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MECHANISMS *of* TUMOR PROMOTION

Volume I
Tumor Promotion in Internal Organs

Thomas J. Slaga

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Tumor Promotion in Internal Organs

Editor

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PREFACE

There has been increased interest in recent years in scientific investigations pertaining to the mechanisms of tumor promotion, and the resulting accumulation of new scientific data is receiving much attention. The promotion phase of carcinogenesis is widely investigated in a variety of systems and a presentation of these studies by the finest scientific minds is especially relevant.

With the increased attention being given to the daily exposure of man to low-level amounts of an assortment of cancer-causing chemicals, their scientific investigation is likewise increasing. Few of these agents are carcinogenic at very low levels; however, subthreshold amounts may become carcinogenic through an additive effect, cocarcinogenesis or by expression through both endogenous and/or natural tumor promoters. The role of these environmental chemicals, as well as diet, radiation, and viruses as possible modifying factors contributing to the increase of human cancers is of added importance. High caloric and high fat diets, cigarette smoking, asbestos, and alcohol are among the suggested promoting factors that may also play a significant part.

An in-depth examination of the phenomena of tumor promotion in various systems will help provide the scientific understanding of the cellular and biochemical mechanisms involved in these processes. As a more thorough comprehension of these mechanisms is developed, procedures can hopefully be devised to intervene in their course of events thereby inhibiting carcinogenesis. Better testing procedures may, in addition, be forthcoming that will be increasingly proficient in assessing the potential of various agents to act as modifiers of the process of carcinogenesis.

These four volumes will attempt to present the more recent accomplishments in the role of tumor promotion in internal organs (Volume I), investigations in the area of tumor promotion and skin carcinogenesis (Volume II), tumor promotion and cocarcinogenesis in vitro (Volumes III and IV). It will be the general aim of Volume I to carefully examine tumor promotion in various internal organs such as the liver, the lung, and the respiratory system, and in addition, studies on the colon, bladder, pancreas, and breast will be presented. The attainment of a better understanding of the two-stage system of carcinogenesis in these organs will be emphasized.

It is indeed hoped that these volumes will encourage further scientific investigations and therefore a better understanding of the multistage nature of tumor promotion, its role in the induction of cancer, and finally, the ultimate goal, its prevention. It should be of interest to all scientists as well as laymen interested in the pursuance of these goals.

THE EDITOR

Thomas J. Slaga, Ph.D., is the Director of The University of Texas System Cancer Center, Science Park — Research division in Smithville, Texas, an associate institute of the M. D. Anderson Hospital and Tumor Institute in Houston, Texas. In addition to his administrative duties at the Science Park-Research Division, Dr. Slaga continues to direct an extensive and highly productive research program on the mechanisms of chemical carcinogenesis both in vivo and in vitro.

Dr. Slaga received his Ph.D. in physiology/biophysics from the University of Arkansas Medical Center, Little Rock, Arkansas, in 1969. He then spent 3 years as a postdoctoral fellow in the laboratory of Dr. R. K. Boutwell at the McArdle Laboratory for Cancer Research, the University of Wisconsin, Madison, Wisconsin. This was indeed a significant period in his early career since it was under Dr. Boutwell's guidance that Dr. Slaga began his investigations of the carcinogenic effects of tumor promoters. In less than 10 years, he has assumed a position of leadership in this important area of investigative research, an area of study that may someday unravel the cancer puzzle.

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Chapter 1

MULTISTAGE HEPATOCARCINOGENESIS*

Carl Peraino, William L. Richards, and Fred J. Stevens

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* Submitted in August, 1981.

I. INTRODUCTION

As discussed in detail in Volume II, the concept of tumorigenesis as a multistage process originated from studies of the characteristics of skin tumor formation.[1-5] The multistage concept was first formalized by Rous,[4] who coined the now classic terms "initiation" and "promotion" to denote, respectively, (1) the production of potentially tumorigenic cells by limited exposure to carcinogen and (2) the completion of the neoplastic transformation as the result of subsequent treatment with appropriate agents that are not intrinsically carcinogenic.

Despite the definitiveness of the evidence for multistage skin tumorigenesis, the relevance of this mechanism to tumorigenesis in general did not have clear empirical support until the last decade, during which the additional initiation-promotion systems described in this volume have been developed. In this article we shall trace the evolution of the multistage carcinogenesis concept in liver, which is the first nonepidermal system in which this phenomenon was demonstrated unequivocally, and shall also provide an overview of the present state of the liver initiation-promotion system, including descriptions and evaluations of the various experimental models and methods that have been developed for the detection of preneoplastic changes. As part of this discussion, we shall identify what we consider to be conceptual ambiguities regarding the mechanistic boundaries between initiation and promotion that in our judgment are a product of experiments utilizing excessively severe tumorigenic protocols. Finally, we shall briefly discuss some of the possible insights into mechanisms of multistage hepatocarcinogenesis that can be gained from the examination of an important issue that has arisen with the newly acquired ability to detect preneoplastic hepatocyte foci, namely the fact that the numbers of such foci far exceed the numbers of hepatic tumors that ultimately appear.

II. HISTORICAL PERSPECTIVE

A. Indirect Evidence for Multistage Hepatocarcinogenesis

Since the first demonstration of chemically induced hepatocarcinogenesis in 1935,[6] the liver has become the focus of investigation by many experimental oncologists engaged in the analysis of tumorigenesis mechanisms. Indirect evidence that liver tumorigenesis occurs in stages was obtained several years ago in a number of these studies. Cole and Nowell[7] observed that administration of the hepatotoxin carbon tetrachloride (CCl_4) to previously X-irradiated mice substantially increased tumor incidence. Examination by Farber and coworkers[8,9] of various types of nodular hepatic lesions occurring during prolonged carcinogen

treatment led to the suggestion that malignant liver tumors evolve from cells contained in certain of the characteristic lesions, termed "hyperplastic nodules," that precede tumor formation under these conditions. Circumstantial evidence for the role of hyperplastic nodules as tumor precursors was obtained by Teebor and Becker[10] who observed that the feeding of 0.06% dietary 2-acetylaminofluorene (AAF) to rats for three separate 3-week intervals, alternating with 1-week intervals on the basal diet, produced a high yield of hyperplastic nodules that regressed, with a low subsequent incidence of hepatic tumors. An additional 3-week interval of AAF treatment, however, yielded both nodules that persisted after carcinogen withdrawal and a high incidence of hepatic tumors. A later variant of this approach involved the administration of a single dose of dimethylnitrosamine (DMN) following the three AAF feeding intervals. This combined treatment produced a high tumor yield, though no tumors were generated by either treatment alone.[11] More recently, studies by Farber and associates have shown that the expression of the tumorigenic potential of hepatocytes exposed to diethylnitrosamine (DEN) is enhanced by subsequent administration of AAF as a cytotoxin, coupled with proliferative stimulation induced by partial hepatectomy or CCl_4 administration.[12-14]

The studies cited above indicated that limited carcinogen treatment produces preneoplastic hepatocytes that require subsequent additional stimuli in order to complete the transformation into frank tumor cells. However, these investigations suffered from a common methodological deficiency, namely the use of substances with carcinogenic and/or mutagenic activity as the additional stimuli. Under such conditions, it is not possible to determine whether progressive stages of tumorigenesis are qualitatively similar (involving, for example, the progressive accumulation of mutations) or dissimilar, as had been demonstrated for skin tumorigenesis. Moreover, the value of the earlier attempts (reviewed by Farber[8]) to identify stages of hepatic preneoplasia was vitiated by the severity of the carcinogenic treatments used in virtually all of the studies. Such procedures made it difficult to distinguish between hepatic responses causally related to neoplasia and those representing the response of the liver to the toxic effects of the prolonged exposure to high levels of carcinogen. This uncertainty was especially burdensome in attempts to evaluate the role of hyperplastic nodule development in the etiology of hepatic neoplasia.[8,15] Despite the exhaustive characterization of the cytological and biochemical properties of these lesions,[8] their identity as obligatory tumor precursors, as opposed to ancillary manifestations of regeneration from carcinogen-induced cytotoxicity, could not be established unequivocally. In view of these ambiguities, the types of protocols leading to the generation of hyperplastic nodules and the investigation of the characteristics of these lesions no longer appear to represent useful experimental approaches to the analysis of tumorigenesis mechanisms.

B. Direct Demonstration of Initiation-Promotion Phenomenon in Liver

The first definitive evidence that the onset of liver neoplasia proceeds in qualitatively distinct sequential stages emerged from an investigation of the effect of phenobarbital on AAF-induced hepatocarcinogenesis.[16] This study compared the effects of two different types of AAF-phenobarbital exposure modalities on tumor incidence. In the first instance, AAF (0.02%) and phenobarbital (0.05%) were present in the diet concurrently (simultaneous treatment protocol). Under these conditions, hepatic tumor incidence was substantially less than that produced by feeding AAF alone. A protective effect of phenobarbital also occurred when it was administered simultaneously with the hepatocarcinogens, 4-dimethylaminoazobenzene[17] and DEN.[18] Since phenobarbital is a potent inducer of enzymes that actively metabolize these carcinogens,[19] it may be concluded that the anticarcinogenic action of phenobarbital in these instances stems from a phenobarbital-mediated shift in the balance of carcinogen metabolism toward detoxification and degradation.

The second type of exposure involved the prolonged feeding of 0.05% dietary phenobarbital after the termination of a brief (2 to 3 week) interval of feeding 0.02% dietary AAF (sequential treatment protocol). This regimen produced a markedly greater tumor incidence than that observed in rats receiving only the brief AAF treatment.[16] Subsequent studies have verified and extended this observation.[20-23]

From a consideration of the contrasting effects of the simultaneous and sequential treatment protocols on tumorigenesis, it is evident that the enhancing effect of phenobarbital cannot be a consequence of increased metabolic activation of the carcinogen. The hypothesis most consistent with the data is that phenobarbital given according to the sequential treatment protocol facilitates the ultimate expression of tumorigenic changes initiated by prior exposure to the carcinogen. This interpretation implies the existence of at least two elements of the tumorigenic process that differ in mechanism as well as in temporal occurrence, and it comprises the basis for the argument that the initiation-promotion concept of tumorigensis applies to liver as well as to skin.

Because the overall carcinogen exposure in the AAF-phenobarbital studies[16,20-23] was greatly reduced in comparison with that in most of the earlier studies,[8,10,11] the AAF-phenobarbital treated livers exhibited none of the injury-related nodular hyperplasia that previously had been a common feature of carcinogen-treated liver.[8] As illustrated most clearly in a recent study of liver tumor promotion,[23] livers remained free of any nodular growth for approximately 4 months following cessation of carcinogen treatment, after which continually growing, nonregressing hepatic tumors began to appear.

Figure 1 shows a typical liver from a rat fed 0.02% dietary AAF during the first 18 days after weaning followed by the feeding of 0.05% dietary phenobarbital for 250 days. Note that the surface of the liver is smooth except for the occurrence of a large tumor in the left lobe. This appearance contrasts sharply with the grossly nodular surface seen prior to the appearance of tumors in rats on more severe carcinogen regimens,[8] and demonstrates clearly, in agreement with the conjecture of Foulds,[15] that hyperplastic nodules are not obligatory precursors of hepatic neoplasia.

III. METHODS FOR ANALYZING MULTISTAGE HEPATOCARCINOGENESIS

A. Treatment Protocols

1. Sequential Feeding of Carcinogen and Promoter

As indicated above, this approach has adapted the treatment strategies used for many years in studies of multistage skin tumorigenesis.[1-5] The main contribution of this procedure is the clarity with which it has revealed the existence of distinct stages of hepatic tumorigenesis and permitted the examination of several characteristics of the promotion stage,[16,20-23] as will be discussed in a subsequent section. Disadvantages of this system include (1) the retention of a significant interval of carcinogen treatment, thereby reducing the ability to detect and resolve the earliest stages of initiation, as well as blurring the distinction between the initiating and promoting action of the carcinogen, and (2) the slowness of the response, requiring a prolonged experimental duration in order to obtain useful information. Thus, whereas the sequential AAF-phenobarbital protocol has been useful as a means of examining the phenomenology of multistage hepatocarcinogenesis, the foregoing deficiencies severely limit its usefulness for more sophisticated mechanistic analyses of liver tumor initiation and promotion.

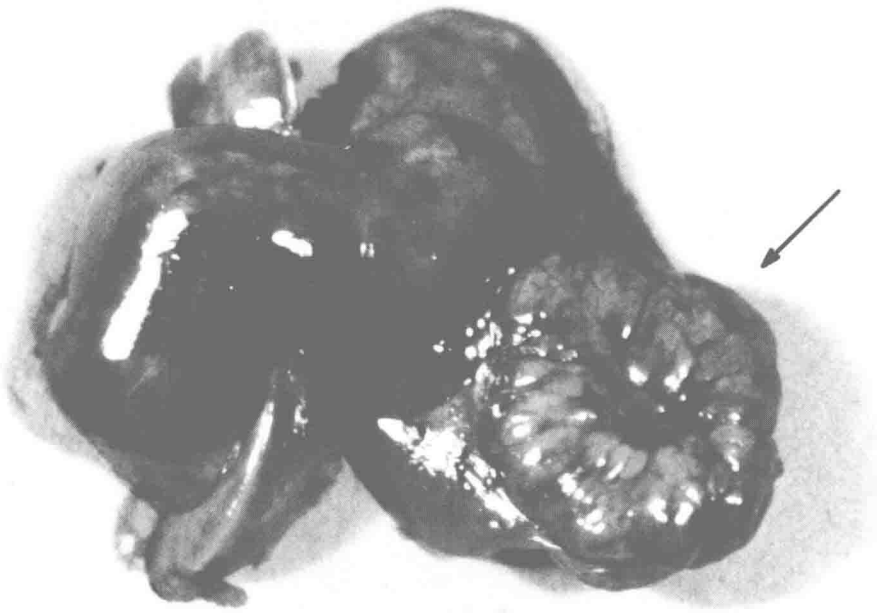

FIGURE 1. Tumor (arrow) in the left lobe of the liver from a rat fed a 0.02% AAF diet for 18 days followed by a 0.05% phenobarbital diet for 250 days. The largest diameter of the tumor is 2.5 cm. The remainder of the liver is free of nodular hyperplasia.

2. Single Treatment with Carcinogen Followed by Proliferative Stimulation in the Presence of a Growth Suppressant (Selection Model)

This procedure and the rationale on which it is based were discussed at length in a recent review by Farber.[14] Briefly, it was postulated that the neoplastic process involves, in part, the acquisition by preneoplastic cells of resistance to the cytotoxic and hence "mitoinhibitory" effects of the carcinogenic stimulus. The strategy for the early detection of such cells in experimental hepatocarcinogensis involves the creation of conditions fostering the selective growth of the putative preneoplastic hepatocytes. These hepatocytes are produced by prior treatment with a necrogenic dose of carcinogen such as DEN. The "selection" environment is produced by the generation of an intense proliferative stimulus, as a result of partial hepatectomy or CCl_4 administration,[24] in the midst of treatment with an agent, AAF, that inhibits the proliferation of normal hepatocytes. While the regeneration of the bulk of the liver is blocked, the hepatocytes rendered resistant by the prior DEN treatment proliferate rapidly and produce visible nodules within 1 week; tumors appear within 8 months. These responses are not observed in the absence of the initiating stimulus in adult rats, and the cells capable of responding to the "selection" procedure persist for at least 9 months.[24]

The major advantage of this procedure is the rapidity and intensity with which the initial response is produced (i.e., the appearance of histochemically altered foci and discrete nodules). This characteristic renders the procedure potentially useful as a rapid method for

screening environmental contaminants for tumor initiating activity, as demonstrated in a recent study wherein 21 carcinogens, including polycyclic aromatic hydrocarbons, gave clear-cut positive responses.[25]

The procedure appears less satisfactory, however, as a means of analyzing mechanisms of multistage hepatocarcinogenesis. The basis for this reservation is the severity of both the initiation and "selection" steps, and the use of AAF as the selecting agent. Thus the administration of a necrogenic dose of initiating agent (DEN), justified on the basis of the need to juxtapose the production of regenerative hyperplasia with the creation of the initial tumorigenic lesion,[26-28] undoubtedly increases the number of tumorigenic molecular lesions borne by the initiated cells, thereby reducing the proportion of those initiated cells bearing the minimum number of changes essential for the acquisition of tumorigenic potential. Moreover, since the probability that the promoting action of an initiator will be expressed increases with increasing initiator dosage,[16] the high DEN level used in the selection procedure raises the possibility, as acknowledged by Solt et al.,[24] that a significant proportion of the response to this treatment is the result of the coincidental interplay of initiation and promotion mechanisms.

The use of AAF in conjunction with partial hepatectomy or CCl_4 administration as the selection procedure also compromises attempts at mechanistic interpretation, since one is inescapably faced with the possibility that the 2-week AAF treatment introduces additional molecular changes into the DEN-initiated cells (in essence exerting additional initiating effects), thereby influencing their growth rates, phenotypic characteristics, and tumorigenic potential. Fully cognizant of this problem, Tsuda et al.[25] have presented evidence that the AAF-CCl_4 treatment does not produce presumptive preneoplastic foci in the absence of the prior initiation treatment unless the AAF feeding interval is extended for 4 weeks or longer.[25] The significance of this observation is undermined, however, by the fact that the livers were examined 1 week after the cessation of AAF feeding, thereby eliminating the possibility of detecting later-emerging lesions. In any case, the occurrence of numerous altered foci per square centimeter of liver after 6 weeks of AAF treatment alone in these rats,[25] strengthens the possibility that the 2-week AAF exposure may subtly influence the character of the previously initiated cells.

Evidence that the AAF selection treatment might exert extraneous initiating effects also derives from studies in which this AAF treatment served as a potent initiator of tumorigenesis when fed to weanling rats,[23] or of altered hepatocyte foci when fed to older animals.[29] Moreover, it has clearly been demonstrated that hepatocytes, altered by prior carcinogen treatment, are subject to further tumorigenic modification by subsequent exposure to the same or different carcinogens.[10,11,30]

Finally, concern must be raised about the interchangeable use of CCl_4 and partial hepatectomy as methods for stimulating hepatocyte proliferation during AAF administration in the selection procedure.[14,24,25] The hepatotoxic effects of CCl_4 undoubtedly generate complex biochemical changes that interact in as yet unknown ways with those produced by hepatocarcinogens; in any case, the spectrum of interactions could very likely be considerably different from that engendered by combining carcinogen treatment with partial hepatectomy. Evidence for the complexity of CCl_4-hepatocarcinogen interactions is provided by several studies in which variable enhancing effects of CCl_4 on tumorigenesis were produced, depending on the nature of the hepatocarcinogen used and the temporal relationships of the carcinogen-CCl_4 treatments.[31-33]

Overall, therefore, the selection procedure appears to represent a valuable method for the rapid production of carcinogen-altered hepatocytes that can then be subjected to further study.[34] However, the multiplicity of complex overlapping effects generated by the various elements of this protocol reduces its value for distinguishing the characteristics of the sequential stages of hepatocarcinogenesis.

3. Single Treatment with Carcinogen During Liver Regeneration, Followed by Phenobarbital Feeding

This highly useful protocol[35-37] was synthesized from elements of a variety of published procedures and has significantly advanced our capacity to examine the stages of hepatic neoplasia. Basically, the protocol involves first, the gastric intubation of a nonnecrogenic dose of DEN (approximately 10 mg/kg body weight) 24 hr after partial hepatectomy; this aspect of the protocol derives from prior observations that such treatment produces foci of presumptive preneoplastic hepatocytes[30,38-40] and liver tumors if the DEN dosage is sufficiently high.[41] The second element of the protocol utilizes prolonged exposures to dietary phenobarbital[20-23] following the DEN intubation to enhance the production of foci and tumors. Finally, the development of altered hepatocyte foci is monitored by the application of histochemical tests to detect the absence of glucose-6-phosphatase[42-44] and canalicular ATPase,[30,38-40] and the presence of γ-glutamyltranspeptidase[44] in foci contained in adjacent frozen liver sections. By superimposing the images of these sections, foci with one to three of the foregoing marker changes are scored,[44] permitting the assessment of the occurrence of seven different focus phenotypes.[36,44]

The advantages of this procedure are (1) the use of a single carcinogen treatment at a relatively low dosage level minimizes uncertainties regarding the possible overlapping of initiating and promoting actions of the carcinogen that may occur with higher dosages or more prolonged administration of carcinogen; (2) the carcinogen treatment regimen allows precise control of carcinogen intake, an important consideration in carcinogen dose-response studies; and (3) the use of histochemical techniques permits the monitoring of relatively early hepatic changes during the onset of neoplasia. However, the protocol suffers from the requirement that carcinogen treatment be preceded by partial hepatectomy, a relatively impractical procedure for large-scale studies. In addition, the experimental duration required for the attainment of a definitive end point, namely the appearance of tumors,[36] is as long as those in other protocols.[16,20-23,45]

4. Production of Lipotrope Deficiency Prior to or Following Exposure to Carcinogen

As reviewed by Rogers and Newberne,[46] a number of studies have shown that the hepatocarcinogenicity of several agents, including aflatoxin, DEN, dibutylnitrosamine, and AAF, is increased in rats previously fed a diet marginally deficient in the lipotropes, choline, methionine, and folic acid. Despite substantial differences between the control and deficient diets with respect to protein composition, fat composition and content, and carbohydrate composition and content, the increase in liver tumorigenesis is largely reversed by lipotrope supplementation of the deficient diet,[46] supporting the role of lipotrope deficiency in tumorigenic enhancement. Thus, far, attempts to determine whether the lipotrope deficient diet alters carcinogen metabolism in a manner consistent with the diet's tumorigenic enhancing effect, have yielded inconclusive results (e.g., aflatoxin activation was not increased in livers of lipotrope deficient rats although urinary excretion of mutagenic aflatoxin metabolites was higher in these animals), raising the possibility that other factors such as diet-induced changes in host susceptibility (e.g., increased proliferation of target cells) may play a role in the enhancement process.[46]

Enhancement of hepatocarcinogenesis was also observed in rats fed a choline-devoid diet during[47-49] or after[50,51] exposure to the carcinogenic stimulus. In the latter instance, the addition of phenobarbital to the choline-devoid diet at a concentration of 0.06% produced a significantly greater enhancement than the sum of the responses to the choline-devoid and phenobarbital diets given individually, indicating a synergistic interaction of the two promoting stimuli.[51] In an investigation of the basis of this synergism, Abanobi et al.[52] reported that the feeding of a choline-devoid diet stimulated liver DNA synthesis and hepatocyte

mitosis (as had been reported previously in the case of lipotrope deficiency[53]), whereas dietary phenobarbital suppressed both end points by 50% in control and choline-deficient rats. On the basis of these opposing effects of the two treatments in rats not treated with carcinogen, it is postulated that the promoting action of the choline-devoid diet involves proliferative stimulation of both initiated and noninitiated hepatocytes, whereas phenobarbital feeding primarily suppresses the proliferation of noninitiated cells.[52] This speculation follows the logic of the selection model outlined earlier (Section III.A.2) and places the choline-devoid diet in the role of proliferative stimulus, currently occupied in the model by partial hepatectomy or CCl_4 treatment,[12-14] with phenobarbital serving as the "selecting" agent instead of AAF. However, Farber and colleagues have, on the basis of preliminary data, suggested that the choline-devoid diet might substitute for AAF as the "selecting" agent in their model.[25] Should this substitution prove feasible, the rationale for the selection protocol would be compromised if the current interpretation[52] of the effect of the choline-devoid diet is correct, since the necessity for the selective "mitoinhibition" of noninitiated cells would be eliminated. The absence of such a requirement would in turn undermine the argument that the promoting action of phenobarbital stems from its selective proliferative suppression of noninitiated cells.[52]

It must be noted that the experimental end point reported in the studies of the promoting effect of choline deficiency, and its interaction with phenobarbital, was restricted to the occurrence of hepatic foci positive for γ-glutamyltranspeptidase with the assumption that this end point represents a valid early indication of the subsequent carcinogenic response.[51] The tentative nature of this assumption should be kept in mind, however, in view of the paucity of information on the constancy of the relationship between focus frequency and tumor frequency under various experimental conditions, especially in view of the fact that focus frequency exceeds tumor frequency by more than three orders of magnitude.[36]

With regard to its utility as a means of investigating multistage hepatocarcinogenesis, the induction of lipotrope deficiency would appear to suffer from the same limitation that characterized most of the early prior experimental hepatocarcinogenesis systems, namely the severity of the treatment, which in this case produces profound disruptions in lipoprotein metabolism, accompanied by fatty infiltration of the liver, leading to hepatic cirrhosis.[54-57] Under such circumstances, the observed hyperplasia[52,53] would be expected as part of the well-known compensatory regeneration that characterizes the response of the liver to chemically or physically induced injury. Thus, in terms of its enhancement of hepatocarcinogenesis, the induction of lipotrope deficiency may act in part as a means of stimulating hepatocyte proliferation, which may in turn play a role in tumorigenic enhancement both during and after carcinogen treatment (see below). In any case, in view of the complex biochemical manifestations of hepatic damage induced by lipotrope deficiency, it is difficult to see how events causally related to tumorigenic enhancement can be distinguished from extraneous injury-related responses; consequently, the value of this approach for mechanistic studies would appear to be limited.

5. Tumorigenic Enhancement by Proliferative Stimulation

a. Proliferative Stimulation Concurrent with Initiation

In this section we shall be concerned only with artificially induced hepatocyte proliferative stimulation at the time of initiation. Initiation of inherently rapidly proliferating hepatocytes in newborn animals will be discussed later (Section III.A.8).

Several studies have shown that the hepatocarcinogenic potential of various agents is greatly enhanced if the agents are administered to animals undergoing liver regeneration as a result of partial hepatectomy.[40,41,58-64] Such enhancement could stem from an increase in

the vulnerability of replicating DNA to alteration by carcinogen,[63] and subsequently to fixation of the carcinogen-induced DNA lesions by replication of the modified DNA prior to repair.[65] In any case, preinitiation partial hepatectomy has become an important part of recent experimental protocols aimed at analyzing mechanisms of multistage hepatocarcinogenesis,[36,40] since, by improving the sensitivity of the carcinogenic response, this procedure has permitted the use of subtoxic initiating dosages of carcinogen. Moreover, this increase in sensitivity has also made possible the detection of hepatocarcinogenic activity in agents such as urethan and 7,12-dimethylbenz[a]anthracene that are virtually ineffective in quiescent hepatic tissues.[58-63]

Replacement of the preinitiation partial hepatectomy by a single necrogenic CCl_4 treatment also enhances subsequent hepatic tumor incidence,[66-68] and the mechanism of this enhancement is presumed to be strictly analogous to that underlying the enhancing effect of partial hepatectomy, namely the stimulation of regenerative hyperplasia.[67] However, as pointed out earlier, there exists the inescapable possibility that the complex biochemical changes accompanying CCl_4-induced hepatotoxicity also influence the response to the carcinogen. The virtual impossibility of assessing the magnitude of this influence vitiates the usefulness of CCl_4 treatment as a substitute for partial hepatectomy in such studies.

b. Proliferative Stimulation Following Initiation

Repeated partial hepatectomies have been performed following carcinogen treatment in an effort to determine whether the repeated proliferative stimulation of hepatocytes exerts a promoting effect on hepatocarcinogenesis.[64] Marginal enhancement of tumorigenesis by this procedure suggests, but does not demonstrate convincingly, that regenerative hyperplasia promotes hepatic tumorigenesis. The equivocal results suggest either that proliferative stimulation does not constitute a potent promoting stimulus, or that surgical removal of clones of dividing initiated cells masks what might be in fact a strong response.

An alternative approach, involving repeated postinitiation treatments with CCl_4 in place of multiple partial hepatectomy, has produced a substantially larger increase in tumor yield.[32,68] It is argued that the greater promoting effectiveness of the CCl_4 treatment stems from a higher proliferative response to this treatment than to partial hepatectomy.[68] However, it is equally likely that other biochemical effects unrelated to proliferative stimulation are predominantly responsible for the promoting activity of CCl_4. This possibility is supported by evidence that phenobarbital, a potent promoter of hepatocarcinogenesis,[16,20-23] produces only a single early transient increase in hepatocyte proliferation,[16,21,69,70] despite prolonged administration, although many other phenobarbital-mediated hepatic changes are sustained as long as this treatment continues.[19] Since extended exposure to phenobarbital, far beyond that required for manifestation of its limited proliferative effect, is required for expression of phenobarbital's promoting activity,[20-22] it is probable that factors other than proliferative stimulation are primary contributors to the mechanism of liver tumor promotion.

6. Hybrid Treatment Protocols

In efforts to develop rapid bioassays for tumor initiators and promoters using liver as the test system, various treatment protocols have been assembled from elements of the procedures described above. Ito and colleagues[71,72] describe a system (based on the selection system discussed in Section III.A.2) in which rats are given a necrogenic dose of DEN (200 mg/kg) by i.p. injection, followed after 2 weeks by administration of the test agent for 2 weeks, with partial hepatectomy performed midway through the latter treatment. Rats killed at the end of the latter 2-week interval are examined histochemically and histologically for the presence of "hyperlastic nodules". In a later variant of this procedure,[29] rats are fed AAF for 2 weeks, followed by an 8-week feeding of the test chemical, the rats being subjected