Chemical Carcinogenesis: A Long-Neglected Field Blossoms

The directions of cancer research have undergone substantial changes during the last few years. In large part, these changes simply reflect the natural progression of research, but there is little question that the pace of that progression was sharply accelerated by the National Cancer Act of 1971, which initiated what politicians then termed a "crusade against cancer." As a result of that act, federal funding for cancer research has nearly tripled—from $190 million in 1970 to an estimated $527 million in 1974—and cancer has become the best-funded area of biological research in this country.

Meanwhile, in biology there has been an increasing emphasis on the study of mammalian cells, rather than on bacteria and other simpler organisms. Hence, with the increased cancer funding and the near stagnation in support for most other types of biological research, many new investigators have been attracted to the study of cancer. A recent estimate from the National Institutes of Health indicates that some 7.7 percent of the total U.S. biomedical manpower pool—more than 7250 scientists—are working on projects that can be directly related to cancer, and many more are working on projects that are peripherally related.

The effect of these stimuli has been to produce distinct changes in emphasis within many of the subdisciplines of cancer research. In viral carcinogenesis, for example, there has been a subtle shift from efforts to isolate a tangible human cancer virus to more sophisticated attempts to detect biochemical traces of such viruses in human tumors. In chemical carcinogenesis, there has been a more marked shift away from simple screening and identification of possible carcinogens to a detailed examination of the interaction of chemical and cell. The investigation of the biochemistry of cancer has increasingly been focused on the role of cellular membranes in tumorigenesis and on the identification of tumor antigens that might permit earlier detection and more precise quantification of the disease. And there has been a strong trend in cancer therapy away from reliance on any single tool (such as surgery, irradiation, and drugs) toward a combined modality approach and a renewed interest in stimulation of the body's own immune defenses.

There is still no consensus about the cause or causes of cancer; indeed, it is often difficult to find a consensus about any aspect of cancer research. The sharply conflicting views of investigators in different subdisciplines has been most aptly summarized by one scientist, Charles Heidelberger of the University of Wisconsin, who argues that "the mechanism of cancer is a mirror into which each man looks and sees himself."

There is also no prospect of an imminent cure for cancer. But there has been progress, and there is the promise of much more to come. In the next few weeks, Research News will present several articles assessing the status of cancer research and examining some of the areas where that progress has been most apparent. These articles cannot pretend to be either comprehensive or exhaustive. Instead, they will focus on what appears to be some of the most interesting and significant developments in the drive to understand the molecular biology of cancer.

Chemicals—in the workplace, in the environment, and in the diet—may be the single most important cause of human cancers. Many scientists estimate that at least 60 percent and perhaps as many as 90 percent of the 655,000 cases of cancer that will be discovered in the United States this year will have been caused by environmental factors, mostly chemicals. Almost 1000 chemicals have been reported to produce tumors in man or other animals, and many times that number are suspect.

Yet despite these statistics, and despite the large amount of effort that has been expended since chimney soot was first identified as a cause of cancer 199 years ago, little is known about the mechanisms of chemical carcinogenesis. In part, this state of affairs results from the greater emphasis that has been placed on viral carcinogenesis. More important, it reflects the fact that, until recently and with only a very few exceptions, the field has been dominated by experimentalists who paint suspect chemicals on the skins of animals or feed the chemicals to animals to determine whether the chemicals induce tumor formation. This process has been useful in identifying carcinogens that should be removed from the environment, but it has been of limited value in elucidating the mechanisms of carcinogenesis or in revealing ways to inhibit or reverse the action of the chemicals.

While a major portion of the resources allotted to the study of chemical carcinogenesis is still devoted to such screening (see accompanying box), a new emphasis is being placed on the molecular biology of carcinogenesis. This emphasis has, in the past 5 years, attracted many new investigators into the field and has stimulated a blossoming of research comparable to that which occurred in viral carcinogenesis several years earlier. This research already has illuminated some of the first steps in the interaction between carcinogen and cell, has demonstrated that most carcinogens must be activated by the host's metabolism, and has led to the tentative identification of certain groups in the population which may be more susceptible to exposure to carcinogens.

A primary contributor to this blossoming has been the development of cell culture systems in which healthy cells can be transformed (converted to malignant cells) by chemicals. As compared to work in vivo, experiments with these systems provide many advantages: the period between the application of a chemical and the appearance of its effect is sharply reduced; the environment can be totally controlled; host factors can be eliminated; and the dosages of chemicals can be controlled.

Most important, the systems permit detailed biochemical examination of the metabolism of the carcinogens and of changes they induce in the cells. Significantly, it was the development some 5 years earlier of comparable cell cultures which can be transformed by viruses that led to many of the discoveries in viral carcinogenesis.

Maintaining healthy, growing cells in culture, some scientists argue, is in many ways more art than science, a factor that has impeded development and implementation of the culture systems. Regulation of the proper balance of nutrients, serum, and growth factors in the medium is a delicate skill best learned by apprenticeship, and developments in the art are often communicated more readily by personal contact than by journal publication.

Hamster Cell Cultures Were First

The first investigator to apply the art successfully to chemical carcinogenesis was Leo Sachs at the Weizmann Institute in Israel, who developed a hamster embryo cell system in the mid-1960's (see box). This system has been refined and used extensively by Joseph A. DiPaolo and his associates at the National Cancer Institute (NCI) in Bethesda, Maryland. Another early entrant in the field was Charles Heidelberger of the McArdle Laboratory for Cancer Research at the University of Wisconsin Medical School, Madison, who developed cell cultures of fibroblasts (connective tissue cells) from mouse prostate glands. At about the same time, Elizabeth K. Weisburger of NCI and John H. Weisburger of the American Health Foundation in New York City, developed cultures of epithelial cells from mouse livers. Several other investigators have subsequently developed systems derived from other types of cells.

The culture systems share the same general characteristics: The parent cells proliferate until they form a confluent monolayer in the culture dish, at which time they cease dividing. Application of appropriate carcinogens produces colonies of morphologically altered cells which do not stop dividing when they contact other cells and which cannot be visually distinguished from malignant cells. The transformed cells acquire antigens and other properties of tumor cells in vivo, but will not grow into a tumorlike mass in culture, partly because of a tumor's requirement for a circulatory system. Because the biochemical characteristics that distinguish malignant from healthy cells are largely unknown, however, the ultimate criterion for transformation is formation of a tumor when the cells are injected into a genetically identical animal or one that has undergone immunosuppression.

Some of the cultures are, by all available criteria, free of oncogenic viruses. Most of the systems are susceptible to transformation by such viruses, but the malignant cells thus formed are antigenically distinguishable from those transformed by chemicals. Some of the cell strains grown in culture are aneuploid; that is, they have an abnormal number of chromosomes, and critics argue that this abnormality creates an intrinsic bias toward transformation. The cultured cells exhibit a very low incidence of spontaneous transformation, however, and the spontaneously transformed cells, unlike those transformed by chemicals or viruses, are generally not antigenically distinct from the parent cells. There is, moreover, a very good correlation between the chemicals that transform cells in culture and those that are oncogenic in vivo.

A crucial reservation in assessing these systems has been that no one has yet satisfactorily demonstrated the transformation by chemicals of human cells in culture. Most of the research findings thus can be applied to humans only by analogy. Another reservation is that many of the cell systems contain only very low concentrations of the enzymes necessary for metabolizing the carcinogens. Perhaps the most important conclusion reached about chemical carcinogens in recent years is that, with a few exceptions, they require activation by the host before they can transform a cell.

A principal tenet of chemical carcinogenesis is that a chemical must react irreversibly with cellular macromolecules such as DNA, RNA, or protein to initiate transformation. Several of the known carcinogens, such as β-propiolactone and uracil mustard, are in fact alkylating agents that react readily with nucleosides or amino acids. But the greatest number of carcinogens, perplexingly, are relatively unreactive chemicals such as polycyclic aromatic hydrocarbons, aromatic amines, and nitrosamines.

Scientists had also been puzzled by the lack of structural or other relations among the highly diverse carcinogens and by the absence of correlation between carcinogenicity and mutagenicity, another phenomenon that involves interaction of chemicals with cellular macromolecules. These seeming paradoxes have now largely been resolved through the pioneering efforts of James A. Miller and Elizabeth C. Miller of the McArdle Laboratory and the contributions of, among others, Eric Boyland and Peter Sims of the Chester Beatty Research Institute in London, Heidelberger, Elizabeth and John Weisburger, and Harry V. Gelboin of NCI.

The Millers' widely accepted conclusion is that the form of the chemical that ultimately reacts with cellular macromolecules (the "ultimate carcinogen") must contain a reactive electrophilic center—an electron-deficient atom which can attack the many electron-rich centers in polynucleotides and proteins. Important electrophilic centers include carbonium ions, free radicals, epoxides, some metal cations, and the nitrogen in esters of hydroxylamines and hydroxamic acids. All recognized carcinogens that are not themselves electrophiles are now known or presumed to be metabolized to electrophilic derivatives that are the ultimate carcinogens.

Assayed in Different Systems

It has thus become clear that the apparent lack of a correlation between the carcinogenicity and mutagenicity of chemicals arises because the two activities are measured in different systems. Mutagenicity is generally determined in bacteria, bacteriophages, and yeasts, which usually do not have the enzymes necessary for activation of the carcinogen. In all instances where known ultimate carcinogens have been tested in such systems, however, they have been found to be mutagenic, and it is now becoming generally accepted that all ultimate carcinogens are also mutagens.

The converse of that conclusion appears to be valid too: With the exception of only two classes, all mutagens are generally considered to be carcinogenic. Those exceptions are the simple frameshift mutagens, such as acridine dyes, that cause mistranscription of DNA by noncovalent intercalation between nucleotides in the double helix, and base analogs, chemicals that can substitute for the purine and pyrimidine bases in DNA synthesis to produce a defective product. Few mutagens from

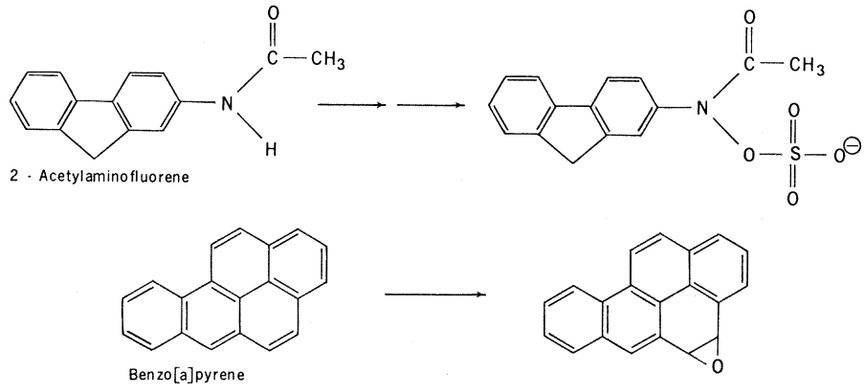

Fig. 1. 2-Acetylaminofluorene and benzo[*a*]pyrene and their activated forms.

these two classes have been demonstrated to be carcinogenic.

Ironically, the enzymes that activate the carcinogens are those whose primary function is the detoxification and disposal of foreign chemicals. The most important of these are several sets of oxidizing enzymes known collectively as microsomal mixed-function oxygenases. Microsomes are subcellular particles that are, among other things, the seat of protein synthesis. The mixed-function oxygenases are found in moderate concentrations in liver and kidney cells, where foreign chemicals are accumulated, and in variable but generally lower concentrations in most other types of cells.

By oxidizing various functional groups of a foreign molecule, the oxygenases make the chemical more polar —and thus more soluble—and provide a point of attachment for sugars and other molecules that help solubilize the chemical so that it can be excreted. Several other enzyme systems accomplish the same objective in other ways, but the oxygenases have been the most thoroughly studied and are believed to be the most important in carcinogenesis.

One of the best examples of the activity of the oxygenases, unraveled largely by James and Elizabeth Miller, involves the metabolism of 2-acetylaminofluorene (AAF), a potential insecticide that was found during routine biological screening to be a potent carcinogen. Any of the ring carbons of AAF can be hydroxylated by the microsomal oxygenases; the resulting alcohols are esterified with glucuronic acid by a second enzyme to produce a series of relatively inert ring-hydroxy-AAF glucuronides that can be detected in the urines of animals fed AAF. Since identical, synthetically prepared alcohols are substantially less carcinogenic than the parent compound, this sequence of reactions is generally accepted to be a detoxification pathway.

One of the oxygenases, however, can also hydroxylate the amide moiety of AAF (Fig. 1), and synthetically prepared *N*-hydroxy-AAF is substantially more carcinogenic than the parent compound. But *N*-hydroxy-AAF does not react with cellular macromolecules in vitro, suggesting that it is a metabolic intermediate rather than the ultimate carcinogen. This conclusion was affirmed by the more recent discovery by the Millers and by Charles King of the Michael Reese Hospital and Medical Center, Chicago, Illinois, of a soluble enzyme, sulfotransferase, which converts *N*-hydroxy-AAF to a strongly electrophilic sulfate ester that is believed to be the major ultimate carcinogen. At least three other pathways for production of electrophilic derivatives of *N*-hydroxy-AAF have been identified, but they appear to be much less important; AAF is, at best, only weakly carcinogenic in species that do not contain the sulfotransferase.

Another good example is found in

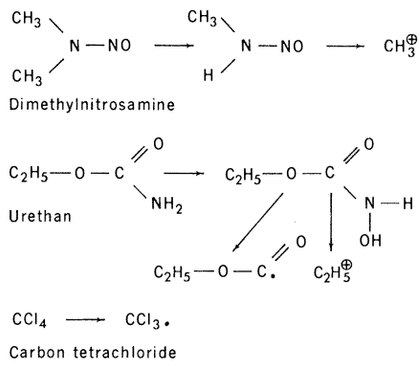

Fig. 2. The presumed routes for activation of some other carcinogens.

the metabolism of polycyclic aromatic hydrocarbons such as benzo[*a*]pyrene, a product of incomplete combustion that is one of the major carcinogens in tobacco smoke. Gelboin has shown that its carcinogenic and toxic effects rely upon its activation by microsomal enzymes. Like AAF, benzo[*a*]pyrene can be detoxified by hydroxylation of the ring carbons by a subgroup of microsomal oxygenases called aryl hydrocarbon hydroxylases (AHH's). Sims and Gelboin have identified several such metabolites, and suggest that there may be as many as 20, all less carcinogenic than the parent compound. Boyland, Sims, and Heidelberger have shown that one AHH in particular can form a reactive epoxide at certain ring positions (Fig. 1) to produce what is believed to be the ultimate carcinogen. Some other examples of carcinogens and their metabolically activated derivatives are shown in Fig. 2.

Although activation of the carcinogen and its reaction with cellular macromolecules are only the first of many steps leading to tumor formation, they offer several highly speculative possibilities for moderating the effect of carcinogens. The Millers have found, for example, that flooding the target cell with chemicals that can react with the ultimate carcinogen inhibits the carcinogen's reaction with the cell. Thus, rats fed a diet supplemented with methionine, cystine, or casein have a lower incidence of liver tumors induced by AAF than do those fed a control diet. Such a supplementation might not be practical for humans, but it is conceivable that undiscerned natural components of the diet might be partially responsible for country-to-country differences in the incidence of some types of cancer.

The activating enzymes can also be blocked with an inhibitor. Gelboin and Leilah Diamond of the Wistar Institute, Philadelphia, Pennsylvania, have found, for instance, that 7,8-benzoflavone inhibits AHH activity. Its application to mouse skin greatly reduces the incidence of tumors induced by the simultaneous application of 7,12-dimethylbenz[*a*]anthracene to the same site. But a broad-spectrum inhibition of the microsomal oxygenases is also not practical because the enzymes are needed for detoxification of other foreign chemicals. Protection from carcinogens in this manner would thus leave an individual increasingly susceptible to

the toxic effects of drugs and other environmental chemicals.

A more promising alternative might lie in alteration of the balance between those enzymes that produce detoxification and those that produce carcinogenic activation, assuming that such a distinction exists. In the case of polycyclic aromatic hydrocarbons, this approach would involve either selective inhibition of the enzyme that forms the epoxide or stimulation of the enzymes that hydroxylate the ring carbons. Or if epoxidation is merely the first step in hydroxylation, as many investigators believe, it might be possible to stimulate the enzymes that convert epoxides to alcohols. This stimulation is possible because of the well-documented phenomenon of induction—an increase in the concentration of the microsomal enzymes in response to exposure of the cell to foreign chemicals.

A large number of polycyclic aromatic hydrocarbons, drugs, and other chemicals have been shown to stimulate the synthesis of microsomal oxygenases, but the results of this stimulation can vary greatly. The Millers, for example, have shown that 3-methylcholanthrene fed to male rats stimulates ring hydroxylation of AAF, and thus inhibits tumor initiation by that compound. Stimulation can be a two-edged sword, however: When the same two compounds are fed to hamsters, *N*-hydroxylation of AAF is stimulated.

Other enzymes might also profitably be induced. Recent results from Elizabeth Weisburger suggest that *p*-hydroxyacetanilide reduces the carcinogenicity of AAF by stimulating the production of enzymes that convert AAF metabolites to inert glucuronides. But it has by now become quite clear

Can Potential Carcinogens Be Detected More Quickly?

Since it is not yet possible to reverse the tumorigenic effects of chemicals in man, a major facet of research in chemical carcinogenesis has been the prevention of exposure by identifying carcinogens and removing them from the environment. Efforts to control recognized carcinogens have met with mixed results—witness the 10 percent increase in U.S. cigarette consumption during the 10 years since the U.S. Surgeon General's report which linked smoking and lung tumors—but their identification has continued at a modest rate.

Nearly 450 chemicals are now being screened for carcinogenicity at 28 different U.S. laboratories under the sponsorship of the National Cancer Institute's (NCI) carcinogenesis program. This screening is expensive and time-consuming. Each chemical tested requires, on the average, 3 years, 500 animals, and $70,000. Moreover, only a fraction of the available chemicals can be tested. Even if all the screening facilities in the country were mobilized, by one estimate, it would still be possible to screen only about 700 chemicals per year. At that rate, it would take many years to screen all the new chemicals that are introduced in any one year, and a much longer time to screen all of those already in the environment.

A promising solution to this problem is to be found in the cell culture systems described in the accompanying story. Exploitation of these systems could sharply reduce the time and expense of screening. Morphological changes indicative of carcinogenicity appear in cultured cells as soon as 2 weeks after application of a chemical, and confirmation that the altered cells cause tumors in animals usually requires no more than 18 weeks. A substantial effort is thus being made to refine the systems for use in screening.

Many investigators are examining this approach, but their work is typified by that of Roman Pienta and his associates at NCI's new Frederick Cancer Research Center in Frederick, Maryland. Pienta's group is now about half way through a 2-year project to develop standardized cell strains and assay techniques that will make it possible to compare results obtained in different laboratories. The cell strains they are examining are the hamster embryo cell cultures developed by Leo Sachs at the Weizmann Institute in Israel and refined by Joseph A. DiPaolo at NCI. Most of Pienta's effort now is being devoted to screening about 100 chemicals whose carcinogenicity is well defined to ensure that the tests do not produce misleading results. His group is also developing stringent criteria for determining whether the test cells have been transformed. If this phase is successful, the cultures and techniques will be made available to other investigators.

Even with success, however, a major problem remains: The hamster cells contain insufficient concentrations of some of the enzymes known to be necessary for activation of most carcinogens. Investigators are thus looking for some way to expose the chemicals to these enzymes before or during the screening.

One approach adopted by Pienta and others relies on liver cells—which have relatively high concentrations of the necessary enzymes—that have been x-irradiated so they can no longer proliferate. The liver cells could thus be added to the cell cultures and presumably would metabolize the test chemicals without interfering with the screening. Insufficient results have been obtained for a proper assessment of this scheme.

An alternative approach, developed by DiPaolo, requires injection of the chemical into a pregnant hamster. The hamster fetuses, which are thus exposed to both the chemical and its metabolites, are subsequently removed, and embryonic cells from them are cultured in the same manner as those in the fibroblast system. DiPaolo has tested 12 carcinogens and 5 noncarcinogens in this fashion with no false negatives or false positives, so the testing is being expanded to include more chemicals.

These or similar systems might finally make it possible to screen all new chemicals before their introduction, but there is one more reservation. The results obtained in animal cells can be applied to humans only by analogy and, while these analogies have generally been good, they are not perfect. The ultimate test, still out of reach, would thus be based on cultures of human cells.—T.H.M.

that the subtlety of the interactions among the various enzymes makes manipulation of their activities a delicate task that will require a great deal more knowledge before it is attempted in humans.

The greatest benefit from the oxygenases, in fact, might come from using them to identify individuals who should limit their exposure to certain types of carcinogens. The capacity for enzyme induction is not uniform, so some individuals have a greater capacity than others.

Gelboin and Charles R. Shaw of the M. D. Anderson Hospital and Tumor Institute, Houston, Texas, have recently demonstrated that AHH activity can be identified and quantified in lymphocytes from human blood—the first reports of the presence of this enzyme in an easily obtainable human tissue. The concentration and inducibility of AHH in the lymphocytes presumably correlates with those characteristics in other tissues such as the lung, where AHH is believed to activate the carcinogenic hydrocarbons in tobacco smoke.

Identify High Risk Smokers

Shaw's preliminary results suggest that about 9 percent of the white population in the United States has the capacity for induction of high concentrations of AHH; moderate concentrations can be induced in about half of the others, and only low concentrations in the rest. His initial findings also indicate that the incidence of lung cancer is about 36 times higher in cigarette smokers with high AHH inducibility than in those with low inducibility, and about 16 times higher in cigarette smokers with moderate AHH inducibility. If this postulated relationship between high AHH inducibility and increased lung tumor incidence can be substantiated, and if the lymphocyte assay can be proved and adapted for large-scale use, then it might for the first time be possible to identify individuals who are at a greater risk from smoking and who should thus be given the greatest encouragement to quit.

Factors other than the microsomal enzymes probably also influence the interaction of carcinogen and cell, but very few have yet been identified. Vitamin A is one of the important factors that has been recognized. Working with hamster tracheas in organ culture, Michael B. Sporn of NCI has shown that the percentage of added benzo[*a*]pyrene that becomes covalently bound to epithelial cell DNA is much higher in tracheas from vitamin A–deficient animals than in those from healthy specimens. The tracheas cannot be maintained in culture long enough to ascertain whether the increased binding is associated with an increased incidence of tumor formation, but all evidence suggests that the association exists.

Sporn's observation is one of the first cases where a carcinogenic response has been associated with a nutritional deficiency. More important, vitamin A deficiency is a common one. Barbara A. Underwood of the Columbia University School of Public Health and Administrative Medicine, New York City, has performed analyses which suggest that 15 to 30 percent of the U.S. population suffers from long-term vitamin A deficiency without overt symptoms. Since there is no evidence that the induction of microsomal enzymes is associated with vitamin A concentrations, it thus appears that individuals with a vitamin A deficiency form a second population subgroup that is more susceptible to carcinogens in tobacco smoke. Presumably, then, the greatest incidence of lung cancer might be found in cigarette smokers who have high AHH inducibility and who are deficient in vitamin A.

Beyond the stage of the carcinogen's activation and interaction with cellular components, very little is known about the course of chemical carcinogenesis. The main theories can be divided into two broad categories, genetic and epigenetic. Postulated genetic mechanisms include modification of existing DNA, modification of RNA which is subsequently transcribed into DNA that is integrated into the host DNA, and modification of proteins to decrease—at least temporarily—the fidelity of copying DNA. The most reasonable epigenetic mechanism is based on protein alterations that effect quasi-permanent changes in the transcription of DNA (that is, gene expression); this process is analogous to the (unknown) mechanism by which a change in gene expression converts embryonic cells into mature cells characteristic of a specific organ.

An alternate epigenetic mechanism invokes alterations of proteins in individual cells or in the immune system that might allow preferential proliferation (selection) of previously existing malignant cells. Many carcinogens are, in fact, also immunosuppressants. Recent evidence from Gelboin's laboratory, moreover, indicates that some of the carcinogen-activating enzymes are present and highly inducible in tissues involved in immunological activity; interaction of the ultimate carcinogen with these tissues could produce immunity impairment without being carcinogenic in itself.

Heidelberger, however, has demonstrated that single mouse prostate cells in culture can be transformed by 3-methylcholanthrene with a high efficiency. This result suggests that selection is probably not a primary mechanism for chemical carcinogenesis, although it may well play a secondary role in promoting tumor growth or in the cocarcinogenesis of chemicals and viruses. Heidelberger has also shown, in collaboration with Robert Nowinski of McArdle, that carcinogens do not transform the prostate cells from some strains of mice by activating a latent oncogenic virus, eliminating that possibility as an epigenetic mechanism.

Genetic or Epigenetic?

There is little conclusive evidence to support either a genetic or an epigenetic mechanism. But many investigators, typified by Emmanuel Farber of the Temple University School of Medicine, Philadelphia, Pennsylvania, favor a genetic explanation because a modification of existing DNA is the simplest possible mechanism and because such an explanation provides a straightforward analogy to mutagenesis. Perhaps most important, Farber argues, modifications of DNA provide the most rational explanation for preservation of necessary information during the prolonged period between application of the carcinogen and appearance of a tumor. This latent period is typically between 10 and 20 percent of the host's life-span, and it is difficult to visualize retention of simple protein alterations during such an extended period.

It is in elucidating the mechanisms of tumor development during this latent period that cell culture systems offer the most promise, and it is for that reason that most investigators consider them so important. But in view of the complexities involved, it may be quite some time before those mechanisms are revealed.

—THOMAS H. MAUGH II