Initiation and Promotion in Cancer Formation: 
The Importance of Studies on Intercellular 
Communication* 

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Received June 9, 1980 

Three major theories of cancer—somatic mutation, virus causation, and faulty differentia-
tion—are proposed to involve alterations in DNA structure. Each results finally in terms of 
failures in the normal intercellular communication that involves feedback between dif-
ferentiated cells acting on less differentiated cells still capable of proliferation. The historical 
background of the latter idea is traced to Osgood, to Weiss and Kavanau, and to Iversen. The 
historical background of concepts of initiation and promotion are traced to Berenblum and 
Mottram and the Boutwell concept of promotion as gene activation is cited. It is proposed that 
gene activation by promoters is a valid concept and that it results from the blocking of the 
normal intercellular communication postulated by Osgood and others. The problem of 
explaining the low probability of cancer following initiators or promoters acting alone is cited 
as a problem in basic science. A hypothesis to solve the problem is proposed: Cancer results 
from two or more relevant mutations; promoters enhance proliferation of cells with one 
relevant mutation, thereby increasing the probability of obtaining a cell with two relevant 
mutations. A new scheme of five stages of hepatocarcinogenesis is proposed in terms of the 
hypothesis and available data. 

INTRODUCTION 

Twenty-six years ago I had the honor to present the Seventh Edgar Allen Memorial Lecture at this University and I am privileged today to be here again to help celebrate another milestone in the history of Yale University and its contributions to cancer research. 

On that earlier occasion I had the temerity to speak on "Ten years of Cancer Research." In a similar vein, today I would have to admit to "Forty years of Cancer Research," but I no longer feel justified in trying to recount all the adventures and misadventures covered in that time span. Instead I have chosen to focus on "Initiation and Promotion in Cancer Formation," which might be subtitled "The Importance of Studies on Intercellular Communication," because, in the framework of this symposium, this is where I think much attention should be focused in "The Decade Ahead."

*Presented at The Dedication of The Yale Comprehensive Cancer Center in The Symposium entitled "Cancer: The Decade Ahead" at the Yale University School of Medicine, New Haven, Connecticut, March 26, 1980. 

Hilldale Professor of Oncology. The author's work has been supported in part by grants CA-07175, 
CA-22484, and CA-17334 from The National Cancer Institute. 

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For some years I have believed that the three major theories on the nature of cancer could be integrated into a single conceptual framework. According to these theories, which have been considered as mutually exclusive, cancer is caused by somatic mutations, by viruses, or by faulty differentiation. At the center is the somatic mutation theory, formerly incompatible with the virus theory and bitterly opposed by Peyton Rous [1]. Today the virus theory is no longer incompatible with the somatic mutation theory because it is comprehended in terms of altered DNA structures, following the brilliant intuition by Howard Temin and the subsequent experiments by Temin and by Baltimore [1].

The third remaining theory is that cancer is merely a case of faulty differentiation, but it is always implied that this is without altered DNA structures, i.e., without somatic mutation [2]. I disagree with this view. I believe that cancer is indeed a case of faulty differentiation, or, as I phrase it, "Oncogeny is blocked or partially blocked Ontogeny" [3]. In contrast to the usual inference, however, I prefer the hypothesis that the faulty differentiation results from alterations in DNA structure, i.e., either somatic mutations in the usual sense, or by viral modifications of the genome, operationally equivalent to mutations.

Thus the three major theories to explain cancer would all be integrated into a single framework in which faulty differentiation is coupled with either one of the two others. This concept can be further elaborated in terms of failures in the normal intercellular communication that involves positive and negative feedback between differentiated cells acting on their less differentiated precursor or stem cells, as I will indicate.

The idea of faulty differentiation leading to breakdown in intercellular communication is presently being illuminated by basic science advances in the understanding of the phenomena of initiation and promotion, which I shall report today. My major conclusion will be that promoters cause activation of genes for growth factors in normal and initiated cells by derepression. This derepression is brought about by blocking the formation, transmission, or reception of repressors (i.e., inhibitors or chalones [4]). Repressors normally act between differentiated cells and cells that are capable of dividing, in order to control proliferation.

I will begin by discussing the concepts of initiation and promotion, which have by now received acceptance by many cancer investigators [5].

Promoters are not carcinogens. At the outset it is important to make clear what we mean by the words promoter, initiator, and carcinogen. In order to be effective as a carcinogen in the usual type of laboratory test, a compound almost certainly acts both as an initiator and as a promoter. On the other hand, at very small ("subcarcinogenic") doses, the so-called "complete" carcinogens can act more like "pure initiators," and will not produce tumors when the usual numbers of animals are employed. Thus in the absence of promotion the dose-response at low levels of a carcinogen should exhibit a threshold effect, because the dose may be too low to elicit promotion and therefore too low to produce cancers. In contrast, when higher doses are applied continuously in diets or in skin painting procedures, promotion as well as initiation occurs and cancers result. My own recent work, as yet unpublished, has dealt with the induction of liver tumors in rats with low doses of a known carcinogen in the presence and absence of a known promoter.

Initiators, or the initiating aspects of complete carcinogens, cause alterations in DNA structure and are mutagenic by virtue of their reactivity as electrophiles or after being metabolized to an electrophilic form, according to the well-known work of James and Elizabeth Miller [6]. Sporn and Newton have recently commented that
"classical strong 'carcinogens' . . . all of which are known to damage DNA and to be highly mutagenic, may be postulated to have the ability to inactivate the genes that normally repress the synthesis of transforming proteins, and thus to activate synthesis of transforming proteins in an irreversible manner" [7]. In other words, they would regard transformation as a derepression that results from a mutation.

Promoters do not damage DNA and are not mutagenic. However, they do affect phenotypic gene expression and favor cell proliferation so long as they are present [8]. Promoters have been shown to affect cell differentiation [9] and to block intercellular communication [10,11,12], of which more will be said later. It is proposed here that promoter action includes the derepression of initiated cells by virtue of the action of the promoter on normal cells, which may decrease the output of chalones or increase the output of growth factors.

The concepts of initiation and promotion are validated in the final analysis by the fact that the phenomena of "pure initiation" and "pure promotion" have been documented (see below). It seems undesirable to call promoters carcinogens and then use the label as evidence [12] that there are exceptions to the expanding generalization [6] that carcinogens are electrophiles and damage DNA.

Thus neither initiators nor promoters are carcinogens. Acting alone they do not produce cancer, or we may say produce cancer at a very low probability. In contrast, while acting together in proper sequence, they can produce cancer in nearly 100 percent of animals, using relatively small experimental groups, e.g., 20 to 30 animals. We thus pose a problem for basic science and a hypothesis upon which to proceed.

Problem: How to explain extremely low cancer probability by initiators or promoters acting alone and high probability when acting in appropriate sequence?

Hypothesis (in simplest form): Cancer results from two or more relevant mutations: Promoters enhance proliferation of cells with one relevant mutation; this increases the probability of obtaining one cell with two relevant mutations.

The development of this hypothesis will be the purpose of this lecture. Relevant literature on the two-hit or multiple-hit mutation theory of cancer is available [1,13–16]. Bell [17] has commented "it is possible that the action of the 'promoter' is simply to expand the mutant clone to a critical size." My hypothesis combines these ideas.

Models for Demonstrating Initiation and Promotion

The experiments that have defined the concept of initiation and promotion were begun by Berenblum, who studied the production of skin tumors in mice [18]. However, the methodology has been extensively refined and extended by R.K. Boutwell of the McArdle Laboratory, who has gone on to explain tumor promotion as gene activation [19]. Figure 1 is taken from a recent report by Boutwell [20] since it describes the classic example of initiation and promotion. It will be noted that no tumors in mouse skin result when only initiator is applied, or only the promoter, or when the sequence is reversed or when promoter is not applied at an appropriate frequency. However, when a single application of initiator is followed by applications of promoter at suitable frequency many skin tumors result. In these experiments the number of mice with tumors is nearly 100 percent when both initiation and promotion occurred. This type of experiment raises several issues not immediately
FIG. 1. Initiation and promotion in mouse skin. From Boutwell [15]. The application of a single subcarcinogenic dose of a carcinogen such as benzantracene or dimethylbenzanthracene can act as an initiator, while croton oil or 12-0-tetradecanoylphorbol-13-acetate (TPA) given repeatedly at suitable intervals can act as promoters. Protocol No. 5 indicates that initiator action is irreversible and considerable intervals of time can intervene between the application of initiator and promoter. Protocol No. 6 indicates that if intervals of time between promoter applications are too long, no tumors will result. Not shown but implied by Protocol No. 6 is the fact that single applications of promoters are ineffective in the case of the classic mouse skin experiments.

apparent. First, it appears that with moderate numbers of animals there can be a threshold effect with no tumors developing with “subcarcinogenic” single doses of a carcinogen employed as an initiator not followed by a promoter. Skin tumors are produced only with larger doses of the carcinogen, in which case the carcinogen needs no second chemical. Second, since application of promoter to skin treated with an initiator or subcarcinogenic doses of a carcinogen will produce many tumors, it is evident that skin treated only with initiator must contain many altered cells that cannot be identified under the microscope. These altered cells are what I will call “operationally normal” in the absence of promotion. This idea is pertinent to the experiments used to argue that tumor cells are not mutants [2] since it proves that mutant cells relevant to cancer can be operationally normal in the appropriate cell microenvironment.

The point is that in real life many people can be exposed to subcarcinogenic or sub-threshold levels of a carcinogen and never develop cancer even though they may have substantial numbers of initiated cells at what I will refer to as Stage I Inhibited, i.e., repressed, single mutant cells. This concept is very important to our understanding of lung cancer in men and women as a result of cigarette smoking. In my opinion lung cancer in the human is the second classic example of initiation and promotion. This possibility has been the subject of an editorial in the journal Lancer [21]. In 1959 Roe, Salaman, and Cohen [22] carried out experiments demonstrating promoter activity in cigarette smoke condensates. They concluded, “The correlation between smoking habits and lung tumour incidence may well be determined not primarily by the carcinogenic effect of tobacco smoke but by its predominantly tumor promoting action on the bronchial epithelium.” Direct support for this conclusion is available on the basis of studies on the bronchial epithelium of 216 carefully matched smokers, former smokers, and non-smokers who had died of causes other than lung cancer [23]. The “former smokers” had smoked for at least ten years but had not smoked for at least five years before the time of death. My conclusions are based on the findings that 93.2 percent of the 3,156 sections from 72 smokers contained atypical cells while only 6 percent of 3,436 sections from 72 “former smokers” contained such cells and only 1.2 percent of the sections from non-smokers showed atypical cells. The study suggests that discarding the smoking habit may be helpful in preventing lung cancer no matter how many times it has to be attempted. In discussing the above results,
E. Cuyler Hammond expressed a theory of carcinogenesis that is almost a paraphrase of the initiation-promotion concept [24] as presented in this paper. Hammond regarded the cells with atypical nuclei to be genetic variants ("I will assume them to be such" he said [24]). He continued, "I next propose that some of the variant cells with atypical nuclei are better adapted to an environment containing cigarette smoke products than are ordinary cells." In the smoke environment the bronchial epithelium can cause both hyperplasia and metaplasia. In the former there is an increase in the number of basal cells from just one or two rows to as many as 20 or more rows with "little or no changes in the size or appearance of individual cells." This is essentially a description of promotion. Hammond's theory of carcinogenesis is essentially a concept that cancer involves both somatic mutation (initiation) and a changed microenvironment that encourages proliferation of initiated cells (promotion). Hammond interpreted the earlier findings [23] to mean that "If the individual stops smoking, the [micro]environment reverts to freedom from cigarette smoke products, the selective process is reversed and ordinary cells replace variant cells" [24]. I agree with his views and merely add a hypothesis that is self-evident, namely, that the proliferation of cells with one or more relevant mutations directly increases the probability of getting a cell with additional relevant mutations, as I will describe below.

The third model of what is now widely regarded as initiation and promotion is the case of liver cancer produced in experiments with rats. Many experiments on liver carcinogenesis have been carried out at the McArdle Laboratory by the Millers, by Henry Pitot, by Brian Laishes, and by me as well as by other groups throughout the world. From among the latter I will present charts adapted from the reports of Roy Albert et al. [25] at New York University and Carl Peraino et al. [26] at the Argonne Laboratory (Fig. 2). Albert fed acetylaminofluorene (AAF) at a variety of levels (0.001, 0.003, 0.01, and 0.03 percent) in the diet for various periods of time (8, 16, and 32 weeks). In terms of the threshold concept, it is interesting to note that even with 32 weeks of treatment and over 80 weeks of observation, no tumors were seen at the two lower levels of AAF. Tumors were observed after exposure to diets containing 0.01 or 0.03 percent AAF but the incidence was greatly delayed at the lower dietary level, and in fact no tumors were observed when 0.01 percent AAF was administered for eight weeks. It is my opinion that in these experiments there were single mutant Stage I cells present in the livers at the ineffective doses of AAF. In order to obtain tumors it was necessary to administer AAF at adequate doses for longer times. This was sufficient to bring about promotion to what I call Stage II, The Critical Mass, and to provide sufficient time to permit progression to the rapidly growing large liver tumor. In terms of the threshold concept, it is not possible to calculate the probability of cancer production at low doses of AAF by using large doses of AAF for longer periods because at the low doses promotion is not present and at the high doses promotion is present. I make this claim because in 1973 Carl Peraino et al. [26] carried out a crucial experiment, also shown in Fig. 2, in which 0.02 percent AAF was given for only 18 days and then followed by either basal diet or a diet containing 0.05 percent phenobarbital, which most people now believe to act as a promoter of liver carcinogenesis in these experiments [27]. The data in Fig. 2 show the incidence of rats with tumors 1 cm in diameter or larger in the Peraino experiment. In my laboratory I have just completed an experiment with 0.015, 0.030, and 0.045 percent AAF for only 14 days, followed by 0.05 percent phenobarbital. The data for this experiment are in process. I wish to emphasize that the goal of my own experiments is to find the lowest amount of AAF that will produce demonstrable lesions in liver when followed by a
promoter and to inquire whether hepatocarcinomas are produced at these exposures. There are still no hard data anywhere on liver tumor incidence as a function of AAF dose in the lowest ranges used by Albert et al. (0.001 and 0.003 percent) followed by maximal promotion. At this time I can state that under conditions as in the Peraino experiment in Fig. 2, phenobarbital alone does not produce any hepatomas; that is, it acts as a pure promoter in the case of rat liver carcinogenesis. I will now turn to some of the background for the modern studies.

**Clinical Origins of the Promoter Concept**

All of the present basic science approaches to chemical carcinogenesis have a background in the past. There were many indications that led people to believe that irritation had something to do with cancer. However, there were only a few observations that seemed to indicate the possibility that irritation per se was not the cause of cancer but that it mattered only when some earlier event had occurred. I am aware of only two such cases:

In 1761 John Hill, an English physician, wrote as follows [28]:

> Whether or not [cancers], which attend Snuff-takers, are absolutely caused by that custom: or whether the principles of the disorders were there before and Snuff only irritated the parts, and hastened the mischief, I shall not pretend to determine . . .

Over 100 years later, in 1874, Francesco Durante, an Italian physician, summarized a series of his clinical experiences with the following statement, in which he drew on an analogy that he related to his own clinical experience [29].
Such elements remain enclosed within well matured tissue for years and years, betraying not the least indication of their presence, until an irritation, a simple stimulus, suffices to rekindle in them movement and cellular activity as is excited by heat within elements of the germinal macula of fowls’ eggs.

Basic Science Origins of the Promoter Concept

In 1974 Boutwell [19] in reviewing the status of the promoter concept noted that, following the observations of John Hill in 1761,

Nearly 150 years elapsed before Yamagiwa and Ichikawa in 1915 showed that similar lesions could be produced experimentally by the repeated application of coal tar condensate to the ears of rabbits. By 1918 Tsutsui had shown that mice were also responsive. Because the coal tar caused inflammation and wounding these men concluded “The repetition or continuation of chronic irritation may cause cancer” in confirmation of Rudolf Virchow’s hypothesis.

Here the point is that when the experimental production of cancer in rabbits and mice with coal tar was first achieved there was no intuition that separated the idea of irritation from the idea of cancer production by chemicals. It took a long time for the isolation of precisely those chemicals that would produce cancer when applied repeatedly in sufficient amounts. The definitive story of their isolation from two tons of coal tar in the 1930s cannot be detailed here but the exact structures were worked out and they could be chemically synthesized. Among them was the aromatic hydrocarbon, 3:4 benzpyrene, which was widely used for the production of skin cancer in mice. An authoritative account of the work by Sir Ernest Kennaway and his collaborators has been published [30] and reference works are available [31]. With pure carcinogenic compounds available investigators could now do experiments that would separate the idea of irritation from the idea of carcinogenesis. The first hint came from the pen of Berenblum in 1941 while still in England even before he had carried out experiments that clearly separated promotion from initiation. He began to suspect that irritation was not inevitably carcinogenic and wrote as follows [18]:

In clinical discussion on the relation of irritation to carcinogenesis, there is a tendency to oversimplify the issue by considering only 2 possibilities, either that all irritants are carcinogenic, or that irritation has nothing whatever to do with carcinogenesis.

In the same paper, he reported experiments with a known irritant, croton resin or croton oil, obtained from the croton bean. Although it was some years before the more elegant experiments were carried out [19,20], Berenblum stated the problem quite clearly:

Was it possible that among [irritants] there were some which, without being themselves carcinogenic, could augment the tumor-producing action of a carcinogenic agent when the degree of irritation by the latter was insufficient?

Berenblum used the word cocarcinogen and originally used a carcinogen and croton oil together. Meanwhile other experimenters used drastic methods to induce wound healing in areas pretreated with a carcinogen. Thus Peyton Rous and coworkers used cork borers to punch holes in pretreated rabbit ears and observed increased cancer
formation on the healing margins. They wrote the first paper with the words\textit{initiation} and \textit{promotion} in the title [32] and commented as follows:

Many of the hidden entities, which ordinarily would not come to anything as the late findings show, can be induced to assert themselves and form visible tumors by agents or conditions which do not themselves initiate neoplastic change . . .

\textit{Origins of the Modern Era}

At almost the same time, in 1944, an English investigator J.C. Mottram [33] performed a key experiment that set the stage for many more elegant experiments by Boutwell and others [19,20]. His report included the idea, not fully exploited, that a single application of a subcarcinogenic dose of a carcinogen could be employed to study the effect of a promoter. He wrote as follows:

The combination of croton oil with benzpyrene provides a much more delicate test than the sledge-hammer treatment of continuous painting . . . By its use very short paintings with benzpyrene—even a single painting—sufficed to produce both benign and malignant epidermal tumors.

From these early beginnings in 1941 and 1944 it required nine more years for another milestone when it was shown by Salaman and Roe [34] in England that ethyl carbamate (urethane), a very simple compound, can act as a pure initiator of skin cancer in mice.

The object of the present work was to test the hypothesis that there exist also initiating agents which are not carcinogens, at any rate for the tissue under treatment. . . . It is concluded that urethane is an initiator of carcinogenesis, but not a carcinogen or a co-carcinogen, for mouse skin.

They showed that urethane alone produced no skin tumors. However, when followed by croton oil in multiple treatments many skin tumors resulted.

Their report was almost immediately repeated and confirmed by Berenblum and Haran who reported two years later [35]:

These results support the findings of Salaman and Roe (1953) that urethane, itself non-carcinogenic for mouse skin, can induce the initiating phase of carcinogenesis in that tissue.

These two reports are extremely significant because they show that promotion can be the deciding factor for cancer production. If a completely non-carcinogenic compound can be capable of producing cancer when followed by a promoter, what about the many compounds that seem to be weak carcinogens because, like urethane, they exert little or no promoting activity? What about the situation in which humans are exposed to small doses of strong carcinogens followed by conditions that are not promoting? The data are also vital to the concept that initiated cells can be "operationally normal."

The point is that a Stage I initiated cell can behave as a normal cell in one microenvironment and as a neoplasm in another, just as John Hill and Francesco Durante suspected over 100 years ago. There are many references in the literature to the concept of the "sleeping" or "dormant" tumor cell [36]. Experiments with initiators and promoters now provide the opportunity to learn how hormone imbalances and life styles may relate to cancer. An example of contrasting life styles is seen in the case of heavy smokers vs. occasional smokers.
Feedback Control by Differentiated Cells

For some time there has been in the literature a fundamental idea that is the parent of much current research. I have elsewhere [3] referred to it as the Osgood Principle and cited Osgood's efforts at the University of Oregon dating back to 1950 [37,38]. In 1957 he stated the principle that "with the majority of cell series the homeostatic regulator is an inhibitor of arithmetic cell division and is probably produced by the most chemically mature of the differentiating cells of that series" [37]. The same idea was put forth at about the same time by Weiss and Kavanau [39] and has been extensively discussed by Iversen [40]. I suggest that there are two models for feedback control of cell proliferation. In the first model (Fig. 3) the organism sends signals

![Diagram](https://example.com/diagram.png)

FIG. 3. Two models for the regulation of progenitor cell proliferation. Modified from Potter [3]. Both models illustrate the role of the progeny cells in regulating the proliferation of stem cells. Model I illustrates the general case, while Model II is more in line with the proposals by Osgood [37,38], Weiss and Kavanau [39] and Iversen [40], all of whom emphasized negative feedback. The inclusion of positive feedback in the scheme [3] is based on the recent work of Kurland et al. [41]. In both models, the cell cycle is shown in the conventional way with M for Mitosis, S for DNA synthetic period, G1 for the time gap before S, and G2 for the time gap between S and M. Gm to GNth represents a series of stages in differentiation that are still able to get back to G1 and move into the cell cycle. The superscripts A to Nth represent special cell properties that characterize a particular stage and may be transiently expressed. GT (from ref. [3]) represents a stage of terminal differentiation beyond Go and no longer able to return to G1. "Organism-serving molecules" such as hemoglobin form the link between the terminally differentiated cells and the organism, and the term is preferred to "luxury molecules" [3].

In Model II CSF is for Colony Stimulating Factor, which is a glycoprotein [41]. There appear to be a number of glycoproteins with this function and with different sources and target stem cells. The balance between G1 and Go is not fixed but can vary according to the balance between positive and negative feedback factors. The prostaglandin PGE shown in Model II is based on a particular cell system [41] and the negative feedback indicated for PGE is not to be generalized, since many other substances perform the chalone function depending on the tissue [40].
from differentiated cells of a different series, e.g., kidney, to the stem cells of a line that gives rise to quite different cells, e.g., in bone marrow. However, this is but a modification of the Osgood Principle, which is shown as model II in Fig. 3. Here it is emphasized that both growth stimulators and growth inhibitors can spring from the more differentiated cells and can act on the progenitor or stem cells, as demonstrated by Kurland et al. in 1978 [41]. These feedback systems are actually the means of control by which normal cells can repress gene expression in initiated cells or in each other. It is this feedback control by normal differentiated cells that I am proposing as the significant locus of promoter action. Feedback may conceivably act either to control gene availability or phenotypic expression of available genes.

That control of gene expression is a many-splendored thing is illustrated by Fig. 4. In this figure it may be noted that at least six categories of regulatory sites of action may be specified. Many more could be documented. In examining differentiation at the molecular level it should be recalled that in any given cell only a small fraction of the total genome is available for transcription. Availability of a DNA sequence does not imply that transcription is occurring at the same rate at all times. Thus modulation of phenotypic expression can occur at four levels:

1. Change in enzyme activity without enzyme synthesis
2. Change in enzyme amount (by synthesis or degradation)
3. Change in enzyme amount with mRNA synthesis from available genes
4. Change in mRNA amount with change in DNA availability

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**Fig. 4.** Six categories for the modulation of phenotypic gene expression. The regulation of cells in G₀ and G₁ stages in Fig. 3 may be seen as physiological adaptations to environmental stress or change. The numbers in the chart indicate to some extent the sequence of responses by cells in the G₀ stage, while cells in G₁ no longer have the sixth response open to them. The scheme includes the action of cyclic AMP via protein kinases that phosphorylate proteins, making them either active or inactive, and it is also indicated that not all protein kinases are cyclic AMP dependent.
It seems likely that in various examples of differentiation the fourth category is involved, while maturation in a cell series may involve only the third category.

*The Stages of Chemical Carcinogenesis in Liver*

As a result of current studies in my own laboratory and of work with which I am acquainted I propose a somewhat new scheme of staging categories based on the concepts of initiation and promotion (Fig. 5). According to this scheme we propose five stages that are relevant either to continuous carcinogen treatment or to initiation-promotion treatments:

- **Stage I**—Inhibited single mutant cells
- **Stage II**—Critical mass of cells
- **Stage III**—Slow growth subject to fluctuations in host repressors
- **Stage IV**—First appearance of double mutant cell
- **Stage V**—Fast autonomous growth and further mutation in double mutant clones

The individual stages can now be discussed in more detail.

*Stages I and II*

The methodology employed is a histochemical stain for demonstrating enzyme action in a thin slice of properly fixed tissue. The method reveals the presence of gamma-glutamyl-transpeptidase (GGT) by the production of a red dye [42]. A slice of normal liver appears almost colorless to the unassisted eye, while microscopic inspection reveals GGT in bile duct epithelium. Our scheme proposes that in Stage I there are numbers of single mutant initiated cells depending on the dose of initiator and the number of susceptible cells. However, these single cells are repressed and held in a non-proliferative state by feedback from adjacent normal cells and possibly by more distant cells. They are virtually impossible to count at the single-cell stage for two reasons: they are difficult to see and identify with certainty, and the probability of hitting any given single cell with only a few slices per liver is very small. The stain for GGT activity is a method for visualizing cells or clones referred to as "enzyme-altered foci" always with reference to GGT or whatever other property that can be visualized histochemically. At this date it is still not clear what alterations are necessary for a cell to be quiescent in the absence of a promoter and to proliferate in the presence of a promoter, and the enumeration of GGT-positive foci is taken only as a measure of the ability of a foreign compound to initiate changes that result in proliferation when a promoter is administered.

The reason for postulating the existence of Stage I single mutant repressed non-proliferating cells is based on two lines of collateral research, in addition to the actual observations of the GGT-positive groups of cells seen when phenobarbital is given. First, there is a large and growing literature on the existence of tissue-specific growth suppressors under the name of *chalones*, for which reports and reviews are available [4,40,43–46]. But more to the point is a new development that deserves considerable attention and further research. In the last week of November 1979 two publications appeared simultaneously, reporting that tumor promoters blocked intercellular communication, thereby strongly supporting the proposition illustrated in Fig. 5. One report was from James Trosko and coworkers at Michigan State University [10] and the other was from Murray and Fitzgerald [11] at the University of South Australia, using different cell types and different measurements, but with both groups emphasizing the property of tumor promoters to block intercellular communication. A third report confirming the phenomenon in liver cell monolayers has been made [12].
FIG. 5. Five stages of development in the production of hepatocellular carcinomas by compounds acting as initiators and as promoters. Experiments with rat liver have employed acetylaminofluorene (AAF) at low levels for brief periods as an initiator [26,27] and phenobarbital as a promoter [26,27]. Enzyme-altered foci have been visualized by a histochemical procedure that reveals gamma-glutamyl transpeptidase (GGT) [42]. In the above scheme, open circles represent single cells or enzyme-altered foci that are subject to chalone action (negative feedback) from neighboring normal cells, indicated by arrows targeted on the open circles. To represent the application of the GGT technique, these open circles might be colored red. The filled circles represent double-mutant or N + 1 mutant cells that are not subject to host controls. The double bars through the arrows represent promotor action blocking the intercellular communication between normal cells and initiated cells. The double bars between arrows and cells represent a stage at which the proliferation of cells in the enzyme-altered foci can proceed even in the absence of promoter, i.e., when the altered cells have reached the stage of "critical mass." The Stage III cells are in the form of neoplastic nodules that are highly differentiated and slowly growing, as in the case of the comparable Morris hepatomas [3]. The "progression through time" is assumed to involve a second or N + 1 mutation to yield the cells represented by filled circles in the chart. This "progression through time" is postulated to occur without the continued presence of a carcinogen or simple initiator and to be the result of single (relevant) mutations that occur spontaneously or as the result of unknown environmental influences. Such single mutations would not result in a neoplasm except when superimposed upon a genome that has already had a relevant mutation. The key to the entire scheme is the idea that a second or N + 1 mutation with a low probability becomes increasingly probable as the population of first-stage altered cells increases. "Conversion" represents the experimental production of a second step in the initiation process acting on one or more cells in the expanded population of Stage I cells (refer to Table 1).

The only way the number of initiated "single-mutant" cells can be estimated is by treating the animal with a promotor or a promoting influence that in my proposal blocks the repression exerted by the normal liver cells. In this situation a clone of from 1,000 to 10,000 cells will develop and form a mass that is readily seen under the
microscope. In the case of the larger clones they can be seen on a microscope slide with the unassisted eye. The probability of hitting a clone with a single liver slice depends in a complex way on the size of the clone. Formulae for taking the probabilities into consideration have been worked out and applied by Scherer et al. in Amsterdam [47]. Other methods are being investigated by Dr. H.A. Campbell in connection with experiments referred to earlier (unpublished). After sufficient time has passed many of the clones will have attained a sufficient mass to be able to survive and grow slowly in the absence of the promoter even though subject to fluctuations in host repression. This is Stage II, the critical mass.

The newer techniques for visualizing clones of enzyme-altered cells are very dramatic and there are now at least four different properties that have been visualized [48–51]. It has been a thrilling experience to look at a liver section and to see masses of altered cells for the first time, using the GGT technique even though subsequent experience has shown that these cells are not necessarily destined to become cancer cells. Work in my laboratory has suggested to me that there may be single mutant cells and clones that cannot be visualized by any of the available techniques, and in fact there can be hepatomas that do not stain for GGT [52].

**Stage III: Slow Autonomous Growth**

Just as the number of initiated cells can be appreciated only by promoting their proliferation and allowing time for them to reach a feasible size, the number of cells that have attained critical mass can be appreciated only by withdrawing the promoter and allowing sufficient time for the regression of the clones that have not attained critical mass. It is possible or even likely that the various clones at this stage include a variety of combinations of mutations and that the designation “single mutant” is an over-simplification, since there must be many kinds of single mutant cells and cells containing two or more mutants. Various combinations of enzyme alterations within single livers have been demonstrated by Pitot et al. [48] and by Goldfarb and Pugh [49]. That the phenomenon of regression to what I would call “operationally normal” cells when carcinogen or promoter is withdrawn was suggested by the earlier work of Teebor and Becker [53] and more recently by the work of Gary Williams and coworkers [51,54] whose tabulated data I have converted to graphic form.

In Fig. 6 are shown the data for the rate of appearance of different sizes of altered enzyme foci as a function of time on a diet containing 0.02 percent AAF, expressed as foci transections per sq cm not corrected for probability of hits (left panel). In the right panel are shown similar data for the disappearance of the altered foci when the animals were placed on a basal diet. It may be seen that nearly all the foci disappeared.

In Fig. 7 further tabulated data from Hirota and Williams [54] are shown graphically. My purpose is to illustrate the change in the numbers of Stage I, Stage II, Stage III, and Stage V cells with time. With continued exposure to a diet containing 0.02 percent AAF the numbers of enzyme-altered foci transections increased. When the AAF diet was discontinued the numbers dropped sharply by 12 weeks, but the numbers increased somewhat by 24 weeks as critical masses of cells continued to expand and become more likely to be counted. But it is emphasized that the number of altered foci would calculate to thousands per liver as reported by Pitot [56], while the number of Stage VI nodules was in the range of 0 to 20 per liver and the number of cancers after 48 weeks off the diet was in the range of 0 to 3 per liver. My interpretation of these findings is in terms of Stages I to III for the assumption of the
need for a further mutation (Stage IV) to account for the much smaller number of cancers in Stage V at the end of the experiment.

Stage IV: The Occurrence of a Second or \((N + 1)\) Mutation

It is well known from our previous work that there are highly differentiated hepatomas that grow very slowly and respond to glucagon by producing cyclic AMP, while other, more rapidly growing hepatomas do not respond to glucagon [57]. We propose that the rapidly growing hepatomas have at least one additional mutation and that the numbers shown in Fig. 7 derive from this possibility. Promotion would enhance the possibility of a second mutation because of the increased number of cells with one mutation. In contrast to this route, the probability of a second mutation without promotion is very small because there are vastly fewer cells available for a hit (Fig. 5).

Thus, the overall scheme (Fig. 5) shows two pathways to the Stage V progressed tumor, or hepatocellular carcinoma in this system. I refer to the low probability pathway in the absence of promotion and the higher probability by a factor of 1,000 or more in the presence of promotion (Table I). In the case of urethane-treated mouse skin, as I mentioned earlier, there were no tumors in the absence of promoter and high incidence in its presence [34,35]. Relevant to this figure I emphasize the
FIG. 7. Enzyme-altered foci transections per cm², neoplastic nodules per liver, and hepatocellular carcinomas per liver as a function of time on or off a diet containing 0.02 percent acetylaminofluorene (AAF). The charts were made by plotting data from Hirota and Williams [49] (with permission from G.M. Williams). The ordinate expresses the number of foci transections per cm² as noted in Fig. 6. "Cycles" refers to periods of AAF feeding followed by a period of basal diets as indicated on the abscissa. Groups of animals received AAF for three cycles (● or ○), four cycles (■ or □), or five cycles (▲ or Δ). Solid lines represent the time during cycling. Dashed lines represent the time when animals were permanently shifted to basal diet. None of the groups in this chart or Fig. 6 received phenobarbital. In a separate publication Williams and Watanabe [55] reported that when phenobarbital was given in place of basal diet following AAF, the marked decline in foci transections seen in Fig. 6 and Fig. 7 did not occur.

experimental fact, never demonstrable before the studies on enzyme-altered foci, that there are thousands of enzyme-altered foci at Stage I even with low doses of AAF, when visualized with the aid of a promoter. Later there are only a few dozen promotion-independent foci as Stage III, and finally there are either no or only a few rapidly growing hepatocellular carcinomas at Stage V. These numbers are very rough and much further work will be required to improve their accuracy and significance. However, I have used the order of magnitude of these numbers, plus the recent findings of Trosko and others [10,11,12] to suggest that promoters act to block the repression of initiated cells by normal cells, thereby leading to their proliferation. Depending on the degree of proliferation of the first stage cells, the probability of further stages is greatly enhanced. Together the available data appear to support the hypothesis I proposed in the beginning. This hypothesis is based on the vast literature now available regarding the mutational nature of initiation and the non-mutational nature of promotion [58,59]. Moreover, the phenomenon appears to be applicable to a variety of tumors [56]. Wider appreciation and understanding of the phenomena of initiation and promotion is important because the available data suggest that (1) there may be a low risk of human cancer at levels of carcinogens too low to elicit promotion, and (2) more effort should be made to evaluate the identification and the role of promoting influences in the overall risk of human cancer. Although the
### TABLE 1

Estimation of Effect of Promotion on Probabilities in Chemical Carcinogenesis in Liver*

1. **Calculate hepatomas per rat with 2 single low doses of initiator separated in time, with no intervening promotion.**

   \[
   \text{Hepatomas per rat} = \frac{\text{Probability of 1st hit} \times \text{Probability of 2nd hit}}{\text{No. of cells per liver}}
   \]

   \[
   \text{Probability of 1st hit} = \frac{\text{No. of clones after 1st hit plus promotion}**}{\text{No. of cells per liver}}
   \]

   Assume \(10^9\) cells/liver.

   Assume number of single hit cells = number of clones after single low dose plus promotion of single one-hit cells to clones**; take 1,000 such clones as an example (= low value)

   then, Probability of 1st hit = \(\frac{1,000}{10^9}\) = \(10^{-6}\)

   Assume Probability of 2nd hit = Probability 1st hit

   Assume 2 hits in one cell = Hepatoma

   then, Probability of 2 hits per liver = \(\frac{10^{-6} \times 10^{-6}}{10^9}\) = \(10^{-3}\) per liver

   = 1 hepatoma per 1,000 rats

2. **Calculate hepatomas per rat with 2 single low doses of initiation with promotion intervening to give clones of average size 1,000 cells.**

   Assume same no. of 1-hit cells after 1st single dose of initiator = 1,000 cells

   Calculate number of 1-hits after promotion = number of clones × number of cells per clone \(f\) (time)]

   Assume 1,000 cells per clone (= low value)

   then, No. of 1-hit cells after promotion = \(1000 \times 1000 = 10^6\)

   then number of cells with 2 hits = No. of hepatomas expected

   = \(10^8\) (probability per cell) \(\times 10^9\) (no. of cells with 1 hit)

   = \(10^9\) (cells per liver)

   = 1 hepatoma per rat, when promotion intervenes between single low doses of initiator, compared to 1 hepatoma per 1,000 rats with no promotion.

*Based on the assumption that two relevant hits are required to give a neoplasm not requiring promotion. (Example calculated on basis of 1,000 single-hit cells after single low dose of initiator, and 1,000 cells per clone after promotion. Both are low values.)

**Promotion is needed to permit the counting of the single-hit cells, but is not employed in a group of comparable rats that receive "2 single low doses of initiation separated in time, with no intervening promotion."

Simplistic form of my hypothesis and of my staging categories may need modification, I am convinced that studies on the molecular mechanisms and practical aspects of initiation and promotion deserve and will get much further attention in the “Decade Ahead.”

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