Volume 34—1981

ADVANCES IN CANCER RESEARCH

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Volume 34—1981

ACADEMIC PRESS

A Subsidiary of Harcourt Brace Jovanovich, Publishers

New York London Toronto Sydney San Francisco

ACADEMIC PRESS, INC.
111 Fifth Avenue, New York, New York 10003

United Kingdom Edition published by
ACADEMIC PRESS, INC. (LONDON) LTD.
24/28 Oval Road, London NW1 7DX

LIBRARY OF CONGRESS CATALOG CARD NUMBER: 52-13360

ISBN 0-12-006634-3

PRINTED IN THE UNITED STATES OF AMERICA

81 82 83 84 9 8 7 6 5 4 3 2 1

CONTENTS

CONTRIBUTORS TO VOLUME 34 ix

The Transformation of Cell Growth and Transmogrification of DNA Synthesis by Simian Virus 40

ROBERT G. MARTIN

I. Introduction 1
II. A Definition of Transformation 4
III. Normal Growth Control 5
IV. Early Steps in Transformation by Virions 11
V. Induction of Host DNA Synthesis and Abortive Transformation 14
VI. Integration of Viral DNA into the Host Genome 18
VII. Sequences Necessary for Transformation by SV40 and PyV 23
VIII. SV40 Functions Required for Maintenance of the Transformed State 25
IX. Comparison of PyV and SV40 Transformants 32
X. The Mutagenic Activity of SV40 34
XI. Gene Activation by SV40 36
XII. Tumorigenicity and Tumor Rejection 38
XIII. Host Control of T-Antigen 40
XIV. Normal DNA Synthesis in Mammalian Cells 42
XV. DNA Synthesis in SV40 Transformants 47
XVI. The Enzymatic Activity of the 90K T-Antigen 51
XVII. Summary 52
References 53

Immunologic Mechanisms in UV Radiation Carcinogenesis

MARGARET L. KRIPKE

I. Introduction 69
II. Carcinogenesis by UV Radiation 70
III. Immunosuppression and UV Carcinogenesis 75
IV. Antigenicity of UV-Induced Tumors 77
V. Immunologic Reactivity of Mice during UV Carcinogenesis 83
VI. Mechanisms in UV-Induced Immunosuppression 91
VII. Conclusions 98
References 103

The Tumor Dormant State

E. FREDERICK WHEELOCK, KENT J. WEINHOLD, AND JUDITH LEVICH

I. Introduction and Definition 107
II. Review of the Clinical Literature on Tumor Dormancy 109
III. Animal Models of Tumor Dormancy 113
IV. Stages of Tumor Dormancy 116
V. Comments 131
VI. Conclusions 134
References 135

Marker Chromosome 14q+ in Human Cancer and Leukemia

FELIX MITELMAN

I. Introduction 141
II. Survey of the Total Material 142
III. Nomenclature 144
IV. General Review of Chromosome Patterns in Cancer 145
V. Survey of Occurrence of 14q+ Marker Chromosomes 149
VI. Survey of Geographic Distribution of 14q+ Marker Chromosomes 158
VII. Relation between the 14q+ Marker Chromosome and EBV 160
VIII. Summary of 14q+ Marker Chromosomes 161
IX. Significance of the 14q+ Marker and Other Specific Chromosome Abnormalities 162
References 167

Structural Diversity among Retroviral Gene Products: A Molecular Approach to the Study of Biological Function through Structural Variability

JAMES W. GAUTSCH, JOHN H. ELDER, FRED C. JENSEN, AND RICHARD A. LERNER

I. Introduction 171
II. Molecular Portraiture: Technique of Peptide Mapping 173
III. Background, Historical Overview 176
IV. The Comparative Structure of the Major Core Protein of Retroviruses 177
V. Xenotropic Viruses of Different Classes Have Different p30s 178
VI. N- and B-Tropism Are Cryptic Phenotypes of X-tropic MuLV 179
VII. Additional Structural Markers in MuLV p30 186
VIII. The Structure of MuLV p30 Is Different from the Major Core Protein of Other Mammalian Type C Viruses 191
IX. Structural Evidence for Interspecies Transfer of Genetic Information 193

X. Two Type C Viruses from a Single Species of Mouse Constitute Members of Both the Woolly Monkey–Gibbon Ape and Murine Groups 193
XI. Structural Markers Associated with MuLV gp70 195
XII. Structural Polymorphism of MuLV p15 196
XIII. Structure–Function Correlates of Fv-1 Tropism 201
XIV. Structural Evidence for Different Fv-1^n Genes 202
XV. The Use of Structural Markers on MuLV Gene Products to Identify Intragenic Recombinations 203
XVI. Summary 205
References 206

Teratocarcinomas and Other Neoplasms as Developmental Defects in Gene Expression

BEATRICE MINTZ AND ROGER A. FLEISCHMAN

I. Introduction 211
II. Stem Cells in Normal Development and in Tumors 213
III. Spontaneous Differentiation in Tumors 221
IV. Induction of Normalization and Differentiation in Tumors and Established Cell Lines 222
V. Effects of Tumor Promoters on Differentiation 264
VI. Anomalous Differentiation in Metaplasia and Neoplasia 265
VII. Expression of "Banal" Cellular Genes in Normalcy vs Malignancy 267
References 272
Note Added in Proof 278

Immune Deficiency Predisposing to Epstein–Barr Virus-Induced Lymphoproliferative Diseases: The X-Linked Lymphoproliferative Syndrome as a Model

DAVID T. PURTILO

I. Introduction 279
II. Immunopathogenesis of Infectious Mononucleosis 280
III. Infectious Mononucleosis Mimics Graft-versus-Host Response (GVHR) 284
IV. Immune Deficiency Predisposing to Fatal Lymphoproliferative Disorders 287
V. X-Linked Lymphoproliferative Syndrome as a Model Illustrating Immunodeficiency Predisposing to Fatal Infectious Mononucleosis and Malignant Lymphoma 288
VI. Ataxia Telangiectasia (AT), Immunodeficiency, and Chromosomal Breakage Predisposing to Malignant Lymphoproliferation 292
VII. Familial Fatal Infectious Mononucleosis and EBV-Induced Lymphoproliferative Diseases 292
VIII. EBV-Induced Lymphoproliferative Diseases in Transplant Recipients 294

IX. Hypothesis: EBV-Induced Oncogenesis in Immunodeficiency 298
X. Techniques for Detecting EBV Infection in Immune-Deficient Patients 304
XI. Summary and Recommendations 306
References 307

INDEX 313
CONTENTS OF PREVIOUS VOLUMES 319

CONTRIBUTORS TO VOLUME 34

Numbers in parentheses indicate the pages on which the authors' contributions begin.

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THE TRANSFORMATION OF CELL GROWTH AND TRANSMOGRIFICATION OF DNA SYNTHESIS BY SIMIAN VIRUS 40

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I. Introduction 1
II. A Definition of Transformation 4
III. Normal Growth Control 5
IV. Early Steps in Transformation by Virions 11
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VIII. SV40 Functions Required for Maintenance of the Transformed State 25
IX. Comparison of PyV and SV40 Transformants 32
X. The Mutagenic Activity of SV40 34
XI. Gene Activation by SV40 36
XII. Tumorigenicity and Tumor Rejection 38
XIII. Host Control of T-Antigen 40
XIV. Normal DNA Synthesis in Mammalian Cells 42
XV. DNA Synthesis in SV40 Transformants 47
XVI. The Enzymatic Activity of the 90K T-Antigen 51
XVII. Summary 52
References 53

I. Introduction

Ernest Hemingway (1960) was impressed by a corrida in Malaga, in the summer of 1959. Antonio Ordoñez, he wrote, "made a perfect and an almost unbearably emotional faena with this bull, holding him controlled in the long slow passes in any one of which, if he had hurried or been even a shade abrupt, the bull would have broken in his charge and left the cloth to gore him. This way of fighting is the most dangerous in the world and on this last bull he gave an entire course in how to do it. . . . It was one of the very greatest bullfights I have ever seen." Alberto Vera, a Spanish bullfight critic wrote of the same fight, "This afternoon we saw two famous matadors fight six bulls, and each animal had two distinctions. It was barely three years old and was therefore more truly a calf. And what horns it did have were mercilessly shaved" (quoted in Macnab, 1959).

ADVANCES IN CANCER RESEARCH, VOL. 34

ISBN 0-12-006634-3

It is the firmly held belief of one group of investigators that an understanding of membrane function is fundamental to an understanding of transformation. According to this view the encyclopedic catalog of membrane-associated activities affected by transformation can best be explained by a fundamental reorganization of the plasma membrane. Yet virtually all of the Simian Virus 40 (SV40) and Polyoma Virus (PyV) transformation-associated "aberrations" can be mimicked by stimulating normal cells to active growth. (For references see pertinent section.) Although the ability of lectins to induce cellular aggregation is often associated with transformation, aggregation is also increased when cells enter mitosis. Plasminogen activator, generally but not invariably released into the medium in large quantities by transformed cells, is also released by actively growing normal cells. Membrane fluidity is changed in transformed cells as in growing normal cells. Transport activities, e.g., 2-deoxyglucose and uracil uptake are often elevated in transformed cells, but are also stimulated when resting cells are induced to resume active growth. Dissolution of actin cables at prophase and reformation at telophase are normal parts of the mitotic process. Thus, the lower proportion of cells containing actin cables in transformed cultures could be a manifestation of the prolongation of these normal processes rather than be a clue to the fundamental change responsible for transformation. There is simply no convincing evidence that the primary target of transformation by papovaviruses is the cellular membrane. It is often forgotten that much of the original impetus for the notion that transformation involved membrane alterations was the hypothesis of contact inhibition. Although this concept has now been questioned, the notion of membrane involvement in transformation remains widespread.

A second group argues that the transforming activity of papovaviruses works at the level of the regulation of DNA synthesis. (Although DNA synthesis continues in transformed cultures beyond the point of confluence this may be a trivial corollary of the fact that transformed cultures continue growing after reaching confluence.) This hypothesis is based principally on the observation that cells transformed by SV40 mutants that make a temperature-sensitive large T-antigen (*tsA* mutants) are frequently temperature-sensitive for expression of the transformed phenotype. It is widely accepted that the large tumor (T)-antigens of SV40 and PyV are "initiator proteins" for the viral replicons (Jacob *et al.*, 1963), i.e., that they carry out some reaction that is essential for the initiation of viral DNA replication. Thus, if T-antigen is an initiator of DNA synthesis and necessary for the maintenance of transformation, it is reasonable to suppose that the

initiation of DNA synthesis is essential for the maintenance of transformation. In support of this hypothesis is the observation that rapidly growing SV40-transformed cells have more origins of DNA replication than nontransformed cells. The most telling among the myriad of arguments that have been used against this hypothesis are: (1) the failure of many laboratories to obtain temperature-sensitive transformants using *tsA* mutants of SV40 and PyV under certain conditions; (2) the observation that most PyV-induced tumors lack the large T-antigen and that PyV-transformed cells can lose T-antigen concurrent with tumor induction; (3) the enhanced ability of DNA lacking half of the coding capacity for the large T-antigen of PyV to cause tumors; (4) the ability of SV40 *tsA* mutants to induce host DNA synthesis at the nonpermissive temperature (40°C); and (5) the observation that some *tsA*-transformed cell lines that are temperature-sensitive for growth are not especially temperature-sensitive for host DNA synthesis (i.e., the cells replicate, die, and slough). However, some of these arguments apply only to PyV, and tumor promotion by SV40 and PyV is clearly different.

A third group, not necessarily in opposition to either of the preceding, believes that the essential factor in transformation by papovaviruses is the reduction in the requirement for serum growth factors. Whether the growth factor-like activity is supplied by the large, middle, or small viral t-antigens or possibly the cellular middle t protein induced by the papovaviruses is unclear (Crawford *et al.*, 1979, 1980; DeLeo *et al.*, 1979; Kress *et al.*, 1979; Lane and Crawford, 1979; Linzer and Levine, 1979; Linzer *et al.*, 1979; McCormick and Harlow, 1980; Melero *et al.*, 1979).

Still others argue that papovavirus transformation can occur in multiple ways, one of which may be the result of an inheritable genetic or epigenetic alteration induced by the mutagenic activity of the virus.

The purpose of this article is to present a simple, coherent model for transformation by SV40 that reconciles many ostensibly disparate observations. Since there remain enormous gaps in our knowledge of papovavirus biology and cell biology, it has been necessary to make a number of assumptions. I have chosen to emphasize certain aspects of mammalian DNA replication and the role of SV40 in perturbing cellular DNA synthesis in transformed cells. Although it is fashionable to consider that SV40 causes transformation through some action on the plasma membrane, there is very little support for this hypothesis. On the other hand, neither is there overwhelming evidence for the model presented here. This model is an extension of that already proposed (Martin *et al.*, 1974) and similar in some aspects to the earlier model of Levine and Burger (1972). There are probably as many vantage points

from which to view the problem of transformation by SV40 as there are papovavirus aficianados. As with the visions of Hemingway and Vera, the truth probably lies somewhere between the *sol y sombra.*

The exposition of each Section is preceded by a precis.

II. A Definition of Transformation

"Transformation" is of necessity a term that compares the growth properties of a cell line to those of its parent. As used here it refers to acquisition of the ability to form dense foci on plastic or colonies in soft agar.

Few investigators have difficulty in associating the terms "nontransformed" and "transformed" with the extremes of growth behavior exhibited in tissue culture by normal primary cells and by cells from highly malignant tumors (Enders, 1964). The former tend to form monolayers on plastic and readily enter a resting state in depleted medium (Aaronson and Todaro, 1968a,b; Baserga 1968, 1969; Martin and Stein, 1976). They tend not to agglutinate readily in the presence of lectins (Burger, 1969; Inbar *et al.*, 1969), not to release plasminogen activator (Ossowski *et al.*, 1973a,b; Pollack *et al.*, 1974; Rifkin and Pollack, 1977), to coat themselves with fibronectin (LETS protein) (Steinberg *et al.*, 1979), to attach tightly to the substrate (Cassiman and Bernfield, 1975), and to exhibit prominent actin cables during interphase (McNutt *et al.*, 1971; Osborn and Weber, 1975; Pollack and Rifkin, 1975; Pollack *et al.*, 1975a). They fail to grow in suspension culture (Macpherson and Montagnier, 1964) or to produce tumors in syngeneic or immunosuppressed animals (Shin *et al.*, 1975). On the other hand, cell lines derived from papovavirus-induced tumors tend to produce tumors in syngeneic or immunosuppressed animals, to form dense monolayers or multilayered cultures on plastic, to grow in suspension culture, and not to enter a resting state rapidly in depleted medium.

Despite this, no universally accepted characteristic or set of characteristics is associated with "nontransformed" vs "transformed" growth. The problem lies in our inability to define "normal" growth.

One aspect of this problem is that the spectrum of behavior of transformed cells overlaps with that of normal cells. For example, cells treated with wild-type SV40 acquire the ability to form foci without necessarily acquiring the ability to grow in agar. However, cells that acquire the ability to grow in agar invariably acquire the ability to form dense foci (Risser and Pollack, 1974). Thus, a hierarchy of transformed phenotypes can be established where the ability to grow in low

serum seems to be a less stringent criterion of transformation than the ability to reach a high saturation density on plastic; this in turn appears to be a less stringent criterion than the ability to overgrow a normal monolayer or the ability to grow in suspension culture and even less so than the ability to produce tumors (Risser and Pollack, 1974; Shin *et al.*, 1975).

A second aspect of the problem in defining "normal" is that normal cells passaged in tissue culture can spontaneously acquire some or all of the characteristics of a transformed cell, including the ability to produce tumors (Diamandopoulos and Enders, 1965; Kuster *et al.*, 1977). Indeed, one of the prototypes of "normality" is the mouse 3T3 line which is aneuploid!

A third aspect of the problem is that changes in the substratum can have enormous effects on biological behavior. "Normal" mouse 3T3 cells when injected into mice are not tumorigenic. However, if the cells are allowed to grow on glass beads and the cells growing on glass beads are injected into syngeneic animals, tumors are induced (Boone *et al.*, 1976).

But perhaps the greatest difficulty in defining "normal" growth derives from our inadequate knowledge of the normal mechanisms of growth control and our misconceptions of that control.

Because of these difficulties, any definition of transformation must be arbitrary. In this article the term "transformation" will be used to indicate the acquisition of growth characteristics not exhibited by the parental cells. Thus, two extreme examples of cells *not considered* to be transformed by papovaviruses are: (1) a cell line that exhibits no change in growth properties following the integration of SV40 into its genome; and (2) a tumor cell line into which SV40 subsequently has integrated and in which the early antigens are then expressed. On the other hand, a cell is considered to have been transformed following exposure to the virus if the progeny merely form dense foci on plastic—even if they fail to grow in suspension culture or to produce tumors in appropriate animals. This, of course, *provided* the parental cells are homogeneous (recently cloned) and fail to give dense foci on plastic.

III. Normal Growth Control

Two of the more commonly accepted principles of cell biology, "contact inhibition" and "the cell cycle," are at best oversimplifications or at worst, entirely inaccurate. The G_1 phase may not be an obligatory interval between M and S but the invariable result of

our inability to provide ideal growth conditions in tissue culture. Cells can be growth arrested at a point immediately prior to S phase, but other arrest points may also exist. Cascade enzyme systems could be of importance in the operation of arrest points and could explain the first-order kinetics of the entry of cells into S phase. Growth factor requirements for further DNA replication may differ between growth-arrested cells and growing cells that have just completed S phase.

Cessation of growth at confluence (density arrest of growth) probably has little if anything to do with cell-to-cell contacts at least for fibroblasts. A significant proportion of the history of cell biology deals with the development of media that allow the proliferation of cells in culture. These media were invariably designed to promote cell growth and to allow the cultures to achieve confluence. I know of no medium intentionally compounded to permit growth at very high densities. Therefore, the observation that cell proliferation ceases at confluence is not itself sufficient to demonstrate that cell contact inhibits cell division. Indeed, the elegant experiments of Stoker (1968, 1973; Clarke *et al.*, 1970) of Dulbecco (1970a,b; Dulbecco and Elkington, 1973; Dulbecco and Stoker, 1970), of Holley (Holley and Kiernan, 1971; Holley *et al.*, 1977), and of others (Kruse *et al.*, 1969; Paul *et al.*, 1971; Roehm and Lipton, 1973; Temin 1967; Todaro *et al.*, 1967) strongly suggest that most, if not all, "contact inhibition of growth" for fibroblasts is nothing more than depletion of the medium in the microenvironment of the cell. The isolation of inhibitory factors from mouse 3T3 fibroblasts (Whittenberger and Glaser, 1977; Whittenberger *et al.*, 1978) does not necessarily negate this conclusion.

Also without solid foundation is the concept of the "cell cycle." This concept implies both that obligatory functions are carried out in each phase of the cycle (Cooper, 1979) and that each phase requires a certain average length of time for completion (Smith and Martin, 1973). There is good evidence from which to conclude that the durations of DNA synthesis (S phase) and of mitosis (M phase) are reasonably constant for a given cell type in a given medium (Basèrga, 1968, 1969, 1976; Pardee *et al.*, 1978; Prescott, 1976; Tobey, 1973). The same is true of G_2, the period that follows S phase and precedes M, although the existence of G_2 growth arrest points have been proposed (Gelfant, 1975, 1977) but also have been disputed (Sauerborn *et al.*, 1978). However, there is no evidence that suggests either that a uniform length of time is required for G_1, the period from M to S, or that certain functions are necessarily carried out during G_1. Indeed, eukaryotic

cells lacking a G_1 phase have been described (Robbins and Scharf, 1967). (Perhaps under "ideal" conditions all cells would pass directly from M to S phase as during early embryogenesis.) Furthermore, kinetic data overwhelmingly suggest that in well-defined media there is no average length for G_1 (Brooks, 1976; Brooks *et al.*, 1980; Shields, 1978; Shields and Smith, 1977; Shields *et al.*, 1978; Smith and Martin, 1973; Stiles *et al.*, 1979a,b).

Part of our difficulty in understanding the cell cycle comes from the fact that our understanding of growth arrest in suboptimal media is also limited. In particular, there is considerable controversy concerning the number of growth arrest points. A large body of data demonstrates that cells arrest with a diploid complement of DNA between M and S phase when placed in suboptimal medium (see Baserga, 1976; Pardee *et al.*, 1979; Prescott, 1976). In a number of systems the length of time it takes arrested cells to enter S phase following enrichment of the medium depends on the conditions that were used to induce growth arrest. For example, when mouse 3T3 cells are density arrested, S phase does not start until 12 hours after adding fresh serum. On the other hand, the same cells arrested by depletion for isoleucine resume DNA synthesis within 6 hours after replenishing the isoleucine (Stiles *et al.*, 1979c). Furthermore, cells arrested by density, stimulated by plasma, and then again growth arrested by removal of plasma before the start of S phase resume DNA synthesis immediately with no measureable lag (Pledger *et al.*, 1978). Experiments of this type have often been interpreted as demonstrating multiple growth-arrest points (Baserga, 1968, 1969, 1976). Such experiments however, are very difficult to interpret because one cannot measure how much of the elapsed time represents the time necessary to complete G_1, and how much of it represents the time required to repair metabolic alterations resulting from the depletion. Thus, all conclusions based on the measurement of elapsed time must be viewed with considerable skepticism. Indeed, Pardee (1974) has demonstrated with a number of nutrients that irrespective of the order in which one first starves for one nutrient and subsequently starves for a second nutrient (having simultaneously replenished the medium for the first nutrient) DNA synthesis never ensues. He has therefore proposed that cells have only a single arrest point between M and S and has termed this the restriction point.

On the other hand, other data support the notion of multiple growth-arrest points. Using cell lines that contain a temperature-sensitive block expressed during G_1, Baserga and Basilico and their colleagues (Ashihara *et al.*, 1978; Burstin *et al.*, 1974; Meiss and

Basilico, 1972; Talavera and Basilico, 1978) have demonstrated that cells arrested by serum deprivation do not enter S phase upon serum stimulation at the nonpermissive temperature. On the other hand, the same cells arrested by isoleucine deprivation do enter S phase upon isoleucine supplementation at the nonpermissive temperature. Furthermore, different temperature-sensitive mutants arrested by the same procedure express different phenotypes (Rossini *et al.*, 1980). The clear implication of these results is that at least two growth-arrest points can be defined.

A similar conclusion has been drawn from other experiments. Stiles *et al.* (1979c, 1980) have purified a polypeptide from human platelets, the platelet-derived growth factor (PDGF), which in combination with platelet-depleted plasma stimulates density-arrested mouse 3T3 cells to enter S phase. They have shown that the order in which the arrested cells are exposed to PDGF and platelet-depleted plasma is crucial. Treatment with PDGF in the absence of plasma primes the cells so that upon subsequent incubation in the absence of PDGF but in the presence of plasma, the cells will enter S phase. [Ca^{2+} will substitute for PDGF (Stiles *et al.*, 1979c).] However, if the order of exposure to PDGF and plasma is reversed, no DNA synthesis ensues. They have therefore suggested that the G_1 phase can be divided into at least two phases with at least two arrest points: the density arrest point from which cells do not emerge in platelet-depleted plasma alone, followed by a phase induced by PDGF; and a second arrest point, the "competence" point, at which the cells remain unless stimulated by plasma to progress toward S phase. Furthermore, when the plasma from hypophysectomized rats was used, they were able to demonstrate a further arrest point that can be overcome by the addition of somatomedin. From these results it seems likely that there is a series of events which occur when density-arrested cells are stimulated to enter S phase. Although these results strongly suggest that there are multiple arrest points, they do not address the question of whether or not these arrest points are intrinsic to G_1. An understanding of this distinction requires an understanding of recent models for growth control (Brooks *et al.*, 1980; Cooper, 1979).

Cooper (1979) has proposed that preparation for the next round of DNA synthesis in growing cells may start as early as immediately after the onset of the preceding S phase. According to this model, there may be a series of sequential events prerequisite to S phase, but these events are not inherently part of G_1. They become a part of G_1 only when suboptimal conditions prevent them from starting before mitosis. According to this model, growth in tissue culture even in the most

enriched medium is almost always "suboptimal." In support of this model are the observations that: (1) G_1-less cells can be isolated (Robbins and Scharf, 1967) and mutated so that they again acquire a G_1 period (Liskay and Prescott, 1978); (2) the length of G_1 is considerably shorter in growing cells (G_1 taken as the time from mitosis to DNA synthesis) than in resting cells (G_1 taken as the time from reversal of density arrest) (see Baserga, 1968); and (3) PDGF added prior to mitosis reduces the duration of G_1 (Scher *et al.*, 1979).

From studies of the kinetics of cell growth Brooks *et al.* (1980) have provided a more detailed version of this model. They propose that the chance of entering a preparative phase, L (which can start immediately after the onset of S phase), is defined by a first-order probability function (rate constant, K_Q), but completion of this phase (unlike the Cooper model) does not commit the cell to S phase. Rather, the cell next enters a holding pattern or "A-state" from which it leaves to enter S phase, again with a probability defined by a first-order function (rate constant, K_A). The rate at which cells enter S phase is thus dependent on two transition probabilities; the constants dictating these probabilities are affected by the "richness" of the medium. Brooks *et al.* (1980) point out that the random transitions could result from the fluctuation of some crucial substance(s) about a mean, the transitions occurring only when some threshold concentration was exceeded. No biochemical prototype was suggested however.

Here, I wish to propose that cascade enzyme systems could account for a number of features known to be associated with growth control. Cascade systems have been described in both prokaryotic and eukaryotic systems and have recently been reviewed (Chock and Stadtman, 1979; Stadtman *et al.*, 1979, 1981). A cascade is formed when an enzyme that carries out a single biochemical reaction can be modified (e.g., by adenylation or phosphorylation) so that the kinetics of its enzymatic activity are altered. A cascade increases in complexity if the enzyme(s) carrying out the modification(s) can themselves be modified by other enzymes. A cascade thus contains the potential for responding to multiple signals at the same time since different modifying enzymes could respond to different affectors (inhibitors or activators). Furthermore, if an affector works on the enzyme and/or on one or more of the modifying enzymes, the cascade takes on a number of biochemically unique features. These include the potential for enormous signal amplification and for a wide range of time-lags. This is true whether the basic enzymatic reaction is reversible or irreversible.

A cascade system operating at the restriction point (or at several arrest points) provides a biochemical model for understanding much of