Biochemistry of Carcinogenesis

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Summary

The highlights of some of the major developments in chemical carcinogenesis have been presented and discussed. The success in elucidating many of the metabolic transformations of several groups of chemical carcinogens, including the nature and mechanism of the interaction with cellular macromolecules, indicate the need for increasing attention upon the next steps in the process. One of the challenges is the development of model systems for the molecular analysis of discrete cell populations at different steps in the neoplastic transformation. The suggested parallelism between viral and chemical carcinogenesis and the possible persistence of fragments of the original viral or chemical causative agent open up new avenues for identification of the essential metabolic steps in the conversion of a normal to a neoplastic cell.

The major objective of studies on carcinogenesis is to understand the essential nature of the neoplastic cell, how it differs from its normal counterpart, and how it becomes abnormal. Although neoplasia is a phenomenon that can only be understood meaningfully at the level of functional integration of the whole cell and at more complex levels, considerable research in this field is based on the hope that the understanding of such altered cell behavior at the molecular level is an attainable goal. Insight into the sequence of chemical events leading to a malignant tumor may not only form a solid base for the prevention and control of cancer in man but will have many implications for many other phases of biology. In fact, carcinogenesis involves so many fundamental properties of biologic systems that, while it is safe to say that formulation of the most penetrating rational hypotheses concerning the mechanisms of carcinogenesis in its various steps will only be possible when we will have more understanding of some of the most basic aspects of biology, it is equally true that studies on the genesis of neoplasia will have a favorable influence on the development of other areas in biology.

Logically, one should have a clear notion of the nature of the biologic problem before one can hope to study its pathogenesis effectively. What is the essence of this biologic problem, neoplasia? Obviously some disturbance in the regulation of cell reproduction is a key manifestation of neoplastic tissues. However, since both the rate of cell proliferation and the pattern of cell differentiation are frequently disturbed in cancer, neoplasia may be either a primary disturbance in the control of cell proliferation with secondary changes in differentiation or a primary disturbance in cell differentiation which secondarily interferes with the control of normal cell replication. This question may become even more important in view of recent work indicating some intimate link between differentiation and cell division. For example, in explants of the mammary gland from pregnant mice, DNA synthesis and cell division, induced in this case by insulin, appear to be essential for initiation of casein synthesis by prolactin and hydrocortisone (36, 69). A similar relationship may also hold for differentiation of embryonic kidney mesenchyme in vitro (64). These findings remind one of the possible requirement of cell division for antibody synthesis, since I am told by immunologists that antibody synthesis has yet to be initiated in the absence of cell proliferation.

Is the ability of the cancer cell to invade and set up satellites in other organs an integral part of an altered state of cell growth or differentiation, or are they separate and discrete properties that the cells acquire in some step subsequent to the primary neoplastic transformation? If the latter, why do the primary stages in this transformation so often lead to the invasive and metastatic capabilities?

Biologic and chemical studies of cancer have failed, so far, to throw much light upon these important questions. Unfortunately, with rare exceptions (76), the focus has not been on the molecular or chemical analysis of each of the biologic properties of cancer cells, such as continual growth and its relationship to different degrees of differentiation. On the contrary, the extensive biochemical studies on cancer have concentrated in the main upon the hope that a sufficient number of varied quantitative analyses of chemical components in tissues containing several cell populations in different physiologic phases of cell behavior may uncover some one or more key qualitative differences between the neoplastic cell and its normal counterpart that can be exploited therapeutically. To my knowledge, no such changes have been found so far.

Another problem concerns the adaptation of tumor cells. A few biologic studies have shown quite clearly that, in cancer, we are never dealing with an end-stage phenomenon at all but with a continually changing system (22). As is now evident in virtually every phase of cell biology, the ability to react and to change in ways compatible with the persistence of cell life is an essential property of the living cell, and the neoplastic cell is no exception. By continual selection or modulation, the
cancer cell keeps changing its properties until it dies along with its host. Clean model systems for the analysis of these phenomena are wanting and may have to be developed before we can hope to learn whether this progression is composed of a linear series of stages which have survival value for the neoplastic cell or a random or parallel series of changes reflecting a heightened instability of the genome in the neoplastic cell. Aberrations in nuclear structure are extremely common in cancer and may even be a hallmark of some prerequisite for neoplasia. Are these a manifestation of the same instability in the organization of the genetic information in the cancer cell? These are some of the key questions about which we are ignorant today. Therefore, at the moment, the current knowledge about "endstage" neoplasia offers little help in formulating a rational analysis of the process of carcinogenesis.

Before going on to discuss carcinogenesis, I should point out one very important development in our knowledge of neoplasia, namely, the acquisition of new cell antigens (32, 51), which, although not yet explored chemically, nevertheless has important implications in our understanding of the molecular basis of carcinogenesis. Virus-induced neoplasias contain cell antigens that are characteristic of the virus and not of the cell transformed (29). In contrast, chemically induced neoplasms, where studied, each contain antigens which appear to be unique to that tumor (55). Whether such different antigens originate from new cell information induced by the chemical or from information that was repressed at some previous stage in the history of the cell and is now derepressed is not established.

Some Biologic Features of Carcinogenesis

Since our knowledge of the neoplastic cell offers little help in formulating testable hypotheses on mechanisms of carcinogenesis, we are forced to concentrate our major efforts upon less clearly defined aspects of the process of carcinogenesis, with the hope of developing systems in which one can pose fundamental questions. In order to do this, we must take stock of what we know of the biology of the process since, after all, our goal is to understand a biologic process.

The work of Rous, Kidd, and their coworkers, and of Berenblum and Shubik early in the 1940's, subsequently refined and confirmed by Boutwell and others, has shown clearly that the neoplastic transformation is not a single step process but rather a multistage one (4, 5, 9). In the case of skin carcinogenesis with chemicals, at least two steps are clearly defined—a short initial one, called initiation, and a subsequent much longer one, called promotion. This suggestion of a multistep process was a radical departure from previous concepts dominant until that time, which held that the carcinogenic process was a rapid irreversible single step process. The multistep nature of carcinogenesis is also evident in many other tissues and systems, including some forms of viral oncogenesis such as mammary cancer in mice, and in a variety of human cancers in which a discrete identifiable "premalignant" carcinoma-in-situ phase has been identified. Also, the existence of benign neoplasms with a great tendency for malignant transformation is further in line with this concept. Thus, any analysis of carcinogenesis must recognize this characteristic of the process (Chart 1).

Another important characteristic of the biology of oncogene-

![Chart 1](chart1.png)

**Chart 1.** Diagrammatic representation of some of the interactions between a susceptible cell and a carcinogen. The obligatory need for a new cell population between the normal cell and the malignant one is suggested. Also, the further transformation of the malignant cell in a stepwise fashion is indicated under the title "progression."

sis is the relatively long period between the time of application and removal of the oncogenic agent and the time of appearance of identifiable neoplastic cells. This was first shown clearly in the skin where a potent carcinogen need only be applied once for a period of minutes or hours in order to initiate the molecular events leading to neoplasia. The eclipse period can be modified and shortened by the use of many compounds called promoters or cocarcinogens, the majority of which are themselves not carcinogenic or only weakly so. In the case of the skin, several phenols and fractions of croton oil are promoting agents. Presumably, with many carcinogens, the promoting agents are endogenous in origin, e.g., hormones (25). This lag phenomenon is the most challenging intellectually and has stimulated a great amount of speculation and theoretical consideration. We will naturally return to this as one of the key problems in need of exploration and clarification.

A third characteristic of the carcinogenic process is the apparent need for cell proliferation or hyperplasia at some time between exposure to the carcinogenic stimulus and the first recognizable neoplastic cell. This requirement is as yet only circumstantial in the case of most carcinogenic processes. However, there is increasing evidence for its need in viral oncogenesis *in vitro* and in at least two instances *in vivo*—mammary cancer in mice induced by virus and liver cancer induced with a variety of chemical agents. This phase of carcinogenesis is also a critical one and will be discussed in greater detail somewhat later.

Let us now consider what we know about the biochemical correlates of these properties of the carcinogenic process.

**The Initial Interaction**

Research over the past fifty years or so has uncovered an increasing array of environmental agents that may induce cancer under the proper circumstances. Hundreds of diverse chemical compounds, some fairly simple and some complex, dozens of viruses with either DNA or RNA as their nucleic acid, and various forms of radiation are each capable of initiating the
carcinogenic process. On the basis of current concepts and from the point of view of the mechanisms of carcinogenesis, all of these may be divided into one of two large groups: (a) agents that carry with them information in a form that is translatable per se by some living cell, and (b) agents that generate such information only after their interaction with the living cell.

The first group are obviously the oncogenic viruses, although theoretically other forms of biologic organization with similar properties might exist. The viruses vary very much in the amount of translatable information they may contribute to the cell. The clearest picture emerging to date in this area of research appears to the outsider to be with the DNA viruses, especially polyoma but also SV40. In the case of polyoma, the virus contains only a relatively small amount of translatable information (16, 17). With both polyoma and SV40, the viral DNA becomes incorporated into the cell structure and appears to persist, at least in part, until neoplastic transformation has been induced. The information which the virus carries with it into the cell also initiates the synthesis of several proteins, including one localized in the nucleus and one related to transplantation. The transplantation antigen may reside in the plasma membrane. The relation of both polyoma and SV40 to DNA synthesis and cell division is especially intriguing. In the case of SV40, it appears that cell replication is needed for the initial transfer of information (71). Subsequently, the number of cells transformed is proportional to the number of cell cycles up to a few. In the case of polyoma, the virus turns on the synthesis of host DNA, apparently as an integral part of its interaction with the host (16, 17). Although the picture is more complex with RNA viruses, it appears as if DNA synthesis is also needed for transformation by at least one such virus, Rous sarcoma virus (2, 70).

These studies have opened exciting possibilities for new understanding of the process of carcinogenesis. The findings of the persistence of at least a portion of the viral genome throughout the process and the induction of new surface and other antigens open up new avenues for further exploration. Some of the critical questions for which answers are anxiously awaited are: (a) Does the viral genome play an active or passive role in the oncogenic process? In other words, does the information contained in the viral DNA contribute vital information, or does it possess oncogenic properties only by virtue of an ability to become incorporated selectively into the host genome and thereby cause its disorganization? (b) If the viral genomic information is actively involved, does it contribute an essential piece of information or the essential information for oncogenesis?

Let us now turn our attention to the second group of oncogenic agents. It is becoming increasingly evident that many chemical carcinogens and various forms of radiation interact with living systems to effect a variety of chemical alterations. The effects of radiation are complex (13) and are beyond the scope of this presentation and will not be considered further.

It would appear from the vantage point of 1967 that chemical carcinogens can be divided into at least two broad categories: (a) agents that appear to be active per se in initiating the carcinogenic process, and (b) agents that no doubt require preliminary metabolic conversion to active metabolites which in turn initiate the neoplastic transformation. Work with the second group has been active in recent years and has given important new insight into the nature of the initial interactions between potential carcinogens and living cells. The first group doubt contains the most active and versatile carcinogens, the polycyclic aromatic hydrocarbons as well as several other carcinogens such as B-propiolactone, S and N mustards, and epoxides (47, 49). The polycyclic aromatic hydrocarbons can affect such a wide variety of cells and tissues in so many different species that one is naturally led to the notion that these are active per se and need no specific preliminary metabolic conversion. In support of this working hypothesis is the fact that no known metabolic derivative of these compounds appears to have enhanced biochemical reactivity and carcinogenicity as compared to the parent compound, and that the majority of the metabolites tested are less active or inactive. These metabolites are predominantly hydroxy derivatives. For the time being, therefore, the polycyclic aromatic hydrocarbons can be considered as the active carcinogenic molecules, at least until someone discovers an active derivative. The other chemical oncogens in this group are chemically sufficiently reactive as to require no prior enzymatic conversion before they interact with cell macromolecules. They are presumably the active carcinogens.

Recent work by Gelboin and associates (3, 26, 27) suggests that actinomycin in doses large enough to inhibit DNA as well as RNA synthesis interferes with the initial interaction of some carcinogens of this group with the skin and inhibits subsequent formation of tumors. Also, Sachs and coworkers (6, 8, 57) have suggested a requirement for a process associated with cell division in cell transformation induced by polycyclic aromatic hydrocarbons or X-irradiation. Although these studies have interesting implications, it would be preferable to delay discussion until the experimental details and results are clarified.

With respect to the second group, here the advances in knowledge in recent years have been most significant. The outstanding progress in our understanding of aromatic amines has been spearheaded by the Millers and their colleagues at Madison. The fascinating developments in the story of the carcinogenic nitrosamines have been pioneered by Magee and coworkers in Carshalton, while insight into some aspects of the action of ethionine as a carcinogen has been obtained through the efforts of Stekol and coworkers and of various collaborators in our group. Since each compound or group of compounds illustrates some interesting features of the initial impact of carcinogens on the living cell, I would like to review briefly this phase of carcinogenesis using these as examples.

Aromatic Amines. Work by the Millers has uncovered an interesting new biochemical reaction, N-hydroxylation, which occurs with many different aromatic amines such as derivatives of aminofluorene,azo dyes, naphthylamine, and aminobiphenyl as well as others (47, 49). With all of these compounds, there is strong evidence to suggest that preliminary metabolic conversion of the administered compound to a more active derivative is a prerequisite for carcinogenicity. Since the enzymatic makeup of cells differs so much, it is probable that the high degree of tissue specificity for cancer induction by these agents...
is merely a reflection of the tissue distribution of the required enzymes. In the case of the aromatic amines and the nitrosamines, some metabolic derivatives called proximate carcinogens (47, 49), are much more potent and versatile oncogenic amines, some metabolic derivatives called proximate carcinogens. In the case of the aromatic amines and the nitrosamines, the probable inactivity of the parent compound, the transformation to a potent alkylating agent by enzymes in the endoplasmic reticulum, at least in the liver, the high degree of tissue specificity for cancer induction by the parent nitrosamines, and the augmented carcinogenic potency and versatility with suggested proximate carcinogens (59-61) and the probable nonenzymatic alkylation of different types of macromolecules by the active derivatives all indicate that a general pattern of interaction between various carcinogens and living cells is emerging which will lay a solid foundation for our understanding of the initial events in the carcinogenic process.

Ethionine. The picture developing with respect to this carcinogen mirrors in part that observed with the first two groups —the probable inactivity of the parent compound per se as a carcinogen and the need for metabolic conversion for biologic activity (19, 68). However, one outstanding difference probably operating is the close link between ethionine and the methionine-metabolizing enzymes which it parasitizes. Although ethionine is also converted to a more active alkylating agent, S-adenosylmethionine, as is the case with the aromatic amines and nitrosamines, it appears that the metabolic interaction with cell macromolecules by this agent is still dependent upon enzyme action and probably does not take place nonenzymatically. Thus, with this agent, the ultimate carcinogenicity may always be a reflection of the endogenous cellular enzymatic specificity. This dependence upon enzymatic mechanisms would obviously restrict the number of possible alkylations and as such offers the possibility of obtaining insight into some highly selective features of the carcinogenic process not present with the nonenzymatic alkylation agents generated from most of the other carcinogens.

Interaction with Cell Constituents

It is now evident that different types of carcinogens interact with several components of cells including proteins, RNA, and DNA. Since the original discovery of the firm binding of DAB or derivatives to liver protein by Miller and Miller in 1947 (46), many oncogenic chemicals have been found to interact with cell proteins of several different organs or tissues. Where studied in liver and skin, this interaction has not been found to be random but rather quite specific in that certain groups of proteins, especially the slow h2 fraction of soluble cytoplasmic proteins, show the greatest degree of binding with some carcinogens (1, 43, 66). However, it should be pointed out that other protein fractions, including some nuclear proteins, also show binding but to a lesser degree. The biologic significance of different degrees of binding remains unknown and, as a consequence, no degree of interaction can be ruled out as insignificant on an a priori basis.

Two recent developments in the field of protein-binding seem particularly pertinent and deserve some special comment. First,
the chemical nature of the bound forms of azo dyes and acetylaminofluorene has been clarified to a considerable degree, thanks again to the efforts of the Millers and their coworkers. The discovery of the interaction of DAB with the S atom of methionine in protein to form a sulfonium compound in which the S atom is bound to CH₃, CH₂CH₂CH(NH₂)-COOH, and to the azo dye via C₂ in the primary benzene ring opens a whole new avenue of study (35, 47, 49, 62). This finding has been followed by further studies showing reaction in vitro at a physiologic pH between esters of N-OH metabolites and some amino acids including methionine. This may be a type reaction for several different kinds of carcinogens (14). The further study of these interactions and of the effects of such alterations on the metabolism and function of proteins offers exciting new possibilities for understanding the cellular metabolic consequences of the initial impact of carcinogens upon living tissues.

The second development is that of Freed and Sorof (23, 24) concerning the possible biologic properties of one of the h₂ proteins of liver. They have found that purified preparations of such fractions have a reproducible growth-inhibiting effect on cells in vitro. Further analysis of this interesting finding has shown that the growth inhibitor is probably arginase which acts by destroying the arginine in the medium. Obviously, the possible role of such a compound in the function of an intact cell remains to be studied. However, one cannot help but wonder whether this discovery might not be related to the tumor-inhibiting properties of asparaginase and to the suggested alterations in arginase in the Shope rabbit papilloma system.

In addition to their obvious interaction with cellular proteins, it is now clear that several carcinogens also interact with various RNA's and with DNA. In some systems, the reaction with RNA is as strong as that with protein. With at least one carcinogen, ethionine, the reaction with RNA is 10 to 20 times that found with total protein (20). However, when cell proteins are fractionated, a saline-soluble protein fraction of rat liver nucleus can be obtained which is labeled with L-ethionine-ethyl-14C to a degree approaching that seen in the tRNA. The tRNA appears to be more heavily labeled also with AAF and DMN than are some other types of cellular RNA.

DNA is also a target for interaction with several chemical carcinogens (Table 1). Although the degree of binding of most compounds to DNA is less than with total RNA, it is nevertheless quantitatively significant. The one possible exception is ethionine, which effectively labels various RNA's and protein fractions but which has only an extremely small affinity for DNA. In fact, the level of ethionine interaction with DNA is so low as to make it difficult to decide with current technics whether it occurs at all. Some quantitative aspects of the interaction of some carcinogens with protein, RNA, and DNA are listed in Table 1.

The existence of the three major targets for chemical carcinogens does not rule out the possible occurrence of other macromolecular target molecules. In fact, the identification of highly reactive chemical derivatives of some of the carcinogens makes it most likely that other types of cellular constituents such as polysaccharide (see below) or lipids may also be altered by interaction with one or another of the diverse oncogenic chemicals. An intriguing possibility is the polysaccharide components of the plasma membrane, especially since it is becoming evident that altered cell membrane function is probably an important property of neoplastic cells.

The increasing evidence for high chemical reactivity of some of the derivatives of several carcinogens raises a possible doubt concerning the biologic significance of positive or negative correlations between degrees of binding to or reaction with various cellular constituents and carcinogenic potency of a particular carcinogen. In the case of those compounds from which are generated highly reactive products, the degree of interaction with any particular cell component may be merely a passive reflection of the extent of production of such derivatives and need not be intimately related to the steps involved in the neoplastic transformation. It may well be that the selection of some carcinogen-cell-constituent interaction for relevance to cancer induction may have to depend upon the findings with less potent agents or less potent derivatives of the most active agents. Such compounds may be more selective in the type and degree of their interaction with cell components and thus may be useful in deciding which interactions are more relevant to the induction of neoplasia.

What Chemical Alterations are Significant in Carcinogenesis

From these developments in the field of chemical carcinogenesis, it is evident that cells, with some selectivity, convert many different tumor-inducing chemicals to active derivatives which can chemically alter some cellular macromolecules, either nonenzymatically or enzymatically, in a reproducible manner (Chart 2). The spectrum of tissue and species specificity of carcinogenic chemicals is probably a reflection of the ability of their target cells to perform these requisite chemical reactions. These developments are providing a foundation of knowledge sufficiently broad and in sufficient depth to warrant an increasing interest in the next important phase of the analysis of the...
Table 1

<table>
<thead>
<tr>
<th>Compound</th>
<th>Dose (µmoles/kg)</th>
<th>Duration of action</th>
<th>Tissue and species</th>
<th>DNA</th>
<th>RNA</th>
<th>PROTEIN</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
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<td>moles of carcinogen/gm</td>
<td>moles of carcinogen/gm</td>
<td>moles of carcinogen/gm</td>
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<tr>
<td>Ethionine</td>
<td>26 i.p.</td>
<td>5 days</td>
<td>Liver, rat</td>
<td>1.8 \times 10^{-10}</td>
<td>2.7 \times 10^{-8}</td>
<td>7.5 \times 10^{-9}</td>
</tr>
<tr>
<td>2-Acetylaminofluorene</td>
<td>2.8 i.p.</td>
<td>1 day</td>
<td>Liver, rat</td>
<td>7.7 \times 10^{-9}</td>
<td>1.2 \times 10^{-9}</td>
<td>25 \times 10^{-9}</td>
</tr>
<tr>
<td>Diethylnitrosamine</td>
<td>175 i.p.</td>
<td>23 hr</td>
<td>Liver, rat</td>
<td>5.3 \times 10^{-6}</td>
<td>6 \times 10^{-4}</td>
<td>8 \times 10^{-6}</td>
</tr>
<tr>
<td>2700 i.p.</td>
<td>24 hr</td>
<td>Liver, rat</td>
<td>5.3 \times 10^{-6}</td>
<td>6 \times 10^{-4}</td>
<td>8 \times 10^{-6}</td>
<td>3.8 \times 10^{-3}</td>
</tr>
<tr>
<td>Dimethylaminoazobenzene</td>
<td>28.5 stomach tube</td>
<td>16 hr</td>
<td>Liver, rat</td>
<td>14 \times 10^{-5}</td>
<td>2.3 \times 10^{-5}</td>
<td>114 \times 10^{-5}</td>
</tr>
<tr>
<td>675 i.p.</td>
<td>16 hr</td>
<td>Liver, rat</td>
<td>14 \times 10^{-5}</td>
<td>2.3 \times 10^{-5}</td>
<td>114 \times 10^{-5}</td>
<td>2.7 \times 10^{-5}</td>
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<tr>
<td>675 i.p.</td>
<td>3 mo.</td>
<td>Liver, rat</td>
<td>14 \times 10^{-5}</td>
<td>2.3 \times 10^{-5}</td>
<td>114 \times 10^{-5}</td>
<td>2.7 \times 10^{-5}</td>
</tr>
<tr>
<td>5.9 skin</td>
<td>22 hr</td>
<td>Skin, mouse</td>
<td>13 \times 10^{-9}</td>
<td>2.4 \times 10^{-9}</td>
<td>72 \times 10^{-9}</td>
<td>4.8 \times 10^{-9}</td>
</tr>
<tr>
<td>7,12-Dimethylbenz(a)anthracene</td>
<td>16 skin</td>
<td>42 hr</td>
<td>Skin, mouse</td>
<td>11 \times 10^{-9}</td>
<td>2.9 \times 10^{-9}</td>
<td>11 \times 10^{-9}</td>
</tr>
<tr>
<td>Benzo(a)pyrene</td>
<td>1.9 \times 10^{5}</td>
<td>4 hr.</td>
<td>Skin, mouse</td>
<td>13 \times 10^{-9}</td>
<td>2.2 \times 10^{-9}</td>
<td>13 \times 10^{-9}</td>
</tr>
<tr>
<td>p-Propiolactone</td>
<td>97 skin</td>
<td>24 hr</td>
<td>Skin, mouse</td>
<td>13 \times 10^{-9}</td>
<td>2.2 \times 10^{-9}</td>
<td>13 \times 10^{-9}</td>
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Interaction of some chemical carcinogens with macromolecules in vivo.

* Soluble RNA.  † Total nuclear protein.  ‡ Ribosomal RNA.

The extent of interaction can be measured in terms of DNA, RNA, or PROTEIN content. The table lists the compounds tested along with their dose and duration of action. The interaction is measured in terms of moles of carcinogen/gm for DNA, RNA, and PROTEIN. The table is designed to help researchers understand the effectiveness of different carcinogens in interacting with cellular macromolecules.
Biochemistry of Carcinogenesis

to this hypothesis, although the key question of translatability of any such new information remains.

However, we must not forget that our current concept of cell differentiation allows an alternate hypothesis—no change in information content of the DNA but rather a change in the "packaging" of the DNA which somehow controls the functional expression of a selected part of the information content of the DNA. If essentially all diploid cells in a single multicellular organism, with the exception of mosaics, possess the same genomic information, then one can have a large number of potential heritable cellular phenotypes without any change in the information content of DNA. Since differentiated cells almost always breed true—liver makes liver makes liver, etc.—then an heritable alteration in a cell can result from some change in the environment in which the DNA functions. Conceivably, some examples of neoplastic cells may represent such an alteration. Could a change in tRNA or other RNA or in one or more of the many cellular proteins be responsible for such a phenomenon?

The possible importance of any cellular macromolecules, other than DNA, to the heritable behavior pattern of an eukaryotic cell is at present in doubt. Yet we can devise plausible speculative schemes whereby altered RNA and/or protein might so change the cellular environment as to favor a new pattern of expression of DNA function (52).

An interesting new development in the chemical carcinogenesis field which might help us to choose between significant and insignificant changes has been discovered by Epstein in our department working with McNary in our biochemistry department. They have found strong evidence to indicate persistence of bound carcinogen throughout the whole carcinogenic process and long after the carcinogen has been removed from the diet. For the past several years, Epstein and I have been working on the development of a model for the study of the biochemical pathology of hyperplastic liver cells (18), since these cells are seen regularly with virtually every hepatic carcinogen during the process of carcinogenesis. We found previously that cancer can be seen to arise within nodules of hyperplastic cells when no cancer could be seen in many sections of other portions of the liver. This observation tends to support the notion of human and experimental pathologists that hyperplastic nodules are a noncancer precursor of liver cell cancer. However, such notions have not been subjected to critical analysis because of the lack of availability of model systems in the liver which satisfy the following three minimal criteria: (a) a cell of origin of cancer can be identified; (b) the cells of origin occur in a localized lesion in sufficiently large numbers to enable gross identification and isolation for standard biochemical as well as histochemical and morphologic investigations; and (c) all the cells in the localized lesion have a uniform genotype and phenotype. By suitable manipulations of the diet, one can induce nodules up to 2 or 3 cm in diameter in well over 50% of the rats with either ethionine or AAF. I should emphasize that the carcinogen is removed several weeks before the animals are killed. During this interval, the nodules persist and grow while the surrounding liver returns almost to normal. With ethionine, no consistent lesion is seen in the remainder of the liver, except an occasional area of cholangiofibrosis. With AAF, some residual small cysts and areas of cholangiofibrosis remain. The liver cells constituting the vast bulk of the hyperplastic nodule are remarkably uniform as judged by light and electron microscopic examination (42).

The nodules induced by either carcinogen contain a new population of liver cells which have distinctively biochemical markers. Analysis to date has shown alterations in glycogen structure and metabolism and in ATP metabolism. The ATP concentration of the nodules is twice that in the surrounding liver. This is of considerable interest since the content of adenine nucleotides in the normal liver is very finely controlled by regulation of the rates of de novo synthesis. Slight increases or decreases in ATP concentration, which constitutes about 80% of the total adenine mononucleotide content in normal liver, are associated with rapid changes in the rate of de novo synthesis. The nodular cells have apparently lost this fine thermodynamic regulation or, less likely, are making adenine nucleotides by other less sensitive pathways.

The developing picture with regard to glycogen is equally intriguing and is pertinent to the present discussion of carcinogenesis. The glycogen in the nodule shows an alteration in metabolism which is probably related to two phenomena, i.e., decrease in some enzymes related to glycogen breakdown and the presence of abnormal components. Whereas the glycogen in the surrounding liver behaves like glycogen in the normal liver in that it virtually disappears on fasting or after the injection of glucagon, the glycogen in the nodule does not respond very much to glucagon and the node always retains significantly more of its glycogen on fasting (18). These changes are probably in part due to a large decrease in the activities of glucose-6-phosphatase and glycogen phosphorylase. However, they are also probably due in part to a change in the structure of the glycogen. By gas and thin-layer chromatography, glycogen from normal liver or from the liver surrounding the nodules shows only glucose after hydrolysis. However, hydrolyzed glycogen from nodules induced by AAF (the glycogen from nodules induced by ethionine have not been analyzed yet) has an additional component which resembles very much an amino-fluorene derivative by spectrophotometric and mass spectrographic analysis. In addition, analysis of the small amount of glycogen present in liver cancers induced by the same carcinogen shows a similar component. The same component is also seen in glycogen from the whole liver of rats fed AAF for a few weeks. If this initial interpretation of the data proves to be valid by further types of analysis, it would indicate the persistence throughout the whole carcinogenic process of at least one carcinogen bound to a macromolecule not hitherto examined for interaction.

The implications of this new finding for carcinogenesis and for pharmacologic mechanisms in general are interesting and potentially quite important, providing, of course, it is not a property unique to AAF. The recent results of Warwick and Roberts (75), showing persistence of DAB or derivatives bound to total liver DNA for three months after a single intraperitoneal injection of DAB, suggests that the property of persistence may not be confined to AAF. Not only might it be possible to identify a possible chemical etiologic agent by examination of the appropriate constituents of a cancer, but it may also

SEPTEMBER 1968

1865
enable us to make more meaningful value judgments as to which macromolecular change may persist and may be related intimately to cancer induction. Also, if the carcinogen persists throughout the process bound to glycogen, perhaps other macromolecules may also have small amounts of bound carci-
nogen which may interfere with their normal metabolic func-
tion or turnover. Since a portion of the glycogen in the hyper-
plastic cells seems to be much less susceptible to enzymatic breakdown, it is tempting to think that this is due to the pres-
ence of the carcinogen at a selective site which interferes with further enzymatic degradation. Could the same phenomenon occur in the carbohydrate moiety of the plasma membrane and thereby interfere with its interaction with the environment, or could it occur in appropriate locations in DNA or RNA mole-
cules or in some selective protein molecules? Obviously, these speculations offer possible fruitful avenues for rigorous testing and experimentation.

These new findings may also offer an explanation of why hyperplastic nodules induced by many different carcinogens have the same apparent aberrations in glycogen when observed histochemically (18) and why the livers of rats fed AAF have glycogen with unusual stability (28, 64).

The finding of an abnormal glycogen was only possible by analyzing the hyperplastic cells free from the bulk of the liver. In my opinion, this offers strong experimental evidence for the validity of the plea that the biochemical analysis of a bio-
logic problem requires not only the availability of pure chemi-
cals but, equally important, the availability of “pure” cells. The molecular analysis of a biologic phenomenon in multicellular organisms demands the development of models and technics whereby uniform or reasonably uniform cell populations can be obtained from a heterogeneous mixture. This need for “cel-

lular purity” is not only critical with regard to cell type but is equally important with regard to the physiologic state of any cell type, especially in problems concerning cellular growth and proliferation, and it is just as important as the need for pure chemicals in many phases of biochemistry.

To return to the problem of carcinogenesis, is the hyper-
plastic nodule just a hyperplastic nodule or is it not an early stage of liver cell neoplasia? My impression is that the irre-
versible nodule can be considered to be a benign neoplasia, per-
haps the “most minimal” or “least deviated” hepatoma. We view the hyperplastic nodule as having acquired some of the properties of the neoplastic cells such as “uncontrolled growth,” but not others such as invasiveness and ability to produce me-
tastases. Consistent with this impression is the unusual degree of cell uniformity (42) and the absence of those nuclear aber-
rations so often seen in unequivocal cancer. If one could develop many more model systems in which cells would show only some of the biologic properties of cancer cells and not others, we would be well along toward having systems in which we can, with hope, pose testable questions at a molecular level.

The analysis of the sequence of metabolic changes from the initial interaction of the carcinogen with the responsive tissue to the appearance of neoplastic cells will require the identification and isolation of the involved population of cells at various times during the process. The hyperplastic liver nodule offers one such system. Another interesting system, devised by DeOme and his coworkers (7, 15, 33), is the hyperplastic nodule in the mammary gland induced originally by viruses and more recently with chemical carcinogens by Prehn (55). The mammary nodules have the advantage of being readily transplantable to the cleared fat pad of a mammary gland of another animal where its course and responsiveness can be studied under a variety of conditions. Other possible systems are the hormone-dependent tumors of some endocrine glands, such as pituitary or thyroid, which have been exploited so well by Durand (25). The meta-

bolic analyses of these systems may offer new approaches in understanding the molecular basis for discrete alterations in the biologic behavior of cells that are steps in the process toward malignant transformation.

These systems, when studied along with the elegant in vitro systems, may offer new leads to the development of more clearly defined hypotheses for mechanisms of carcinogenesis. Hope-

fully the intensive study of such systems with the accompany-
ing dissection of the biologic processes may enable us to begin to understand the essential nature of the fascinating phenome-
non of neoplasia.

I have said nothing so far about the biochemistry of pro-
motion. What is the chemical nature of promoting agents and how do they work? Obviously the main reason for ignoring them is not their unimportance but our lack of knowledge about them. To date, many chemical agents such as simple and com-
pex phenois, croton oil, and other stimuli such as mechanical injury hasten the carcinogenic process. Presumably, they work in part by facilitating the development of localized areas of hyperplasia of altered cells. A potentially interesting develop-
ment in this field is the beginning identification of some of the active components of croton oil by Hecker and his coworkers in Germany (30, 31) and by Van Duuren and his colleagues in New York (73, 74). Hopefully, the availability of the pure components active in croton oil, many of which appear to be derivatives of the polyhydroxyphenolic phorbol, coupled with the exploitation of new model systems may enable a more con-
certed attack on the carcinogenic process.

Finally, I would be remiss if I did not mention briefly one other complicating phenomenon which could be important in any molecular analysis of carcinogenesis—adaptation. Work by Shull et al. (68), as well as by Turner and Reid (72), clearly show that the liver rapidly becomes adapted to at least one carcinogen, ethionine, such that a dose of ethionine injected into a rat fed ethionine for 2 to 3 weeks has quite a different metabolic reaction than it does in an uninitiated rat. The mechanism of such an adaptation is only now being explored. However, it may be important in the eventual understanding of how the hyperplastic cell differs fundamentally from the surrounding nonhyperplastic liver.

In summary, the past several years have seen the rapid de-
velopment of knowledge in the initial phase of carcinogenesis. The success in the elucidation of many of the metabolic trans-
formations of several groups of chemical carcinogens and the nature and mechanism of their interaction with cellular com-
ponents, especially macromolecules, naturally encourages us to focus increasing attention upon the next steps in the process. One of the challenges which must be met is the development of model systems for the molecular analysis of discrete cell
populations at different steps in the neoplastic transformation. The preliminary results on one such system appear sufficiently interesting to encourage their more rapid and intensive study. The suggested parallelisms between viral and chemical carcinogenesis offer real hope that valid generalizations about biochemical mechanisms of carcinogenesis may be forthcoming. Finally, the possible persistence of fragments of the original viral or chemical causative agent may open up new avenues for identification of the essential metabolic steps in the conversion of a normal to a malignant cell.

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