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SHORT-TERM in vivo INVESTIGATION OF INITIATING AND PROMOTING ACTIVITIES OF ENVIRONMENTAL CHEMICALS ON HEPATOCARCINOGENESIS

DISSertation

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By

Michael Millard Milks, B.S. Pharmacy

* * * * *

The Ohio State University

1982

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To Deborah

Always the flower, the sunshine, the song
Always the caring, the trusting, the calm
Always the light, the reason, the meaning
Always my love ... Always
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So that I may have achieved whatever present and future accomplishments, both within and without the realm of science, innumerable people have unselfishly extended to me their support, encouragement, and guidance. To them I am forever indebted.

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# TABLE OF CONTENTS

| TITLE PAGE | i |
| DEDICATION | ii |
| ACKNOWLEDGEMENTS | iii |
| VITA | v |
| LIST OF TABLES | xii |
| LIST OF FIGURES | xiii |
| LIST OF PLATES | xiv |
| INTRODUCTION | 1 |
| A. TWO-STAGE MODEL OF CARCINOGENESIS | 2 |
| 1. Initiation | 3 |
| 2. Promotion | 4 |
| B. ENZYME-ALTERED FOCI BIOASSAY | 5 |
| 1. Evidence That Enzyme-Altered Foci are Indicative of Hepatocarcinogenesis | 7 |
| 2. Role of Partial Hepatectomy | 10 |
| 3. Role of Liver Tumor Promotion | 11 |
| a.) 2-Acetylaminofluorene | 12 |
| b.) Choline-Deficient Diet | 13 |
| c.) Liver Regeneration | 15 |
| d.) Phenobarbital | 17 |

viii
C. SELECTION OF CHEMICALS FOR STUDY ......... 18
   1. Methapyrilene .......................... 19
   2. 1,2-Dibromoethane ..................... 20
   3. Trichloroethylene ...................... 21

STATEMENT OF THE PROBLEM .......................... 25

METHODS ............................................. 27
   A. ANIMALS .................................... 27
   B. PARTIAL HEPATECTOMY ...................... 27
   C. HISTOCHEMICAL TECHNIQUES ................. 29
       1. Tissue Preparation ..................... 29
       2. Cryostatic Sectioning .................. 29
       3. Histochemical Staining ................. 30
       4. Light Microscopy ....................... 32
       5. Calculation of Foci Per Unit Area ...... 33
   D. TREATMENT PROTOCOLS ....................... 36
       1. Methapyrilene .......................... 39
       2. 1,2-Dibromoethane ..................... 40
       3. Trichloroethylene ...................... 43
          a.) Initiation .......................... 43
          b.) Promotion .......................... 46
APPENDIX

ADDITIONAL STUDIES INVOLVING THE ENZYME-ALTERED FOCI BIOASSAY AS AN INDICATOR OF HEPATOCARCINOGENESIS ................................. 107

A. DOSE-RESPONSE EFFECTS OF AFLATOXIN B, ON THE FORMATION OF ENZYME-ALTERED FOCI .......................................................... 107

B. PRELIMINARY STUDIES INVESTIGATING THE CARCINOGENIC POTENTIAL OF VARIOUS HALOGENATED HYDROCARBONS USING THE ENZYME-ALTERED FOCI BIOASSAY ........................................... 109

C. PRELIMINARY INVESTIGATION OF THE PROMOTING EFFECTS OF TRICHLOROETHYLENE ......................................................... 110

D. PROMOTING EFFECTS OF PHENOBARBITAL AND METHAPYRILENE AFTER THIRTEEN MONTHS OF CONTINUED PROMOTION ......................... 111

E. PHENOBARBITAL DOSE-RESPONSE PROMOTER EFFECTS ON THE FORMATION OF ENZYME-ALTERED FOCI ........................................ 113

F. TUMOR PROMOTING EFFECTS OF CHLOROFORM ASSESSED BY THE ENZYME-ALTERED FOCI BIOASSAY ........................................... 115

G. CHLOROFORM/N-NITROSODIETHYLAMINE COCARCINOGENESIS STUDY ................................................................. 116

H. THE EFFECTS OF SELENIUM ON THE INDUCTION OF ENZYME-ALTERED FOCI BY CHEMICAL CARCINOGENS ........................................ 116
# LIST OF TABLES

<table>
<thead>
<tr>
<th>TABLE</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Comparison of the General Features of Initiation and Promotion</td>
</tr>
<tr>
<td>2</td>
<td>Experimental Protocol: Initiating and Promoting Effects of Methapyrilene on the Formation of Enzyme-Altered Foci in Rat Liver</td>
</tr>
<tr>
<td>3</td>
<td>Experimental Protocol: Initiating Effects of Trichloroethylene on the Formation of Enzyme-Altered Foci in Rat Liver</td>
</tr>
<tr>
<td>4</td>
<td>Experimental Protocol: Promoting Effects of Trichloroethylene on the Formation of Enzyme-Altered Foci in Rat Liver</td>
</tr>
</tbody>
</table>
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>FIGURE</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>31</td>
</tr>
<tr>
<td>2</td>
<td>38</td>
</tr>
<tr>
<td>3</td>
<td>45</td>
</tr>
<tr>
<td>4</td>
<td>52</td>
</tr>
<tr>
<td>5</td>
<td>56</td>
</tr>
<tr>
<td>6</td>
<td>62</td>
</tr>
<tr>
<td>7</td>
<td>66</td>
</tr>
<tr>
<td>PLATE</td>
<td>PAGE</td>
</tr>
<tr>
<td>-------</td>
<td>------</td>
</tr>
<tr>
<td>I</td>
<td>34</td>
</tr>
<tr>
<td>II</td>
<td>35</td>
</tr>
</tbody>
</table>

I  Representative Photomicrograph of a Gamma-Glutamyl Transpeptidase Positive Focus (mag. 30X) .......................... 34

II  Representative Photomicrograph of a Gamma-Glutamyl Transpeptidase Positive Focus (mag. 120X) .......................... 35
INTRODUCTION

Chemical carcinogenesis as a recognized scientific concept had its origins in late eighteenth-century London, England through the separate observations of John Hill and Sir Percival Pott. Hill reported an association between the development of nasal cancer and the excessive use of tobacco snuff. Pott, on the other hand, described the unusually high incidence of skin cancer of the scrotum in chimney sweeps. Since that time, much has become known about the process of tumor formation, and about how chemical or physical agents can affect this process. Over four decades ago the phenomenon of tumor causation by a chemical was shown to be composed of at least two separate activities or stages (Rous and Kidd, 1941; Berenblum, 1941; Friedewald and Rous, 1944).

This multi-stage concept of carcinogenesis has been extended from the original rabbit and mouse skin models to many different tissues and organs (Colburn, 1980; Berenblum and Armuth, 1981) including liver (Farber and Solt, 1978; Peraino et al, 1978; Pitot, 1977; Pitot et al, 1978b; Scherer and Emmelot, 1976; Solt et al, 1977; Williams, 1978), lung (Witschi and Lock, 1978), colon (Reddy et al, 1978), and
bladder (Hicks et al, 1978). With the recognition that a chemical or physical agent may increase tumor production by at least two distinctly different mechanisms, it becomes important to distinguish which mechanism is predominantly involved in the formation of tumors. With this knowledge, a more meaningful approach may be designed for elucidating the molecular fundamentals of carcinogenesis rather than obtaining a limited yes/no answer to the singular question "Does this agent cause tumors?" The major emphasis of this thesis, then, was to attempt to evaluate several carcinogens for initiating and promoting activity using a two-stage model of rat hepatocarcinogenesis.

A. TWO-STAGE MODEL OF CARCINOGENESIS

The current concept of carcinogenesis as a multi-stage model was derived, in part, from the early work of Rous and Kidd (1941), Berenblum (1941), and Friedewald and Rous (1944) using rabbit and mouse skin models. Such phenomena as the apparent irreversibility of neoplastic initiation and the requirement for "encouraging" or promoting conditions in order to allow proliferation of neoplastic cells into tumors were recognized in these early works. Since that time, considerable additional information has been obtained regarding the processes of initiation and promotion.
1. Initiation

The process by which a normal cell becomes altered in such a way as to gain the potential for neoplastic growth is referred to as initiation. This process is largely, but not unanimously, believed to involve a mutational change in the genome of such an "initiated" cell. This "genotoxic" interaction between carcinogen and DNA is generally considered to occur via electrophilic attack of the ultimate carcinogen on the macromolecular nucleophile, DNA (Miller, 1978). The pioneering work of the Millers in the area of metabolic activation of chemical carcinogens provided a unifying hypothesis for the genotoxicity of most chemical carcinogens (Miller and Miller, 1966). The exact mechanism by which this genetic lesion gives rise to a population of cancerous cells is not known. One attractive theory is that the genotoxic damage alters the genetic information which codes for various "regulatory elements" which, in a normal cell, would suppress the expression of certain genes or groups of genes. In these initiated cells, the loss of cellular regulation would then allow the unrestrained growth and clonal expansion of such cells into tumorous masses (Weinstein et al, 1978). Other theories, perhaps equally tenable, have also been proposed to explain the nature of the initiating process.
(Neubert et al, 1981; Huebner and Todaro, 1969; Miller, 1978). However, until more is learned about the basic mechanisms of initiation, an operational definition of the process may be superior to a mechanistic one with respect to a discussion of the multi-stage concept of carcinogenesis. Initiation, then, may be conceived as the alteration of the genetic information of a cell (genotoxicity) in such a way that the initiated cell becomes predisposed toward neoplastic development.

Implicit in the designation of an agent as an initiator is a contrast between that agent and tumor promoters. For this reason, the discussion will now be shifted toward addressing the phenomenon of tumor promotion.

2. Promotion

A promoting agent, by definition, is an agent which is devoid of initiating or genotoxic activity of its own, but when applied or administered repeatedly to an animal which has received a subcarcinogenic treatment with an initiating agent, will allow the development of tumors as evidenced by an enhancement in the rate of tumor formation and by a decrease in the latency of tumorigenic expression. Because of the apparent lack of a direct chemical interaction between cellular DNA and a promoting agent, promotion is often referred to as an "epigenetic" phenomenon, although the exact
nature of the process remains obscure. It seems reasonable that gene activation, perhaps through a "derepression" effect, may be fundamental in explaining the mechanism of promotional activity (Boutwell, 1974; Berenblum and Armuth, 1981). As was the case for the process of initiation, an operational definition of the phenomenon of tumor promotion may better serve the conceptual discussion of the multi-stage hypothesis of carcinogenesis. Toward this end, the following summary chart (Table 1) is given to illustrate the salient features of both processes, initiation and promotion.

B. ENZYME-ALTERED FOCI BIOASSAY

It has been estimated that up to 90% of all human cancers are environmentally induced (Weisburger et al, 1977; Wynder et al, 1978), with chemicals, no doubt, constituting a major class of environmental carcinogens. The designation of an agent as a carcinogen has previously relied upon the whole animal lifetime bioassay which requires hundreds (if not thousands) of animals, a sizeable commitment of research dollars, as well as a disturbing investment of time and laboratory space. For these reasons, alternative systems for carcinogenesis testing were sought and are currently being developed and/or refined. One such alternative for assessing the carcinogenic activity of a compound is the in vivo
<table>
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<th>INITIATION</th>
<th>PROMOTION</th>
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<tr>
<td>- IRREVERSIBLE AND ADDITIVE</td>
<td>- REVERSIBLE (AT EARLY STAGES) AND NOT ADDITIVE</td>
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<td>- MUST PRECEDE THE PROMOTING REGIMEN</td>
<td>- MUST OCCUR AFTER THE INITIATING EVENT(S)</td>
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<td>- SINGLE EXPOSURE IS SUFFICIENT</td>
<td>- REPEATED OR PROLONGED EXPOSURE IS REQUIRED</td>
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<td>- NO APPARENT THRESHOLD</td>
<td>- THRESHOLD FOR EFFECTS SEEMS PROBABLE</td>
</tr>
<tr>
<td>- INVOLVES THE COVALENT BINDING OF ELECTROPHILES TO CELLULAR MACROMOLECULES</td>
<td>- Macromolecular binding is not apparent</td>
</tr>
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<td>- HIGHLY CORRELATED TO MUTATIONAL ACTIVITY</td>
<td>- NOT CORRELATED TO MUTATIONAL ACTIVITY</td>
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rat hepatic enzyme-altered foci bioassay. In this assay alterations in the normal occurrence of certain biochemical events, especially the activities of various enzymes, are demonstrated histochemically in clusters or clones of putative preneoplastic hepatocytes (Scherer and Emmelot, 1975; Solt et al, 1977; Pitot, 1977; Pitot et al, 1978a; Hirota and Williams, 1979; Ogawa et al, 1980; Solt et al, 1980). The use of such a short-term in vivo system has been repeatedly reviewed and evaluated with respect to sensitivity, accuracy, relevance, and advantages (Boelsterli, 1979; Bannasch et al, 1980, Emmelot and Scherer, 1980; Farber, 1980; Pitot and Sirica, 1980; Williams, 1980; Ford and Pereira, 1980; Tsuda et al, 1980; Pereira, 1982). Using the focal appearance of enzyme activity of gamma-glutamyl transpeptidase (GGT), various aspects of tumor initiation and promotion were investigated and are reported in this dissertation.

1. Evidence That Enzyme-Altered Foci Are Indicative of Hepatocarcinogenesis

Perhaps the best evidence that hepatocellular carcinomas may develop from enzyme-altered foci is the observation that within these hyperplastic foci, hepatocytes are seen to progress from a benign to a malignant state (Goldfarb and
Pugh, 1982). Additional evidence includes the proportionalities that exist between the development of enzyme-altered foci and the eventual appearance of liver tumors in animals administered various dosages of carcinogens (Scherer and Emmelot, 1975; Pitot, 1977; Solt et al, 1977, Farber, 1980; Goldfarb and Pugh, 1982). Furthermore, not only do enzyme-altered foci precede the appearance of frank liver tumors, but they also demonstrate atypical phenotypes which are often characteristics of tumor cells (Scherer and Emmelot, 1975). The histochemical demonstration of GGT activity in adult rat liver is one such example of an atypical phenotype expressed by both putative preneoplastic hepatocytes and hepatic tumor cells. In fact, approximately 90% of primary hepatocellular carcinomas induced in rats by the administration of 2-acetylaminofluorene display the GGT-positive phenotype (Pugh and Goldfarb, 1978). Many other chemical carcinogens have also been shown to produce enzyme-altered foci including N-nitroso-diethylamine, aflatoxin B₁, benzo(a)pyrene, urethane (ethyl carbamate), 2-naphthylamine, and ethionine among others (Scherer and Emmelot, 1976; Tsuda et al, 1980; Pereira, 1982).

Hepatocytes within enzyme-altered foci are also known to proliferate at a much higher rate than normal surrounding liver parenchymal cells (Williams et al, 1976; Schulte-Hermann et al, 1981) as well as being resistant to the
cytotoxic effects of various carcinogens (Williams et al, 1976; Farber et al, 1976).

One very interesting finding which supports a precursor relationship of enzyme-altered foci to hepatocellular carcinomas is that when liver cells obtained from donor rats initiated with 2-acetylaminofluorene were injected into isogeneic recipient rats, altered foci were observed in as short a time as five days and hepatocellular carcinomas within seventeen months (Laishes and Rolfe, 1980).

In later studies, hepatocytes were obtained from N-nitrosodiethylamine-initiated rats and were purified by centrifugation such that over 70% of the hepatocytes demonstrated GGT activity. In these studies, after only four days following transplantation of these purified GGT-positive cells, enzyme-altered foci were observed in the livers of recipient rats (Miller et al, 1982).

Additionally, hepatocytes from enzyme-altered foci have been shown to proliferate in vitro, retaining the enzyme-altered phenotype, and upon successive subculture, becoming transplantable and capable of growth in soft agar (Kitagawa et al, 1980).

Indeed, that enzyme-altered foci may represent one possible stage of hepatocarcinogenesis appears to be quite well established. However, it should be noted that this stage is not an absolute requirement for the development of
all tumors which may arise within hepatic tissue.

2. Role of Partial Hepatectomy

That partial hepatectomy can enhance the tumorigenic response of mice and rats to a chemical carcinogen has been known for over a decade (Pound, 1968; Hollander and Bentvelzen, 1968; Chernozemski and Warwick, 1970; Lane et al, 1970; Craddock, 1971; Craddock, 1973; Craddock and Frei, 1974; Pound and Lawson, 1974; Pound and McGuire, 1978a) and, therefore, that partial hepatectomy was shown to likewise increase the incidence of enzyme-altered foci in rats which were administered carcinogens (Scherer et al, 1972) is certainly a logical expectation in view of these earlier reports. The optimal time for carcinogen administration in relation to the surgical excision of two-thirds of the liver was shown to be six to twelve hours post-surgery for benzo(a)-pyrene, six to eighteen hours post-hepatectomy for dimethylhydrazine, and twenty-four hours following surgery for N-nitrosodiethylamine (Emmelot and Scherer, 1980; Tsuda et al, 1980). For the carcinogen N-nitrosodiethylamine a significant foci-enhancing effect of partial hepatectomy was observed even when the carcinogen was administered seven days prior to two-thirds hepatectomy or up to 72 hours post-surgery (Ishikawa et al, 1980; Emmelot and Scherer, 1980). In addition to a
quantitative increase in the tumorigenic response of the liver to various carcinogens, partial hepatectomy was also observed to change the site(s) of predilection for numerous non-hepatocarcinogens so as to include the liver as a major target organ. The lung and skin carcinogen benzo(a)pyrene, the mammary and skin carcinogen 7,12-dimethylbenzanthracene, the bladder carcinogen 2-naphthylamine, and the potent carcinogen for many tissues and organs (but notably excluding intact liver) N-methyl-N-nitrosourea, all have been shown to be hepatocarcinogenic when administered in conjunction with a two-thirds partial hepatectomy (Cayama et al, 1978; Tsuda et al, 1980). For these reasons, to increase and to expand the susceptibility of the liver to the carcinogenic effects of chemical agents, partial hepatectomy has become an integral component of the hepatic enzyme-altered foci bioassay.

3. Role of Liver Tumor Promotion

To facilitate the detection of carcinogens using the hepatic enzyme-altered foci bioassay, most laboratories have incorporated into their experimental protocols a "promoting" treatment or regimen which, by itself, will not substantially increase the tumor burden of a "promoted" animal. However, if experimental animals are first "initiated" with a genotoxic carcinogen and then subsequently exposed to the
promoting regimen, tumorigenesis is greatly enhanced. Various promoting regimens have also been shown to increase the sensitivity of the liver tissue to the carcinogen, to enhance the emergence of putative preneoplastic foci, and to alter the organ or tissue susceptibility such that liver becomes included as a vulnerable organ for chemical carcinogenesis. The major modes by which liver tumor promotion is accomplished include liver regeneration, dietary 2-acetylaminofluorene, choline-devoid diet, and phenobarbital administration, all of which will be discussed below.

a.) 2-Acetylaminofluorene

A resistance to the cytotoxicity of several hepatocarcinogens was noted in cell populations of putative premalignant hepatocytes (Laws, 1959; Kitagawa, 1971; Solt and Farber, 1976; Farber, 1980; Farber et al, 1976). Furthermore, normal hepatocytes of rats administered 2-acetylaminofluorene in their diets were found not to respond significantly to the proliferative stimulus provided by partial hepatectomy in contrast to the preneoplastic cells which were observed to proliferate vigorously. This difference in proliferative response was soon exploited as dietary 2-acetylaminofluorene was observed to apparently endow the "resistant" hepatocytes with considerable
proliferative advantage. In such a way, the premalignant hepatocytes were encouraged to grow and tumors could be obtained at a relatively early time point (Emmelot and Scherer, 1980). Together with partial hepatectomy, this selection process has become widely used in many laboratories for "promoting" the emergence of carcinogen-induced enzyme-altered foci (Solt et al, 1977; Tsuda et al, 1980; Emmelot and Scherer, 1980; Farber, 1980). A considerable disadvantage to using this selection procedure, however, is the use of one carcinogen (2-acetylaminofluorene) in the testing of another, not only because it complicates discerning between initiation and promotion, but also because it imposes a certain health risk to those persons involved in the treatment and care of the experimental animals. With this consideration in mind, the feasibility of using an alternate regimen for promoting the appearance and growth of enzyme-altered foci in the studies presented in this thesis was investigated.

b.) Choline-Deficient Diet

More than three decades ago, the induction of hepatic tumors in rats was shown to be enhanced by the feeding of a choline-deficient diet (Copeland and Salman, 1946). Furthermore, that a diet deficient in lipotropes could
affect the process of chemical carcinogenesis was demonstrated over ten years ago with an observed enhancement of the tumorigenic effects seen in rats which were administered the potent liver carcinogen aflatoxin B₁ (Rogers and Newberne, 1969; Rogers and Newberne, 1971). Since these early reports, lipotrope-deficient diets have also been shown to augment the hepatocarcinogenic effects of numerous other agents as well, including N-nitrosodiethylamine, 2-acetylaminofluorene, N-nitrosodibutylamine, ethionine, and azaserine (Rogers, 1975; Rogers and Newberne, 1975; Shinozuka et al, 1978a; Shinozuka et al, 1978b; Lombardi and Shinozuka, 1979). Similarly, an enhancing effect of a choline-devoid diet on the emergence of gamma-glutamyl transpeptidase (GGT)-positive foci in response to carcinogen treatment has been demonstrated (Shinozuka et al, 1978c; Sells et al, 1979; Shinozuka et al, 1979; Shinozuka and Lombardi, 1980). This enzyme-altered foci enhancing effect of a choline-devoid diet has also been shown by Shinozuka and Lombardi (1980) to act synergistically with the promoting effects of phenobarbital, an agent which will be further discussed below.

In order to avoid the introduction of additional complicating features within the experimental protocol used in the present studies, however, a choline-deficient diet was not used, either with or without phenobarbital
promotion.

c.) Liver Regeneration

Partial hepatectomy performed on rats which were fed 2-acetylaminofluorene (Laws, 1959) or dimethylaminoazobenzene (Glinos, 1951) was found to reduce the latency of tumor appearance. Also, carbon tetrachloride-induced liver regeneration was found to increase tumor yield when given subsequent to a single dose of X-irradiation or neutron bombardment (Cole and Nowell, 1965; Curtis et al, 1968). Similarly, partial hepatectomy performed after a dose of radiation was found to marginally increase the yield of hepatomas in rats (Haran-Ghera et al, 1962). Repeated doses of $\text{CCl}_4$ administered after a single dose of N-nitroso diethylamine were also shown to greatly enhance tumor formation in mice (Pound and McGuire, 1978a) and repeated partial hepatectomy was demonstrated to increase the carcinogenic response in the liver of rats which had received prior administration of N-nitroso diethylamine (Pound and McGuire, 1978b). The collective results of these experiments are illustrative of the promoting ability of liver regeneration following administration of a carcinogen, and suggest the possibility of using this promotional activity to enhance the sensitivity of the rat
hepatic enzyme-altered foci bioassay.

Several laboratories currently perform partial hepatectomy or administer a hepatonecrotic dose of carbon tetrachloride in conjunction with the administration of 2-acetylaminofluorene as a promotional regimen within their experimental protocols (Solt and Farber, 1976; Solt et al, 1977; Cayama et al, 1978; Tsuda et al, 1980). The induced liver regeneration is believed to afford a generalized growth stimulus to the liver, while the dietary 2-acetylaminofluorene is thought to provide an inhibitory effect on cellular proliferation in normal hepatocytes but not in those putative preneoplastic liver cells within the enzyme-altered foci. In such a way, the preneoplastic cells are endowed with a substantial growth advantage over the normal liver parenchyma and thusly they become more readily detectable in a shorter period of time with such treatment.

However, because a single partial hepatectomy performed during the promotional phase following carcinogen administration afforded only a limited promoting stimulus to liver, and because repeated partial hepatectomy poses substantial technical difficulty in addition to requiring considerable expenditures of time, neither of these modalities of liver tumor promotion were included in the design of the experiments presented here. The administration of hepatonecrotic
doses of $\text{CCl}_4$ were also avoided as several proposed test compounds were themselves halogenated hydrocarbons. For these reasons, another method of liver tumor promotion was considered.

d.) Phenobarbital

The effects of the drug phenobarbital on hepatic microsomal enzymes and on the mitotic activity of hepatocytes led investigators to study the effects of dietary phenobarbital on hepatocarcinogenesis in rats which were administered the liver carcinogen 2-acetylaminofluorene (Peraino et al, 1971). Phenobarbital, when fed simultaneously with 2-acetylaminofluorene, exerted a protective effect which was in accord with results of earlier studies with the carcinogens 4-dimethylaminoazo-benzene (Ishidate et al, 1967) and N-nitrosodiethylamine (Kunz et al, 1969). A stimulation of the detoxification of the carcinogen was the apparent mechanism by which phenobarbital reduced 2-acetylaminofluorene-induced tumorigenesis.

In contrast to this protective effect of concomitant administration of phenobarbital and 2-acetylaminofluorene was the dramatic enhancement of the incidence of hepatomas in rats which received 2-acetylaminofluorene in their diet and were subsequently fed a diet containing phenobarbital
Phenobarbital, in and of itself, has not been shown to be a genotoxic agent and was not determined to be mutagenic in the Ames Salmonella mutagenesis test, the sperm abnormality test, or the micronucleus assay (Kunz et al, 1969; Peraino et al, 1975; McCann et al, 1975; Heddle and Bruce, 1977). Thus phenobarbital has become the prototype of liver tumor promoting agents and has likewise been adopted into the protocols of this and other laboratories for the promotion of enzyme-altered foci (Pitot, 1977; Pugh and Goldfarb, 1978; Ford and Pereira, 1980; Emmelot and Scherer, 1980; Couri et al, 1982).

C. SELECTION OF CHEMICALS FOR STUDY

The intent of this research effort was to discern, if possible, the genotoxic (initiating) from the epigenetic (promoting) activities of various carcinogens using the enzyme-altered foci bioassay. For these studies, then, three compounds of pharmacological and/or toxicological interest were chosen, namely methapyrilene, 1,2-dibromoethane, and trichloroethylene, all of which have been reported to be carcinogenic in conventional lifespan animal studies.
1. Methapyrilene

In 1979, the FDA announced a voluntary recall of those drug products containing the $H_1$-histamine antagonist methapyrilene which had recently been shown to produce liver tumors when fed to rats (Bohardt, 1979; Lijinsky and Taylor, 1977; Lijinsky et al, 1980). Although methapyrilene was shown to bind to nucleic acids and protein when administered to rats, a causal relationship between the macromolecular binding of this compound and its tumorigenic effects has not been established (Underwood and Lijinsky, 1980; Lijinsky and Muschik, 1982). Autoradiographic subcellular binding studies showed that methapyrilene bound principally to the mitochondria of hepatocytes with only minor localization over the nuclei of the cells (Reznik-Schuller and Lijinsky, 1981). Furthermore, methapyrilene was not found to be mutagenic when tested in the Ames Salmonella/mammalian-microsome mutagenicity assay, with or without microsomal activation (Andrews et al, 1980). Neither was methapyrilene active in morphologically transforming hamster embryo cells in culture (Lijinsky et al, 1980; Pienta et al, 1977). From these reports it became apparent that methapyrilene would be an excellent candidate for further investigation into its mechanism of carcinogenic action, with special emphasis placed upon elucidating potential epigenetic mechanisms.
2. 1,2-Dibromoethane

The halogenated hydrocarbon 1,2-dibromoethane (ethylene dibromide) is a widely used compound with U.S. production figures for 1977 of nearly 250 million pounds (NTP, 1982). The principal uses of 1,2-dibromoethane are as a lead scavenger in gasoline and as a pesticidal fumigant for soil and grain, although the compound is also used as an industrial solvent, a chemical intermediate in the syntheses of dyes, pharmaceuticals, and other organic compounds, and in some types of fire extinguishers (IARC, 1977; NTP, 1982). The toxic effects of 1,2-dibromoethane have been reported to include central nervous system depression, pulmonary irritation, hepatic and renal injury in rats, decreased egg laying in hens, and degeneration of spermatozoa in bulls (Rowe et al, 1952; Bondi et al, 1955; Amir and Volcani, 1965; Nachtomi et al, 1968; Broda et al, 1976; NTP, 1982). The halogenated hydrocarbon was also shown to be mutagenic (Brem et al, 1974; Vogel and Chandler, 1974; Sparrow et al, 1974; Buselmaier et al, 1973; Fishbein, 1976; Rannug, 1980) and carcinogenic (Olson et al, 1973; Powers et al, 1975; IARC, 1977; Van Duuren et al, 1979; Plotnick et al, 1979; Stinson et al, 1981; Wong et al, 1982).
1,2-Dibromoethane was also shown to exert a marked mitogenic effect on rat hepatocytes, causing considerable proliferation within the liver parenchyma of rats administered even a single dose of the compound (Nachtomi and Farber, 1978). The documented genotoxic effects of 1,2-dibromoethane, coupled with its proliferative stimulation of hepatocytes were compelling reasons to investigate the initiating and promoting effects of the halogenated hydrocarbon in the rat hepatic enzyme-altered foci bioassay.

3. Trichloroethylene

The extensively used chlorinated hydrocarbon trichloroethylene has been reported to be carcinogenic in mice but not in rats (DHEW, 1976). However, because this NCI carcinogenesis study used the technical grade of the solvent, considerable debate has arisen over the actual declaration of trichloroethylene as a carcinogen. The technical grade trichloroethylene was shown to contain 0.65% impurities among which were the potent mutagens epichlorhydrin and 1,2-epoxibutane (Henschler et al, 1977). In fact, the carcinogenic effects observed with the technical grade of the chlorinated hydrocarbon have been suggested to be predominantly, if not entirely, due to the epoxides which are routinely added as preservatives to some
brands of trichloroethylene (Henschler et al, 1977).

Commercial grade trichloroethylene which was stabilized with epichlorhydrin and butylene oxide was found to be slightly mutagenic in an Ames mutagenicity testing system using an _E. coli_ K 12 tester strain and phenobarbital-induced mouse microsomes (Greim et al, 1975; Henschler et al, 1977). Trichloroethylene stabilized with triethylamine and tested at the 1% level was not observed to cause mutations in a _S. typhimurium_ tester strain (Jones and Hathway, 1978; Hathway, 1980).

Several studies suggest that trichloroethylene is metabolized via an epoxide intermediate (Bonse and Henschler, 1976; Leibman and Ortiz, 1977; Uehleke et al, 1977; Hathway, 1980) which may account for the sometimes observed mutagenic activity of the compound. Furthermore, in the mouse, the rearrangement of this postulated epoxide metabolite _in vivo_ is thought to yield dichloroacetyl chloride to a measurable extent in addition to the chloral which is produced in other mammals. This reactive dichloroacetyl chloride would be expected to react with DNA nucleotides and may be responsible for the tumorigenic effects seen in mice but not in rats (Hathway, 1980). Although this is an attractive theory, it is currently without sufficient experimental evidence to be generally accepted.
Purified trichloroethylene gave consistently negative results in several tests of carcinogenicity using Ha:ICR Swiss mice (Van Duuren et al., 1979). In this study, trichloroethylene was investigated for potential initiating activity in mouse skin when the compound was applied singly and promoted with phorbol myristate acetate (PMA; TPA; a known skin tumor promoter) or when applied repeatedly to the backs of the mice. The halogenated hydrocarbon was also administered to other groups of mice by intragastric feeding and by subcutaneous injection. In all instances, no significant tumorigenic effects of trichloroethylene were observed (Van Duuren et al., 1979). Furthermore, trichloroethylene was not found to induce foci of nucleoside-5'-triphosphatase-deficient hepatocytes (enzyme-altered foci) when newborn rats were exposed in inhalational chambers for ten weeks to 2000 ppm of the chlorinated hydrocarbon (Laib et al., 1979).

The obvious conclusion from the above cited studies is that trichloroethylene is without significant tumor initiating activity. It was of interest, therefore, to investigate the tumor promoting activity of trichloroethylene to determine if the earlier reported positive results in the NCI carcinogenesis bioassay might not be explained by such a promoting property of the compound. By using the short-term in vivo rat hepatic enzyme-
altered foci bioassay, both the initiating and promoting activities of trichloroethylene were evaluated as part of this thesis effort.
STATEMENT OF THE PROBLEM

With the exquisitely sensitive analytical methods currently available (e.g. gas chromatography/mass spectrometry) it is becoming readily apparent that a "zero" level of exposure to most environmental contaminants is simply not possible. For this reason regulatory agencies need to define and adopt a sufficiently low level of exposure such that an acceptable risk-to-benefit ratio may be achieved. In the area of chemical carcinogenesis, the establishment of an acceptable level of carcinogen exposure becomes an extremely difficult task indeed. To further complicate the decision process, risk assessment calculations should logically be linked to the molecular mechanism(s) by which an agent exerts its tumorigenic effect. That is, for irreversible, heritable alterations in the genetic constitution of a cell, obviously the acceptable limits of exposure to the offending compound would be appreciably less than those limits which might be permissible for a chemical associated with only reversible, non-heritable effects. In this light, it becomes essential to ascertain the mechanism of a chemical's cancer-producing effects so as to facilitate
a reasonable risk assessment for that compound. The basic difficulty is, however, that to date precious little detail has been paid to determining the exact mechanism of cancer induction by environmental chemicals. Particularly deficient in this regard is the lifetime carcinogenesis bioassay adopted by the National Cancer Institute. For this reason, a suitable method for detecting and discriminating between the initiating (genotoxic) and promoting (epigenetic) activities of chemical agents is essential. The rat hepatic enzyme-altered foci bioassay may well provide such a system of detection and discrimination. In order to evaluate the utility of this system as such, a series of three chemicals which had been previously designated as carcinogens was tested in the enzyme-altered foci bioassay for any component initiating and promoting activity. These compounds included methapyrilene, 1,2-dibromoethane, and trichloroethylene. The results of these experiments were carefully interpreted and considered with regard to the agents' mechanisms of carcinogenic actions and with respect to evaluating the enzyme-altered foci bioassay as a method for assessing both initiating and promoting activities.
METHODS

A. ANIMALS

Male Sprague-Dawley rats (130±10g) were obtained from Harlan Sprague-Dawley (Indianapolis, IN) and were acclimated to the animal housing facilities for three to seven days prior to treatment. The Ohio State University Medical School vivarium is an American Association for Accreditation of Laboratory Animal Care (AAALAC)-approved facility and provides climate-controlled conditions of a twelve hour light cycle (6 AM - 6 PM), room temperature of 22-23°C, and relative humidity of 45%. All animals received Purina Lab Chow and tap water ad libitum except where specified differently in the experimental protocols.

B. PARTIAL HEPATECTOMY

Partial hepatectomy or sham operation was performed between 8 and 10 AM under light ether anesthesia according to the procedure of Higgins and Anderson (1931). Partial
hepatectomy consisted of secure ligation of the large left lateral lobe and of the smaller median lobe of the liver and surgical excision of those portions of liver five to ten millimeters distal to the ligature. In those animals receiving a sham hepatectomy, the liver was momentarily exteriorized, manipulated, and returned to the abdominal cavity. The peritoneum and abdominal muscles were closed with several sutures of type 000 silk. The incision through the skin was then closed with stainless steel wound clips placed one to three centimeters apart. No special treatment was given to the animals postoperatively and survival of these animals routinely exceeded 90%.

The removal of the left lateral and median lobes of the liver effectively reduced the remaining liver mass to one-third the original mass, thusly constituting a two-thirds hepatectomy. Liver regeneration following this procedure is rapid, the liver regaining nearly 100% of its original mass within only one week. Indeed, this pronounced proliferation has been proposed to account for much of the increased carcinogenic response of the regenerating liver to the actions of carcinogens.
C. HISTOCHEMICAL TECHNIQUES

1.) Tissue Preparation

Upon completion of the experimental treatments, the rats were ether-euthanized, the remaining portions of liver excised, and two-millimeter sections of liver were obtained and embedded in O.C.T. Compound (VWR Scientific Inc., Columbus, OH). The tissue molds were then rapidly frozen by placing them on dry ice. The frozen blocks were stored at -80°C until cryostatic sections were prepared.

2.) Cryostatic Sectioning

The frozen tissue blocks were allowed to warm up to -20°C just prior to cryostatic sectioning. Six-micron sections of the blocks were sliced in the cryostat and mounted on glass microscope slides. Well wrapped in aluminum foil, the mounted sections have been stored in a refrigerator at 0 to 10°C for several weeks with no apparent loss of enzyme (gamma-glutamyl transpeptidase) activity.
3.) Histochemical Staining

The slide-mounted tissue sections were fixed in acetone for two seconds and then air-dried. According to the methods of Rutenburg et al (1969), a freshly prepared solution of dye-substrate was placed onto the tissue sections and incubated at room temperature for thirty minutes. The dye-substrate mixture consisted of 5 mg gamma-glutamyl-4-methoxy-2-naphthylamide mixed to a smooth paste with 50 μl of dimethylsulfoxide. To this, 50 μl of 1.0 N NaOH was added, followed by the addition of 900 μl of distilled water. This mixture was stirred well to a uniform solution to which 16 mg of fast blue BBN in 20 ml of a stock buffer solution was added. The stock buffer consisted of 250 ml of 0.1 M tris buffer (pH 7.4), 700 ml of 0.85% NaCl, and 500 mg of glycylglycine. The combination of the dye-substrate solution with the fast blue BBN dye-coupling solution would yield a fine suspension which was applied to the slides before significant settling occurred (15-20 min.).

Gamma-glutamyl transpeptidase activity liberates 4-methoxy-2-naphthylamine from the dye-substrate. This product is then promptly coupled with the diazonium salt (fast blue BBN) to yield a red-orange insoluble azo dye. (See Figure 1).
FIGURE 1

HISTOCHEMICAL REACTION FOR DETECTING
ENZYME ACTIVITY OF GAMMA-GLUTAMYL TRANSPEPTIDASE
Following incubation, the slides were washed for two minutes in saline and then immersed in a solution of 0.1 M cupric sulfate for two minutes. The cupric cation chelates with the red-orange insoluble dye to yield an even more insoluble and intensely red dye. The slides were rinsed again in saline for two minutes and then counterstained in hematoxylin for thirty seconds. Finally the slides were washed free of excess hematoxylin by several rinses in distilled water. The slides were air-dried and coverslipped using a heated glycerol-gelatin solution which consisted of:

- 40 g gelatin
- 210 ml distilled water
- 250 ml glycerol
- 100 mg thymol

The prepared slides were microscopically examined either immediately following coverslipping or within 48 hours of refrigerated storage as the staining would dissipate with time.

4.) Light Microscopy

Under bright field light microscopy, the stained slides were examined for foci of gamma-glutamyl transpeptidase activity, seen as bright red-orange clusters of hepatocytes.
To ensure certainty and uniformity in declaring foci of liver cells as GGT-positive, a minimum of nine hepatocytes (which were easily counted according to the darker hematoxylin-stained nuclei) was required for scoring as a GGT-positive focus. Furthermore, because bile ducts often showed some enzyme activity, it was important to discern morphologically the biliary epithelial cells from hepatocytes. A representative photomicrograph of a GGT-positive focus is shown at two magnifications in Plates I and II. The area of densely red-staining hepatocytes marking activity of gamma-glutamyl transpeptidase demonstrates an enzyme-altered focus. Notice the virtually complete absence of red staining in the surrounding liver parenchyma. The lack of interfering background staining and the obvious nature of the histochemical detection made the quantitative determination of GGT-positive foci relatively facile even without extensive training in cellular histopathology.

5.) Calculation of Foci Per Unit Area

Each tissue section was carefully scored for the number of GGT-positive foci. The images of the liver sections were projected under low magnification onto paper and the outlines traced with a planimeter in order to calculate the areas of the liver sections. Final results were expressed as the number
PLATE I

REPRESENTATIVE PHOTOMICROGRAPH OF A GAMMA-GLUTAMYL TRANSPEPTIDASE POSITIVE FOCUS (mag. 30 X)
PLATE II

REPRESENTATIVE PHOTOMICROGRAPH OF A GAMMA-GLUTAMYL TRANSPEPTIDASE POSITIVE FOCUS (mag. 120 X)
of GGT-positive foci per square centimeter of tissue examined.

D. TREATMENT PROTOCOLS

In each experiment, although the exact treatment protocol differed somewhat, the basic protocol consisted of:

1.) At Day -5 the animals were received and were acclimated for five days prior to surgery.

2.) Partial hepatectomy (or sham operation) was performed on Day 0.

3.) On Day 1, the animals were given the oral dose of initiator, either N-nitrosodiethylamine or the appropriate test compound.

4.) The animals were allowed to more fully recover from surgery until Day 5, at which time they began to receive the promoting regimen of phenobarbital or test compound.

5.) After eight weeks of promotion, the rats were sacrificed and the liver sections were prepared as previously described.

A summary chart of this general treatment protocol is shown in Figure 2.
FIGURE 2

GENERAL EXPERIMENTAL PROTOCOL: ENZYME-ALTERED FOCI BIOASSAY

Groups of male Sprague-Dawley rats (130 ± 10 g) are received on Day -5, and acclimated to the animal quarters for 5 days. On day 0 the rats are subjected to a two-thirds partial hepatectomy or sham operation. Twenty-four hours following surgery, the animals are gavaged with the initiating agent (N-nitrosodiethylamine or test compound). Three days following initiation, the rats are started on the promoting regimen (phenobarbital or test compound) and are continued for eight weeks, after which time the animals are sacrificed, the livers excised, and cryostatic sections of liver prepared and histochemically stained for gamma-glutamyl transpeptidase activity.
GENERAL EXPERIMENTAL PROTOCOL

ENZYME-ALTERED FOCI BIOASSAY

FIGURE 2

GENERAL EXPERIMENTAL PROTOCOL: ENZYME-ALTERED FOCI BIOASSAY
1.) Methapyrilene

In this experiment seven groups of animals were treated. Group 1, the positive control group, received two-thirds partial hepatectomy followed 24 hours later with 30 mg/kg of the initiating carcinogen N-nitrosodiethylamine by oral gavage. Three days subsequently, the animals were started on 500 ppm of the liver tumor promoter phenobarbital administered in their drinking water. After eight weeks of promotion, the animals were sacrificed and the liver sections prepared as described earlier.

Group 2 represented the methapyrilene promotion group which received the identical treatment as did Group 1 except that methapyrilene (200 ppm) replaced the promoter phenobarbital in the drinking water.

Group 3 served as the initiator control group and received two-thirds partial hepatectomy, was initiated with distilled water vehicle only, and was subsequently promoted with 200 ppm methapyrilene in drinking water.

The promoter control animals, Group 4, received partial hepatectomy and 30 mg/kg N-nitrosodiethylamine initiation but were promoted only with normal tap water.

Group 5 (sham control) was given a sham operation and 24 hours subsequently was initiated with 30 mg/kg N-nitrosodiethylamine. Promotion was accomplished with 200 ppm methapyrilene in drinking water.
Group 6 was one of the methapyrilene-initiation groups and received partial hepatectomy followed by two doses of 130 mg/kg methapyrilene by oral gavage at 24 and 72 hours postoperatively. Three days following this initiation treatment, the animals were started on a promoting regimen of 500 ppm phenobarbital in drinking water.

The final group in this experiment, Group 7, was the lower-dose methapyrilene-initiation group receiving a single oral dose of 50 mg/kg methapyrilene 24 hours following partial hepatectomy. This group also received phenobarbital promotion.

The experimental treatment protocol for this experiment is given in Table 2.

2. 1,2-Dibromoethane

To thoroughly investigate the initiating effects of the mutagen and carcinogen 1,2-dibromoethane, several different initiation groups were treated. Two groups of rats were initiated prior to partial hepatectomy to study the temporal relationship of 1,2-dibromoethane and partial hepatectomy. Groups 1 and 2 received four consecutive daily oral doses of 60 mg/kg 1,2-dibromoethane in corn oil, and on the day following the last dose, each animal was subjected to surgical excision of two-thirds of its liver. Group 1 then received phenobarbital promotion (500 ppm phenobarbital
**TABLE 2**

**EXPERIMENTAL PROTOCOL: INITIATING AND PROMOTING EFFECTS OF METHAPYRILENE ON THE FORMATION OF ENZYME-ALTED FOCI IN RAT LIVER**

Male Sprague-Dawley rats (130 ± 10 g) were received, acclimated for five days in the vivarium, and subjected to either partial hepatectomy (P.H.) or sham hepatectomy. Twenty-four hours following surgery, the animals were administered the initiator (NDEA - N-nitrosodiethylamine, 30 mg/kg; MPY - methapyrilene hydrochloride) in distilled water by oral gavage. Three days subsequently, the rats were begun on a promoting regimen of 500 ppm phenobarbital (ØB) or 200 ppm methapyrilene hydrochloride (MPY) in drinking water and maintained on such solutions for eight weeks, after which time they were sacrificed.

<table>
<thead>
<tr>
<th>GROUP NO.</th>
<th>DESCRIPTION</th>
<th>N</th>
<th>P.H.</th>
<th>INITIATOR</th>
<th>PROMOTER</th>
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<tbody>
<tr>
<td>1</td>
<td>POSITIVE CONTROL</td>
<td>9</td>
<td>+</td>
<td>NDEA</td>
<td>ØB</td>
</tr>
<tr>
<td>2</td>
<td>MPY PROMOTER</td>
<td>10</td>
<td>+</td>
<td>NDEA</td>
<td>MPY</td>
</tr>
<tr>
<td>3</td>
<td>INITIATOR CONTROL</td>
<td>10</td>
<td>+</td>
<td>--</td>
<td>MPY</td>
</tr>
<tr>
<td>4</td>
<td>PROMOTER CONTROL</td>
<td>9</td>
<td>+</td>
<td>NDEA</td>
<td>--</td>
</tr>
<tr>
<td>5</td>
<td>SHAM CONTROL</td>
<td>9</td>
<td>--</td>
<td>NDEA</td>
<td>MPY</td>
</tr>
<tr>
<td>6</td>
<td>MPY INITIATOR (2X 130 MG/KG)</td>
<td>3</td>
<td>+</td>
<td>MPY</td>
<td>ØB</td>
</tr>
<tr>
<td>7</td>
<td>MPY INITIATOR (50 MG/KG)</td>
<td>6</td>
<td>+</td>
<td>MPY</td>
<td>ØB</td>
</tr>
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</table>
in drinking water) beginning three days postoperatively, while Group 2 was presented with normal tap water only.

Three other groups of rats, on the other hand, were initiated after having been surgically operated. Groups 3 and 4 received partial hepatectomy and Group 5 sham hepatectomy, and 24 hours subsequently were administered four consecutive daily doses of 60 mg/kg 1,2-dibromoethane. For Groups 3 and 5, promotion with phenobarbital was begun three days following the final initiating dose of the halogenated hydrocarbon, whereas for Group 4, only normal tap water was given during the promoting phase of the experiment.

Two more groups of rats (Groups 6 and 7) were initiated with a single high dose of 120 mg/kg 1,2-dibromoethane 24 hours subsequent to partial hepatectomy. Group 6 received phenobarbital promotion while Group 7 was given tap water to drink.

The positive control group (Group 8) was given partial hepatectomy, 30 mg/kg N-nitrosodiethylamine, and phenobarbital promotion.

Groups 9 and 10 were partially hepatectomized and 24 hours later, initiated with 30 mg/kg N-nitrosodiethylamine, and were subsequently promoted with oral doses of 10 mg/kg (Group 9) or 30 mg/kg (Group 10) 1,2-dibromoethane in corn oil five times weekly for eight weeks.
Non-initiated animals which had been gavaged with a single dose of water vehicle 24 hours following partial hepatectomy, were also given the promoting regimen of 1,2-dibromoethane of either 10 mg/kg (Group 11) or 30 mg/kg (Group 12) five times weekly.

A sham hepatectomy group, Group 13, received N-nitroso-diethylamine (30 mg/kg) and was promoted with 30 mg/kg 1,2-dibromoethane five times weekly.

Groups 14 and 15 were the promoter control groups and received partial hepatectomy and N-nitrosodiethylamine initiation (30 mg/kg). Three days subsequent to initiation, Group 14 received oral doses of corn oil vehicle five times weekly and Group 15 received normal tap water to drink.

The experimental protocol for the 1,2-dibromoethane initiation and promotion groups is summarized in Figure 3.

3.) Trichloroethylene

   a.) Initiation

   Group 1 of the trichloroethylene initiation experiment was the sham hepatectomy control group which was given sham hepatectomy and 24 hours later was initiated with 30 mg/kg N-nitrosodiethylamine by oral gavage. Three days subsequently, the animals were given a promoting regimen of 500
FIGURE 3

EXPERIMENTAL PROTOCOL: INITIATING AND PROMOTING EFFECTS OF 1,2-DIBROMOETHANE ON THE EMERGENCE OF ENZYME-ALTERED FOCI IN RAT LIVER
EXPERIMENTAL PROTOCOL: INITIATING AND PROMOTING EFFECTS OF 1,2-DIBROMOETHANE ON THE EMERGENCE OF ENZYME-ALTERED FOCI IN RAT LIVER
ppm phenobarbital in drinking water.

Group 2 was the promoter control group of rats which in addition to partial hepatectomy and N-nitrosodiethylamine initiation, received normal tap water during the promotional phase of the experiment.

The positive control group (Group 3) was partially hepatectomized, initiated with N-nitrosodiethylamine, and promoted with phenobarbital.

Group 4, the initiator vehicle control group, received partial hepatectomy followed 24 hours later by a single oral dose of corn oil (5.0 ml/kg). After three days, these rats were given phenobarbital in their drinking water.

The trichloroethylene initiation group, Group 5, was administered 490 mg/kg trichloroethylene (approximating 1/10 the LD$_{50}$) in corn oil 24 hours following partial hepatectomy. This group was promoted with phenobarbital beginning three days after initiation.

A summary of the experimental protocol for this trichloroethylene-initiation experiment appears in Table 3.

b.) Promotion

The experiment designed to examine the promoting effects of trichloroethylene included five additional groups of rats. Group 6, the sham control group, was subjected to
**TABLE 3**

**EXPERIMENTAL PROTOCOL: INITIATING EFFECTS OF TRICHLOROETHYLENE ON THE FORMATION OF ENZYME-ALTERED FOCI IN RAT LIVER**

Groups of male Sprague-Dawley rats received either two-thirds partial hepatectomy (P.H.) or sham operation as indicated. Twenty-four hours later the nominal initiator was administered by oral gavage (NDEA - N-nitrosodiethylamine, 30 mg/kg in distilled water; TCE - trichloroethylene, 490 mg/kg in corn oil). The initiator control group (Group 4) was given corn oil vehicle alone as the initiating agent. The promoting regimen was started three days following initiation and was continued for eight weeks (ØB - phenobarbital, 500 ppm in drinking water). Group 2, the promoter control group received normal tap water to drink.

<table>
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<tr>
<th>GROUP NO.</th>
<th>DESCRIPTION</th>
<th>N</th>
<th>P.H.</th>
<th>INITIATOR</th>
<th>PROMOTER</th>
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<tr>
<td>1</td>
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<td>NDEA</td>
<td>ØB</td>
</tr>
<tr>
<td>2</td>
<td>PROMOTER CONTROL</td>
<td>4</td>
<td>+</td>
<td>NDEA</td>
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<td>POSITIVE CONTROL</td>
<td>6</td>
<td>+</td>
<td>NDEA</td>
<td>ØB</td>
</tr>
<tr>
<td>4</td>
<td>INITIATOR CONTROL</td>
<td>5</td>
<td>+</td>
<td>--</td>
<td>ØB</td>
</tr>
<tr>
<td>5</td>
<td>TCE INITIATOR</td>
<td>5</td>
<td>+</td>
<td>TCE</td>
<td>ØB</td>
</tr>
<tr>
<td></td>
<td>(490 MG/KG)</td>
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sham hepatectomy, initiated with 30 mg/kg N-nitrosodiethylamine, and promoted with 200 mg trichloroethylene in 0.5 ml of corn oil five times weekly for eight weeks. This trichloroethylene regimen approximated the maximum tolerated dosing which was used in the NCI carcinogenesis bioassay.

Group 7, the initiator control group, was given an oral dose of distilled water vehicle 24 hours after partial hepatectomy. Subsequently, this group was administered 200 mg trichloroethylene five times weekly by oral gavage.

The promoter control group (Group 8) was given two-thirds partial hepatectomy, a single initiating dose of N-nitrosodiethylamine, and 0.5 ml corn oil vehicle five times weekly for eight weeks.

Group 9, the trichloroethylene promoter group, was partially hepatectomized, initiated with 30 mg/kg N-nitrosodiethylamine, and promoted with 200 mg trichloroethylene in corn oil five times weekly.

Another positive control group (Group 10) was given partial hepatectomy, N-nitrosodiethylamine initiation, and phenobarbital promotion.

The experimental protocol for the trichloroethylene promotion experiment is given in Table 4.
Male Sprague-Dawley rats received partial hepatectomy (P.H.) or sham operation as indicated. Twenty-four hours later they were gavaged with 30 mg/kg N-nitrosodiethylamine (NDEA) in distilled water. Group 7, the initiator control group was given distilled water vehicle alone. Three days subsequently, the animals were begun on their respective promoting regimens and continued for eight weeks. Trichloroethylene (TCE) was dissolved in corn oil and administered by oral gavage five times weekly. Group 8, the promoter control group, received five times weekly doses of corn oil vehicle alone. The positive control group (Group 10) was administered 500 ppm phenobarbital (0B) in drinking water throughout the eight-week promotional phase.

<table>
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<tr>
<th>GROUP NO.</th>
<th>DESCRIPTION</th>
<th>N</th>
<th>P.H.</th>
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<td>TCE</td>
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<td>7</td>
<td>INITIATOR CONTROL</td>
<td>9</td>
<td>+</td>
<td>--</td>
<td>TCE</td>
</tr>
<tr>
<td>8</td>
<td>PROMOTER CONTROL</td>
<td>9</td>
<td>+</td>
<td>NDEA</td>
<td>--</td>
</tr>
<tr>
<td>9</td>
<td>TCE PROMOTER</td>
<td>8</td>
<td>+</td>
<td>NDEA</td>
<td>TCE</td>
</tr>
<tr>
<td>10</td>
<td>POSITIVE CONTROL</td>
<td>8</td>
<td>+</td>
<td>NDEA</td>
<td>0B</td>
</tr>
</tbody>
</table>
RESULTS

A. INITIATING AND PROMOTING EFFECTS OF METHAPYRILENE

The effects of methapyrilene on the formation of enzyme-altered foci in rat liver are depicted in Figure 4. The positive control group (Group 1) gave results which were consistent with previous experiments conducted in this laboratory and which were comparable to the results of Ford and Pereira (1980). When methapyrilene was substituted for the liver tumor promoter phenobarbital in the animals' drinking water (Group 2), an equal if not greater foci-enhancing effect was seen as compared to the positive controls.

It had been previously reported that intact, non-treated rats and rats which had received only partial hepatectomy or sham operation developed few, if any, enzyme-altered foci (0-0.5 foci/cm²) (Ford and Pereira, 1980; Tsuda et al, 1980; Ogawa et al, 1981; Pereira, 1982; Herren et al, 1982). Additionally, only a low level of foci were produced when a low dose of N-nitrosodiethylamine was administered without subsequent promotion, even if partial hepatectomy was
FIGURE 4

INITIATING AND PROMOTING EFFECTS OF METHAPYRILENE

Animals received the treatment regimen as outlined in Table 2. Histochemical staining of cryostatic sections of liver revealed areas of GGT activity indicating enzyme-altered foci. Results are expressed as the mean number of foci (± SEM) per square centimeter of liver tissue examined.
<table>
<thead>
<tr>
<th>GROUP</th>
<th>DESCRIPTION</th>
<th>GGT- Positive Foci cm^{-2} (Mean ± S.E.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Positive Control</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Methapyrilene Promoter</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Initiator Control</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Promoter Control</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Sham Control</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Methapyrilene Initiator 2 x 130mg/kg</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Methapyrilene Initiator 50mg/kg</td>
<td></td>
</tr>
</tbody>
</table>

FIGURE 4
INITIATING AND PROMOTING EFFECTS OF METHAPYRILENE
performed 24 hours prior to initiation (Scherer and Emmelot, 1975; Pereira, 1982). The results from the experiment presented here are consistent with these earlier reports in that the initiator control group (Group 3) which received only distilled water as the initiating agent but which was promoted with 200 ppm methapyrilene in drinking water showed only a low level of occurrence of GGT-positive foci. Similarly, the promoter control group (Group 4) which was given an initiating dose of N-nitrosodiethylamine 24 hours following partial hepatectomy but which did not receive a promoting regimen (they received only normal tap water to drink) subsequent to initiation, demonstrated only a low level of enzyme-altered foci as well.

Curiously, the sham hepatectomy treatment group (Group 5) which was initiated with N-nitrosodiethylamine and promoted with methapyrilene, gave a dramatic response in the enzyme-altered foci bioassay. Apparently, according to these results, partial hepatectomy is not an essential feature for the induction of GGT-positive foci when promotion is accomplished with methapyrilene.

The results obtained when methapyrilene was tested as the initiator were decidedly negative. The high dose of 130 mg/kg administered twice within a three day period as well as the single dose of 50 mg/kg methapyrilene administered 24 hours following partial hepatectomy and promoted with
phenobarbital did not result in an increase in the formation of GGT-positive foci when compared to background levels. Indeed, when these observed low levels of occurrence are compared to the initiating effects of N-nitrosodiethylamine, the lack of significant initiating activity of methapyrilene is most apparent. It should be reiterated, however, that methapyrilene did produce significant numbers of enzyme-altered foci when administered as a promoter (See Figure 4, Group 2) indicating that the promoting activity of this compound is equal to or greater than that obtained with phenobarbital.

B. 1,2-DIBROMOETHANE EFFECTS ON THE INITIATION AND PROMOTION OF ENZYME-ALTERED FOCI

The results of the 1,2-dibromoethane initiation/promotion studies are summarized in Figure 5.

1. Initiation

Of the groups of animals in which 1,2-dibromoethane was tested for its initiating activity (Groups 1-7), none showed a significant induction of enzyme-altered foci. Whether given before (Groups 1 and 2) or after (Groups 3 and 4) partial hepatectomy, four consecutive daily doses of 60 mg/kg 1,2-dibromoethane did not cause a significant number of
FIGURE 5

INITIATING AND PROMOTING EFFECTS OF 1,2-DIBROMOETHANE

Rats were administered their respective group treatments as outlined in Figure 3. Histochemical staining of cryostatic sections of liver revealed areas of GGT activity indicating enzyme-altered foci. Results are expressed as the mean number of foci (± SEM) per square centimeter of liver tissue examined.
FIGURE 5

INITIATING AND PROMOTING EFFECTS OF 1,2-DIBROMOETHANE
GGT-positive foci to form either with (Groups 1 and 3) or without (Groups 2 and 4) phenobarbital promotion.

Because of the lack of response in the groups discussed above, it was not surprising that the sham hepatectomy group (Group 5) which was given four consecutive daily doses of 60 mg/kg 1,2-dibromoethane following sham hepatectomy and which was subsequently promoted with 500 ppm phenobarbital in drinking water likewise did not demonstrate significant numbers of enzyme-altered foci.

Furthermore, a single oral dose of 120 mg/kg 1,2-dibromoethane administered 24 hours following partial hepatectomy with (Group 6) or without (Group 7) phenobarbital promotion, resulted in similarly negative results.

The positive control group (Group 8) on the other hand, demonstrated a substantially increased number of GGT-positive foci, averaging 4.7 foci/cm$^2$ of liver tissue examined. This level of response is well within the expected range of formation of enzyme-altered foci for this positive control experimental protocol.

Collectively, these results suggest that the halogenated hydrocarbon 1,2-dibromoethane at doses up to 120 mg/kg was without significant initiating activity in rat liver. These results are somewhat unexpected as 1,2-dibromoethane has been reported to be both mutagenic (Brem et al, 1974; Vogel and Chandler, 1974; Sparrow et al, 1974; Buselmaier et al, 1973;
Fishbein, 1976; Rannug, 1980) and carcinogenic (Olson et al, 1973; Powers et al, 1975; IARC, 1977; Van Duuren et al, 1979; Plotnick et al, 1979; Stinson et al, 1981; Wong et al, 1982). Nevertheless, 1,2-dibromoethane tested as an initiator in rat liver gave consistently negative results in this experiment using the enzyme-altered foci bioassay as an indicator of hepatocarcinogenesis.

2. Promotion

As summarized in Figure 5, (Groups 9 and 10), 1,2-dibromoethane was seen to possess considerable promoting activity. When administered to rats which had previously received partial hepatectomy and N-nitrosodiethylamine initiation, 1,2-dibromoethane in repeated oral doses of 10 mg/kg (Group 9) or 30 mg/kg (Group 10) significantly enhanced the formation of GGT-positive foci above their respective control levels (Groups 11 and 12). The lack of difference between the 10 and 30 mg/kg dosages is an interesting finding and suggests that a maximal promoting effect of 1,2-dibromoethane may have been reached in this dosage range.

As would be expected, the group of rats which received N-nitrosodiethylamine initiation following sham hepatectomy and which was promoted with 30 mg/kg 1,2-dibromoethane (Group 13) demonstrated only a low background level of GGT-positive
foci formation.

In the promoter control animals (Groups 14 and 15) which were partially hepatectomized and subsequently initiated with 30 mg/kg N-nitrosodiethylamine, vehicle promotion with corn oil (Group 14) or with normal tap water (Group 15) resulted in only about one GGT-positive focus per square centimeter of liver tissue examined. These low incidences of enzyme-altered foci are consistent with the results of other experiments from this laboratory (Couri et al, 1982) and from other laboratories (Pitot et al, 1978a; Sells et al, 1979; Pereira, 1982; Herren et al, 1982).

These results show that 1,2-dibromoethane when administered to rats having previously received partial hepatectomy and N-nitrosodiethylamine initiation can markedly enhance the formation of GGT-positive foci in rat liver. This enhancing effect of 1,2-dibromoethane on the development of nitrosamine-induced hepatocytic foci is suggestive of an appreciable tumor promoting activity of the halogenated hydrocarbon. These promoting effects are especially interesting in light of the apparent lack of initiating activity of 1,2-dibromoethane in rat liver.
C. INITIATING AND PROMOTING EFFECTS OF TRICHLOROETHYLENE

1. Initiating Effects of Trichloroethylene

Figure 6 summarizes the results of the enzyme-altered foci bioassay in which the initiating effects of trichloroethylene in rat liver were examined. To assess the initiating effects of the chlorinated hydrocarbon, trichloroethylene was administered by gavage to rats 24 hours following partial hepatectomy (Group 5). Three days subsequently, the animals were given a promoting regimen of 500 ppm phenobarbital in drinking water for eight weeks. The average number of GGT-positive foci per square centimeter of liver tissue examined was 0.23 foci/cm², which does not represent an elevation of foci formation above non-treated animals, and is in sharp contrast to the 6.16 foci/cm² observed in the positive control group of rats (Group 3) which received 30 mg/kg N-nitrosodiethylamine by gavage 24 hours subsequent to partial hepatectomy.

Group 1, the sham hepatectomy control group, displayed approximately one GGT-positive focus/cm² and demonstrated the necessity of performing partial hepatectomy in order to maximize the yield of enzyme-altered foci under these experimental conditions.
The treatment protocol for these animals is outlined in Table 3. Histochemical staining of cryostatic sections of liver revealed areas of GGT activity indicating enzyme-altered foci. Results are expressed as the mean number of foci (+ SEM) per square centimeter of liver tissue examined.
## TRICHLOROETHYLENE INITIATION

<table>
<thead>
<tr>
<th>GROUP</th>
<th>DESCRIPTION</th>
<th>GGT-POSITIVE FOCI CM(^{-2}) (MEAN ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>SHAM CONTROL</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>PROMOTER CONTROL</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>POSITIVE CONTROL</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>INITIATOR CONTROL</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>TCE INITIATOR (490 MG/KG)</td>
<td></td>
</tr>
</tbody>
</table>

**FIGURE 6**

TRICHLOROETHYLENE INITIATION
Similarly, the crucial role of phenobarbital promotion is exemplified in the results of the promoter control treatment (Group 2) in which partially hepatectomized rats, initiated with N-nitrosodiethylamine but not promoted with phenobarbital, resulted in 1.92 foci/cm$^2$. This number is somewhat higher than has been reported by others (Sells et al, 1979; Pereira, 1982) but the small number of animals (n=4) used in this group may account for a certain degree of experimental error associated with these results. Regardless of this potentially inflated number, phenobarbital was still seen to enhance foci formation three-fold (6.16 foci/cm$^2$; Group 3).

Serving as an initiator control group, Group 4 received only gavage vehicle (corn oil) for the initiating treatment, and yielded 0.63 foci/cm$^2$. The importance of an initiating agent in the formation of enzyme-altered foci is demonstrated by the very low number of GGT-positive foci observed in this treatment group.

Even with the inclusion of partial hepatectomy and phenobarbital promotion in the treatment of the trichloroethylene initiation group (Group 5), no evidence of a substantial initiating effect was seen with the chlorinated hydrocarbon at the 490 mg/kg dose administered.
2. Promoting Effects of Trichloroethylene

The promoting activity of trichloroethylene was examined in rat liver using the hepatic enzyme-altered foci bioassay. The results are illustrated in Figure 7.

Trichloroethylene, when administered as a promoter (five times weekly in doses of 200 mg/rat) to rats which had previously received sham hepatectomy and 24 hours later were administered an initiating dose of 30 mg/kg N-nitrosodiethylamine (Group 6), produced virtually no GGT-positive foci in the livers of any of the animals. These results were considerably lower than for the N-nitrosodiethylamine-initiated sham hepatectomized rats which were promoted with phenobarbital (See Figure 6, Group 1). Likewise, only very low numbers of enzyme-altered foci were observed in the initiator control group (Group 7) which received distilled water 24 hours following partial hepatectomy and which was also subsequently promoted with five times weekly oral doses of 200 mg trichloroethylene.

The promoter control group (Group 8) was partially hepatectomized, initiated with N-nitrosodiethylamine, but received five times weekly doses of vehicle only (corn oil) throughout the eight weeks of the promotional phase of the experiment. These animals exhibited slightly greater, although still only low levels of occurrence of GGT-positive
FIGURE 7

TRICHLOROETHYLENE PROMOTION

The treatment protocol for these animals is outlined in Table 4. Histochemical staining of cryostatic sections of liver revealed areas of GGT activity indicating enzyme-altered foci. Results are expressed as the mean number of foci (+ SEM) per square centimeter of liver tissue examined.
TRICHLOROETHYLENE PROMOTION

<table>
<thead>
<tr>
<th>GROUP</th>
<th>DESCRIPTION</th>
<th>GGT-POSITIVE FOCI CM(^{-2}) (MEAN ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>SHAM CONTROL</td>
<td>0</td>
</tr>
<tr>
<td>7</td>
<td>INITIATOR CONTROL</td>
<td>1</td>
</tr>
<tr>
<td>8</td>
<td>PROMOTER CONTROL</td>
<td>2</td>
</tr>
<tr>
<td>9</td>
<td>TCE PROMOTER</td>
<td>3</td>
</tr>
<tr>
<td>10</td>
<td>POSITIVE CONTROL</td>
<td>4</td>
</tr>
</tbody>
</table>

FIGURE 7

TRICHLOROETHYLENE PROMOTION
foci. In the trichloroethylene promotion group (Group 9) which received partial hepatectomy, N-nitrosodiethylamine initiation, and trichloroethylene promotion, an average of 1.30 foci/cm² was observed. This figure is not statistically different from the vehicle control group (Group 8). Interestingly, however, two of the eight rats receiving the trichloroethylene promotion treatment developed over 2.5 foci/cm², an incidence not usually observed in control treatment groups. Whether this occurrence is suggestive of a weak promoting activity of trichloroethylene or whether, simply, the phenomenon of chance intervened, cannot be conclusively decided based upon these data.

The positive control group (Group 10) was partially hepatectomized, initiated with N-nitrosodiethylamine, and promoted with phenobarbital. This treatment resulted in a considerable response (5.47 foci/cm²) which was consistent with previous positive control results (See Figure 6, Group 3).

Clearly, at a dose approximating 1000 mg/kg administered by oral gavage five times weekly, trichloroethylene has, at best, meager or no promoting activity compared to 500 ppm phenobarbital in drinking water. In any case, these data are not indicative of a substantial effect of trichloroethylene as a tumor promoter in rat liver.
A. INITIATING AND PROMOTING EFFECTS OF METHAPYRILENE

With the unequivocal evidence that chronic administration of methapyrilene to rats produced virtually a one-hundred percent incidence of liver tumors (Lijinsky and Taylor, 1977; Lijinsky et al, 1980), the carcinogenic risk posed to man by this agent becomes an issue of considerable gravity. Methapyrilene, an H₁-histamine antagonist, was an ingredient contained in numerous prescription and over-the-counter drug preparations including cough and cold remedies, allergy relieving products, antipruritic and local anesthetic creams, sleep aids, and sedative products. Although widespread exposure to this agent was discontinued in this country in 1979, the expectancy of tumor development in those individuals having taken the antihistamine is a function of the mode or mechanism by which methapyrilene produces its obvious carcinogenic effects. That is, if the drug were strictly a genotoxic carcinogen, one would reasonably assume that the initiating event (i.e. DNA lesion) has already occurred and, depending upon the latency for tumor induction, a previously exposed individual would
be at risk for developing liver cancer at some time in the future. Fortunately, the current data do not suggest a prominent genotoxic activity for methapyrilene. The anti-histamine was not found to be mutagenic in the Ames Salmonella mutagenicity assay, with or without microsomal activation (Andrews et al, 1980), and methapyrilene was also found to be inactive in morphologically transforming hamster embryo cells in culture (Lijinsky et al, 1980; Pienta et al, 1977).

Clearly, at the doses used in the experiments presented in this dissertation, methapyrilene exhibited no significant initiator activity (Figure 4, Groups 6 and 7).

Alternatively, then, methapyrilene may function as a tumor promoter, enhancing the expression of some already existing genetic aberation. This pre-existing initiating lesion may have been inherited (oncogenes), possibly induced by a viral, chemical, or physical agent, caused by an error in DNA replication, or may have resulted from an as yet obscure cause. If methapyrilene were indeed purely a promoter of hepatocarcinogenesis, a considerably lesser risk may be assumed for those same exposed individuals. The reason for the expected reduction of cancer risk lies with the chronicity of exposure which tumor promoters classically require to produce frank neoplasms. Accordingly, only those individuals who regularly and repeatedly used the methapyrilene-containing products would be at risk for
developing liver tumors subsequent to their exposure to the antihistamine. Furthermore, as tumor promoters tend to exhibit organ specificity (Nakanishi et al, 1982) only a genotoxic lesion in the target organ (in this case liver) would be subjected to the putative promotional activity of methapyrilene.

That a promoting agent can increase the tumor burden in previously initiated animals was demonstrated by the increased liver tumor incidence observed in rats which were given long-term administration of phenobarbital or DDT following initiation with 2-acetylaminofluorene (Peraino et al, 1975). Other researchers have reported that long-term administration of phenobarbital or DDT also increased the tumor burden in rats which had not received prior initiating treatment (Rossi et al, 1977). However, because no compelling evidence has been proffered to support an initiator role for either phenobarbital or DDT, these observed results could represent promotion of spontaneous initiation or unsuspected prior exposure to environmental carcinogens in diet, water, air, or bedding. Furthermore, phenobarbital has been reported to enhance tumor formation in mice which are prone to spontaneous hepatic tumorigenesis and to not produce tumors in mice which are not predisposed to spontaneous tumorigenesis (Peraino et al, 1973; Thorpe and Walker, 1973; Kunz et al, 1969; Peraino et al, 1975). It is
therefore entirely conceivable that the carcinogenic effects of methapyrilene may also be the result of pronounced promotional activity of the compound.

The results from this thesis research would support such a supposition, especially in view of the dramatically enhanced formation of N-nitrosodiethylamine-initiated enzyme-altered foci even in sham hepatectomized animals. Ultimately, if prospective epidemiologic studies are conducted which would investigate methapyrilene exposures and eventual liver tumor incidences, a considerable amount of information could be obtained which should help to elucidate the mechanism of carcinogenic action of the drug. In the interim, much additional information could be gained from animal studies designed to investigate such parameters as reversibility, additivity, dose/response, temporal relationships, and interactions with other initiating and promoting agents. Elucidating these and other aspects of the mechanisms of promoting action of methapyrilene may afford some insight into the biochemical nature of tumor promotion and of carcinogenesis in general. If the biochemical mechanisms of tumor promotion can be revealed, the potential for pharmacologic intervention will be greatly enhanced. In such a way, the risk of developing cancer might be significantly reduced by attenuating or even reversing the promotional status of an otherwise imminent neoplasm. Indeed the
importance of such possibilities cannot be overstated.

One interesting aspect of methapyrilene promotion which deserves further discussion is the marked promoter activity of the drug in sham hepatectomized N-nitrosodiethylamine-initiated rats, (Figure 4, Group 5). This effect is in contrast to the promoting activity of phenobarbital which does require partial hepatectomy to produce significant results in animals initiated with a relatively low dose of N-nitrosodiethylamine (See Figure 6, Group 1 vs. Group 3). It is not known exactly why partial hepatectomy is not an obligatory element in the formation of enzyme-altered islands in rats initiated with N-nitrosodiethylamine and promoted with methapyrilene. Perhaps it is the intensity of the promoting activity of methapyrilene which obviates the necessity of partial hepatectomy. Alternatively, the mechanism of promoter action of methapyrilene may be quite dissimilar to that of phenobarbital. At any rate, further research will be required to define the precise biochemical pathways of tumor promotion, and maybe the actions of methapyrilene as described in these experiments will prove useful in designing future investigations.
B. INITIATING AND PROMOTING EFFECTS OF 1,2-DIBROMOETHANE

The halogenated hydrocarbon 1,2-dibromoethane, which is widely used as a lead scavenger in gasoline and as a pesticide in soil and grain fumigants (IARC, 1977) has been previously shown to be both mutagenic (Brem et al, 1974; Fishbein, 1976; Rannug, 1980) and carcinogenic (Olson et al, 1973; Van Duuren et al, 1979; Stinson et al, 1981; Wong et al, 1982). Other researchers have also reported that 1,2-dibromoethane covalently binds to DNA, RNA, and protein (Nachtomi and Sarma, 1977; Hill et al, 1978; Banerjee et al, 1979). These studies all would appear to support a genotoxic mechanism of carcinogenic action for 1,2-dibromoethane. However, because 1,2-dibromoethane was also shown to provide a prominent proliferative stimulus to rat hepatocytes after oral administration of the compound (Nachtomi and Farber, 1978), it was of interest to investigate any potential promoter activity of the halogenated hydrocarbon in addition to its apparent genotoxicity. For these reasons, the initiating (genotoxic) and promoting (epigenetic) activities of 1,2-dibromoethane were examined using the rat hepatic enzyme-altered foci bioassay to assess the carcinogenic potential of the various treatments.

When 1,2-dibromoethane was tested as an initiator in this system, uniformly negative results were obtained (Figure
5, Groups 1-7). These results were quite unexpected in view of the earlier reports of mutagenicity, carcinogenicity, and macromolecular binding of 1,2-dibromoethane. Even though partial hepatectomy has been shown to greatly enhance initiation of chemical carcinogenesis (Scherer and Emmelot, 1975; Cayama et al, 1978; Hirakawa et al, 1979; Ishikawa et al, 1980; Columbano et al, 1981), this procedure did not increase the formation of GGT-positive foci whether performed before or after the administration of 1,2-dibromoethane as an initiator. The reason for the apparent lack of initiator activity of 1,2-dibromoethane in the enzyme-altered foci bioassay is not yet clear. Possibly, in liver, rapid and efficient DNA-repair mechanisms may undo the initiating or genotoxic events induced by the halogenated hydrocarbon. Alternatively, the specific nature or the chemical identity of the liver DNA adducts formed by the interaction of 1,2-dibromoethane or an activated metabolite may not confer a carcinogenic predisposition to the affected hepatocytes. That is, although covalent binding to cellular DNA may have occurred, the introduction of an initiating lesion within the genome of the cell may not have transpired. Other plausible explanations include the inability of the enzyme-altered foci bioassay to detect the initiating events at the doses used, or the failure of the promoting agent phenobarbital to enhance the expression of the specific genotoxic lesion(s)
produced by 1,2-dibromoethane. In any event, when tested as an initiator in the enzyme-altered foci bioassay, 1,2-dibromoethane was without significant initiating activity.

When 1,2-dibromoethane was administered as a promoter to N-nitrosodiethylamine-initiated rats, on the other hand, a substantial induction of GGT-positive foci was observed. These results might have been reasonably anticipated as tumor promotion has been associated with cellular hyperplasia (Boutwell, 1974; Schulte-Hermann, 1974; Schulte-Hermann et al, 1981) and as 1,2-dibromoethane was previously reported to exert a pronounced mitogenic effect on rat hepatocytes (Nachtomi and Farber, 1978). Collectively then, this evidence suggests that 1,2-dibromoethane is a tumor promoter in rat liver and that this promoting activity of the halogenated hydrocarbon may be related to its mitogenic effects. Furthermore, the promoter activity of 1,2-dibromoethane may contribute significantly to the carcinogenic effects seen upon repeated administration or exposure of animals to this compound.

As was the case for methapyrilene, further studies will be necessary before a final declaration can be made as to the mechanism of carcinogenic action of 1,2-dibromoethane. One very important point which is supported by the data presented in this thesis is that the halogenated hydrocarbon 1,2-dibromoethane, the antihistamine methapyrilene, and most
probably numerous other carcinogens, possess substantial promoter activity which may be related to their observed carcinogenicity. Based upon this possible involvement of tumor promotion in the process of carcinogenesis, it becomes pivotal to the understanding of a compound's mechanism of carcinogenic action to first assess the initiating and/or promoting activities of such a compound. Toward this end, the enzyme-altered foci bioassay may serve as one important method of investigating these two distinctly different activities as was presented for methapyrilene and 1,2-dibromoethane, and as will now be addressed for trichloroethylene.

C. INITIATING AND PROMOTING EFFECTS OF TRICHLOROETHYLENE

Trichloroethylene is widely used as an industrial solvent, primarily in degreasing operations, as well as having been utilized quite extensively in dry cleaning and food processing. The production of trichloroethylene in the U.S. has been declining since 1970, largely due to the legislative restrictions placed on the uses and permissible emissions of the halogenated hydrocarbon (IARC, 1976). Results from the NCI carcinogenesis bioassay of trichloroethylene indicated that repeated oral doses of the compound induced hepatocellular carcinomas in B6C3F1 mice but not in Osborne-Mendel rats (DHEW, 1976). It had been reported that
various promoting agents such as phenobarbital and DDT which are without apparent genotoxicity were observed to produce liver tumors in chronically treated rats which either were (Peraino et al, 1975) or were not (Rossi et al, 1977) previously initiated with a genotoxic carcinogen. Thus, the possibility that trichloroethylene may likewise possess promoting activity seemed an entirely tenable hypothesis. Therefore in an attempt to better understand the disparate results of earlier carcinogenesis testing in mice versus rats, trichloroethylene was investigated for its initiating and promoting activity in the enzyme-altered foci bioassay.

Several reports have appeared in the literature which indicate a slightly positive (Greim et al, 1975; Henschler, 1977) to negative (Jones and Hathway, 1978; Hathway, 1980) mutagenic activity of trichloroethylene when assayed by an Ames mutagenicity test. The discrepancy between these reported results may be due to the various stabilizing agents which are routinely added to preserve the chemical integrity of many commercial grades of trichloroethylene, or due to a difference in tester strains of bacteria used in the Ames tests, or possibly due to some as yet undetermined variable. However, other studies which have investigated the genotoxicity of trichloroethylene have not evidenced a significant initiator role for this halogenated hydrocarbon (Van Duuren et al, 1979; Laib et al, 1979). Similarly, the results
presented in this dissertation do not support a significant initiating activity of trichloroethylene.

When tested as a promoter in N-nitrosodiethylamine-initiated rats, trichloroethylene gave somewhat equivocal results. Two of the eight rats which received partial hepatectomy, N-nitrosodiethylamine initiation, and trichloroethylene promotion demonstrated an increased occurrence of enzyme-altered foci. The other six animals exhibited only a low background incidence of GGT-positive foci, and when the average of the entire group was computed, it was not statistically different from that calculated for the rats receiving corn oil vehicle instead of trichloroethylene as a promoting regimen. Due to the uncertainty of concluding from these results whether or not trichloroethylene possesses promoting activity, further studies will be required to settle the issue. Perhaps if larger groups of animals were used and/or if higher or more prolonged dosing with trichloroethylene were executed, the ambiguity of results might be avoided. Furthermore, by extending the enzyme-altered foci bioassay to the mouse species, additional information might be obtained to help explain the species variation seen with regard to the carcinogenic effects of trichloroethylene in the mouse as compared to the lack of tumorigenic effects in the rat. Ultimately, a more accurate estimate of the carcinogenic risk which trichloroethylene may
pose to man might be derived from such proposed experiments.

D. EVALUATION OF THE ENZYME-ALtered FOCI BIOASSAY AS A VALID METHOD FOR DETECTING THE INITIATING AND PROMOTING ACTIVITIES OF CHEMICALS

Because the lifetime carcinogenesis bioassay is so time-consuming and costly, alternate methods of assessing the carcinogenic potential of chemicals, methods which could reduce both the time and expense of testing, would be preferred. However, any new method to be used for investigating carcinogenesis rightfully needs to be carefully evaluated both for accuracy and sensitivity. The hepatic enzyme-altered foci bioassay is one such alternate method which is currently receiving rather exhaustive examination. Although several modifications have arisen from the general scheme of the bioassay, the basic premise that putative preneoplastic foci of hepatocytes represent an early indication of carcinogenesis remains fundamental to all the enzyme-altered foci bioassay systems, modified or not.

These various systems have been repeatedly reviewed, evaluated, and reassessed with regard to accuracy, sensitivity, relevance, and potential advantages and disadvantages when compared to other carcinogenesis testing systems (Bannasch et al, 1980; Emmelot and Scherer, 1980;
The enzyme-altered foci bioassay has also been proposed as a detection system for initiators and promoters of chemical hepatocarcinogenesis (Ford and Pereira, 1980; Pereira, 1982; Couri et al, 1982; Herren et al, 1982; Sivak, 1982). The initiation/promotion experiments presented in this thesis support the validity of the enzyme-altered foci bioassay as a test system for detecting and discriminating between the initiating and promoting activities of known or suspected carcinogens. Additional studies which also support the validity of the bioassay are briefly summarized in the Appendix. Furthermore, by investigating compounds in a two-stage system of carcinogenesis, a better understanding may be gained as to how some of these compounds (which appear to lack substantial genotoxicity) are found to increase the tumor yield upon repeated administration (i.e. promoters).

Several unresolved questions pertinent to judging the validity of the enzyme-altered foci bioassay as an alternative to the lifetime carcinogenesis bioassay involve the generalizability of the system to the detection of non-liver initiators and promoters.

The positive results which have been obtained with benzo(a)pyrene, urethane, 2-naphthylamine, azaserine, and dimethylbenzanthracene are encouraging with respect to the generality of detecting non-hepatic initiating compounds.
However, further experience with many different classes of carcinogens is probably still warranted.

With regard to detecting non-liver promoters, it seems unlikely that the enzyme-altered foci bioassay will be of any utility. However, as the lifetime carcinogenesis bioassay cannot detect promoters in either hepatic or non-hepatic tissue, the fact that the enzyme-altered foci bioassay can at least detect liver tumor promoters is an asset rather than a liability.

Overall, the enzyme-altered foci bioassay appears to have greater utility, reduced requirements of time and money, and similar if not greater sensitivity than the lifetime carcinogenesis bioassay. Its growing acceptance as a valid indicator of carcinogenic potential of chemicals is further advanced by the studies presented in this thesis.

E. SIGNIFICANCE OF IDENTIFYING INITIATING OR PROMOTING MECHANISMS IN CHEMICAL CARCINOGENESIS

Ideally, the absolute avoidance of exposure to any chemical or physical agent which causes, contributes to, or enhances the formation of cancer would constitute a prudent course of action by which man could reduce his risk of developing neoplastic disease. Obviously this simply is not possible due to the ubiquitous nature of such agents. What
is practicable and desirable, however, is the establishment of exposure limits which would be expected to incur only an acceptably low risk for cancer development. Although the definition of an "acceptably low" risk may involve considerable discussion and debate, a careful analysis of the risks incurred and the benefits derived from such exposures is essential in establishing any regulatory limits. Germaine to an accurate assessment of risk are several factors which may characterize the biological event or events resulting from a given exposure to an agent under consideration. These factors include reversibility, dose-response relationships, threshold phenomena, and temporal requirements. With respect to the processes of initiation and promotion, most of these factors are related differently, even oppositely, to the molecular events associated with each process. For this reason, identification of the mechanism of carcinogenic action of a compound should allow for a more accurate evaluation of the risks posed by that compound. For example, a threshold or "no effect" level is generally believed to be a characteristic of tumor promoters but not of genotoxic carcinogens, and therefore in promulgating regulatory exposure limits for promoting agents, a higher exposure may be considered "acceptable" while for a genotoxic initiator, a much lower limit would be understandably justified. Accordingly, it becomes apparent that a genotoxic agent "requires
entirely distinct regulatory actions" than does an epigenetic or promoting agent (Weisburger, 1980).

In addition to aiding in regulatory functions, the identification of promoting agents should facilitate the systematic investigation of those biochemical events causally related to the process of tumor promotion. In such a way the potential for eventual pharmacologic or therapeutic intervention should be increased. Furthermore, it has been stated that promoting or modifying agents play an important role in human carcinogenesis and may actually be more amenable to intervention than the classical genotoxic carcinogens (Wynder et al, 1978). Ultimately, with a more complete understanding of the basic mechanisms of tumor promotion, perhaps even safer and more effective modalities of cancer treatment may be made available. From the above discussion, the far-reaching significance of the identification and elucidation of tumor promoting mechanisms has hopefully been made apparent.

F. SPECULATION ON THE POSSIBLE ROLE OF GAMMA-GLUTAMYL TRANSPEPTIDASE IN NEOPLASTIC DEVELOPMENT

Although the appearance of the enzyme gamma-glutamyl transpeptidase may well serve in the early detection of preneoplastic foci of hepatocytes, the
physiological significance of the phenotypic reacquisition of this enzyme is not fully understood. There cannot be a strict causal relationship between GGT activity and liver neoplasia because not all neoplastic liver nodules and hepatic tumors exhibit increased gamma-glutamyl transpeptidase activity (Pugh and Goldfarb, 1978; Hirota and Williams, 1979; Farber, 1980). Furthermore, although an association has been shown to exist between GGT activity and hepatic neoplasia, there is no compelling data to conclude that the reacquisition of this enzyme actually leads to tumor development. On the contrary, it is more generally believed that in the early stages of hepatocarcinogenesis, the liver tissue may revert to a fetal or embryonal stage of development as has been evidenced by the reappearance of alpha-fetoprotein and several fetal isozymes (Fiala and Fiala, 1970; Abelev, 1971; Fiala and Fiala, 1973; Taniguchi et al, 1974; Kalengayi et al, 1975; Solt et al, 1977). This reversion to fetal phenotypic expression most likely involves an impairment of gene control which is characterized by a derepression or a "switching on" of fetal protein synthesis (Taniguchi et al, 1975; Boelsterli, 1979; Selvaraj et al, 1981). However, if the derepression of the fetal gene for GGT is simply a chance consequence of an alteration in genetic control, why would there be such a high correlation between GGT activity
and neoplasia? Two plausible explanations (but by no means the only possible ones) for this observed association are:

1.) The genetic lesion which results in the derepression of GGT synthesis may also cause a derepression of mechanism(s) normally controlling cellular proliferation (possibly the loss of growth control precedes the phenotypic expression of GGT and other fetal proteins); and

2.) The increased activity of gamma-glutamyl transpeptidase may endow the "derepressed" hepatocyte(s) with a substantial proliferative advantage over normal liver cells, and thereby allow clonal expansion of these altered cells into foci, and for some, perhaps into tumors.

In discussing the first possibility, a consideration of the normal genetic regulation of these activities should precede a hypothesis of an alteration in the expression of these genes. Although major advances are being made in molecular biology and genetic biochemistry as it relates to carcinogenesis (O'Brien and Rice, 1979), considerably more information is needed to provide a reasonable understanding of mammalian gene regulation. On a theoretical basis, however, this hypothesis appeals to one's sense of logic in view of our current understanding of gene expression and regulation. Such a coincidental occurrence of the
derepression of both GGT activity and cellular growth regulation could account for those cases of liver neoplasia which do not express gamma-glutamyl transpeptidase activity. But this hypothesis does not serve to explain the occurrence of the preponderance of cases which are GGT-positive.

The second possibility, which is not necessarily exclusive of the first, is that GGT-activity may in some way contribute to the process of hepatocarcinogenesis. This possibility receives support from the apparent physiological role of gamma-glutamyl transpeptidase as a component enzyme of an amino acid transport system (Meister, 1973; Griffith and Meister, 1979; Griffith et al, 1979; Allison and Meister, 1981). Because rapidly growing cells need to constantly replenish their synthesis precursors, and because active transport processes are often involved in supplying the cells with these essential compounds, an increased capacity for amino acid transport (i.e. GGT induction) might reasonably be expected to afford these cells a certain growth advantage. This is not to suggest that the capacity for amino acid transport is causally related to carcinogenesis, but rather in those cells which have been altered with regard to growth regulation, an increased capacity to obtain amino acids could possibly enhance their proliferation (provided that growth is in some way limited by amino acid availability). In such a way, the expression of the fetal GGT-positive phenotype could
be related to the eventual development of autonomous liver tumors. Obviously, if a cell possesses other modes of nutrient and substrate provision, gamma-glutamyl transpeptidase would not be essential for neoplastic development, which could account for the occasionally observed GGT-negative hyperplastic foci and hepatic tumors.

An admitted shortcoming of both of these hypotheses is that they do not address the causality of the genetic derepression. Is it a single genotoxic lesion which results in a cascade of preneoplastic events or are more than one "hit" required to confer upon a cell a neoplastic destiny? Although the exact genetic basis of carcinogenesis remains to be determined, the possibility that the expression of gamma-glutamyl transpeptidase activity might contribute to neoplastic growth seems reasonable and may be quite useful in explaining the observed association between this histochemical marker and the process of hepatocarcinogenesis.
SUMMARY

This dissertation examined and used the rat hepatic enzyme-altered foci bioassay to evaluate the initiating and promoting activities of several chemical compounds. These experiments also served to further assess the validity of the system for detecting carcinogens. The principal findings of these investigations were as follows:

1.) The rat hepatic enzyme-altered foci bioassay has been proposed as an alternate method to the lifetime carcinogenesis bioassay for assessing chemical carcinogenicity. The results of the investigations performed as part of this thesis effort support the short-term enzyme-altered foci bioassay as a sensitive and accurate indicator of the carcinogenic potential of environmental chemicals, as both methapyrilene and 1,2-dibromoethane gave positive results as potential carcinogens, although their mechanisms of action appear to be promotional in nature rather than genotoxic.

2.) The enzyme-altered foci bioassay was utilized to discern between the initiating and promoting activities of
several compounds. As such a method of discrimination, this short-term in vivo system of carcinogenicity testing appeared capable of discerning between such genotoxic and epigenetic activities. Based upon a careful assessment of these two component activities, the carcinogenic risk posed by other chemicals may be more accurately estimated and the promulgation of appropriate regulations may be greatly facilitated by the use of this bioassay.

3.) The once commonly used antihistamine methapyrilene was detected in the enzyme-altered foci bioassay as a potential carcinogen. Although methapyrilene was without significant initiating activity in this system, the compound was shown to possess marked promoter activity. The significance of this promoting activity with regard to the production of liver tumors in rats which were chronically administered methapyrilene was considered. In addition, the occurrence of liver tumors in the human population which was exposed to this drug might be expected to be very low based upon the apparent epigenetic effects of this carcinogen in contrast to the observed lack of genotoxicity.

4.) Partial hepatectomy was seen to not be required for the production of gamma-glutamyl transpeptidase-positive foci
in rats initiated with N-nitrosodiethylamine and promoted with methapyriline. This protocol (methapyriline promotion without prior partial hepatectomy) may provide a simpler and more sensitive method for detecting the genotoxicity of drugs, industrial chemicals, and environmental contaminants when the enzyme-altered foci bioassay is used in carcinogenicity testing.

5.) The mutagen and carcinogen 1,2-dibromoethane was shown to be without significant initiating activity in rat liver. The halogenated hydrocarbon was shown, however, to possess considerable promoting activity in the enzyme-altered foci bioassay.

6.) The widely used industrial solvent trichloroethylene was not found to possess significant initiating or promoting activity in rat liver. Trichloroethylene does not appear to have substantial carcinogenic potential, and any carcinogenic potential which this compound may have might be associated with a weak promoting activity.

7.) The observed association between the activity of gamma-glutamyl transpeptidase and the process of neoplasia might be explained in terms of GGT-positive hepatocytes possessing an increased capacity for amino acid transport.
which in turn could conceivably endow them with a certain proliferative advantage. In this way, GGT-positive clones would be enabled to proliferate at a faster rate than normal liver cells, and thus the progression of these preneoplastic foci into frank liver tumors might be facilitated.


APPENDIX

ADDITIONAL STUDIES INVOLVING THE ENZYME-ALTERED FOCI BIOASSAY AS AN INDICATOR OF HEPATOCARCINOGENESIS

In addition to the major experiments presented in this dissertation, several other important investigations of both a preliminary and/or ancillary nature were conducted as part of this thesis research, most of which have been (or will be) published in the scientific literature. A brief discussion of the major findings of these experiments is given below.

A. DOSE-RESPONSE EFFECTS OF AFLATOXIN B₁ ON THE FORMATION OF ENZYME-ALTERED FOCI

In order to provide additional support to the enzyme-altered foci bioassay as a valid method for detecting hepatocarcinogens, and to show that the methodology used in this laboratory was adequate for gamma-glutamyl transpeptidase (GGT) detection, a dose-response experiment was performed using the potent hepatocarcinogen aflatoxin B₁. The
experimental protocol used in this experiment differed somewhat from that which was used in the later experiments presented in the main text of this dissertation.

Male Sprague-Dawley rats were administered various oral doses of aflatoxin B₁ (0.1 - 3.0 μmole/kg) dissolved in dimethylsulfoxide. After one week, the rats received a promoting regimen of 500 ppm phenobarbital in drinking water for seven days. Partial hepatectomy was then performed and the phenobarbital removed for 24 hours, after which time the animals were again presented with phenobarbital in drinking water, but at a reduced concentration (100 ppm) and continued for four weeks. The histochemical staining of fresh-frozen sections of liver was performed as previously described.

The results from this experiment showed a linear increase in the formation of GGT-positive foci with increasing doses of aflatoxin B₁ over the range of 0.1 to 3.0 μmole/kg. Furthermore, these results established the methodology as being appropriate for assessing the production of enzyme-altered foci. The requirements for phenobarbital promotion and for partial hepatectomy in optimizing the induction of GGT-positive foci were also noted.
B. PRELIMINARY STUDIES INVESTIGATING THE CARCINOGENIC POTENTIAL OF VARIOUS HALOGENATED HYDROCARBONS USING THE ENZYME-ALTERED FOCI BIOASSAY

To assess the carcinogenic potential of various halogenated hydrocarbons, a single oral dose of each compound was administered to partially hepatectomized male Sprague-Dawley rats at approximately one-tenth the LD<sub>50</sub>. These compounds and dosages included: trichloroethylene (490 mg/kg); tetrachloroethylene (20 mg/kg); 1,2-dibromoethane (12 mg/kg); and 1,2-dichloroethane (68 mg/kg). Promotion in this experiment consisted of an eight-week regimen of phenobarbital in drinking water. Several control groups were also included in this study such as a vehicle control group, a promoter control group, a sham hepatectomy group, and a positive control group which received 30 mg/kg N-nitrosodiethylamine as an initiating carcinogen.

Except for the positive control group, no significant formation of enzyme-altered foci was observed in any of the groups. These results suggested that these chlorinated hydrocarbons were without significant initiating activity at the dosages tested. Alternatively, phenobarbital promotion may not have sufficiently promoted the genotoxic damage (if any) introduced by these compounds at the levels of dosing used (one-tenth LD<sub>50</sub>) in this experiment. In any event, the
necessity of further investigation was made apparent by these negative results. For example, further studies with the halogenated hydrocarbon 1,2-dibromoethane (thesis text) revealed an absence of initiator activity despite higher and repeated dosing with the compound, while significant promoter activity was evidenced. Similar types of experiments may be warranted for the other agents discussed above as well.

C. PRELIMINARY INVESTIGATION OF THE PROMOTING EFFECTS OF TRICHLOROETHYLENE

Due to the apparent lack of initiating activity of trichloroethylene in the enzyme-altered foci bioassay, it was of interest to investigate the potential promoting activity of this compound. This preliminary experiment was designed to investigate any foci-enhancing effects that trichloroethylene might exert on N-nitrosodiethylamine-initiated rats.

Groups of male Sprague-Dawley rats were partially hepatectomized and 24 hours subsequently were initiated with an oral dose of 30 mg/kg N-nitrosodiethylamine. Three days following initiation, the animals were given 1000 mg/kg trichloroethylene in corn oil by oral gavage three times weekly for eight weeks. A positive control group was also included which received partial hepatectomy and 30 mg/kg
N-nitrosodiethylamine, followed by phenobarbital promotion (500 ppm phenobarbital in drinking water) throughout the eight-week promoting period.

The results obtained from this experiment did not evidence a statistically significant increase in the formation of GGT-positive foci in rats promoted with trichloroethylene as compared to those promoter control animals which were administered only corn oil vehicle three times weekly. However, because a slight elevation in the mean number of foci was observed in the trichloroethylene promotion group, the experiment was repeated with larger groups of animals and with more frequent dosing with trichloroethylene in an attempt to ascertain whether the suggested trend, but statistically insignificant increase in foci formation was an experimentally reproducible effect. This repeated experiment was presented in the main text of this thesis, and the results indicated that trichloroethylene did not exhibit significant promoting activity in these experiments.

D. PROMOTING EFFECTS OF PHENOBARBITAL AND METHAPYRILENE

AFTER THIRTEEN MONTHS OF CONTINUED ADMINISTRATION

The experiment designed to investigate the initiating and promoting effects of methapyrilene which was previously
discussed in the main text of this dissertation, showed
dramatic promoter effects of the antihistamine methapyrilene
in N-nitrosodiethylamine-initiated rats. To further investig­
ate these promoter effects, another experiment was designed
to evaluate the tumor promoting effects of long-term adminis­
tration of phenobarbital and methapyrilene. The original
intent of this experiment was to examine actual tumor for­
mation after 24 months of promotion, but due to substantial
mortality (Mycoplasma pneumonia), the study was terminated
after thirteen months. At this time, no grossly observable
hepatic lesions were seen. Histochemical staining of fresh-
frozen sections of liver demonstrated a pattern of occurrence
of GGT-positive foci similar to that reported in the short-
term experiment. In this long-term experiment, the promoting
activity of 200 ppm methapyrilene in drinking water led to a
nearly two-fold greater incidence of GGT-positive foci than
was observed for 500 ppm phenobarbital. That partial hepa­
tectomy was not required for the induction of GGT-positive
foci in N-nitrosodiethylamine-initiated and methapyrilene-
promoted rats was noted, and this observation was consistent
with the results obtained in the short-term experiment.
Furthermore, this thirteen month experiment corroborated
the results of the foci-enhancing effects of methapyrilene
and phenobarbital which were seen in the shorter-term
eight-week study.
E. PHENOBARBITAL DOSE-RESPONSE PROMOTER EFFECTS ON THE FORMATION OF ENZYME-ALTED FOCI

To investigate the dose-response nature of the foci-enhancing effects which phenobarbital exerts in the livers of N-nitrosodiethylamine-initiated rats, various concentrations of phenobarbital (10 to 1000 ppm) were administered in drinking water to male Sprague-Dawley rats which, three days prior to promotion, had been given a single oral dose of 30 mg/kg N-nitrosodiethylamine. These rats had not received prior partial hepatectomy.

The results of this experiment obtained after eight weeks of promotion did not show significant foci-formation effects in any of the groups, even though a dose-response effect on liver weight was clearly evident. Initiation with N-nitrosodiethylamine was not seen to affect body weight gain or liver weight increases in these animals. The lack of formation of GGT-positive foci after eight weeks of promotion was attributed to the omission of partial hepatectomy from the experimental protocol. These results are consistent with other eight-week studies in which partial hepatectomy was not performed.

After 24 weeks of phenobarbital promotion, a dose-response relationship between phenobarbital and GGT-foci production was
observed. The rats which did not receive N-nitrosodiethylamine initiation did not show significant numbers of GGT-positive foci, although the dose-related increase in liver weight was still apparent in both the initiated and non-initiated animals. The lowest promoting dose (10 ppm) of phenobarbital in drinking water did not result in an elevation in the yield of GGT-positive foci per square centimeter of liver tissue examined, suggesting the possibility of a threshold phenomenon being associated with phenobarbital promotion. A longer time course (52 weeks) of phenobarbital promotion is currently in progress, the results of which should allow for a more decisive conclusion as to the possibility of such a threshold phenomenon.

F. TUMOR PROMOTING EFFECTS OF CHLOROFORM ASSESSED BY THE ENZYME-ALTERED FOCI BIOASSAY

The promoting activity of chloroform was assessed using the enzyme-altered foci bioassay. In these experiments, male Sprague-Dawley rats which were not partially hepatectomized were given a single initiating dose of 30 mg/kg N-nitrosodiethylamine and three days later, received solutions of chloroform (900 and 1800 ppm) in distilled water to drink throughout the promoting periods (8 and 24 weeks). The hypothesis was that if chloroform possessed tumor promoting activity, it should produce an increase in the number of GGT-positive foci observed in the
livers of rats initiated with N-nitrosodiethylamine.

The results of these experiments did not suggest an appreciable tumor promoting activity of chloroform at the dosages used and over the specified periods of time, as only low background levels of GGT-positive foci were observed across all the chloroform treatment groups, with or without N-nitrosodiethylamine initiation. The performance of partial hepatectomy prior to N-nitrosodiethylamine initiation, if it had been included, might have increased the susceptibility of the hepatic tissue to the initiating effects of the genotoxic carcinogen. In such a way, the promoting effects of chloroform might have been investigated with greater sensitivity. If the experiment were ever to be repeated, partial hepatectomy and perhaps higher doses of chloroform should probably be considered.

G. CHLOROFORM/N-NITROSODIETHYLAMINE COCARCINOGENESIS STUDY

In order to examine a possible cocarcinogenic action of chloroform administered concomitantly with low doses of N-nitrosodiethylamine, two groups of rats were given weekly oral doses of 0.15 mg/kg N-nitrosodiethylamine for 8 or 24 weeks. In addition, one group received a solution of 1800 ppm chloroform in distilled water to drink throughout the course of the experiment. At neither time point (8 or 24
were any significant foci-enhancing effects observed, suggesting that chloroform is without substantial cocarcinogenic effects when administered concomitantly with low doses of N-nitrosodiethylamine.

Two important points of consideration which should be recognized, however, are that this dosage of chloroform does not induce substantial hepatotoxicity, and that the repeated 0.15 mg/kg doses of N-nitrosodiethylamine may not have induced sufficient genotoxic damage to result in observable preneoplastic changes within the time frame of this experiment. Further investigation will be required to definitively rule out potential cocarcinogenic effects of chloroform.

H. THE EFFECT OF SELENIUM ON THE INDUCTION OF ENZYME-ALTERED FOCI BY CHEMICAL CARCINOGENS

Because selenium has been implicated in a reduction of cancer-associated human mortality, and because selenium has been demonstrated to inhibit carcinogenesis in several tissues, it was of interest to investigate the effects of this trace element on the induction by various carcinogens of GGT-positive foci in rat liver.

Groups of rats were placed on selenium-deficient diets and received various levels of selenium supplementation in drinking water (0, 0.2, 2.0, and 5.0 ppm selenium as sodium
selinite). Twenty-four hours following partial hepatectomy, the rats were administered either 0.6 mg/kg aflatoxin B1, 150 mg/kg benzo(a)pyrene, or 30 mg/kg N-nitrosodiethylamine by oral gavage. The animals were then returned to a normal diet and were also discontinued from selenium supplementation. One week subsequently, the rats were given a promoting regimen of 500 ppm phenobarbital in drinking water for eight weeks. Histochemical staining of fresh-frozen sections of liver revealed foci of GGT-positive hepatocytes in a pattern consistent with the level of selenium supplementation among the three carcinogens tested. The 0.2 ppm selenium supplementation group (which approximates normal dietary intake of selenium) gave the greatest response with regard to the formation of enzyme-altered foci. The 5.0 ppm selenium group gave the lowest number of foci among the carcinogen-treated groups, but was still significantly above background levels. The 0 and 2.0 ppm selenium treatments resulted in intermediate levels of enzyme-altered foci production. Thus, variation of selenium supplementation was seen to affect the process of chemical carcinogenesis as assessed by the enzyme-altered foci bioassay.

A similar pattern of effects was seen in a DNA-binding experiment which investigated the covalent binding of $^3$H-aflatoxin B1 to the liver DNA of rats maintained on these given levels of selenium supplementation. The 5.0
ppm selenium group had the lowest level of binding among the four selenium treatment groups.

One important point which should be considered before interpreting a "protective" effect of 5.0 ppm selenium supplementation, is that this level of selenium intake has been associated with mild to moderate hepatotoxic effects which in their own right might have caused a reduction in the metabolic activation of the carcinogen. Further investigation is warranted to elucidate this or alternative mechanisms for the reduced carcinogenic effects seen at the highest level of selenium intake.