenesis by chemicals: an overview. G. H. A. Clowes Memorial Lecture. Cancer Res. 30:559-576.
- MINTZ, B., & ILLMENSEE, K. (1975). Normal genetically mosaic mice produced from malignant teratocarcinoma cells. Proc. Natl. Acad. Sci. USA 72:3585-3589.
- MITCHISON, J. M. (1969). Enzyme synthesis in synchronous cultures. Science 165:657-663.
- MITCHISON, J. M. (1971). "The Biology of the Cell Cycle". Cambridge University Press, London.
- MITSUI, Y., & SCHNEIDER, E. L. (1976). Increased nuclear sizes in senescent human diploid fibroblast cultures. Exp. Cell Res. 100:147-152.
- MITSUI, Y., SMITH, J. R., & SCHNEIDER, E. L. (1981). Equivalent proliferation potential of different size classes of human diploid fibroblasts. J. Gerontol. 36:416-419.
- MONNAT (Jr.), R. J., & LOEB, L. A. (1983). Mechanisms of neoplastic transformation. Cancer Invest. 1:175-183.

- MONTESANO, R., DEVRON, C., KUROKI, T., SAINT-VINCENT, L., HANDLEMAN, S., SANFORD, K. K., DEFAO, D., & WEINSTEIN, I. B. (1977). Test for malignant transformation of rat liver cells in culture: cytology, growth in soft agar, and production of plasminogen activator. *J. Natl. Cancer Inst.* 59:1651-1658.
- MOOLENAAR, W. H., BONNSTRA, J., van der SAAG, P. T., & de LAAT, S. W. (1981). Sodium/proton exchange in mouse neuroblastoma cells. *J. Biol. Chem.* 256:12883-12887.
- MOOLGAVKAR, S. H., KNUDSON, A. G. (1981). Mutation and cancer: a model for human carcinogenesis. *J. Natl. Cancer Inst.* 66:1037-1052.
- MORELL, B., & FROESCH, E. R. (1973). Fibroblasts as an experimental tool in metabolic and hormone studies. I. Growth and glucose metabolism of fibroblasts in culture. *Eur. J. Clin. Invest.* 3:112-118.
- MORGAN, R. A., INGE, K. L., & CHRISTOPHER, C. W. (1981). Localisation and characterisation of N-ethyl maleimide sensitive inhibitor(s) of thiol cathepsin activity from cultured Nil and polyoma virus-transformed Nil hamster cells. *J. Cell. Physiol.* 108:55-66.
- MORRIS, H. P., WAGNER, B. P., & MERANZE, D. R. (1970). Transplantable adenocarcinomas of the rat kidney possessing different growth rates. *Cancer Res.* 30:1362-1369.
- MOSCATELLI, D., SANUI, H., & RUBIN, H. (1975). Effects of depletion of K^+ , Na^+ , or Ca^{2+} on DNA synthesis and cell cation content in chick embryo fibroblasts. *J. Cell. Physiol.* 101:117-128.
- MOTT, D. M., FABISCH, P. H., SANI, B. P., & SOROF, S. (1974). Lack of correlation between fibrinolysis and the transformed state of cultured mammalian cells. *Biochem. Biophys. Res. Commun.* 61:621-627.
- MOYER, M. P., & MOYER, R. C. (1981). Virus-induced transformation. In: "The Transformed Cell", (I. L. Cameron & T. B. Pool, eds.), pp. 189-234. Academic Press, New York.
- MUFSON, R. A., FISHER, P. B., & WEINSTEIN, I. B. (1978). Phorbol esters produce a delay in the expression of melanogenesis by B-16 melanoma cells. *Proc. Am. Assoc. Cancer Res.* 19:183.
- MULLINS, D. E., & ROHRLICH, S. T. (1983). The role of proteinases in cellular invasiveness. *Biochem. Biophys. Acta* 695:177-214.
- MURPHY, G., CAWSTON, T. E., GALLOWAY, W. A., BARNES, M. J., BUNNING, R. A. D., MERCER, E., REYNOLDS, J. J., & BURGESSON, R. E. (1981). Metalloproteinases from rabbit bone culture medium degrades types IV and V collagens, laminin and fibronectin. *Biochem. J.* 199:807-811.

- MURRAY, V. & HOLLIDAY, R. (1981). Increased error frequency of DNA polymerases from senescent human fibroblasts. *J. Mol. Biol.* 146:55-76.
- NATRAJ, C. V., & DATTA, P. (1978). Control of DNA synthesis in growing BALB/c3T3 mouse cells by a fibroblast growth regulatory factor. *Proc. Natl. Acad. Sci. USA* 75:6115-6119.
- NELSON, R. L., & BRANTON, P. E. (1984). Identification, purification, and characterisation of phosphotyrosine-specific protein phosphatases from cultured chicken embryo fibroblasts. *Mol. Cell. Biol.* 4:1003-1012.
- NEVILLE, D. M., & GLOSSMAN, H. (1974). Molecular weight determination of membrane protein and glycoprotein subunits by discontinuous gel electrophoresis in dodecyl sulfate. In: "Methods in Enzymology", (S. Fleischer & L. Packer, eds.), Vol. 32, pp. 92-102. Academic Press, New York.
- NEWBOLD, R. F., OVERALL, R. W., & CONNELL, J. R. (1982). Induction of immortality is an early event in malignant transformation of mammalian cells by carcinogens. *Nature* 299:633-635.
- NEWCOMB, E. W., SILVERSTEIN, S. C., & SILAGI, S. (1978). Malignant mouse melanoma cells do not form tumors when mixed with cells of a nonmalignant subclone: relationships between plasminogen activator expression by the tumor cells and the host's immune response. *J. Cell. Physiol.* 95:169-178.
- NIELSEN, P. J., & RYAN, J. M. (1981). Cumulative population doublings as the determinant of chick cell lifespan in vitro. *J. Cell. Physiol.* 107:371-378.
- NISHIZUKA, Y. (1984a). The role of protein kinase C in cell surface signal transduction and tumor promotion. *Nature* 308:693-698.
- NISHIZUKA, Y. (1984b). Protein kinases in signal transduction. *Trends Biochem. Sci.* 9:163-166.
- NORWOOD, T. H., PENDERGRASS, W. R., SPRAGUE, C. A., & MARTIN, G. M. (1974). Dominance of the senescent phenotype in heterokaryons between replicative and post-replicative human fibroblast-like cells. *Proc. Natl. Acad. Sci. USA* 71:2231-2235.
- NOWELL, P. C. (1974). In: "Chromosomes and Cancer", pp. 267-285. John Wiley, New York.
- O'BRIEN, T. G., & DIAMOND, L. (1978). Ornithine decarboxylase polyamines and tumor promoters. In: "Carcinogenesis, Vol. 2: Mechanisms of Tumor Promotion and Cocarcinogenesis", (T. J. Slaga, A. Sivak, & R. K. Boutwell, eds.), pp. 273-287. Raven Press, New York.

- O'BRIEN, T. G., SIMSIMAN, R. C., & BOUTWELL, R. K. (1975a). Induction of the polyamine biosynthetic enzymes in mouse epidermis by tumour promotion agents. *Cancer Res.* 35:1662-1670.
- O'BRIEN, T. G., SIMSIMAN, R. C., & BOUTWELL, R. K. (1975b). Induction of the polyamine biosynthetic enzymes in mouse epidermis and their specificity for tumor promotion. *Cancer Res.* 35:2426-2433.
- OHNO, S. (1974). In: "Chromosomes and Cancer", pp. 77-94. John Wiley, New York.
- OKADA, Y., & TODOKORO, J. (1962). Analysis of giant polynuclear cell formation caused by HVJ virus from Ehrlich's ascites tumor cells. *Exp. Cell Res.* 26:108-118.
- OLDEN, K., PRATT, R. M., & YAMADA, K. M. (1979). Role of carbohydrate in biological function of the adhesive glycoprotein fibronectin. *Proc. Natl. Acad. Sci. USA* 76:3343-3347.
- O'MEARA, R. A. Q. (1958). Coagulative properties of cancers. *Ir. J. Med. Sci.* 394:474-479.
- OREN, M., MALTZMAN, W., & LEVINE, A. J. (1981). *Mol. Cell. Biol.* 1:101-110.
- ORGEL, L. E. (1963). The maintenance of the accuracy of protein synthesis and its relevance to aging. *Proc. Natl. Acad. Sci. USA* 49:517-521.
- ORGEL, L. E. (1973). Ageing of clones of mammalian cells. *Nature* 243:441-445.
- OSSOWSKI, L., QUIGLEY, J. P., KELLERMAN, G. M., & REICH, E. (1973). Fibrinolysis associated with oncogenic transformation. I. Requirement of plasminogen for correlated changes in cellular morphology, colony formation in agar, and cell migration. *J. Exp. Med.* 138:1056-1064.
- OUTZEN, H. C., & CUSTER, R. P. (1975). Growth of human normal and neoplastic mammary tissues in the cleared mammary fat pad of the nude mouse. *J. Natl. Cancer Inst.* 55:1461-1463.
- OVERGAARD, J. (1977). Effect of hyperthermia on malignant cells in vivo: a review and a hypothesis. *Cancer* 39:2637-2646.
- OVERGAARD, J., & NIELSEN, O. S. (1980). The role of tissue environmental factors on the kinetics and morphology of tumor cells exposed to hyperthermia. *Annals N.Y. Acad. Sci.* 335:254-280.
- OWENS, O., GEY, G. O., & GEY, M. K. (1953). A new method for the cultivation of mammalian cells suspended in agitated fluid medium. *Proc. Am. Assoc. Cancer Res.* 1:41.

- OYOMADA, I., KITAOKO, M., ARAKI, R., YOSHIOKA, H., & USUKU, G. (1983). Collagenase from the culture medium of rabbit colon wall with special reference to the latent type and substrate susceptibility. *Gastroenterology* 85:376-383.
- OZER, H., & JHA, K. (1977). Malignancy and transformation: expression in somatic cell hybrid variants. *Adv. Cancer Res.* 25:53-94.
- PAINTER, R. H., & CHARLES, A. F. (1962). Characterisation of a soluble plasminogen activator from kidney cell cultures. *Amer. J. Physiol.* 202:1125-1130.
- PALL, M. L. (1981). Gene amplification model of carcinogenesis. *Proc. Natl. Acad. Sci. USA* 78:2465-2468.
- PARANJPE, M., ENGEL, L., YOUNG, N., & LIOTTA, L. A. (1980). Activation of breast carcinoma collagenase through plasminogen activator. *Life Sci.* 26:1223-1231.
- PARDEE, A. B. (1964). Cell division and a hypothesis of cancer. *Natl. Cancer Inst. Monogr.* 14:7-10.
- PARDEE, A. B. (1974). A restriction point for control of normal animal cell proliferation. *Proc. Natl. Acad. Sci. USA* 71:1286-1290.
- PARDEE, A. B., DUBROW, R., HAMLIN, J. L., & KLEITZIEN, R. F. (1978). Animal cell cycle. *Ann. Rev. Biochem.* 47:715-750.
- PARKER, L., & SMITH, J. R. (1974). Extension of the lifespan of cultured normal diploid cells by vitamin E. *Proc. Natl. Acad. Sci. USA* 71:4763-4767.
- PATAKI, J., & HUGGINS, C. (1969). Molecular site of substituents of benz(a)anthracene related to carcinogenicity. *Cancer Res.* 29:506-509.
- PATEK, P. Q., COLLINS, J. L., & COHN, M. (1978). Transformed cell lines susceptible or resistant to in vivo surveillance against tumorigenesis. *Nature* 276:510-511.
- PATTERSON (Jr.), M. K. (1972). Uptake and utilisation of amino acids by cell cultures. In: "Growth, Nutrition and Metabolism of Cells in Culture", (G. H. Rothblath and V. J. Cristofalo, eds.). Academic Press, London.
- PATTERSON (Jr.), M. K. (1979). Measurement of growth and viability of cells in culture. In: "Methods in Enzymology", Vol. 58, (W. B. Jakoby & I. H. Psatan, eds.), pp. 141-152. Academic Press, New York.
- PAULI, B. U., SCHWARTZ, D. E., THONAR, E. J. M., & KUETTNER, K. E. (1983). Tumor invasion and host extracellular matrix. *Cancer Metastasis Rev.* 2:129-152.

- PEDERSON, T. (1976). In: "Protein Synthesis, Vol. 2", (E. H. McConkey, ed.), pp. 69-123. Marcel Dekker, New York.
- PEEHL, D. M., & STANBRIDGE, E. J. (1981). Anchorage-independent growth of normal human fibroblasts. *Proc. Natl. Acad. Sci. USA* 78:3053-3057.
- PEGG, A. E. (1984). Methylation of the O⁶ position of guanine in DNA is the most likely initiating event in carcinogenesis by methylating agents. *Cancer Invest.* 2:223-231.
- PENDERGRASS, W. R., SAULEWICZ, A. C., BURMER, G. C., RABINOVITCH, P. S., NORWOOD, T. H., & MARTIN, G. M. (1982). Evidence that a critical threshold of DNA polymerase-alpha activity may be required for the initiation of DNA synthesis in mammalian cell heterokaryons. *J. Cell. Physiol.* 113:141-151.
- PETERSON, H. I., PETRUSSON, B., & KORSAN-BENGSTON, K. (1973). Fibrinolytic activity of human carcinomas. *Throm. Diath. Haemorrh.* 30:133-137.
- PFHOL-LESZKOWICZ, A., SALAS, C., FUCHS, R. P. P., & DIRHEIMER, G. (1981). Mechanism of initiation of enzymatic deoxyribonucleic acid methylation by 2-(acetylamino)fluorene bound to deoxyribonucleic acid. *Biochemistry* 20:3020-3024.
- PIEZ, K. A. (1984). Molecular and aggregate structures of the collagens. In: "Extracellular Matrix Biochemistry", (K. A. Piez and A. H. Reddi, eds.), pp. 1-39. Elsevier, New York.
- POILEY, J. A., SCHUMAN, R. F., & PIENTA, J. R. (1978). Characterisation of normal human embryo cells grown to over 100 population doublings. *In Vitro* 14:405-412.
- POLLACK, R., & RIFKIN, D. (1975). Actin-containing cables within anchorage-dependent rat embryo cells are dissociated by plasmin and trypsin. *Cell* 6:495-506.
- POLLACK, Y., STEIN, R., RAZIN, A., & CEDAR, H. (1980). Methylation of foreign DNA sequences in eukaryotic cells. *Proc. Natl. Acad. Sci. USA* 77:6463-6467.
- PONDER, B. A. J. (1980). Genetics and cancer. *Biochem. Biophys. Acta* 605:369-410.
- PONTECORVO, G. (1975). Production of mammalian somatic cell hybrids by means of polyethylene glycol treatment. *Somat. Cell Genet.* 1:397-400.
- PONTEN, J. (1976). The relationship between in vitro transformation and tumor formation in vivo. *Biochem. Biophys. Acta.* 458:397-422.

- PORTER, K., PRESCOTT, D., & FRYE, J. (1973). Changes in surface morphology of Chinese hamster ovary cells during the cell cycle. *J. Cell Biol.* 57:815-836.
- POTT, P. (1775). Chirurgical observations. Reprinted in: *Natl. Cancer Inst. Monogr.* 10:7-13 (1963).
- POTTER, V. R. (1983). Cancer as a problem in intercellular communication: regulation by growth-inhibiting factors (chalones). *Prog. Nucleic Acid Res. Mol. Biol.* 29:161-173.
- PREHN, R. T. (1975). Relationship of tumor immunogenicity to concentration of the oncogen. *J. Natl. Cancer Inst.* 55:189-190.
- PRESCOTT, D. M. (1976). The cell cycle and the control of cellular reproduction. *Adv. Genet.* 18:99-177.
- PRITCHARD, R. H., BARTH, P. T., & COLLINS, J. (1969). Control of DNA synthesis in bacteria. *Symp. Soc. Gen. Microbiol.* 19:263-297.
- PUCK, T. T., & MARCUS, P. I. (1955). A rapid method for viable cell titration and clone production with HeLa cells in tissue culture: the use of X-irradiated cells to supply conditioning factors. *Proc. Natl. Sci. USA* 41:432-437.
- QUIGLEY, J. P. (1979). Phorbol ester-induced morphological changes in transformed chick fibroblasts: evidence for direct catalytic involvement of plasminogen activator. *Cell* 17:131-141.
- RAAPHORST, G. P., & AZZAM, E. J. (1982). Comparisons of fixation of heat radiation and heat plus radiation damage by anisotonic sodium chloride solutions. *Natl. Cancer Inst. Monogr.* 61:95-97.
- RAAPHORST, G. P., & AZZAM, E. I. (1983). Thermal sensitivity of Chinese hamster V79 cells related to time after plating and cell cycle redistribution. *J. Therm. Biol.* 8:327-332.
- RAAPHORST, G. P., AZZAM, E. I., SARGENT, M. D., EINSPENNER, M., & BORSA, J. (1981). The oncogenic potential of X-rays and/or hyperthermia in C3H-10T $\frac{1}{2}$ cells. *Br. J. Radiol.* 54:638-639.
- RAAPHORST, G. P., FREY, H. E., & KRUUV, J. (1978). Effects of salt solutions on the radiosensitivity of mammalian cells. IV. Treatment with NaCl solutions containing ouabain, NEM, PNAPP, cysteamine or DMSO. *Int. J. Radiat. Biol.* 33:341-356.
- RAAPHORST, G. P., SZEKELY, J., LOBREAU, A., & AZZAM, E. I. (1983). A comparison of cell killing by heat and/or X-rays in Chinese hamster V79 cells, Friend erythroleukemia mouse cells, and human thymocyte MOLT-4 cells. *Radiat. Res.* 94:340-349.
- RABINOVITZ, M., OLSON, M. E., & GREENBURG, D. M. (1956). Role of

- glutamine in protein synthesis by the Ehrlich ascites cells. *J. Biol. Chem.* 222:879-893.
- RADMAN, M., VILLANI, G., BOITEUX, S., DEFAIS, M., CAILLET-FAUQUET, P., & SPADARI, S. (1977). On the mechanism and genetic control of mutagenesis induced by carcinogenic mutagens. In: "Origins of Human Cancer", (H. H. Hiatt, J. D. Watson, & J. A. Winsten, eds.), pp. 903-922. Cold Spring Harbor Laboratory, New York.
- RAICK, A. N. (1974). Cell differentiation and tumor promoting activity in skin carcinogenesis. *Cancer Res.* 34:2915-2925.
- RAICK, A. N., & BURDZY, K. (1973). Ultrastructural and biochemical changes induced in mouse epidermis by a hyperplastic agent, ethylphenylpropiolate. *Cancer Res.* 33:2221-2230.
- RAO, P. N., & JOHNSON, R. T. (1970). Mammalian cell fusion: studies on the regulation of DNA synthesis and mitosis. *Nature* 225:159-164.
- RAO, P. N., SUNKARA, P. S., & WILSON, B. A. (1977). *Cell Biol.* 74:2869-2873.
- RAPP, F. (1983). Viral carcinogenesis. *Int. Rev. Cytol.*, Supplement 15:203-244.
- RASMUSSEN, H. (1970). Cell communication, calcium ion, and cyclic adenosine monophosphate. *Science* 170:404-412.
- RAZIN, A., & RIGGS, A. (1980). DNA methylation and gene function. *Science* 210:604-610.
- RECHAVI, G., GIVOL, D., & CANAANI, E. (1982). Activation of a cellular oncogene by DNA rearrangement: possible involvement of an IS-like element. *Nature* 300:607-611.
- RECKLIES, A. D., MORT, J. S., & POOLE, A. R. (1982). Secretion of a thiol proteinase from mouse mammary carcinomas and its characterisation. *Cancer Res.* 42:1026-1032.
- REICH, E., RIFKIN, D. B., & SHAW, E. (eds.) (1975). "Protease and Biological Control". Cold Spring Harbor Laboratory, New York.
- REITZER, L. J., WICE, B. M., & KENNEL, D. (1979). Evidence that glutamine, not sugar, is the major energy source for cultured HeLa cells. *J. Biol. Chem.* 254:2669-2676.
- RENNER, E. D., PLAGEMANN, P. G. W., & BERNLOHR, R. W. (1972). Permeation of glucose by simple and facilitated diffusion by Novikoff rat hepatoma cells in suspension culture and its relationship to glucose metabolism. *J. Biol. Chem.* 18:5765-5776.
- REZNIKOFF, C. A., BRANKOW, D. W., & HEIDELBERGER, C. (1973a).

Establishment of a cloned line of C3H mouse embryo cells sensitive to postconfluence inhibition of division. *Cancer Res.* 33:3231-3238.

REZNIKOFF, C. A., BERTRAM, J. S., BRANKOW, D. W., & HEIDELBERGER, C. (1973b). Quantitative and qualitative studies of chemical transformation of cloned C3H mouse embryo cells sensitive to postconfluence inhibition of cell division. *Cancer Res.* 33:3239-3249.

RICK, W. (1974). Measurement (of protease activity) with N-p-toluene-sulfonyl-L-arginine methyl ester as substrate. In: "Methods of Enzymatic Analysis, Vol. 2", (H. U. Bergmeyer, ed.), pp. 1021-1024. Verlag Chemie Weinheim/Academic Press, New York.

RIFKIN, D. B., & POLLACK, R. (1977). Production of plasminogen activator by established cell lines of mouse origin. *J. Cell Biol.* 73:47-55.

RIFKIN, D. B., & LOEB, J. N., MOORE, G., & REICH, E. (1974). Properties of plasminogen activators formed by neoplastic human cell cultures. *J. Exp. Med.* 139:1317-1328.

RIGGS, A. D., & JONES, P. A. (1983). 5-methylcytosine, gene regulation, and cancer. *Adv. Cancer Res.* 40:1-30.

RINGER, S. (1882). *J. Physiol.* 4:29-42.

RISTOW, H. J., & PAUL, D. J. (1979). Cell cycle control by Ca^{2+} ions in mouse 3T3 cells and in transformed 3T3 cells. *J. Cell. Physiol.* 98:31-40.

ROBBINS, E., & SCHARFF, M. D. (1967). The absence of a detectible G_1 phase in a cultured strain of Chinese hamster lung cell. *J. Cell Biol.* 34:684-686.

ROBBINS, E., LEVINE, E. M., & EAGLE, H. (1970). Morphologic changes accompanying senescence of cultured human diploid cells. *J. Exp. Med.* 131:1211-1222.

ROBERTS, E., & SIMONSEN, D. G. (1960). Free amino acids and similar substances in normal and neoplastic tissue. In: "Amino Acids, Proteins and Cancer Biochemistry", (J. T. Edsall, ed.), pp. 127-145. Academic Press, New York.

ROBERTS, J. W., & ROBERTS, C. W. (1975). Proteolytic cleavage of bacteriophage lambda repressor in induction. *Proc. Natl. Acad. Sci. USA* 72:147-151.

ROBINS, D. M., RIPLEY, S., HENDERSON, A. S., & AXEL, R. (1981). Transforming DNA integrates into the host chromosome. *Cell* 23:29-39.

- ROBINSON, J. E., WIZENBERG, M. J., & MCCREADY, W. A. (1974). Combined hyperthermia and radiation suggest an alternative to heavy particle therapy for reduced oxygen enhancement ratio. *Nature* 251:521-522.
- ROBINSON, J. H., SMITH, J. A., & DEE, L. A. (1976). Synthesis and degradation of proteins in cultured, androgen-responsive tumor cells. *Exp. Cell Res.* 102:117-126.
- ROBLIN, R., CHOU, I-N., & BLACK, P. H. (1975). Proteolytic enzymes, cell surface changes, and viral transformation. *Adv. Cancer Res.* 22:203-260.
- ROHME, D. (1981). Evidence for a relationship between longevity of mammalian species and lifespans of normal fibroblasts in vitro and of erythrocytes in vivo. *Proc. Natl. Acad. Sci. USA* 78:5009-5013.
- ROSS, R., & VOGEL, A. (1978). The platelet-derived growth factor. *Cell* 14:203-210.
- ROSS, R., RAINES, E., BOWEN-POPE, D., & GLENN, K. (1982). General concepts of growth factors: a progress report on platelet-derived growth factor. In: "Growth of Cells in Hormonally Defined Media", (G. H. Sato, A. B. Pardee, & D. A. Sirbasku, eds.). Cold Spring Harbor Conferences on Cell Proliferation, Vol. 9, pp. 27-35. Cold Spring Harbor Laboratory, New York.
- ROSSMAN, T. G., & TROLL, W. (1980). Protease inhibitors in carcinogenesis: possible sites of action. In: "Carcinogenesis, Vol. 5: Modifiers of Chemical Carcinogenesis", (T. J. Slaga, ed.), pp. 127-143. Raven Press, New York.
- ROSSOW, P. W., RIDDLE, V. G. H., & PARDEE, A. B. (1979). Synthesis of labile serum-dependent protein in early G₁ controls animal cell growth. *Proc. Natl. Acad. Sci. USA* 76:4446-4450.
- ROST, T. L. (1977). In: "Mechanisms and Control of Cell Division", (T. L. Rost and E. M. Gifford Jr., eds.), pp. 111-143. Dowden, Hutchinson & Ross, Stroudsburg, Pennsylvania.
- ROTHFIELD, L. I. (1971). "Structure and Function of Biological Membranes". Academic Press, New York.
- ROUS, P. (1911). A sarcoma of the fowl transmissible by an agent separable from the tumor cells. *J. Exp. Med.* 13:397-411.
- ROUS, P., & JONES, F. S. (1916). A method for obtaining suspensions of living cells from the fixed tissues, and for the plating out of individual cells. *J. Exp. Med.* 23:549-555.
- ROWLEY, J. (1982). Identification of the constant chromosome regions involved in human hematologic malignant disease. *Science* 216:749-751.

- ROZENGURT, E. (1982a). Adenosine receptor activation in quiescent Swiss 3T3 cells. *Exp. Cell Res.* 139:71-78.
- ROZENGURT, E. (1982b). Monovalent ion fluxes, cyclic nucleotides and the stimulation of DNA synthesis in quiescent cells. In: "Ions, Cell Proliferation, and Cancer", (A. L. Boynton, W. L. McKeehan, & J. F. Whitfield, eds.), pp. 259-281. Academic Press, New York.
- RUBIN, H. (1971). pH and population density in the regulation of animal cell multiplication. *J. Cell Biol.* 51:686-702.
- RUBIN, A. H., & SANUI, H. (1977). Complexes of inorganic pyrophosphate, orthophosphate, and calcium as stimulants of 3T3 cell multiplication. *Proc. Natl. Acad. Sci. USA* 74:5026-5030.
- RUBIN, A. H., & SANUI, H. (1979). In: "Hormones and Cell Culture". Cold Spring Harbor Conferences on Cell Proliferation, Vol. 6, pp. 741-750. Cold Spring Harbor Laboratory, New York.
- RUBIN, A. H., TERASAKI, M., & SANUI, H. (1978). Magnesium reverses inhibitory effects of calcium deprivation on coordinate response of 3T3 cells to serum. *Proc. Natl. Acad. Sci. USA* 75:4379-4383.
- RUBIN, A. H., TERASAKI, M., & SANUI, H. (1979). Major intracellular cations and growth control: correspondence among magnesium content, protein synthesis, and the onset of DNA synthesis in BALB/c3T3 cells. *Proc. Natl. Acad. Sci. USA* 76:3917-3921.
- RUDDLE, F. H. (1973). Linkage analysis in man by somatic cell genetics. *Nature* 242:165-169.
- RYAN, J. M. (1975). Alterations in chromatin functions during aging in vitro. In: "Cell Impairment in Aging and Development", (V. J. Cristofalo & E. Holeckova, eds.), pp. 123-136). *Advances in Experimental Medicine and Biology*, Vol. 53. Plenum Press, New York.
- RYAN, J. M., DUDA, G., & CRISTOFALO, V. J. (1974). Error accumulation and aging in human diploid cells. *J. Gerontol.* 29:616-621.
- RYGAARD, J. (1973). "Thymus and Self", pp. 1-193. F.A.D.L., Copenhagen.
- SABIN, A. B. (1981). Suppression of malignancy in human cancer cells: issues and challenges. *Proc. Natl. Acad. Sci. USA* 78:7129-7133.
- SACHS, L. (1979). Constitutive uncoupling pathways of gene expression that control growth and differentiation in myeloid leukemia: a model for the origin and progression of malignancy. *Proc. Natl. Acad. Sci. USA* 77:6152-6156.
- SACHS, M. M., FREELING, M., & OKIMOTO, R. (1980). The anaerobic

- proteins of maize. Cell 20:761-767.
- SACK (Jr.), G. H. (1981). Human cell transformation by Simian virus 40 - a review. In Vitro 17:1-19.
- SAKAGAMI, H., MITSUI, Y., MUROTA, S., & YAMADA, M. (1979). Cell Struct. Funct. 4:215-225.
- SALO, T., LIOTTA, L. A., & TRYGGVASON, K. (1983). Purification and characterisation of a murine basement membrane collagen-degrading enzyme secreted by metastatic tumor cells. J. Biol. Chem. 258:3058-3063.
- SANFORD, K. K. (1965). Malignant transformation of cells in vitro. Int. Rev. Cytol. 18:249-311.
- SANFORD, K. K., BARKER, B. E., PARSHAD, R., WESTFALL, B. B., WOODS, M. W., JACKSON, J. L., KING, D. R., & PEPPERS, E. V. (1970). Neoplastic conversion in vitro of mouse cells: cytologic, chromosomal, enzymatic, glycolytic, and growth properties. J. Natl. Cancer Inst. 45:1071-1096.
- SANFORD, K. K., EARLE, W. R., & LIKELY, G. D. (1948). The growth in vitro of single isolated cells. J. Natl. Cancer Inst. 9:229-246.
- SANUI, H., & RUBIN, H. (1982). The role of magnesium in cell proliferation and transformation. In: "Ions, Cell Proliferation, and Cancer", (A. L. Boynton, W. L. McKeehan, & J. F. Whitfield, eds.), pp. 517-537. Academic Press, New York.
- SAPARETO, S. A., HOPWOOD, L. E., DEWEY, W. C., RAJU, M. R., & GRAY, J. W. (1978). Effects of hyperthermia on survival and progression of Chinese hamster ovary cells. Cancer Res. 38:393-400.
- SATO, G. H. (1975). The role of serum in cell culture. In: "Biochemical Action of Hormones, Vol. III, (G. Litwick, ed.), pp. 391-396. Academic Press, New York.
- SCALABRINO, G., & FERIOLI, M. E. (1982a). Polyamines in mammalian tumors (Part I). Adv. Cancer Res. 35:151-268.
- SCALABRINO, G., & FERIOLI, M. E. (1982b). Polyamines in mammalian tumors (Part II). Adv. Cancer Res. 36:1-102.
- SCARLETTA, L. J., & EPHRUSSI, B. (1965). Hybridisation of normal and neoplastic cells in vitro. Nature 205:1169-1171.
- SCHAFER, R., DOEHMER, J., DRUGE, P. M., RADEMACHER, I., & WILLECKE, K. (1981). Genetic analysis of transformed and malignant phenotypes in somatic cell hybrids between tumorigenic Chinese hamster cells and diploid mouse fibroblasts. Cancer Res. 41:1214-1221.

- SCHIMKE, R. T. (1984). Gene amplification in cultured animal cells. *Cell* 37:705-713.
- SCHIMKE, R. T., & BARILE, M. F. (1964). Arginine breakdown in mammalian cell culture contaminated with pleuropneumonia-like organisms (PPLO). *Exp. Cell Res.* 30:593-596.
- SCHLESINGER, M. J., ASHBURNER, M., & TISSIERES, A. (eds.) (1982). "Heat Shock: From Bacteria to Man". Cold Spring Harbor Laboratory, New York.
- SCHNEBLI, H. P. (1975). The effects of protease inhibitors on cells in vitro. In: "Proteases and Biological Control", (E. Reich, D. Rifkin, & E. Shaw, eds.), pp. 785-794. Cold Spring Harbor Laboratories, New York.
- SCHNEIDER, E. L., MITSUI, Y., TICE, R. R., SHORR, S., & BRAUNSCHWEIGER, K. (1975). Alteration in cellular RNAs during the in vitro lifespan of cultured human diploid fibroblasts. II. Synthesis and processing of RNA. *Mech. Ageing Dev.* 4:449-458.
- SCHULDINER, S., & ROZENGURT, E. (1982). Na^+/H^+ antiport in Swiss 3T3 cells: mitogenic stimulation leads to cytoplasmic alkalisation. *Proc. Natl. Acad. Sci. USA* 79:7778-7782.
- SCHWARTZ, W. N., & BARRETT, A. J. (1980). Human cathepsin H. *Biochem. J.* 191:487-497.
- SCRIVER, C. R., & ROZENBERG, L. E. (1973). Amino acid metabolism and its disorders. W. B. Saunders, Philadelphia.
- SELKIRK, J. K. (1980). Chemical carcinogenesis: a brief overview of the mechanism of action of polycyclic hydrocarbons, aromatic amines, nitrosamines, and aflatoxins. In: "Carcinogenesis, Vol. 5: Modifiers of Chemical Carcinogenesis", (T. J. Slaga, ed.), pp. 1-31. Raven Press, New York.
- SHAKESPEARE, V., & BUCHANAN, J. H. (1976). Increased degradation rates of protein in aging human fibroblasts and in cells treated with an amino acid analogue. *Exp. Cell Res.* 100:1-8.
- SHARKEY, F. E., & FOGH, J. (1984). Considerations in the use of nude mice for cancer research. *Cancer Metastasis Rev.* 3:341-360.
- SHARKEY, F. E., FOGH, J. M., HAJDU, S. I., FITZGERALD, P. J., & FOGH, J. (1978). Experience in surgical pathology with human tumor growth in the nude mouse. In: "The Nude Mouse in Experimental and Clinical Research", (J. Fogh and B. Giovanella, eds.), pp. 187-214. Academic Press, New York.
- SHATOS, M. A., & STANULIS-PRAEGER, B. A. (1980). Hydrocortisone effect upon surface morphology of aging human diploid fibroblasts. In *Vitro* 16:238.

- SHIER, W. T., DuBOURDIEU, D. J., & HULL, L. A. (1982). Phospholipase and prostaglandin synthesis in the stimulation of cell proliferation by platelet-derived growth factor: the role of calcium. In: "Ions, Cell Proliferation, and Cancer", (A. L. Boynton, W. L. McKeehan, & J. F. Whitfield, eds.), pp. 347-357. Academic Press, New York.
- SHIH, T. Y., & WEEKS, M. O. (1984). Oncogenes and cancer: the p21 ras genes. *Cancer Invest.* 2:109-123.
- SHIH, T. Y., PAPAGEORGE, A. G., STOKES, P. E., WEEKS, M. O., & SCOLNICK, E. M. (1980). Guanine nucleotide binding and autophosphorylation activities associated with p21^{src} protein of Harvey murine sarcoma virus. *Nature* 287:686-691.
- SHIN, S., FREEDMAN, V. H., RISSER, R., & POLLACK, R. (1975). Tumorigenicity of virus-transformed cells in nude mice is correlated specifically with anchorage independent growth in vitro. *Proc. Natl. Acad. Sci. USA* 72:4435-4439.
- SIMINOVITCH, L. (1976). On the nature of hereditary variation in cultured somatic cells. *Cell* 7:1-11.
- SIMONS, J. W. I. M. (1967). The use of frequency distributions of cell diameters to characterise cell populations in tissue culture. *Exp. Cell Res.* 45:336-350.
- SIMS, P., & GROVER, P. L. (1974). Epoxides in polycyclic aromatic hydrocarbon metabolism and carcinogenesis. *Adv. Cancer Res.* 20:165-274.
- SINGER, B. (1984). Alkylation of the O⁶ of guanine is only one of many chemical events that may initiate carcinogenesis. *Cancer Invest.* 2:233-238.
- SISKEN, J. E., & KINOSITA, R. (1961). Timing of DNA synthesis in the mitotic cycle in vitro. *J. Biophys. Biochem. Cytol.* 9:509-518.
- SIVAK, A., & WOLMAN, S. R. (1972). Classification of cell types: agglutination and chromosomal properties. *In Vitro* 8:1-6.
- SIVAK, A., MOSSMAN, B. T., & van DUUREN, B. L. (1972). Activation of cell membrane enzymes in the stimulation of cell division. *Biochem. Biophys. Res. Commun.* 46:605-609.
- SKEHAN, P., & FRIEDMAN, S. J. (1981). Malignant transformation: in vivo methods and in vitro correlates. In: "The Transformed Cell", (I. L. Cameron and T. B. Pool, eds.), pp. 7-65. Academic Press, New York.
- SLAGA, T. J. (1980). Cancer: etiology, mechanisms, and prevention - a summary. In: "Carcinogenesis, Vol. 5: Modifiers of Chemical Carcinogenesis", (T. J. Slaga, ed.), pp. 243-262. Raven Press, New York.

- SLAGA, T. J., SIVAK, A., & BOUTWELL, R. K. (eds.) (1978). "Mechanism of Tumor Promotion and Carcinogenesis, Vol. 2, Carcinogens: A Comprehensive Survey". 588 pp. Raven Press, New York.
- SLOANE, B. F., & HONN, K. V. (1984). Cysteine proteinases and metastasis. *Cancer Metastasis Rev.* 3:249-263.
- SLOANE, B. F., BAJKOWSKI, A. S., DAY, N. A., HONN, K. V., & CRISSMAN, J. D. (1984). Purification and properties of a cathepsin B-like enzyme from human tumors: comparison with cathepsin B isolated from human liver and spleen. (Abstract). *Proc. Am. Assoc. Cancer Res.* 25:4.
- SLOANE, B. F., DUNN, J. K., & HONN, K. V. (1981). Lysosomal cathepsin B: correlation with metastatic potential. *Science* 212:1151-1153.
- SLOANE, B. F., HONN, K. V., SADLER, J. G., TURNER, W. A., KIMPSON, J. J., & TAYLOR, J. D. (1982). Cathepsin B activity in B16 melanoma cells: a possible marker for metastatic potential. *Cancer Res.* 42:980-986.
- SMETS, L. A. (1971). Medium depletion and contact inhibition of replication: absence of a specific inhibitor. *Cell Tissue Kinet.* 4:233-240.
- SMITH, A. E., SMITH, R., & PAUCHA, E. (1979). Characterisation of different tumor antigens present in cells transformed by Simian virus 40. *Cell* 18:335-346.
- SMITH, J. B., & ROZENGURT, E. (1978). Serum stimulates the Na⁺, K⁺ pump in quiescent fibroblasts by increasing Na⁺ entry. *Proc. Natl. Acad. Sci. USA* 75:5560-5564.
- SMITH, J. R., & LINCOLN II, D. W. (1984). Aging of cells in culture. *Int. Rev. Cytol.* 89:151-177.
- SMITH, R. J., LARSON, S., STRED, S. E., & DURSCHLAG, R. P. (1984). Regulation of glutamine synthetase and glutaminase activities in cultured skeletal muscle cells. *J. Cell. Physiol.* 120:197-203.
- SNYDER, M. A., BISHOP, J. M., COLBY, W. W., & LEVINSON, A. D. (1983). Phosphorylation of tyrosine-416 is not required for the transforming properties and kinase activity of pp60^{v-src}. *Cell* 32:891-901.
- SONG, C. W., RHEE, J. G., RYN, H. L., & LEVITT, H. (1982). Heat-induced change in intratumor pH and its implications in thermosensitivity of tumor cells in vivo. *Strahlentherapie* 158:389-395.
- SPECTOR, A. A., MATHUR, S. N., KADUCE, T. L., & HYMAN, B. T. (1980). Lipid nutrition and metabolism in cultured cells. *Prog. Lipid Res.* 19: 155-192.

- SPECTOR, D. H., VARMUS, H. E., & BISHOP, J. M. (1978). Nucleotide sequence related to the transforming gene of avian sarcoma virus are present in DNA of uninfected vertebrates. *Proc. Natl. Acad. Sci. USA* 75:4102-4106.
- STANBRIDGE, E. J. & WILKINSON, J. (1978). Analysis of malignancy in human cells: malignant and transformed phenotypes are under separate genetic control. *Proc. Natl. Acad. Sci. USA* 75:1466-1469.
- STANBRIDGE, E. J., DER, C. J., DOERSEN, C., NISHIMI, R. Y., PEEHL, D. M., WEISSMAN, B. E., & WILKINSON, J. E. (1982). Human cell hybrids: analysis of transformation and tumorigenicity. *Science* 215:252-259.
- STANCEL, G. M., PRESCOTT, D. M., & LISKAY, R. M. (1981). Most of the G₁ period in hamster cells is eliminated by lengthening the S period. *Proc. Natl. Acad. Sci. USA* 78:6295-6298.
- STANNERS, C. P., ADAMS, M. E., HARKINS, J. L., & POLLARD, J. W. (1979). Transformed cells have lost control of ribosome number through their growth cycle. *J. Cell. Physiol.* 100:127-138.
- STANNERS, C. P., ELICEIN, G. L., GREEN, H. (1971). Two types of ribosomes in mouse-hamster hybrid cells. *Nature* 230:52-54.
- STARKEY, P. M., & BARRETT, A. J. (1973). Human cathepsin B1. Inhibition by alpha₂-macroglobulin and other serum proteins. *Biochem. J.* 131:823-831.
- STEELE, V. E., MARCHOK, A. C., & NETTESHEIM, P. (1978). An organ culture system for studying multistage carcinogenesis in respiratory epithelium. In: "Carcinogenesis: Vol. 2. Mechanisms of Tumor Promotion and Cocarcinogenesis", (T. J. Slaga, A. Sivak, and R. K. Boutwell, eds.), pp. 289-300. Raven Press, New York.
- STEHELIN, D., VARMUS, H. E., BISHOP, J., & VOGT, P. K. (1976). DNA related to the transforming gene(s) of avian sarcoma viruses is present in normal avian DNA. *Nature* 260:170-173.
- STEIN, G. H. (1975). DNA-binding proteins in young and senescent normal human fibroblasts. *Exp. Cell Res.* 90:237-248.
- STEINBERG, M., & DEFENDI, V. (1981). Patterns of cell communication and differentiation in SV40-transformed human keratinocytes. *J. Cell. Physiol.* 109:153-159.
- STENBACK, F., GARCIA, H., & SHUBIK, P. (1973). Studies on the influence of ultraviolet light on initiation in skin tumorigenesis. *J. Invest. Dermatol.* 61:101-104.
- STEVEN, F. S., GRIFFIN, M. M., ITZHAKI, S., & AL-HABIB, A. (1980). A

- trypsin-like neutral proteinase on Ehrlich ascites cell surfaces: its role in the activation of tumor-cell zymogen of collagenase. *Br. J. Cancer* 42:712-721.
- STEVENSON, R. M., & LeJOHN, H. B. (1978). Possible regulation of nucleoside transport and RNA synthesis in Achlya. *Can. J. Biochem.* 56:207-216.
- STEWART, F. A., & DENEKAMP, J. (1978). Combined X-rays and heating: is there a therapeutic gain? In: "Cancer Therapy by Hyperthermia and Radiation", (C. Streffer, ed.), pp. 249-250. Urban and Schwarzenberg, Baltimore-Munich.
- STIKER, M., O'NEILL, C., BERRYMAN, S., & WAXMAN, V. (1968). Anchorage and growth regulation in normal and virus-transformed cells. *Int. J. Cancer* 3:683-693.
- STOKER, A. W., ENRIETTO, P. J., & WYKE, J. A. (1984). Functional domains of the pp60v-src protein as revealed by analysis of temperature-sensitive Rous sarcoma virus mutants. *Mol. Cell Biol.* 4:1508-1514.
- STOKER, M. G. P. (1973). Role of diffusion boundary layer in contact inhibition of growth. *Nature* 246:200-203.
- STOKER, M. G. P., & RUBIN, H. (1967). Density-dependent inhibition of cell growth in culture. *Nature* 215:171-172.
- STONER, G. D., & MERCHANT, D. J. (1972). Amino acid utilisation of L-M strain mouse cells in a chemically defined medium. *In Vitro* 7:330-343.
- STREFFER, C. (ed.) (1978). "Cancer Therapy by Hyperthermia and Radiation". Urban and Schwarzenberg, Baltimore, Md.
- SUMMERHAYES, I. C., & FRANKS, L. M. (1979). Effects of donor age on neoplastic transformation of adult mouse bladder epithelium in vitro. *J. Natl. Cancer Inst.* 62:1017-1023.
- SWIM, H. E., & PARKER, R. F. (1957). Culture characteristics of human fibroblasts propagated serially. *Am. J. Hyg.* 66:235-243.
- SYLVEN, B. (1968). Cellular detachment by purified lysosomal cathepsin B. *Eur. J. Cancer* 4:559-562.
- SZPIRER, C., & SZPIRER, J. (1980). Suppression of the transformed phenotypes in hepatoma cells after hybridisation with normal diploid fibroblasts. *Exp. Cell Res.* 125:305-312.
- SZYBALSKI, W., & SZYBALSKI, E. H. (1962). Drug sensitivity as a genetic marker for human cell lines. *U. Mich. Med. Bull.* 28:277-293.

- TABIN, C. J., BRADLEY, S. M., BARGMANN, C. I., WEINBERG, R. A., PAPAGEORGE, A. G., SCOLNICK, E. M., DHAR, R., LOWY, D. R., & CHANG, E. H. (1982). Mechanism of action of a human oncogene. *Nature* 300:143-149.
- TANAKA, K., APPELLA, E., & JAY, G. (1983). Developmental activation of the H-2K gene is correlated with an increase in DNA methylation. *Cell* 35:457-465.
- TANAKA, N., OGAWA, H., TANAKA, K., KINGO, M., & KOHGA, S. (1981). Effects of tranexamic acid and urokinase on hematogenous metastases of Lewis lung carcinoma in mice. *Invasion Metast.* 1:149-157.
- TANKSLEY, S. D., & JONES, R. A. (1981). Effects of O₂ stress on tomato alcohol dehydrogenase activity: description of a second ADH coding gene. *Biochem. Genet.* 19:397-409.
- TAPAROWSKY, E., SUARD, Y., FASANO, O., SHIMIZU, K., GOLDFARB, M., & WIGLER, M. (1982). Activation of the T24 bladder carcinoma transforming gene is linked to a single amino acid change. *Nature* 300:762-765.
- TATE, S. S., & MEISTER, A. (1971). Regulation of rat liver glutamine synthetase: activation by alpha-ketoglutarate and inhibition by glycine, alanine and carbamoyl phosphate. *Proc. Natl. Acad. Sci. USA* 68:781-785.
- TAUTT, J. W., ANUSZEWSKA, E. L., & KOZIOROWSKA, J. H. (1982). Methionine regulation of N-5-methyltetrahydrofolate: homocysteine methyltransferase and its influence on the growth and protein synthesis in normal, neoplastic, and transformed cells in culture. *J. Natl. Cancer Inst.* 69:9-14.
- TEMIN, H. M. (1966). Studies on carcinogenesis by avian sarcoma viruses. III. The differential effect of serum and polyanions on multiplication of uninfected and converted cells. *J. Natl. Cancer Inst.* 37:167-175.
- TEMIN, H. M. (1970). Malignant transformation of cells by viruses. *Perspect. Biol. Med.* 14:11-26.
- TEMIN, H. M., PIERSON, R. W., & DULAK, N. C. (1972). The role of serum in the control of multiplication of avian and mammalian cells in culture. In: "Growth, Nutrition and Metabolism of Cells in Culture, Vol. 1", (G. Rothblat and V. J. Cristofalo, eds.), pp. 50-81. Academic Press, New York.
- TENNANT, R. W., & RASCATI, R. J. (1980). Mechanisms of cocarcinogenesis involving endogenous retroviruses. In: "Carcinogenesis, Vol. 5: Modifiers of Chemical Carcinogenesis", (T. J. Slaga, ed.), pp. 185-205. Raven Press, New York.

- TERZAGHI, M., & LITTLE, J. B. (1976). X-radiation-induced transformation in a C3H mouse embryo-derived cell line. *Cancer Res.* 36:1367-1374.
- THOMAS, G. P., WELCH, W. J., MATHEWS, M. B., & FERAMISCO, J. R. (1981). Molecular and cellular effects of heat shock and related treatments of mammalian tissue culture cells. *Cold Spring Harbor Symp. Quant. Biol.* 46:985-996.
- THORGIERSSON, U. P., LIOTTA, L. A., KALEBIC, T., MARGUILES, I. M., THOMAS, K., RIOS-CANDELORE, M., & RUSSO, R. G. (1982). Effect of natural protease inhibitors and a chemoattractant on tumor cell invasion in vitro. *J. Natl. Cancer Inst.* 69:1049-1054.
- TIDWELL, T., ALLFREY, V. G., & MIRSKY, A. E. (1968). The methylation of histones during regeneration of the liver. *J. Biol. Chem.* 243:707-715.
- TODARO, G. J., & GREEN, H. (1963). Quantitative studies of the growth of mouse embryo cells in culture and their development into established lines. *J. Cell Biol.* 17:299-313.
- TODARO, G. J., GREEN, H., & GOLDBERG, B. D. (1964). Transformation of properties of an established cell line by SV40 and polyoma virus. *Proc. Natl. Acad. Sci. USA* 51:66-73.
- TODARO, G. J., LAZAR, G. K., & GREEN, H. (1965). The initiation of cell division in a contact-inhibited mammalian cell line. *J. Cell. Comp. Physiol.* 66:325-334.
- TRITSCH, G. L., & MOORE, G. E. (1962). Spontaneous decomposition of glutamine in cell culture media. *Exp. Cell Res.* 28:360-364.
- TROLL, W., KLASSEN, A., & JANOFF, A. (1970). Tumorigenesis in mouse skin: inhibition by synthetic inhibitors of proteases. *Science* 169:1211-1213.
- TROLL, W., ROSSMAN, T., KATZ, J., LEVITZ, M., & SUGIMURA, M. (1975). Proteinases in tumor promotion and hormone action. In: "Proteases and Biological Control", (E. Reich, D. B. Rifkin and E. Shaw, eds.), pp. 977-987. Cold Spring Harbor Laboratory, New York.
- TROLL, W., WITZ, G., GOLDSTEIN, B., STONE, D., & SUGIMURA, T. (1982). The role of free oxygen radicals in tumor promotion and carcinogenesis. In: "Cocarcinogenesis and Biological Effects", (E. Hecker, N. E. Fusenig, W. Kunz, F. Marks, & H. W. Thielmann, eds.), pp. 593-597. Raven Press, New York.
- TROSKO, J. E., & CHANG, C. C. (1980). An integrative hypothesis linking cancer, diabetes and atherosclerosis: the role of mutations and epigenetic changes. *Med. Hypoth.* 6:455-468.

- TROSKO, J. E., CHANG, C., & MEDCALF, A. (1983). Mechanisms of tumor promotion: potential role of intercellular communication. *Cancer Invest.* 1:511-526.
- TUPPER, J. T., KAUFMAN, L., & BODINE, P. V. (1980). Related effects of calcium and serum on the G₁ phase of the human WI-38 fibroblasts. *J. Cell. Physiol.* 104:97-103.
- TUY, F. P. D., HENRY, J., ROSENFELD, C., & KAHN, A. (1983). High tyrosine kinase activity in normal and non-proliferating cells. *Nature* 305:435-438.
- ULLRICH, R. L. (1980). Interactions of radiation and chemical carcinogens. In: "Carcinogenesis, Vol. 5: Modifiers of Chemical Carcinogenesis", (T. J. Slaga, ed.), pp. 169-184. Raven Press, New York.
- UMEZAWA, H. (1972). "Enzyme Inhibitors of Microbial Origin", pp. 15-52. University of Tokyo Press, Tokyo, pp. 15-52.
- UNKELESS, J. C., DANO, K., KELLERMAN, G. M., & REICH, E. (1974). Fibrinolysis associated with oncogenic transformation. *J. Biol. Chem.* 249:4295-4305.
- UNKELESS, J. C., TOBIA, A., OSSOWSKI, L., QUIGLEY, J. P., RIFKIN, D. B., & REICH, E. (1973). An enzymatic activity associated with transformation of fibroblasts by oncogenic viruses. I. Chick embryo fibroblast cultures transformed by avian RNA tumor viruses. *J. Exp. Med.* 137:85-111.
- van DUUREN, B. L. (1969). Tumor-promoting agents in two-stage carcinogenesis. *Prog. Exp. Tumor Res.* 11:31-68.
- VARMUS, H. E. (1982). Form and function of retroviral proviruses. *Science* 216:812-827.
- VARSHAVSKY, A. (1983). Diadenosine 5',5'''-p₁,p₄-tetrphosphate: a pleiotropically acting alarmone? *Cell* 34:711-712.
- VEDEL, M., GOMEZ-GARCIA, M., & SALA-TREPAT, J. (1983). Changes in methylation pattern of albumin and alpha-fetoprotein genes in developing rat liver and neoplasia. *Nucleic Acids Res.* 11:4335-4354.
- VELAZQUEZ, J. M., & LINDQUIST, S. (1984). hsp70: nuclear concentration during environmental stress and cytoplasmic storage during recovery. *Cell* 36:655-662.
- VIAJE, A., SLAGA, T. J., WIGLER, M., & WEINSTEIN, B. (1977). Effects of antiinflammatory agents on mouse skin tumor promotion, epidermal DNA synthesis, phorbol ester-induced cellular proliferation, and production of plasminogen activator. *Cancer Res.* 37:1530-1536.

- VICEPS-MADORE, D., & CRISTOFALO, V. J. (1978). Age-associated changes in glutamine synthetase activity in WI-38 cells. *Mech. Ageing Dev.* 8:43-50.
- VILLEREAL, M. L., & OWEN, N. E. (1982). The involvement of Ca^{2+} in the serum stimulation of Na^+ influx in human fibroblasts. In: "Ions, Cell Proliferation and Cancer", (A. L. Boynton, W. L. McKeehan and J. F. Whitfield, eds.), pp. 245-257. Academic Press, New York.
- WACKER, W. E. C. (1969). The biochemistry of magnesium. *Annals N.Y. Acad. Sci.* 162:717-726.
- WANG, C., GOMER, R. H., & LAZARIDES, E. (1981). Heat shock proteins are methylated in avian and mammalian cells. *Proc. Natl. Acad. Sci. USA* 78:3531-3535.
- WANG, J. L., STECK, P. A., & KURTZ, J. W. (1982). Growth control in cultured 3T3 fibroblasts: molecular properties of a growth regulatory factor isolated from conditioned medium. In: "Growth of Cells in Hormonally Defined Media", (G. H. Sato, A. B. Pardee & D. A. Sirbasku, eds.). Cold Spring Harbor Conferences on Cell Proliferation, Vol. 9, pp. 305-317. Cold Spring Harbor Laboratories, New York.
- WARBURG, O. (1926). "Über den Stoffwechsel der Tumoren". Springer Verlag, Berlin. (Translation: "The Metabolism of Tumors". Arnold Constable, London, 1930).
- WARBURG, O., POSENER, K., & NEGELEIN, E. (1924). *Biochem. Z.* 152:309-344.
- WARTERS, R. L., & ROTI ROTI, J. L. (1979). Excision of X-ray-induced thymine damage in chromatin from heated cells. *Radiat. Res.* 79:113-121.
- WATTENBERG, L. W. (1980). Inhibition of chemical carcinogenesis by antioxidants. In: "Carcinogenesis, Vol. 5: Modifiers of Chemical Carcinogenesis", (T. J. Slaga, ed.), pp. 85-98. Raven Press, New York.
- WEINSTEIN, I. B. (1981). Current concepts and controversies in chemical carcinogenesis. *J. Supramol. Struct. Cell. Biochem.* 17:99-120.
- WEINSTEIN, I. B. (1983). Protein kinase, phospholipid and control of growth. *Nature* 302:750.
- WEINSTEIN, I. B., GATTONI-CELLI, S., KIRSCHMEIR, P., LAMBERT, M., HSAIO, W., BACKER, J., & JEFFREY, A. (1984). Multistage carcinogenesis involves multiple genes and multiple mechanisms. *J. Cell. Physiol. Suppl.* 3:127-137.
- WEINSTEIN, I. B., HOROWITZ, A. D., FISHER, P. B., IVANOVIC, V.,

- GATTONI-CELLI, S., & KIRSCHMEIER, P. (1982). Mechanisms of multistage carcinogenesis and their relevance to tumor cell heterogeneity. In: "Tumor Cell Heterogeneity: Origins and Implications", (A. H. Owens Jr., D. S. Coffey, & S. B. Baylin, eds.), pp. 261-283. Academic Press, New York.
- WEINSTEIN, I. B., HOROWITZ, A. D., JEFFREY, A., & IVANOVIC, V. (1983). Cellular events in multistage carcinogenesis. In: "Genes and Proteins in Oncogenesis", (I. B. Weinstein and H. J. Vogel, eds.), pp. 99-110. Academic Press, New York.
- WEINSTEIN, I. B., WIGLER, M., & PIETROPAOLO, C. (1977). The action of tumor-promoting agents in cell culture. In: "Origins of Human Cancer", (H. H. Hiatt, J. D. Watson, & J. A. Winsten, eds.), pp. 751-772. Cold Spring Harbour Laboratory, New York.
- WEISS, M. C., & GREEN, H. (1967). Human-mouse hybrid cell lines containing partial complements of human chromosomes and functioning human genes. *Proc. Natl. Acad. Sci. USA* 58:1104-1111.
- WEISS, R., TEICH, N., VARMUS, H. E., COFFIN, J. (eds.) (1982). "RNA Tumor Viruses". Cold Spring Harbor Laboratories, New York.
- WELCH, W. J., & FERAMISCO, J. R. (1982). Purification of the major mammalian heat shock proteins. *J. Biol. Chem.* 257:14949-14959.
- WELCH, W. J., GARRELS, J. I., THOMAS, G. P., LIN, J. J., & FERAMISCO, J. R. (1983). Biochemical characterisation of the mammalian stress proteins and identification of two stress proteins as glucose- and Ca^{2+} -ionophore-regulated proteins. *J. Biol. Chem.* 258:7102-7111.
- WHITFIELD, J. F. (1982). The roles of calcium and magnesium in cell proliferation: an overview. In: "Ions, Cell Proliferation and Cancer", (A. L. Boynton, W. L. McKeehan, & J. F. Whitfield, eds.), pp. 283-294. Academic Press, New York.
- WHITFIELD, J. F., BOYNTON, A. L., MacMANUS, J. P., SIKORSKA, M., & TSANG, B. K. (1979). The regulation of cell proliferation by calcium and cyclic AMP. *Mol. Cell. Biochem.* 27:155-179.
- WIEBEL, F. J. (1980). Activation and inactivation of carcinogens by microsomal monooxygenases: modification by benzoflavones and polycyclic aromatic hydrocarbons. In: "Carcinogenesis, Vol. 5: Modifiers of Chemical Carcinogenesis", (T. J. Slaga, ed.), pp. 57-84. Raven Press, New York.
- WIENER, F., KLEIN, G., & HARRIS, H. (1973). The analysis of malignancy by cell fusion. IV. Hybrids between tumor cells and a malignant L cell derivative. *J. Cell. Sci.* 12:253-261.
- WIENER, F., KLEIN, G., & HARRIS, H. (1974). The analysis of malignancy by cell fusion. V. Further evidence of the ability of normal diploid cells to suppress malignancy. *J. Cell. Sci.* 15:177-183.

- WIERENGA, R. K., & HOL, W. G. J. (1983). Predicted nucleotide-binding properties of the p21 protein and its associated cancer variant. *Nature* 302:842-844.
- WIKE-HOOLEY, J. L., van der ZEE, J., van RHOON, G. C., van den BERG, A. P., & REINHOLD, H. S. (1984). Human tumor pH changes following hyperthermia and radiation therapy. *Eur. J. Cancer Clin. Oncol.* 20:619-623.
- WOLFF, S. (1977). Sister chromatid exchange. *Ann. Rev. Genet.* 11:183-201.
- WOLFF, S., RODIN, B., & CLEAVER, J. E. (1977). Sister chromatid exchanges induced by mutagenic carcinogens in normal and xeroderma pigmentosum cells. *Nature* 265:347-349.
- WOOLEY, D. E. (1984a). Collagenolytic mechanisms in tumor cell invasion. *Cancer Metastasis Rev.* 3:361-372.
- WOOLEY, D. E. (1984b). Mammalian collagenases. In: "Extracellular Matrix Biochemistry", (K. A. Piez & A. H. Reddi, eds.), pp. 119-157. Elsevier, New York.
- WOOLEY, D. E. (1985). Proteolytic enzymes of invasive cells. In: "Invasion: Experimental and Clinical Implications", (M. M. Mareel and K. C. Calman, eds.), pp. 228-251. Oxford University Press, Oxford.
- WOOLEY, D. E., TETLOW, L. C., & EVANSON, J. M. (1980a). Collagenase immunolocalisation studies of rheumatoid and malignant tissues. In: "Collagenase in Normal and Pathological Connective Tissues", (D. E. Wooley & J. M. Evanson, eds.), pp. 105-125. John Wiley & Sons, Chichester.
- WOOLEY, D. E., TETLOW, L. C., MOONEY, C. J., & EVANSON, J. M. (1980b). Human collagenase and its natural inhibitors in relation to tumor invasion. In: "Proteinases in Tumor Invasion", (P. Strauli, J. C. Barrett, & A. Baici, eds.), pp. 97-115. EORTC Monograph Vol. 6. Raven Press, New York.
- WOOLEY, D. S. (1940). A new dietary essential for the mouse. *J. Biol. Chem.* 136:113-118.
- WRIGHT, J. A., LEWIS, W. H., & PARFETT, C. L. J. (1980). Somatic cell genetics: a review of drug resistance, lectin resistance and gene transfer in mammalian cells in culture. *Can. J. Genet. Cytol.* 22:443-496.
- YAMADA, K. M., & OLDEN, K. (1978). Fibronectin-adhesive glycoproteins of cell surface and blood. *Nature* 275:179-184.
- YAMAMOTO, R. S., UMEZAWA, H., TAKEUCHI, T., MATSUSHIMA, T., HARA, K., &

- SUGIMURA, T. (1975). Preventive role of protease inhibitors on lung metastasis of ascites hepatoma. *Proc. Am. Assoc. Cancer Res.* 16:69.
- YANG, T. C. H., & TOBIAS, C. A. (1980). Radiation and cell transformation in vitro. *Adv. Biol. Med. Physics* 17:417-461.
- YANISHEVSKY, R. M., & STEIN, G. H. (1981). Regulation of the cell cycle in eukaryotic cells. *Int. Rev. Cytol.* 69:223-259.
- YATVIN, M. B. (1977). The influence of membrane lipid composition and procaine on hyperthermic death of cells. *Int. J. Radiat. Biol.* 32:513-521.
- YEN, A., & PARDEE, A. B. (1978). Exponential 3T3 cells escape in mid-G₁ from their high serum requirement. *Exp. Cell Res.* 116:103-113.
- YOFFE, J. R., TAYLOR, D. J., & WOOLEY, D. E. (1985). Mast cell products and heparin stimulate the production of mononuclear cell factor by cultured human monocyte-macrophages. *Biochem. J.* 230:83-88.
- YOSHIDA, M. C. (1972). *Jpn. J. Genet.* 47:211-223.
- YUSPA, S. H., LICHTI, U., HENNINGS, H., BEN, T., PATTERSON, E., & SLAGA, T. J. (1978). Tumor promoter stimulated proliferation in mouse epidermis in vivo and in vitro: mediation by polyamines and inhibition by the anti-promoter steroid fluocinolone acetonide. In: "Carcinogenesis, Vol. 2: Mechanisms of Tumor Promotion and Cocarcinogenesis", (T. J. Slaga, A. Sivak, and R. K. Boutwell, eds.), pp. 145-155. Raven Press, New York.
- ZETTERBERG, A., & ENGSTROM, W. (1981a). Glutamine and the regulation of DNA replication and cell multiplication in fibroblasts. *J. Cell. Physiol.* 108:365-373.
- ZETTERBERG, A., & ENGSTROM, W. (1981b). Mitogenic effect of alkaline pH on quiescent, serum-starved cells. *Proc. Natl. Acad. Sci. USA* 78:4334-4338.
- ZETTERBERG, A., ENGSTROM, W., & LARSSON, O. (1982). Growth activation of resting cells. Induction of balanced and imbalanced growth. *Annals N.Y. Acad. Sci.* 397:130-147.
- ZIEGLER, M. L. (1978). Phenotypic expression of malignancy in hybrid and cybrid mouse cybrid mouse cells. *Somat. Cell Genet.* 4:477-489.
- ZIELKE, R. H., OZAND, P. T., TILDEN, J. T., SEVDALIAN, D. A., & CORNBLATH, M. (1976). Growth of human diploid fibroblasts in the absence of glucose utilisation. *Proc. Natl. Acad. Sci. USA* 73:4110-4114.