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EFFECT OF ETHANOL INGESTION AND ACETALDEHYDE ON O^6-METHYLGUANINE TRANSFERASE (O^6 MEGT)

by

NOEL G. ESPINA

A dissertation submitted to the Graduate Faculty in Biomedical Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

1987

This manuscript has been read and accepted for the Graduate Faculty in Biomedical Sciences in satisfaction of the dissertation requirement for the degree of Doctor of Philosohpy.

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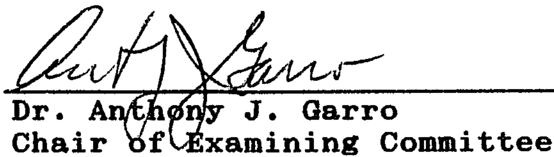
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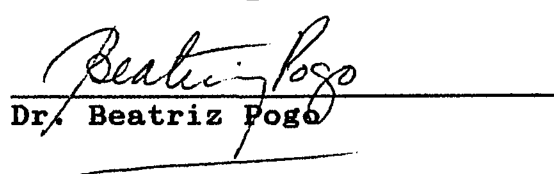
Dr. Beatriz Pogo

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Abstract

EFFECT OF ETHANOL INGESTION AND ACETALDEHYDE ON O^6 METHYLGUANINE TRANSFERASE (O^6 MEGT)

by

Noel G. Espina

Advisor: Professor Anthony J. Garro

Heavy alcohol consumption is one of several dietary factors which have been linked epidemiologically to an increased risk of cancer. One possible mechanism by which alcohol abuse might lead to an increased risk of cancer is to inhibit the repair of carcinogen-DNA adducts thereby increasing the number of somatic mutations associated with carcinogen exposure. The results presented here show that chronic ethanol consumption leads to a decreased capacity for repair of dimethylnitrosamine induced alkylation at the O^6 position of guanine. O^6-methylguanine transferase (O^6MeGT) activity is decreased following both chronic and acute ethanol administration. This in vivo decrease in O^6MeGT activity is primarily due to acetaldehyde, the reactive metabolite, that is generated during ethanol

metabolism. In vitro, rat and human O^6MeGT are inhibited by acetaldehyde at 0.01-1.0uM concentrations. Ethanol at 10-50mM concentrations also inhibited O^6MeGT. These concentrations are physiologically significant in that these alcohol and acetaldehyde levels are found in blood of alcoholics or animals fed ethanol. Although a direct effect cannot be ruled out, the in vitro inhibitory effect of ethanol may be due to trace levels of acetaldehyde that is generated spontaneously or produced from residual alcohol dehydrogenase activity. The in vivo and in vitro inhibitory effect on O^6MeGT, mediated through its metabolite acetaldehyde, may contribute to ethanol's capacity to act as a cocarcinogen.

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And finally, I would like to thank the three most influential people in my life - my mother, my late father and my wife, Wendy. Their love and encouragement made all this a reality.

FORMAT OF DISSERTATION

This is written according to the new guidelines of The City University of New York which permit direct incorporation of published research articles as chapters. This thesis has a general introduction, followed by two chapters, one of which is a published research article and the other a paper submitted for publication. A third chapter is included for unpublished results and new methods that were developed during this study. Each chapter has a specific introductory statement, materials and methods, results and discussion sections. A general discussion section is included in the final chapter and all references are pooled to avoid redundancy. It should be noted that Table I of the first chapter was not part of this research project. It is included here since it was part of the published research article.

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INTRODUCTION

According to recent American Cancer Society figures, 930,000 people will be diagnosed with cancer this year and approximately 472,000 will die. Of those cancer deaths, two to four percent can be attributable to alcohol abuse (American Cancer Society, 1987). Many epidemiological studies have demonstrated a link between consumption of alcoholic beverages and increased cancer risk at various sites. Studies which show a synergistic relationship between alcohol consumption and tobacco use indicate that drinking alcoholic beverages involves an increased risk for cancer of the oral cavity, pharynx, larynx and lungs (Wynder and Bross, 1961; Vincent and Marchetta, 1963; Pollack et al., 1984). Esophageal carcinoma has been associated with various local alcoholic drinks in different countries. In China a popular drink, pai-kan, and in the Transkei region of South Africa, Xhosa-beer, both have been associated with increased incidence of esophageal cancers (Broitman et al. 1983; McGlashan et al., 1982). Several recent studies also have linked alcohol consumption with increased risk for cancer at other sites such as breast, urinary tract, colon and rectum. While some studies demonstrate a positive association only at high alcohol consumption (Pottern et

al., 1981; Pollack et al., 1984), others have suggested that even moderate amounts of alcohol lead to an increased risk of cancers at these various sites (Kunze et al., 1986; Harvey et al., 1987; Schatzkin et al., 1987; O'Connell et al., 1987).

Although the epidemiological evidence is clear, the biochemical mechanisms for this increased cancer risk is not known and may be due to a number of factors associated with alcohol abuse including: 1) Presence of carcinogens in alcoholic beverages; 2) Nutritional and dietary deficiencies associated with alcohol abuse; 3) Immunosuppression and effects of alcohol on the immune system; and 4) Ethanol acting as a cocarcinogen by altering metabolism (activation and detoxification) of carcinogens and its effect on repair of carcinogen damaged DNA.

Carcinogens in alcoholic beverages:

Low levels of carcinogens such as nitrosamines, polycyclic hydrocarbons and even asbestos fibers have been found in alcoholic beverages (Walker et al. 1979; Cunningham and Pontefract, 1971; Masuda et al. 1966). For example, nitrosamine levels of 3ug/kg and 6ug/kg have been reported for German and U.S. beers, respectively (Spiegelhalder et

al., 1979; Scanlan et al. 1980). The general significance of these low levels of carcinogens is not clear. There are, however, several studies which suggest that carcinogens present in some alcoholic beverages can contribute to an increased incidence of cancers. In the Normandy region of France, consumption of a locally prepared brandy has been associated with a high incidence of esophageal cancers (Tuyns, 1970) and Walker and coworkers (1979) have shown that the apple brandies popular in Normandy have relatively high levels of both dimethylnitrosamine (DMN) and diethylnitrosamine (DEN). The high DEN concentrations found in these alcoholic beverages may be significant with respect to the incidence of esophageal cancer since in rodents, DEN acts predominantly as an esophageal carcinogen while DMN is a hepatic carcinogen.

Similar studies of brewery workers in Denmark (Jensen, 1979) and Ireland (Dean et al. 1979) support the possibility that carcinogens present in some alcoholic drinks contribute to the development of cancer. Both groups regularly consume large amounts of beer but only the Irish brewery workers exhibit an increased risk of large bowel cancer. This was attributed to the fact that Danish beers have a significantly lower DMN content than Irish beers (Tuyns, 1979). Whether the DMN is the actual carcinogen responsible for the increased cancer incidence is still open

to question, however, since there is no indication from animal studies that DMN can act as a bowel carcinogen.

Dietary Deficiencies and Alcohol Abuse:

Chronic alcohol abusers may ingest up to 50% of their daily caloric intake in the form of ethanol. Therefore, nutritional deficiencies are common among this population. A case control study among black males in Washington D.C. (Ziegler et al. 1981), reported an 80% causal association between the incidence of esophageal cancer and alcohol consumption. In addition, poor nutritional status was an important risk factor in esophageal cancer rates within this group (Ziegler et al. 1981).

Several dietary deficiencies have been associated with an increased risk of cancer. Corn and wheat based diets can lead to deficiencies of several vitamins and minerals including: riboflavin; nicotinic acid; magnesium and zinc. Deficiencies in these vitamins and minerals have been linked to an increased risk of esophageal cancer (Breeden, 1984). Similarly the Plummer-Vinson syndrome, in which there is an increased incidence of oral and esophageal cancers, has been linked to a dietary deficiency in iron and vitamin B2 (Wynder and Fryer, 1958). Experimental animals

fed a zinc-deficient diets had an enhanced tumor induction by the esophageal carcinogen, methylbenzylnitrosamine (MBN) (Gabrial et al. 1982). Ethanol itself also can affect nutritional status by lowering intestinal absorption of folate, thiamine, vitamin B12 and zinc (Broitman et al. 1983; Langman and Bell, 1982). Therefore, nutritional deficiencies and malnutrition may play a key role in the development of cancers in the alcoholic.

In experimental animals, vitamin A deficiency often increases cancer risk in a number of organs such as the lungs, bladder, stomach and uterus (Breeden, 1984). This increased risk can be abolished by feeding of small amounts of vitamin A. It has been observed that vitamin A deficiency enhances the binding of benzo(a)pyrene to tracheal DNA. Four times more carcinogen is bound to trachea of vitamin A deficient rats than control rats (Genta et al. 1974). This may be due to mucosal surface abnormalities observed in vitamin A depleted rats (Mak et al., 1984). Retinoids also have been reported to suppress chemical or radiation induced transformation of cells in culture (Sporn and Newton, 1979). In vivo, synthetic analogs of vitamin A inhibit methylnitrosurea induced bladder carcinogenesis in rats (Sporn et al., 1976).

Recent experiments by Lieber and coworkers have demonstrated that heavy alcohol consumption may lead to a severe reduction in hepatic vitamin A levels (Leo and

Lieber, 1983; Lieber and Leo, 1985). At the early stages of alcoholic injury (i.e. fatty liver stage), alcoholics have very low hepatic vitamin A levels despite normal plasma levels of vitamin A (Leo and Lieber, 1982). In rats, ethanol administration depresses vitamin A levels in the liver even when diets contain adequate amounts of this vitamin (Sato and Lieber, 1982). When rats are shifted to a vitamin A deficient diet, the rate of depletion of hepatic stores of vitamin A was two to three-fold faster in ethanol fed rats as compared to controls and this may be due to a novel pathway of vitamin A metabolism induced by ethanol feeding (Leo and Lieber, 1985). Vitamin A (retinol) may be mobilized from the liver to other tissues by binding to retinol binding proteins. It can also be degraded to retinoic acids and excreted as a glucuronide conjugate (Sporn et al., 1984; Sato and Lieber, 1982). Reconstituted cytochrome P-450's isolated from microsomes of ethanol fed rats, degraded retinol at a much greater rate than uninduced microsomes (Leo et al., 1984). Chronic ethanol consumption may lead to stimulation of these degradative pathways and increased mobilization of vitamin A from the liver resulting in a significant depletion of hepatic vitamin A levels. Finally, with respect to microsomal activation of nitrosamines retinol may exert a protective effect against nitrosamine induced cancers in that it competitively inhibits DMN demethylation. Therefore, vitamin A deficiency

may be particularly detrimental vis a vis nitrosamine-associated cancers in view of the fact that ethanol consumption also induces a specific cytochrome P-450 that selectively activates DMN.

Immunosuppression and alcohol abuse:

It has long been thought that tumors are recognized and eliminated by the immune system particularly by T-cell mediated responses. Since several studies have shown an association between heavy drinking and decreased immune responsiveness (Berenyi et al. 1974; Bernstein et al. 1974), alcohol associated immunosuppression has been considered a possible contributing factor for increased risk of cancer (Daynes et al. 1979; MacGregor, 1986; Jerrells et al. 1987). For the most part, however, the studies which associated alcohol abuse with decreased immune responses were conducted with patients who already had alcoholic liver disease and therefore it is unclear whether the decreased immune effects were due directly to ethanol or whether they reflected other aspects of the ongoing disease process. Furthermore, there is reason to question the significance of immunosuppression on general oncogenesis.

Immunosuppressed patients or animals that are immunosuppressed have an increased incidence of cancer but the cancers observed are mostly lymphorecticular neoplasms

(Daynes et al. 1979; Baird et al. 1982). Nude mice, which have a defect in their T-cell mediated immune response have been used as a model for studying the relationship between tumorigenesis and immunosuppression. These animals, at latter stages of their lifespan, have a higher incidence of lymphoreticular tumors but do not have an increased incidence of spontaneous tumors in other organs nor are they more prone to develop tumors when treated with chemical carcinogens relative to normal mice (Stutman, 1979; Rygaard and Povlson, 1974). It seems that T-cell mediated immunity plays a minor role in the control of spontaneous or chemically induced tumors of organs other than the lymphoid system. It therefore seems unlikely that immunosuppression is an important factor for increased risk of chemically induced cancers associated with alcohol as a cocarcinogen.

The immune system may, however, play a vital role in the defense against virally induced tumors. Observations in animals where T and B cell numbers and functions are impaired after chronic ethanol feeding (Roselle and Mendenhall, 1984) and where a single dose of ethanol decreased leucocyte mobilization (Rimland, 1984) may be important in virally induced hepatocellular carcinoma which has been associated with Hepatitis B infections (Yarrish et al., 1980; Shafritz and Kew, 1981).

Ethanol as a cocarcinogen:

There is no evidence from animal feeding studies that ethanol per se is carcinogenic (Schottenfeld, 1979). Ethanol on the other hand may act as a cocarcinogen in the complex multistep process of chemical carcinogenesis. By altering the metabolism and/or distribution of carcinogens, ethanol may increase the susceptibility of various tissues to chemical carcinogens (Swann, 1984).

Epidemiologic studies suggest that ethanol is acting as a cocarcinogen for some smoking-associated cancers. The synergistic interaction of alcohol consumption and smoking with respect to risk of developing upper alimentary tract (UAT) or upper respiratory tract (URT) cancers has been recognized for many years (Tuyns, 1979). An example of this interaction is typified in the results of a case-control study (Reif, 1984) in which the increase in URT and UAT cancer risk associated with combination of smoking and drinking was 6 to 15 times background, whereas smoking alone was associated with a 2 to 4-fold increased risk and drinking alone with a 1 to 5-fold increase. In several similar studies the effect of drinking on cancer risk was related to the total amount of alcohol consumed rather than any particular type of drink, such as whiskey, beer or wine (Williams and Horm, 1977).

One of the hypothetical mechanisms by which ethanol may act as a cocarcinogen is through its solvent effect. By solubilizing carcinogens, ethanol may allow higher concentrations of carcinogens to reach target cells within tissues (Kuratsune et al. 1965). Although this is a frequently mentioned mechanism, there is little in the way of experimental data to support it. Benzo(a)pyrene, a known carcinogen, did penetrate mouse esophageal mucosa better when dissolved in alcohol than when dissolved in olive oil; however, an aqeous solution was about as effective as alcohol in penetrating the mouse mucosal surface (Kuratsune et al. 1965). Nevertheless, an increased number of esophageal cancers have been observed in mice given DEN-alcohol solutions as compared to those given carcinogen in aqeous solution.

In addition to a possible solvent effect, diminished peristalsis and mucosal surface damage following alcohol exposure may increase the susceptibility of different organs to various carcinogens (Swann,1984). Organs such as the esophagus, stomach and small intestine may be subjected to high local ethanol concentrations. There is overt tissue damage observed in these organs after ethanol ingestion which is followed by regenerative cell proliferation (Gottfried et al., 1978; Baraona et al., 1974; Willems et al., 1971). However, ethanol may also stimulate epithelial cell regeneration in the absence of overt mucosal damage

(Mak et al., 1986). The increase in DNA synthesis which is associated with cellular proliferation would provide a more susceptible target to chemical carcinogens (Columbano et al., 1982).

Most investigations of the potential mechanism whereby ethanol may act as a cocarcinogen, have been focused on ethanol's capacity to induce the cytochrome P-450 biotransformation system. It is well known that this enzyme system is involved in the metabolic activation of many structurally diverse chemical carcinogens (Miller and Miller, 1977; Gillette, 1976). Highly reactive electrophilic intermediates are formed during carcinogen metabolism and these electrophiles are capable of reacting with critical macromolecules such as DNA, RNA, and proteins.

The general approach used in these studies has been to prepare microsomes from different tissues (liver, esophagi, lungs and intestines) of rats which had been pair-fed alcohol-containing and control liquid diets for a period of four weeks. Isolated microsomes are then assayed for their ability to metabolically activate several different carcinogens to genetically active intermediates and end products as measured in the Ames mutagenesis assay (Maron and Ames, 1983)

Among the carcinogens studied in this laboratory are a number of compounds found in tobacco smoke namely benzo(a)pyrene (BP), nitrosopyrolidine and tobacco

pyrolyzate and two compounds which may be considered models of dietary carcinogens, namely, dimethylnitrosamines (DMN) and tryptophan pyrolyzate. Most of the results can be summarized as follows: Chronic ethanol consumption enhanced the microsomal activation of all the test compounds and pyrolyzates to genetically active metabolites. Moreover, tissue specific effects were seen in the intestines and lungs. For example, in the intestine, ethanol increased microsomal activation of BP and tryptophan pyrolyzate but not tobacco pyrolyzate. In contrast, lung microsomes from ethanol fed rats exhibited an enhanced capacity to activate the premutagens contained in tobacco pyrolyzate but did not exhibit any increased activity towards BP or the tryptophan pyrolyzate (Garro et al., 1981; Seitz et al., 1981a, 1981b; Farinati et al., 1984).

In order to study the cocarcinogenic effects of ethanol, it is necessary to have an appropriate animal model where it is possible to isolate ethanol as the only dietary variable. In the Lieber-DeCarli feeding model, animals are pair-fed nutritionally adequate isocaloric liquid diets with ethanol accounting for 36% of total calories while the control diet contains additional carbohydrates or fats in place of the ethanol (Lieber and DeCarli, 1982). It is well established that rats maintained on the ethanol-containing diet exhibit many of the biochemical changes associated with early stages of alcoholism including: development of fatty

liver; microsome proliferation; induction of microsomal enzymes; and mitochondrial damage (Lieber and DeCarli, 1970a). In addition, in longer-lived species such as baboons approximately 25% of the ethanol-fed animals develop cirrhosis (Lieber et al., 1975).

This feeding model has been used in a variety of studies of the cocarcinogenicity of ethanol in different organs. Results from these studies show that ethanol enhanced the carcinogenic effect in different tissues of several carcinogens. Examples of these studies include: the induction of nasopharyngeal cancers by nitrosopyrolidine (NPY) in hamsters (McCoy et al., 1986); N'-nitrosonornicotine-induced nasal mucosal tumors in F344 rats (Castonguay et al., 1984); dimethylhydrazine-induced rectal cancers in rats (Seitz et al., 1984) and enhancement of acetoxymethylmethylnitrosamine induced colorectal tumors also in rats (Garzon et al., 1986). Ethanol in drinking water led to increased induction of liver tumors by vinyl chloride in rats (Radike et al., 1977, 1981). There are, however, studies that did not show an enhanced carcinogenic effect resulting from ethanol feeding. Ethanol in drinking water (25%) did not enhance DEN induction of liver tumors (Habs and Schmahl, 1981) nor did ethanol in liquid diet (35% of calories) change the rate of hepatocarcinogenesis by DMN (Teschke et al., 1983).

Dimethylnitrosamine: A model carcinogen

Studies in this laboratory have utilized DMN as a model carcinogen since it is a simple alkylating agent which requires microsomal activation. In rats, DMN administered in low doses primarily induces hepatic angiosarcomas which appear to be initiated by DNA alkylation (Lewis and Swenberg, 1980; Lijinsky and Reuben, 1984). The major sites of methylation are the N^7 and O^6 positions of guanine. Other sites on adenine, thymine, cytosine as well as the phosphodiester groups also are methylated by DMN (Faustman-Watts and Goodman, 1984).

Much attention has focused on alkylation at the O^6 position of guanine as alkylation at this site interferes with normal base pairing. G-C to A-T transition mutations may occur and this is thought to be a major factor in the carcinogenic effect of DMN and similar alkylating agents (Eadie et al. 1984; Newbold et al. 1980). Persistence of O^6-methylguanine (O^6MeG) in DNA of various organs has been associated with carcinogenicity of several alkylating agents (Kleihues and Cooper, 1976; Kleihues et al. 1979; Lindamood et al. 1984). Alkylation of the O^4 position of thymine also may be significant (Swenberg et al., 1984; Dyroff et al., 1986) while alkylation of the N atoms of purine bases appears to be less important in carcinogenesis. N^7-methylguanine (N^7MeG) does not interfere with base pairing

and its presence in DNA does not alter transcription by RNA polymerase (Ludlum, 1970). It is however, repaired by an N^7MeG glycosylase and also is lost from DNA by spontaneous depurination. The effect of N^7MeG on induction of DNA repair and changes in DNA structure and coding is still unclear (Pegg, 1977).

Effect of ethanol on DMN metabolism:

Microsomes from ethanol fed rats exhibit a greater capacity to metabolically activate DMN than those of pair-fed control rats (Garro et al. 1981). This is seen over a broad range of DMN concentrations (0.3 to 100mM) and is particularly striking at relatively low DMN concentrations (<1mM). In contrast to other microsomal enzyme inducers, ethanol pre-feeding increases a low Km form of microsomal cytochrome P-450 dependent DMN demethylase (Peng et al. 1982). Ethanol however also can inhibit DMN activation when both ethanol and DMN are present at the same time. Ethanol at a concentration of 1mM acts as a competitive inhibitor of the low Km form (Km = 0.07mM) of DMN demethylase. Propanol and butanol, when added in vitro, also inhibit microsomal metabolism of DMN (Peng et al. 1982)

There are several conflicting reports regarding the effects of in vivo ethanol administration on DMN mediated

tumorigenesis (Belinsky et al., 1982; Teschke et al., 1983). In one study, pretreatment of ethanol had a protective effect against induction of liver tumors (Habs and Schmahl, 1981) while in another, it changed the organotropism of DMN resulting in an increased incidence of esophageal cancers and nasal cavity tumors (Griciute et al., 1981). The basis for these conflicting observations may be related to the opposing effects of ethanol on DMN metabolism noted above i.e. although ethanol exposure induces DMN activating enzyme activity, when present at the same time ethanol may prolong the half-life of circulating DMN resulting in increased exposure of organs other than the liver (Tomera et al. 1984). This may be particularly important for organs which have a lesser DNA repair capacity relative to the liver.

The dose and route of DMN also are important in considering the inhibitory and inductive effects of ethanol on DMN metabolism. When DMN is administered orally it passes through the liver via the portal circulation before entering the general circulation. The liver can effect a "first pass clearance" up to a DMN dose of 30ug/kg (Swann, 1984). At higher doses the hepatic enzymes are saturated and methylation of the kidneys and other organs occur. Ethanol, when given to rats in low concentrations equivalent to a man drinking 0.5 L of beer, prevents this "first pass

clearance" by competing for hepatic microsomal enzymes. In ethanol fed rats given DMN at 30ug/kg, methylation of kidney DNA is 5 times more than the controls (Swann, 1984).

Effect of ethanol on repair of DMN induced O^6-methylguanine residues in hepatic DNA:

Another mechanism by which alcohol abuse may increase the risk of developing cancer is by inhibiting the capacity of cells to repair DNA damage. DNA repair processes are important in protecting the cell from chemical carcinogens that alter DNA structure and sequences that can ultimately lead to uncontrolled cellular growth characteristic of tumors. Cells possess a number of enzyme systems capable of repairing different types of DNA damage. Patients with DNA repair deficiencies such as Xeroderma pigmentosum and ataxia-telangiectasia are in fact at greater risk of developing cancer (Cleaver, 1980; Becker, 1986).

This study focused on the DNA repair enzyme, O^6-methylguanine transferase (O^6MeGT) which removes alkyl groups (methyl and ethyl) from the O^6 position of guanine and transfers them to cysteine residues on an acceptor protein which may be the enzyme itself (Craddock et al. 1982; Bogden et al. 1981; Lemaitre et al. 1982). This

provides cells with a protective mechanism against carcinogenesis by numerous alkylating agents. N^7-methylguanine is lost by spontaneous depurination and by an N^7MeG DNA glycosylase. However, the rate of loss of N^7MeG is much slower than the loss of O^6MeG. Therefore the levels of N^7MeG can be used as a measure of the extent of DNA alkylation in animals given carcinogens such as DMN (Schwartz et al. 1980).

O^6methylguanine transferase activity is an inducible enzyme in bacteria and some mammals. *Escherichia coli* when pre-exposed to low concentrations of methylating or ethylating agents, upon subsequent challenge are resistant to the mutagenic and lethal effects of higher doses of these agents. O^6MeGT activity is induced in *E. coli* as part of what is termed the adaptive response. Extracts from *E. coli* which had been pre-exposed to methylating agents are much more active in removing O^6MeG from DNA than extracts from cells which are not pre-exposed (Demple et al. 1982; Warren and Lawley, 1980). It is firmly established for this bacterial enzyme that the methyl group is transferred to a cysteine residue on the bacterial O^6MeGT. Similar inducible O^6MeGT activity also has been demonstrated in *Micrococcus luteus* (Ather et al. 1984) and *Bacillus subtilis* (Hadden et al. 1983).

In rats and humans, repair of O^6MeG also proceeds via an O^6MeGT. In rodents O^6MeGT activity has been

demonstrated in a variety of tissues with liver having the highest activity. The methyl group of O^6MeG is also transferred to a cysteine residue of an acceptor protein in rodents but it is still not clear whether the acceptor is the enzyme itself (Pegg and Balog, 1979; Pegg and Perry, 1981a, 1981b; Pegg and Hui, 1978; Pegg and Wiest, 1983; Pegg et al. 1983). Human tissues exhibit a 2 to 10 fold higher O^6MeGT activity than the corresponding rat tissues. Among the different human tissues tested, the levels of O^6MeGT activity varied as follows: liver > colon> esophagus > lung > brain. Cultured human epithelial cells, fibroblasts and peripheral blood lymphocytes also have measurable O^6MeGT activities (Pegg et al. 1982; Wiestler et al. 1984; Gafstrom et al. 1984; Cohen and Leung, 1986).

There is little information on the physiological factors which regulate the activity of O^6 MeGT in the rat. In this species it is an inducible enzyme and its levels tend to be increased following exposure to hepatotoxins, DNA alkylating agents and partial hepatectomy (Pegg and Perry, 1981a; Buckley et al. 1979; Pegg et al. 1981). The enzyme is believed to be inactivated as a consequence of the methyl transfer reaction (Pegg et al. 1983), therefore, the rate of resynthesis of this enzyme may be an important factor in the resistance of cells to alkylating agents (Waldstein et al. 1982).

The work described here shows that chronic ethanol feeding inhibits O^6MeGT apparently by reaction of the enzyme with the ethanol metabolite, acetaldehyde. This could contribute to the mechanisms by which alcohol consumption leads to an increased risk of cancers in different organs.

CHAPTER 1

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The Effects of Chronic Ethanol Consumption on Carcinogen Metabolism and on O^6 Methylguanine Transferase-Mediated Repair of Alkylated DNA.

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ABSTRACT

This article presents a review and update of recent experiments conducted in collaboration with Dr. C. S. Lieber, on mechanisms underlying the increased cancer risk associated with alcohol abuse. Ethanol has been found to be a potent inducer of microsomal enzymes involved in carcinogen metabolism in a variety of rat tissues including liver, esophagus, lungs, and intestines. In some of these tissues ethanol's inductive effect on microsomal cytochrome P-450 enzyme activity may result in enhanced levels of electrophilic metabolites of procarcinogens which are not readily detoxified. In addition, chronic ethanol feeding has been found to depress the activity of O^6-methylguanine transferase, an enzyme involved in the repair of carcinogen-induced DNA alkylation. The effects of ethanol on carcinogen metabolism and on DNA repair, would be expected to enhance the initiation phase of chemically induced cancers.

INTRODUCTION

Over the last eight years my coworkers and I have had the pleasure of collaborating with Dr. Charles Lieber on a series of investigations into the mechanisms underlying the increased cancer risk associated with alcohol abuse. At the time we started these investigations, it had been well established through epidemiologic studies conducted in many countries, that alcohol abuse was associated with an increased incidence of upper alimentary tract (UAT) and upper respiratory tract (URT) cancers (Wynder and Bross, 1961; Martinez, 1970; Tuyns, 1970; Williams and Horm, 1977; McMichael, 1978; Chilvers et al., 1979; Kono and Ikeda, 1979; Jensen, 1979) and also with an increased risk for hepatic cancer (Tuyns, 1978; Lieber et al., 1979) and cancers of the large bowel (Williams et al., 1977; Breslow et al., 1974), the pancreas (Burch et al., 1968; Lin et al., 1981), and breast (Williams et al., 1977; Breslow and Enstrom, , 1974).

An understanding of the biochemical basis for these observations is complicated both by the overall complexity of alcoholic beverages, several of which have been shown to

contain low levels of various carcinogens (Masuda et al., 1966; Cunningham and Pontefract, 1971; Walker et al., 1979) and by the dietary deficiencies associated with alcohol abuse some of which also have been associated with increased cancer risk (for reviews, see Lieber et al., 1979, and Lieber et al., 1986). In addition to these factors, there also was reason to suspect, based on epidemiologic studies, that ethanol itself acted as a cocarcinogen which enhanced the carcinogenic potential of other agents. There is, for example, strong evidence of a synergistic interaction between alcohol consumption and tobacco use in the production of UAT and URT cancers (Martinez, 1970; Williams et al., 1977; Wynder et al., 1961; Feldman et al., 1975).

These epidemiologic observations have been supported over the years by animal studies in which ethanol was reported to exhibit cocarcinogenic activity with a variety of carcinogens such as aromatic hydrocarbons (Protzel et al., 1964; Elzay, 1966; Capel et al., 1978), vinyl chloride (Radike et al., 1977) and dimethylnitrosamine (DMN) (Gibel, 1967). More recently, using the type of pair feeding regimen and isocaloric liquid diets, pioneered by Lieber and DeCarli (Lieber et al., 1970a) to study the toxic effects of ethanol in animals receiving nutritionally adequate diets, ethanol was shown to act as a cocarginogen in

nitrosopyrrolidine (NPY) induced URT cancers in hamsters (McCoy et al., 1981) and dimethylhydrazine-induced bowel cancer in rats (Seitz et al., 1984). These latter studies are particularly important as they clearly demonstrate that ethanol's cocarcinogenic activity is not limited to solvent effects or local tissue damage at the site of carcinogen and ethanol administration but rather to systemic effects associated with alcohol consumption.

ETHANOL'S EFFECTS ON CARCINOGEN ACTIVATION AND DETOXIFICATION

One of the mechanisms by which ethanol may act as a cocarcinogen, and which has been of particular interest to us is through its effects on enzyme systems involved in carcinogen activation and detoxification. Ethanol is a known inducer of the microsomal cytochrome P-450 biotransformation system (Lieber and DeCarli, 1968; Lieber and DeCarli, 1970b; Ohnishi and Lieber, 1977; Koop et al., 1982) and while this enzyme system functions primarily to detoxify xenobiotics, metabolic intermediates in this detoxification process are often electrophiles with mutagenic and carcinogenic activity (Miller and Miller, 1977).

The general approach we have utilized in studying ethanol's effect on microsomal P-450 mediated carcinogen metabolism has been to determine the capacity of microsomes, (prepared from tissues of rats which had been pair-fed alcohol containing and control liquid diets (Lieber et al., 1970b) to activate procarcinogens to mutagens detectable in the Ames mutagenesis assay (Ames et al., 1975) or to produce defined metabolic endproducts. The tissues from which microsomes have been prepared and studied include: the liver, which is the principal site of xenobiotic

metabolism; the lungs and intestines, which are major portals of entry for tobacco and dietary carcinogens, and the esophagus, a site at which ethanol consumption is a major risk factor.

Among the compounds utilized in these studies were a number of compounds found in tobacco smoke, namely benzo(a)pyrene (BAP) (Lieber et al., 1979; Seitz et al., 1978; Seitz et al., 1981), tobacco pyrolyzate (Seitz et al., 1981b), NPY (Farinati et al., 1985c), and two compounds which may be considered models of dietary carcinogens, namely, DMN (Garro et al., 1981) and tryptophan pyrolyzate (Seitz et al., 1981b). The results of these studies have recently been reviewed (Lieber et al., 1986) and can be summarized as follows: Chronic ethanol consumption generally enhances cytochrome P-450 mediated activation of procarcinogens and promutagens to genetically active metabolites. However, tissue specific effects can also be seen such as in the intestines and lungs. In the intestine, for example, ethanol increased microsomal activation of BAP and tryptophan pyrolyzate, but not tobacco pyrolyzate. In contrast, lung microsomes from ethanol-fed rats exhibited an enhanced capacity to activate the promutagens contained in tobacco pyrolyzate, but did not exhibit any increased activity towards tryptophan pyrolyzate.

In light of the marked synergism between smoking and alcohol consumption on UAT and URT cancers, which may also extend to the lower respiratory tract (Pollack et al., 1984), the enhanced capacity of lung microsomes from ethanol-fed animals to activate promutagens in tobacco pyrolyzate was of particular interest. The lack of an inductive effect for the activation of tryptophan pyrolyzate in the lung suggested that it was not simply protein degradation products that were being recognized in the pyrolyzed tobacco. The lung microsomes were therefore tested against two additional smoking-associated promutagens, namely, BAP and NPY. While no effect of ethanol was detected on the activation of BAP (Seitz et al., 1981a), the lung microsomes from ethanol treated rats exhibited a significantly enhanced capacity to activate NPY which was detectable in vitro at concentrations of 1mM (Farinati et al., 1985c). Moreover, this enhanced capacity for NPY activation, following chronic alcohol consumption, was also seen with microsomal fractions isolated from esophageal tissue (Farinati et al., 1985c) the site at which the combination of alcohol consumption and smoking have perhaps their greatest effect on cancer risk. The potential significance of these in vitro observations with respect to a mechanism for ethanol's cocarcinogenicity is strengthened by the fact that ethanol does indeed act as a cocarcinogen for NPY in URT tissue (McCoy et al., 1981).

DMN is another nitroso compound whose metabolism was found to be uniquely affected by chronic ethanol consumption. In contrast to other microsomal enzyme inducers such as phenobarbitol, 3-methylcholanthrene, and polychlorinated byphenyls, which act as inducers of high Km DMN demethylases and actually repress the activity of low Km isozymes (Venkatesan et al., 1968; Czygan et al., 1973; Guttenplan et al., 1977), ethanol increases cytochrome P-450 mediated DMN demethylase activity for both high (>40mM) and low (<1mM) isozymes (Garro et al., 1981). Enhanced activation of DMN to a mutagen by microsomes from ethanol-fed animals, actually was detectable in the Ames assay at DMN concentrations as low as 0.3mM (Garro et al., 1981).

It is likely that some of these effects on carcinogen metabolism are related to ethanol's induction of a unique species of cytochrome P-450 (Ohnishi and Lieber, 1977; Koop et al., 1982). Yang et al. (1985) have in fact demonstrated that an ethanol induced cytochrome P-450 has a selective affinity for DMN.

Whether this enhanced capacity for microsome-mediated procarcinogen activation actually results in an increased level of reactive metabolites either at the site of activation or at the other target sites will be influenced by a number of factors including: the route of carcinogen exposure; the presence of ethanol at the time of exposure, and the carcinogen detoxifying capacity of the tissues.

With respect to the latter, we have examined ethanol's overall effect on carcinogen activation and detoxification in the esophagus, which is not particularly rich in xenobiotic detoxifying systems and, as previously noted, is a target site of alcohol abuse-associated cancer. In order to obtain an overview of ethanol's effect on the capacity for carcinogen activation and detoxification in this tissue the following enzyme systems were studied: cytochrome P-450, because of its participation in both carcinogen activation and detoxification; glutathione (GSH) levels and GSH transferase (GSHT) activity, an important detoxifying system particularly in esophageal mucosa (Harris et al., 1979) and UDP-glucuronic acid transferase (UDPGT), another detoxifying enzyme which conjugates electrophiles with UDP-glucuronic acid. The results obtained (Farinati et al., 1985b), which are illustrated in Table 1, show that dietary ethanol has a much greater effect on cytochrome P-450 levels (which are increased approximately 5 fold following ethanol feeding) relative to GSH levels and GSHT and UDPGT activities. These results are consistent with the hypothesis that the increase in esophageal cancer associated with alcohol abuse is due, at least in part, to a shift in the balance between carcinogen activating and detoxifying capacity in this tissue.

ETHANOL'S EFFECT ON O^6-METHYLGUANINE TRANSFERASE (O^6-MeGT)

The DNA repair enzyme O^6-MeGT removes alkyl groups (methyl and ethyl) from the O^6 position of guanine, transferring them to cysteine residues, which appear to be located on the enzyme itself (Pegg and Perry, 1981b; Pegg et al., 1983; Harris et al., 1983). Since alkylation at the O^6 position of guanine is associated with both mutagenesis and carcinogenesis (Lewis and Swenberg, 1980; Newbold et al., 1980), this activity provides protection against carcinogenic alkylating agents, such as DMN and diethylnitrosamine.

In experiments, the purpose of which was to examine the effects of chronic ethanol consumption on DMN induced hepatic DNA alkylation in vivo, we were surprised to observe, in light of the aforementioned inductive effect of ethanol on DMN demethylase activity, that there were no significant differences between alcohol and control diet animals, in the initial extent of hepatic DNA alkylation in rats receiving DMN at a dose of 1 or 10 mg/kg (Farinati et al., 1984). In retrospect, this lack of effect of ethanol on initial levels of DNA alkylation is probably explainable by the complete metabolism of these DMN doses by both the ethanol and control animals within the initial 4-hour observation period. These studies did lead, however, to an

unexpected finding, namely, that hepatic O^6-MeG DNA adducts persisted for longer periods in ethanol-fed animals relative to controls. Moreover, this effect appeared to be specific for O^6-MeG repair as removal of acetylaminofluorene (AAF) DNA adducts, which are repaired by an excision pathway, were unaffected (Farinati et al., 1984). Representative data illustrating this differential effect of dietary ethanol on the two DNA repair pathways is given in Table 2.

This experiment suggested that chronic ethanol consumption produces a decrease in O^6-MeGT activity in hepatic tissue. This was a totally unexpected consequence of ethanol consumption and suggested yet another mechanism through which ethanol might act as a cocarcinogen. We pursued this observation and verified that chronic ethanol consumption does indeed depress O^6-MeGT activity. It was found that after 4 weeks on an ethanol-containing (36% of total caloric intake as ethanol) diet, O^6-MeGT activity was reduced approximately 40% relative to controls (Table 3) (Farinati et al., 1985a). Other experiments have shown that 50mM ethanol, a concentration which corresponds to a blood level seen in alcohol abusers, directly inhibits O^6-MeGT activity (Farinati et al., 1984). Methanol also was observed to inhibit O^6-MeGT activity and results illustrating the inhibitory effect of ethanol and methanol are shown in Table 4. In contrast to this inhibition, ethanol at in vitro concentrations up to 100mM did not

produce detectable inhibition of GSHT activity. In addition, neither butanol at 50mM nor the solvent dimethylsulfoxide at concentrations as high as 100mM inhibited O^6-MeGT activity. These results indicate that ethanol's effect on O^6-MeGT activity is not due simply to a change in dielectric constant of the solution.

The direct inhibition of O^6-MeGT activity by ethanol does not totally explain the depression in O^6-MeGT activity illustrated in Table 2, since in that experiment the ethanol-containing diet was withdrawn 18 h prior to sacrifice and removal of the livers. In 18 h all blood alcohol would have been cleared and thus the experiment indicates that the effect of chronic ethanol feeding persists even after ethanol has been removed from the circulation. One possible explanation is that ethanol also depresses O^6-MeGT synthesis, and this may be an important factor in terms of basal O^6-MeGT activity present in tissue. Alternatively the enzyme may be irreversibly inactivated by ethanol or its metabolite acetaldehyde. O^6-MeGT activity has in fact been shown to be particularly sensitive to formaldehyde inhibition (Grafstrom et al., 1985).

It should be noted that there have been studies which failed to detect an effect of dietary ethanol on the repair of DMN induced O^6MeG (Schwarz et al., 1982; Belinsky et. al, 1982). In one of these studies, however, the last time

point at which O^6-MeG levels were examined was only 4 hours after a single injection of ^{14}C-labeled DMN (Schwarz et al., 1982). That no differences were observed in O^6-MeG levels between ethanol-fed and control diet animals in this particular study (Schwarz et al., 1982) is actually consistent with our data over this time period. In the second study (Belinsky et al., 1982), the levels of O^6-MeG induced by the administration of DMN at a dose of 25mg/kg to rats which had been fed ethanol or control diets declined at the same rate for both diet groups, over a 72 h period. This observation conflicts with our results. The basis for this difference is not known with certainty, but may be due to differences in feeding protocols. In the study where no effect of ethanol was seen on O^6-MeG repair, it appears that the rats were maintained on diets with approximately 50% the caloric intake of our animals, and thus the animals may have been undernourished. Since in the rat O^6-MeGT is an inducible enzyme for which the effects of nutrition on inducibility are not known, the lack of effect of ethanol in this study may be due to low or reduced levels of O^6-MeGT in both diet groups.

SUMMARY

Through the use of pair feeding nutritionally adequate, isocaloric liquid diets, a procedure pioneered by Lieber and his coworkers, it has been possible to isolate ethanol as a dietary variable and study its effects on processes relevant to carcinogenesis. In the experiments described above, several mechanisms whereby dietary ethanol may act as a cocarcinogen and thus contribute to the increased cancer risk associated with alcohol abuse have been illustrated. Dietary ethanol has been shown to be a potent inducer of microsomal enzymes involved in carcinogen activation in a variety of tissues. This effect may be particularly important in tissues which do not contain high levels of xenobiotic detoxifying systems and in which ethanol may shift the balance between carcinogen activation and detoxification towards activation. In addition, dietary ethanol has been found to interfere with repair of alkylated DNA mediated by O^6-MeGT and persistence of O^6-MeG has been associated with carcinogenicity of several alkylating agents.

TABLE 1

Effects of Chronic Ethanol Consumption on Carcinogen Activating and Detoxifying Enzyme Systems in Rat Esophagus

Diet[a] group	Cytochrome P-450	GSH	GSH transferase	UDP-glucuronic acid transferase
	pmol/mg protein	umol/g tissue	nmol/mg protein/min	nmol/mg protein/min
Ethanol	220 ± 22*	0.6 ± 0.2**	69 ± 7**	6 ± 0.5
Control	38 ± 22	0.4 ± 0.2	46 ± 4	5 ± 0.3

[a] Mucosa was scraped from esophagi of rats which had been pair-fed ethanol (36% total calories as ethanol) or dextrimaltose-containing diets for 4 weeks. At least 5 pairs of rats were used for each determination and for P-450 levels three pools of mucosal tissue, each pool obtained from five pairs were used. Results are expressed as means ± SEM.

* $p<0.01$

** $p<0.05$

TABLE 2

Effects of Dietary Ethanol on the in Vivo Repair of DMN and Acetylaminofluorene-Induced DNA Alkylation.

Diet Group*	Fraction of original adduct remaining 36 hr after DMN or acetylaminofluorene exposure	
	O^6-Methylguanine	N-Deoxyguanosine-8-yl-AAF
	Percent	Percent
Ethanol	71%	27%
Control	11%	27%

* Rats were pair fed either the ethanol or control diet for 4 weeks; 18 hr prior to carcinogen administration, both groups were provided with the control diet, which was continued to the end of the experiment.
[^{14}C] DMN (0.62mCi/mmol) was administered as a single intraperitoneal injection of 10mg/kg; ^3H-labeled acetylaminofluorene (11.2mCi/mmol) was administered as a single intraperitoneal injection of 30mg/kg.

TABLE 3

Effect of Dietary Ethanol on Hepatic O^6-MeGT Activity

Diet Group*	O^6-Methylguanine removed
	fmol/min/mg protein
Control	4.42 ± 0.17
Ethanol	2.52 ± 0.31

Results are expressed as means ± SEM and are significantly different with a $p<0.01$.

* Five pairs of rats were pair-fed isocaloric ethanol-containing (36% of total calories as ethanol) or dextrimaltose control diets for a period of 4 weeks; 18 hr prior to deaths the ethanol containing diet was removed and both groups of animals were fed the control diet.

TABLE 4

Direct Effect of Ethanol and Methanol on O^6-MeGT Activity

	O^6-Methylguanine removed
	pmol/30 min
Liver Extract	2.85 ± 0.36
+ 50mM ethanol	1.74 ± 0.41*
+ 50mM methanol	1.36 ± 0.35**

O^6-MeGT was partially purified by ammonium sulfate fractionation of liver homogenates prepared from three hepatectomized rats. Enzyme activity was assayed in 50mM Tris-HCl, ph 8.3, 1mM dithiothreitol, 0.1mM EDTA. The DNA substrate contained 10pmol of O^6-methylguanine and 5 mg of liver homogenate protein were used in the assays.

* $p<0.05$
** $p<0.01$

CHAPTER 2

Submitted to *Carcinogenesis* for publication

Title: **In Vitro and In Vivo Inhibitory Effect of Ethanol and Acetaldehyde on O^6-Methylguanine Transferase**

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ABSTRACT

Human and rat O^6methylguanine transferase (O^6MeGT) is inhibited in vitro by ethanol at concentrations of 10 to 50mM and by acetaldehyde, the first metabolite of ethanol, at concentrations as low as 0.01uM. Several other enzymes, including glyceraldehyde-3-phosphate dehydrogenase and yeast alcohol dehydrogenase, which like O^6MeGT have cysteines in their active sites were not inhibited by acetaldehyde at the levels that inhibited O^6MeGT. Disulfiram, an acetaldehyde dehydrogenase inhibitor, enhanced the inhibitory effect of ethanol in vivo. These results indicate that the inhibitory effect of ethanol on O^6MeGT activity is mediated primarily via its metabolite, acetaldehyde.

INTRODUCTION

Epidemiological studies have shown that alcohol abuse, particularly in association with smoking, is linked to an increased risk of upper alimentary tract and upper respiratory tract cancers (Williams and Horm, 1977; Chilvers et al., 1979; Tuyns, 1978; MacGlashan et al., 1982). This association together with the increased cancer incidence observed at several other sites in alcohol abusers (Creech and Johnson, 1974; Kunze et al., 1986; O'Connell et al., 1987; Harvey et al., 1987) has prompted studies of possible mechanisms through which ethanol may affect carcinogenesis. One result of these studies has been the demonstration that ethanol enhances microsome mediated activation of several procarcinogens such as dimethylnitrosamine (DMN) (Garro et al., 1981) and nitrosopyrrolidine (NPY) (Farinati et al., 1985c) at several tissue sites, apparently as a result of a specific ethanol induced cytochrome P-450 (Lieber and DeCarli, 1970b; Ohnishi and Lieber, 1977; Koop et al., 1982). More recently, we have observed a decreased capacity (in ethanol-fed rats) for the repair of DMN-induced alkylation of the O^6 position of guanine (Garro et al., 1986b). The persistence of this DNA adduct, O^6-methylguanine (O^6MeG) has been correlated with the induction of base substitution mutations in bacteria (Loveless, 1969; Newbold et al., 1980), cell transformation

in cultured human cells (Yarosh, 1985; Sklar et al., 1981) and with organ susceptibility to carcinogenic alkylating agents (Goth and Rajewsky, 1975; Montesano, 1981). The production of O^6MeG adducts appears to be a major initiating event in the induction of cancers by dimethylnitrosamine, methylnitrosourea and other similar alkylating agents.

O^6MeG is repaired by O^6-methylguanine transferase (O^6MeGT) also referred to as alkylguanine transferase which removes the alkyl group from the O^6 position of guanine, transferring it to a cysteine residue on the enzyme itself which in turn is inactivated in the process (Pegg and Perry, 1981a; Mehta et al., 1981; Hora et al., 1983).

The persistence of O^6MeG adducts in DMN treated rats which had been fed an ethanol-containing diet (36% of total calories as ethanol) for 4 weeks prior to DMN exposure has been associated with a decrease in O^6MeGT activity which persists for at least 18 hours following the termination of ethanol consumption (Garro et al., 1986b). Ethanol, at concentrations that are physiologically significant (10 to 50mM) with respect to blood levels produced by drinking, has been observed to inhibit O^6MeGT activity in vitro (Garro et al., 1986b). This apparent direct inhibitory effect of ethanol on O^6MeGT activity did not explain, however, the in vivo observations (Garro et al., 1986b) in which there was a persistence of O^6MeG and depression of O^6MeGT activity since, in these studies, the ethanol diet was withdrawn 18

hours prior to O^6MeGT assay or DMN exposure. There appeared therefore, to be a persistent effect of ethanol feeding on O^6MeGT-mediated DNA repair. Since such an effect could be due to irreversible inactivation of O^6MeGT we examined the ability of acetaldehyde, the reactive first metabolite of ethanol, to inhibit O^6MeGT activity at acetaldehyde concentrations that occur in vivo following ethanol consumption.

MATERIALS AND METHODS

Sources of Chemicals

O^6MeG was a generous gift from Dr. J. B. Guttenplan, Department of Biochemistry, New York University Dental Center, New York, N.Y. 10010. Calf thymus DNA, methylnitrosourea (MNU), disulfiram (DSF), dimethylsulfoxide (DMSO) and other chemical reagents were obtained from Sigma Chemical Company, St. Louis, MO. Ethanol (HPLC grade) and acetaldehyde (HPLC grade) were obtained from Fisher Scientific Company, Fairlawn, N.J.

Preparation of Alkylated DNA Substrate

80mg of calf thymus DNA was methylated by reaction with 5mg of MNU in 15ml of 80mM sodium phosphate buffer, pH 8.0, for 30 minutes at 37°C. The DNA was precipitated with ethoxyethanol, washed four times with ethanol, once with ether and dried. Alkylated DNA was dissolved in TDE assay buffer (50mM Tris-HCl pH 8.3, 1mM DTT, 0.1mM EDTA) and dialyzed overnight before use. This treatment generally produced 0.2 to 2.2 pmol O^6-MeG per ug DNA.

Preparation of O^6-Methylguanine Transferase

O^6MeGT was partially purified, by the method of Pegg et. al. (Pegg et al., 1981; Pegg et al., 1983) both from autopsy samples from patients that showed no sign of liver disease and from male Sprague-Dawley rats that were subjected to two thirds partial hepatectomy and sacrificed three days later. In summary, rat and human liver samples were homogenized in 3 vol of ice cold TDE buffer and the homogenate centrifuged at 12,000 x g for 10 min. The supernatant was removed and the pellet was resuspended in buffer and sonicated. The sonicated extract was then combined with the original supernatant and centrifuged at 22,000 x g for 30 min. The resultant supernatant was fractionated by ammonium sulfate precipitation and the pellet from the 25% to 55% ammonium sulfate cut was dissolved in 1 vol of the original tissue homogenate and dialyzed overnight. The dialysate then was centrifuged at 22,000 x g for 30 min and the supernatant was collected and used in the assays.

Assay of O^6-Methylguanine Transferase

The assay procedure used was of that described by Pegg et.al. (Pegg et al., 1981; Pegg et al., 1983). The assay mixture consisted of 25-75ug of methylated DNA (containing 0.2 to 2.2pmol O^6MeG/ug DNA) and 1-3mg protein extract in 1ml of TDE buffer. Ethanol and acetaldehyde were diluted in this buffer and added to the reaction mixtures to produce final concentrations of 10mM-50mM and 0.01uM-5uM respectably. All of the above reactants were mixed at 4°C with the enzyme being the last component added. The reaction mixture was then transferred to 37°C and incubated for 30 min. The reactions were stopped by the addition of 200ul of 1.5N perchloric acid. The resulting precipitates were collected by centrifugation at 10,000 x g for 10 min and acid hydrolyzed in 1ml 0.1N HCl at 80°C for 30 minutes. The hydrolysates were then chilled and centrifuged at 10,000 x g for 10 minutes. The supernatants were removed, passed through a 0.45um filter and 200ul samples were subjected to HPLC separation on a Waters RCM-100 Partisil SCX column. Purines, including O^6MeG, were eluted with 50mM ammonium formate, pH 2.5 at a flow rate of 2ml per min. Guanine and adenine were monitored at 254nm using a Waters Model 440 absorbance detector. O^6MeG and N^7MeG were monitored by fluorescence using a McPherson Model 750 fluorescence detector equipped with a high sensitivity accessory (288nm

excitation; 360nm cut-off emmission filter). The absorbance and fluorescence detectors were interfaced with an Apple II computer using the Model AI13 Data Aquisition System (Interactive Microware, Inc. PA). A computer program was developed to quantitate peak areas. Standard curves with known amounts of O^6-MeG and other bases were constructed and compared with amounts present in assay DNA hydrolysates. The limit of detection for O^6-MeG was 1 pmol per injection.

Other Enzyme Assays

Glyceraldehyde-3-phosphate dehydrogenase (GAPD), yeast alcohol dehydrogenase (YADH), both of which contain -SH groups in their active sites (Vallee and Hoch, 1955; Bloch et al., 1971;) and isocitrate dehydrogenase (IDH) and glutathione-S-transferase (GSHT) were assayed as follows: GAPD activity was measured as a coupled reaction with phosphoglycerate kinase using 3-phospho-glycerate as substrate and recording the decrease in absorbance at 340nm resulting from the oxidation of NADH (Bloch et al., 1971). YADH activity was monitored by the increase in absorbance at 340nm resulting from the reduction of NAD (Vallee and Hoch, 1955). IDH activity was measured by monitoring the increase in absorbance at 340nm upon conversion of NADP to NADPH (Hanson and Cox, 1967). GSHT activity was assayed according to the method of Habig et.al (Habig et al., 1974).

1-chloro-2,4 dinitrobenzene (CDNB) was used as the substrate for this reaction and the increase in absorbance at 340nm which results from conjugation of CDNB was determined.

Treatment of Animals

Sprague-Dawley rats were lightly anesthetized with ether to facilitate gastric intubation. Dilutions of ethanol in distilled water were made so that a constant volume was administered to each rat. Disulfiram was dissolved in DMSO (100mg/ml) and then injected i.p. (generally less than 0.25ml) at a dose of 100mgdisulfiram/kg four hours prior to ethanol administration. Animals were sacrificed by cervical dislocation at various times after ethanol administration.

RESULTS

Addition of ethanol to human O^6MeGT produced a level of inhibition of enzyme activity similar to that previously reported (Garro et al., 1986b) for the rat enzyme (Table 1). This level of inhibition, 38% to 39% at 50mM ethanol is statistically significant with a $p<0.003$ for the human enzyme preparation and a $p<0.001$ for the rat enzyme preparation which was included for comparison. Other enzymes such as isocitrate dehydrogenase, glutathione transferase and UDPG transferase were not inhibited by 50mM ethanol (data not shown, see also Garro et al., 1986b).

The same in vitro approach was used to determine the sensitivity of human and rat liver O^6MeGT to acetaldehyde, the first metabolite of ethanol. The inhibitory effect of acetaldehyde, over a concentration range of 0.01 to 1.0uM, on human and rat O^6MeGT is shown in Figure 1. The extent of inhibition is similar for both rat and human liver O^6MeGT. No inhibition was observed for GAPD and YADH, both SH-active site enzymes nor for cytosolic enzymes, IDH and GSHT.

Considering the low levels of acetaldehyde which produced an inhibitory effect comparable to that produced by 50mM ethanol, it seemed possible that the inhibitory effect of ethanol itself might be due to trace amounts of acetaldehyde normally present in stock solutions of ethanol. To test this, the effect of freshly distilled ethanol on rat

O^6MeGT was compared to that of stock ethanol. As seen in Figure 2, the inhibitory effect of ethanol is reduced but not totally eliminated by distillation. At 10mM ethanol, there was no inhibitory effect with freshly distilled ethanol while the stock ethanol produced a significant level ($p<0.002$) of inhibition. At 50mM ethanol, significant levels of inhibition were produced by both freshly distilled and stock ethanol solutions although the level of inhibition with the distilled ethanol was significantly lower ($p<0.002$) than that produced by the stock ethanol.

As previously noted chronic ethanol feeding results in decreased O^6MeGT activity in rat liver which persists for up to 18hr post ethanol. It was of interest, therefore , to see the effect of an acute dose of ethanol on hepatic O^6MeGT and the time needed to regenerate O^6MeGT activity. The effect of an intragastrically administered dose of ethanol (3g/kg), which should produce blood alcohol concentrations of 50-60mM and blood acetaldehyde levels up to 5uM within 30 minutes (Gordon et al., 1985; Guerri and Sanchis, 1986) is shown in Figure 3. At 1 hour after ethanol administration, O^6MeGT activity was significantly decreased ($p<0.005$) to a level 60% that of the control values. Partial recovery of O^6MeGT is apparent at 12 hours post ethanol, although the levels of O^6MeGT activity remain significantly depressed up through 24 hours post ethanol.

In order to determine if inhibiting the breakdown of acetaldehyde to acetate would potentiate the inhibitory effect of ethanol on O^6MeGT, rats were pre-treated with disulfiram, an acetaldehyde dehydrogenase inhibitor (Graham, 1951) 4 hours prior to ethanol exposure. Ethanol was administered orally at doses of 0.03, 0.1 and 0.3g/kg and the rats were sacrificed 16 hours later. Figure 4 shows both a dose-dependent ethanol-mediated decrease in O^6MeGT activity and that disulfiram pretreatment further enhances the inhibitory effect of ethanol on O^6MeGT activity at all concentrations tested.

DISCUSSION

The results presented in this study demonstrate that acetaldehyde, the first metabolite of ethanol, inhibits both rat and human O^6MeGT at concentrations that are physiologically significant. Although there has been controversy over the actual blood levels of acetaldehyde that are produced in humans and animals following ethanol consumption (Eriksson, 1980; DeMaster et al., 1983), acetaldehyde measurements have been reported in the range of 0.1uM to 30uM (Lundquist, 1983). Our results indicate that O^6MeGT is sensitive to this range of acetaldehyde concentrations with 0.1uM acetaldehyde producing approximately a 50% decrease in O^6MeGT activity which is further decreased at 1.0uM acetaldehyde to 10% of the original activity in an in vitro reaction mixture.

It is known that aldehydes react with several functional groups on proteins including the thiol groups of cysteine residues. O^6MeGT has a cysteine residue in its active site which acts as the acceptor for the alkyl group removed from DNA during the repair reaction and is a potential target for acetaldehyde. If this is the target on O^6MeGT, however, it appears to be particularly sensitive to acetaldehyde since at least two other enzymes which have cysteines in their active sites, namely glyceraldehyde-3-phosphate dehydrogenase and yeast alcohol dehydrogenase are

not inhibited by acetaldehyde at the concentrations used in this study.

Previous studies have shown that other aldehydes including formaldehyde and acrolein also inhibit O^6MeGT activity when these aldehydes are added either to human lung fibroblast cell cultures or in vitro assay mixtures using fibroblast O^6MeGT (Krokan et al., 1985; Grafstrom et al., 1985). However in these studies, no inhibition of O^6MeGT activity was observed with acetaldehyde at concentrations up to 300uM. The basis for these differences in results is not clear but may be due either to differences in the source of O^6MeGT, liver vs. fibroblasts or to procedural differences in assay conditions. In this study, the enzyme assay mixtures were kept ice cold and pre-mixed with all components before transfer to 37°C in contrast to the referred to study in which the aldehydes were added to cultures or enzyme assays at 37°C. Alternatively the noted differences in observations may be the result of differences in the specific activities of the O^6MeGT preparations. The human fibroblast preparations had O^6MeGT specific activities in the range of 0.14 to 0.19pmol/mg protein in contrast to the human hepatic preparations used here which ranged from 2 to 14 pmol/mg. The extraneous proteins in the fibroblast study may have reacted with the acetaldehyde thus lowering the effective concentration available for reaction with O^6MeGT.

With respect to the previously reported inhibitory effect of ethanol on rat O^6MeGT activity (Garro et al., 1986b) and the effect on human O^6MeGT shown in Table 1, it appears likely that this effect is due largely to the presence of trace levels of acetaldehyde that either spontaneously formed from the ethanol itself or was formed from traces of alcohol dehydrogenase (ADH) present in the O^6MeGT preparations. Dilutions of our ethanol stocks were assayed for acetaldehyde and while none was detected at the limits of detection (1uM), results presented here demonstrate that 0.05uM acetaldehyde produces a level of inhibition of O^6MeGT comparable to 50mM ethanol. ADH activity was detectable in the O^6MeGT extracts but only following the addition of its cofactor, NAD, and while the O^6MeGT preparations were extensively dialyzed, there is a possibility that trace levels of NAD were not removed.

An acute dose of ethanol (3g/kg) results in 50-60mM blood alcohol levels and up to 5uM blood acetaldehyde levels in rats (Gordon et al., 1985; Guerri and Sanchis, 1986). The time course (Figure 3) shows that after an acute dose of ethanol, O^6MeGT activity is reduced to 60% of control values at 1 hour after ethanol administration and remains significantly depressed for up to 24 hours. The O^6MeGT activity recovers to its control levels by 48 hours.

The effect of disulfiram on ethanol-mediated inhibition of O^6MeGT activity (Figure 4) provides additional

evidence that the inhibitory effect of ethanol is mediated by its metabolite acetaldehyde. The results presented in Figure 4 also demonstrate that ethanol ingested at a dose as low as 0.03g/kg produces a significant inhibition of hepatic O^6MeGT activity and that the levels of inhibition increases in a dose dependent manner.

In summary, the results presented here indicate that the inhibitory effects of dietary ethanol on O^6MeG repair is mediated primarily through its reactive metabolite, acetaldehyde. This effect of alcohol may contribute to the increased cancer risk that has been associated with alcohol abuse. By inhibiting the repair of O^6MeG, chronic ethanol consumption may increase the mutagenic and carcinogenic potential of various nitroso compounds either present in alcoholic beverages (Goff and Fine, 1979; Walker et al., 1979) and other environmental sources or formed endogenously (Correa and Tannenbaum, 1981).

TABLE 1

Effect of Ethanol on Rat and Human Liver O^6-Methylguanine Transferase

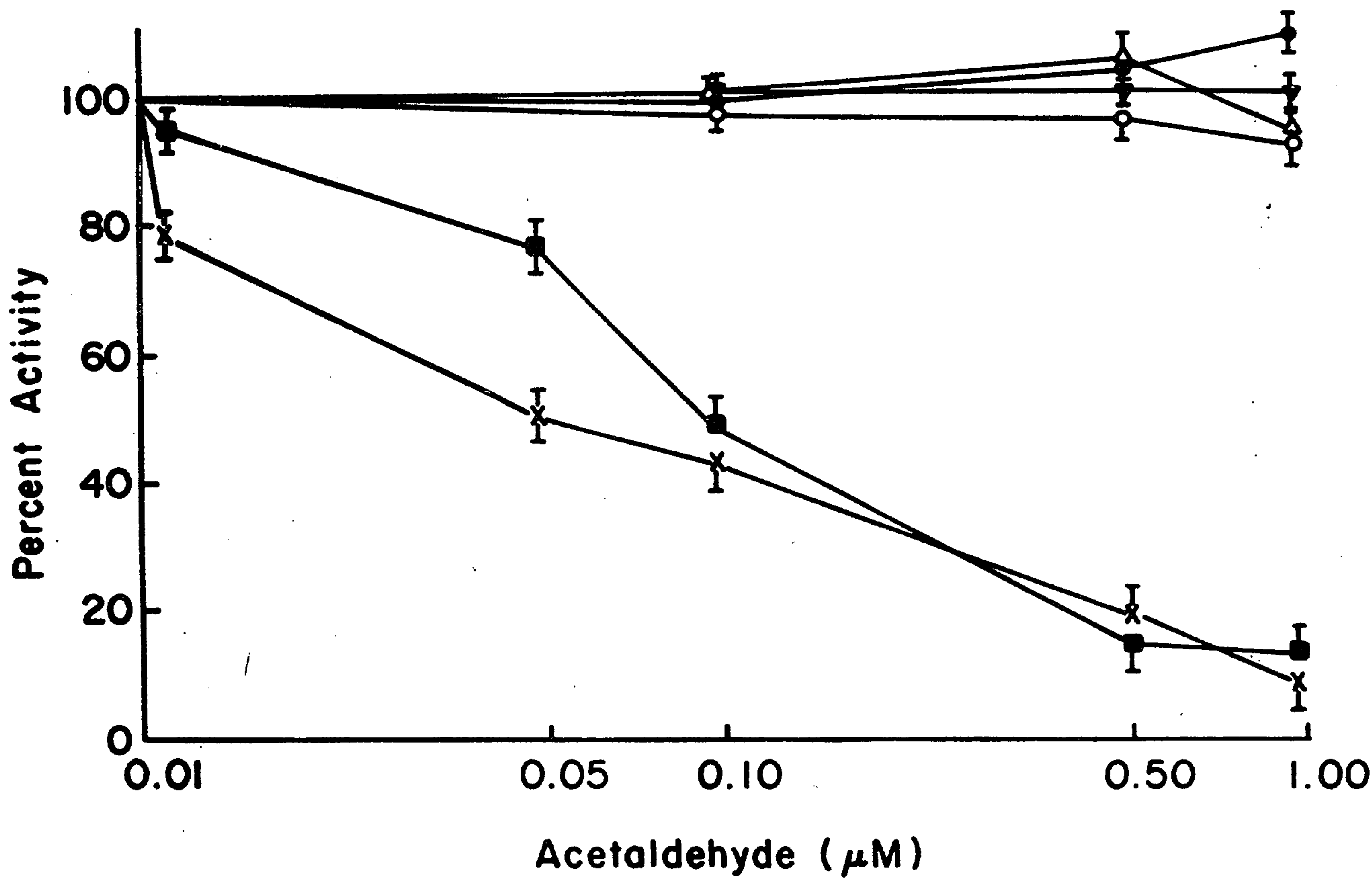
	pmol O^6-MeG removed/30min.	Percent Inhibition
Rat Liver (RL) Extract	4.69 ± 0.25	---
RL Extract + 10mM EtOH	4.25 ± 0.12 *	9%
RL Extract + 50mM EtOH	2.85 ± 0.13 **	39%
Human Liver (HL) Extract	14.39 ± 0.93	---
HL Extract + 10mM EtOH	13.30 ± 0.56	8%
HL Extract + 50mM EtOH	8.93 ± 0.98 ***	38%

O^6-MeGT assays were carried out as described in the "Materials and Methods" section. The assay mixtures contained 2mg O^6MeGT extract protein. The DNA substrates contained 15pmol O^6MeG/50ug and 55pmol O^6MeG/25ug DNA for the rat and human O^6MeGT assays, respectively. Under these conditions, the repair reaction was linear with respect to protein concentration and time of reaction.

* $p<0.05$
** $p<0.001$
*** $p<0.003$

Figure 1. Inhibition of O^6-MeGT Activity by Acetaldehyde.

The acetaldehyde inhibition values are expressed as percent of enzyme activities observed in the absence of acetaldehyde; (●) Isocitrate Dehydrogenase (IDH), (○) Glutathione-S-Transferase (GSHT), (▲) Glyceraldehyde-3-phosphate Dehydrogenase (GAPD), (△) Yeast Alcohol Dehydrogenase (YADH). The O^6MeGT activities of rat liver extract (■) and human liver extract (X) were 4.89 pmol O^6MeG/mg and 14.09 pmol O^6MeG/mg, respectively. The activities of IDH, GSHT, GAPD and YADH were 302, 875, 7.30 and 1.65 U/mg respectively where 1 unit (U) equals the conversion of 1nmol substrate per minute.



Percent Activity
100
80
60
40
20
0
0.01
0.05
0.10
0.50
1.00
Acetaldehyde (μM)

Figure 2. Effect of Stock Ethanol and Freshly Distilled Ethanol on O^6MeGT Activity.

Results are expressed as the MEAN ± SEM of triplicate assays. The assay mixtures contained 1mg rat hepatic extract protein and 62ug of DNA containing 12.4pmol O^6MeG.

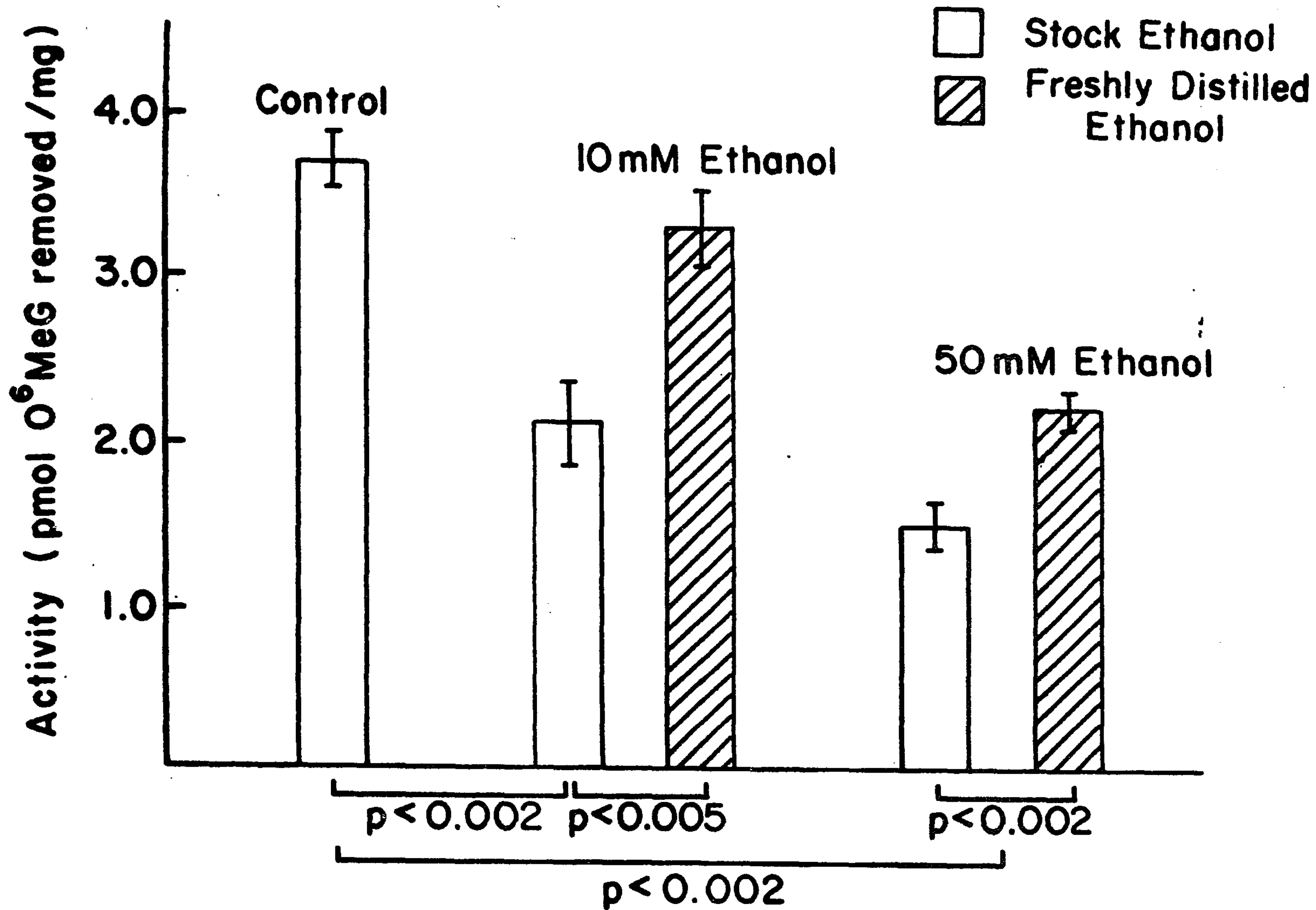
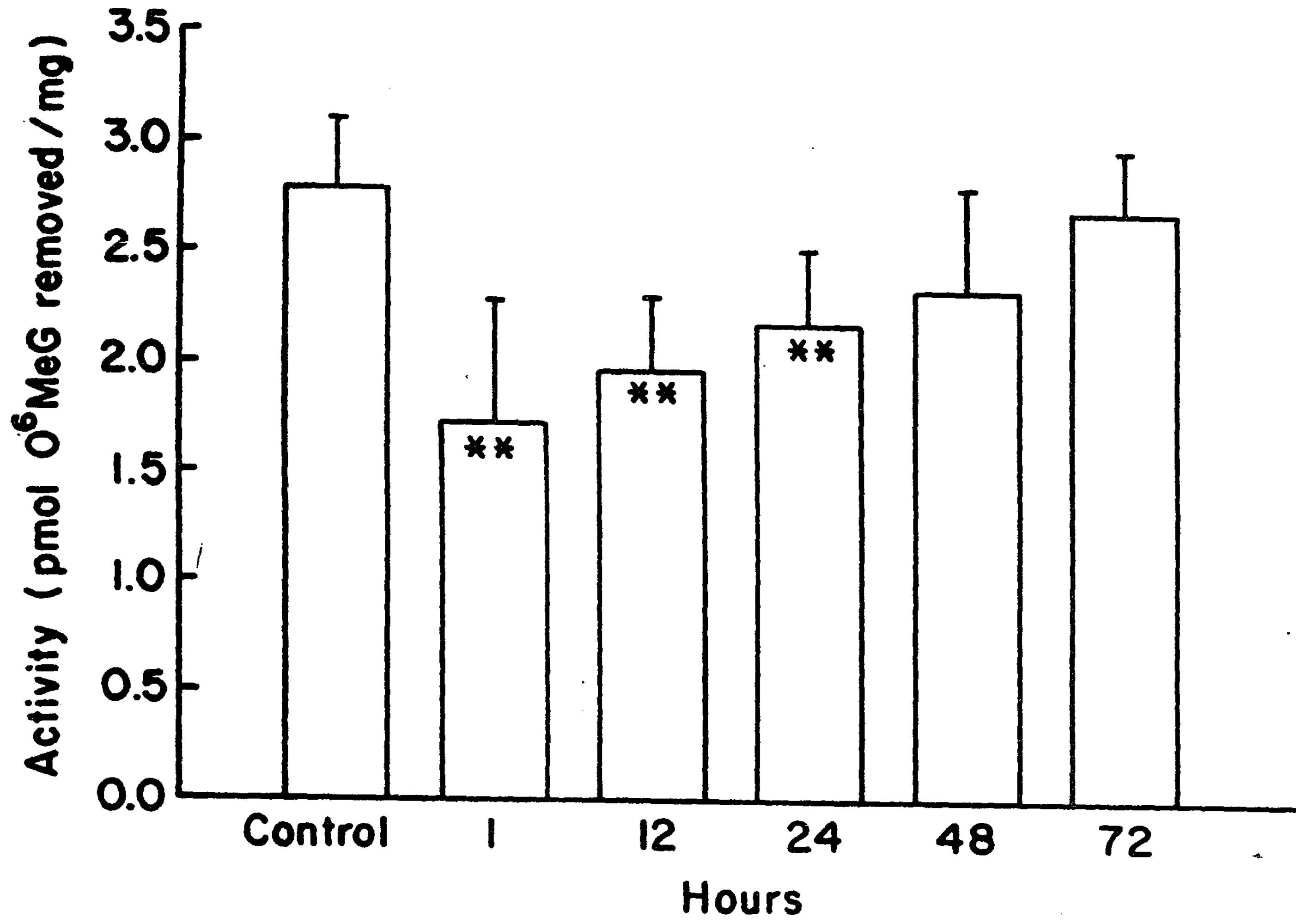

Stock Ethanol
Freshly Distilled Ethanol
Control
10mM Ethanol
50mM Ethanol
Activity (pmol O6MeG removed/mg)
4.0
3.0
2.0
1.0
p<0.002
p<0.005
p<0.002
p<0.002

Figure 3. Effect of Oral Administration of Ethanol on O^6MeGT Activity.

Sprague-Dawley rats were given an acute dose of ethanol (3g/kg) p.o. and sacrificed at various times. O^6MeGT was prepared from livers and assays were done as described in "Materials and Methods". Results are the MEAN $\pm$ SEM of triplicate assays from 3 rats at each time point.

** $p<0.005$

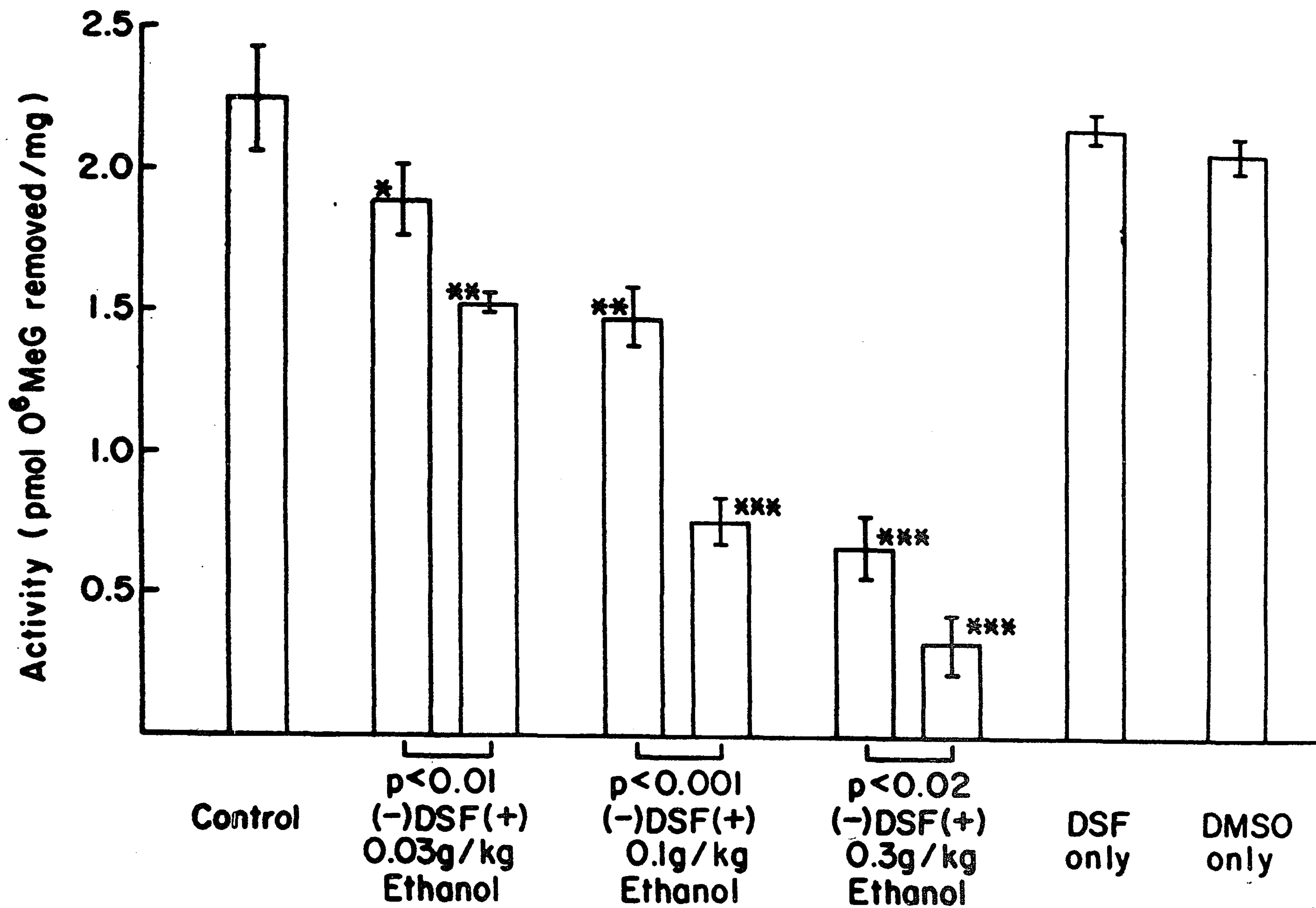


Activity (pmol O6MeG removed/mg)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
**
**
**
Control
1
12
24
48
72
Hours

Figure 4. Effect of Disulfiram and Ethanol on O^6 Methylguanine Transferase.

Sprague-Dawley rats were treated with 0.03, 0.1 and 0.3g/kg ethanol (p.o.). Disulfiram treated rats were injected (100mg/kg i.p.) at four hours prior to ethanol administration. Control groups received either distilled water (p.o.), disulfiram or solvent DMSO alone (i.p.). Results shown are the MEAN±SEM of triplicate assays from 3 rats from each group.

* $p<0.05$
** $p<0.003$
*** $p<0.001$


Activity (pmol O6MeG removed/mg)
2.5
2.0
1.5
1.0
0.5
*
**
**
***
***
***
Control
p<0.01
(-)DSF(+)
0.03g/kg
Ethanol
p<0.001
(-)DSF(+)
0.1g/kg
Ethanol
p<0.02
(-)DSF(+)
0.3g/kg
Ethanol
DSF
only
DMSO
only

Chapter 3

Unpublished Data and Methods Development

INTRODUCTION

This chapter will focus on some data that were not included in previous chapters but that answered such basic questions as: 1. What are the best storage conditions for liver tissue samples and for O^6MeGT extracts? 2. Does ethanol inhibit O^6MeGT activity in a dose dependent fashion? 3. Is ethanol inhibition reversible? 4. What is the effect of other alcohols on O^6MeGT and on other soluble and microsomal enzymes? 5. Does ethanol also affect excision repair of adducts such as those formed by acetylaminofluorene (AAF).

Finally, the development of an HPLC assay for O^6MeGT will be described. This includes the development of a program written in Basic for the Apple II computer that was interfaced with the absorbance and fluorescence detectors and which allowed for a more rapid assay of O^6MeGT activity.

Storage Conditions for Liver Extracts:

In the original method for assaying O^6MeGT activity, the DNA substrate was alkylated with N-^{3}H-methyl-nitrosourea and Sephadex G-10 column chromatography was used to separate the purine bases which were quantified by absorbance spectroscopy (A and G) and scintillation counting (N^7MeG and O^6MeG). This procedure while providing good separation of alkylated and non-alkylated bases (Figure 1), required a chromatography run time of 15-17 hours to elute the radioactive O^6MeG. In addition, counting the radioactivity in the approximately 30 fractions of the O^6MeG peak required another 3 hours. Therefore, the total processing time for assaying the enzyme took almost 20 hours.

Because of the length of time required for each assay, we needed to determine storage conditions under which O^6MeGT preparations would remain stable. In anticipation of our studies with human liver O^6MeGT, we also sought to determine suitable storage conditions for pieces of liver that we would acquire. Human liver slices were obtained from autopsies that were performed at Mount Sinai Hospital. Twenty to fifty gram samples of the liver were removed within 16 hours after the patient expired.

Table 1 shows that overnight storage of rat liver slices at -20°C or 4°C maintains 85% and 78% activity, respectively. Storage of O^6MeGT extracts at -20°C for up to

four weeks maintains 82% of its activity whereas activity was lost during overnight storage of enzyme or liver slices at -70°C. These storage conditions for the rat O^6MeGT was extrapolated to the human liver enzyme. Due to the difficulty of obtaining fresh human liver samples, as close to the time of death as possible, we had to rely on the pathologist who performed the autopsy to remove liver slices and store them under conditions that we established. Based on the storage conditions for the rat liver O^6MeGT, human liver slices were stored at -20°C before processing for the enzyme.

Figure 1. Separation of purine bases from acid hydrolyzed DNA using Sephadex G-10 as per original method for assay of O^6MeGT.

O^6MeGT was prepared from partially hepatectomized rats as described in the previous chapter. The radiolabeled DNA substrate was prepared by reacting calf thymus DNA with 1mCi N-^{3}H-methyl-nitrosourea (specific activity 1Ci/mmol). The assay mixture contained 0.85mg of ^{3}H-methylated DNA (containing 14.5 pmol of O^6-methylguanine), 1-10mg of liver extract in a total of 3 ml assay buffer. The subsequent steps were essentially the same as described in the previous chapter. The radiolabeled DNA present in the reaction pellet was acid hydrolyzed, neutralized with NaOH and then applied onto a Sephadex G-10 column (1cm X 80cm) which was equilibrated in 50mM ammonium formate pH 6.8 and 0.02% NaN_3. 120 fractions (5ml each) were collected at a flow rate of 18ml/hr. Guanine and adenine peaks were measured by absorbance at 260nm. The N^7 and O^6 methylguanine were then determined by counting the radioactively labeled methyl group in a liquid scintillation counter. The levels of O^6methylguanine observed were about 12% that of the N^7 methylguanine. Roughly one guanine base per 4 x 10^5 guanine bases is alkylated at the O^6 position. In this assay, recovery of N^7methylguanine, adenine and guanine was over 95%.

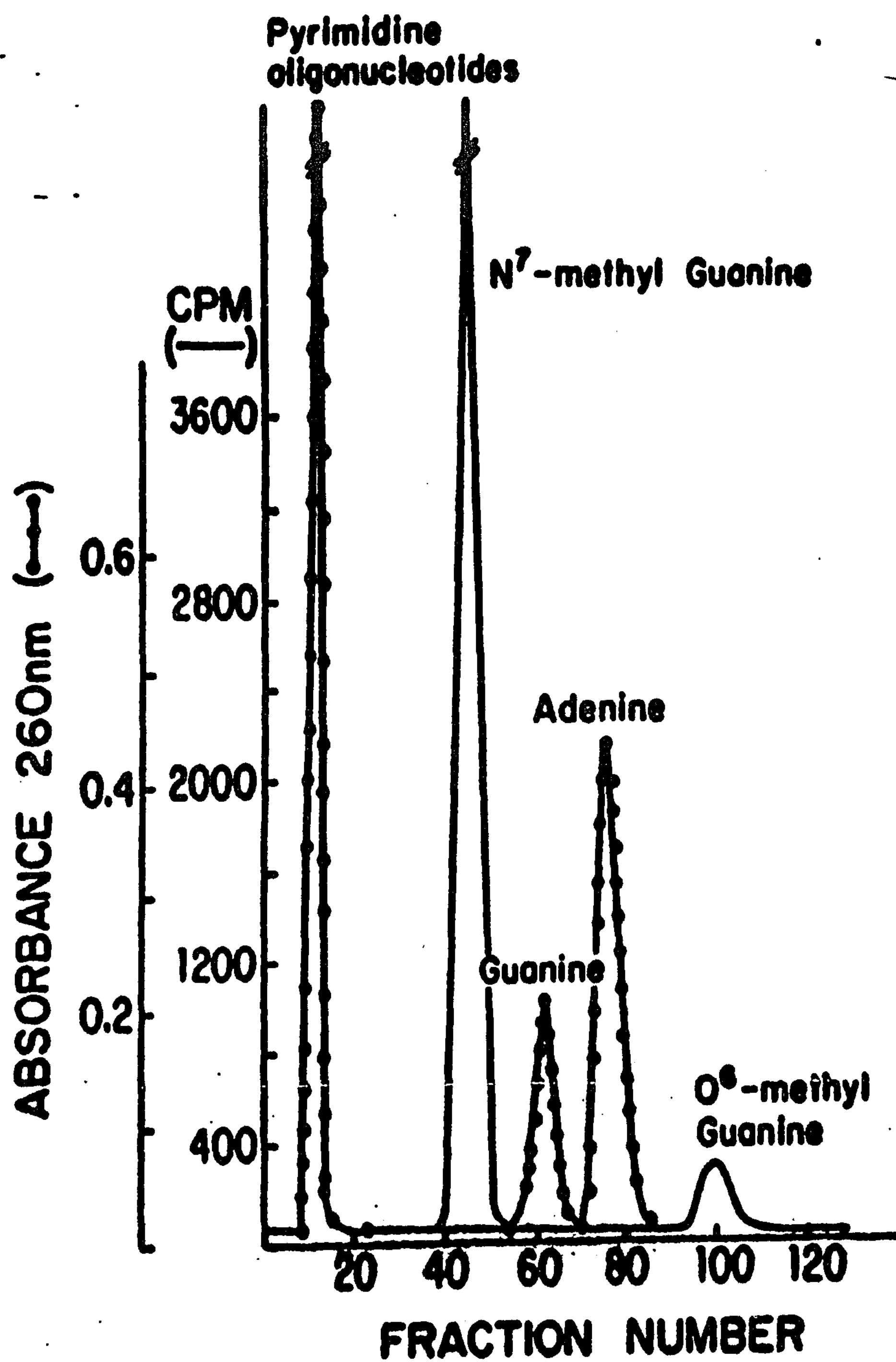

Pyrimidine oligonucleotides
N7-methyl Guanine
CPM (—)
3600
2800
2000
1200
400
ABSORBANCE 260nm (•—•)
0.6
0.4
0.2
Adenine
Guanine
O6-methyl Guanine
20
40
60
80
100
120
FRACTION NUMBER

Table 1

Effect of Different Storage Conditions on Rat Liver and O^6 Methylguanine Transferase Extracts on Enzyme Activity.

O^6 MeGT Source	Percent Activity
Fresh rat liver extract (RLE)	100%
[1] Rat liver slices (RLS) stored overnight at 4° C	78%
RLS stored overnight at -20° C	85%
RLS stored overnight at -70° C	31%
[2] RLE stored overnight at -70° C	21%
RLE stored four weeks at -20° C	82%

[1] RLS - Rat liver slices (3-5gm) in 50mM Tris-HCl pH 7.5 1mM DTT, 0.1mM EDTA under different conditions as stated above. These RLS were then processed and assayed for O^6 MeGT.

[2] RLE - Rat Liver (O^6 MeGT) extracts prepared as described previously and frozen in 1ml aliquots.

Dose Dependent Effect of Ethanol on O⁶MeGT and Other Enzymes

The in vitro inhibition of O^6MeGT by different concentrations of ethanol is shown in Figure 2. The activity decreases in a dose dependent fashion from 10mM-50mM ethanol concentrations. 50mM ethanol concentration caused approximately 40% inhibition of the O^6methylguanine transferase activity. At these ethanol concentrations, three other enzymes isocitrate dehydrogenase, glutathione-S-transferase and UDP-glucuronyl transferase were not inhibited. At higher concentrations of ethanol (100mM), there was still no effect on the activities of these three enzymes (data not shown). These results suggested that ethanol's inhibitory effect on O^6MeGT is not due to a general solvent effect.

Irreversible Inhibition of Ethanol on O^6MeGT Activity:

To determine whether the inhibitory effect of ethanol can be reversed, we added 50mM ethanol to an O^6MeGT preparation, dialysed it for 4 hours and assayed for recovery of activity. As shown in Table 2, the dialysis of the ethanol-treated O^6MeGT preparation did not lead to recovery of activity. Ethanol, therefore, appeared to produce an irreversible inhibition of O^6MeGT activity.

Figure 2. In Vitro Inhibitory Effect of Ethanol on O^6MeGT Activity

O^6MeGT was prepared from livers of partially hepatectomized rats and assayed as described in legend of Figure 1. Isocitrate Dehydrogenase (IDH) activity was assayed by monitoring the increase in absorbance at 340nm upon conversion of NADP to NADPH (Hanson and Cox, 1967). Glutathione-S-Transferase (GSHT) was assayed spectrophotometrically (Habig et al., 1974) using 1-chloro-2,4 dinitrobenzene as substrate. UDP-glucuronyl Transferase (UDPGT) activity was measured using p-nitrophenol as substrate and monitoring the absorbance at 405nm (Burchell and Weatherhill, 1981). Data are expressed as percent of enzyme activity observed when no ethanol was added. In each reaction mixture, ethanol was first diluted in buffer then mixed together with the various substrates before enzyme was added to start the reaction. The activity of O^6MeGT (■) was 480 fmol O^6MeG removed/mg. The activities of IDH (●), GSHT (○) and UDPGT (△) were 1082, 790 and 1196 U/mg, respectively, where 1 unit (U) equals the conversion of 1nmol substrate per minute.

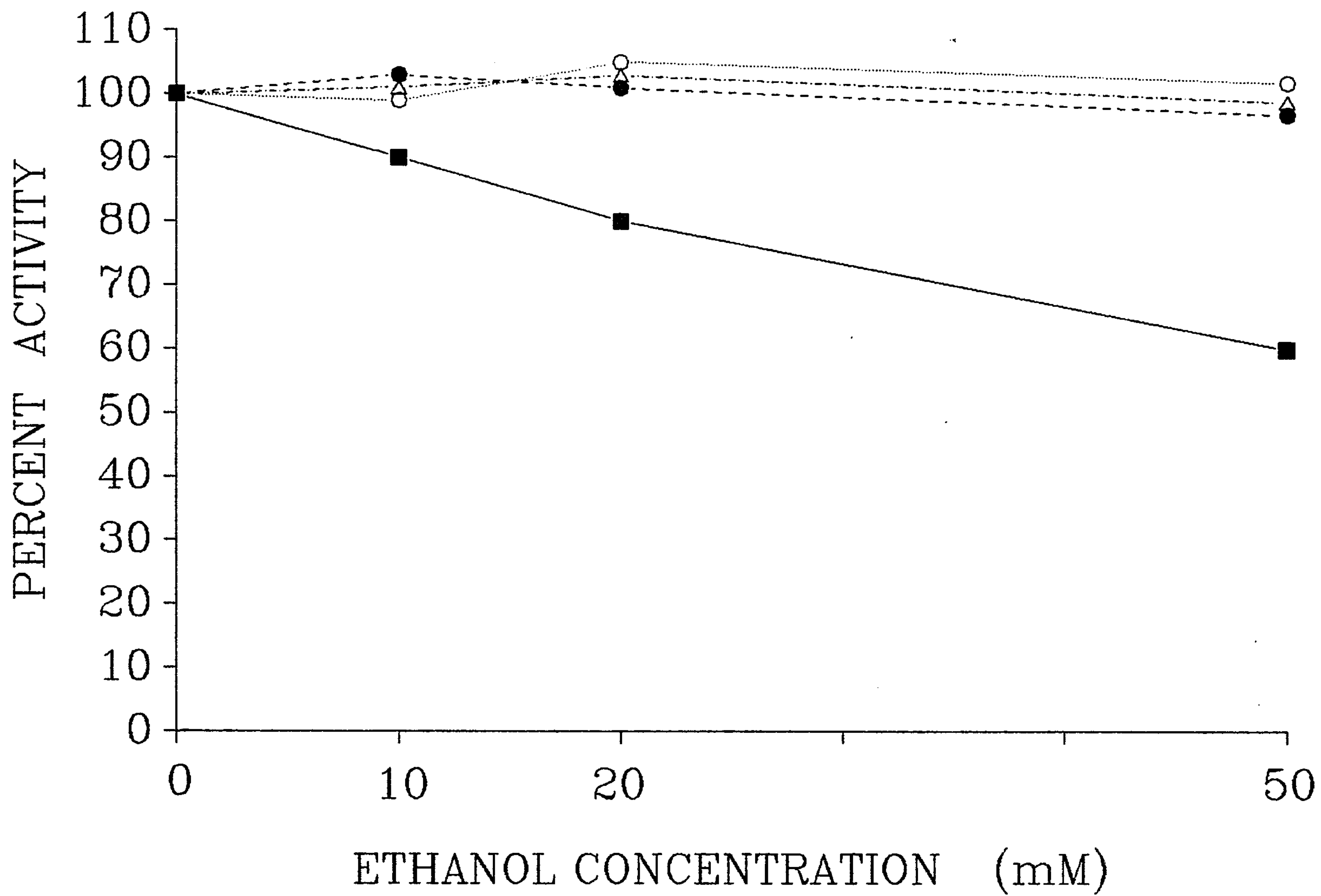

PERCENT ACTIVITY
110
100
90
80
70
60
50
40
30
20
10
0
0
10
20
50
ETHANOL CONCENTRATION (mM)

Table 2

Irreversible Inhibition of Ethanol on O^6MeGT Activity

Sample	Activity (pmol/30min.)
O^6MeGT	2.74
O^6MeGT plus 50mM ethanol	1.96
Dialysed O^6MeGT	2.14
Ethanol treated dialysed O^6MeGT	1.86

O^6MeGT was prepared from two partially hepatectomized rats and assayed with and without 50mM ethanol. A 20ml aliqout of O^6MeGT was treated with 50mM ethanol and dialysed for 4 hours at 4°C in 50mM Tris-HCl ph 7.5, 1mM DTT, 0.1mM EDTA. A second 20ml aliqout of O^6MeGT was also dialysed and served as the control.
O^6MeGT assay was as described in Fig. 1. 2mg of O^6MeGT was used in this assay and the DNA substrate contained 14.5pmol O^6MeG.

Effect of other alcohols on O^6 Methylguanine Transferase.

The effects of different alcohols on O^{6}MeGT is shown in Table 3. There is roughly 40-60% inhibition of O^{6}MeGT by ethanol, methanol, and isopropanol. Butanol does not appear to inhibit the enzyme while isoamyl alcohol strongly inhibits the O^{6}MeGT assay. Isocitrate dehydrogenase is not inhibited by DMSO or other alcohols tested here, however, 50mM methanol stimulated its activity by 30 percent. Glutathione transferase was not affected by any of these alcohols except for isoamyl alcohol at 50mM where there was a 25 percent inhibition of activity. UDP-glucuronyl transferase was also unaffected by DMSO and other alcohols with the exception of butanol which inhibited UDPGT activity by 18 percent. DMSO at 100mM did not exert an inhibitory effect on any of the enzymes tested.

Table 3

Effect of Different Alcohols on Activities of O^6 Methylguanine Transferase (O^6 MeGT), Isocitrate Dehydrogenase (IDH), Glutathione-S-Transferase (GSHT) and UDP-Glucuronyl Transferase (UDPGT).

	Percent Activity relative to Controls			
	O^6 MeGT	IDH	GSHT	UDPGT
Controls	100%	100%	100%	100%
Methanol (50mM)	50	130	98	91
Ethanol (50mM)	58	105	101	103
Isopropanol (50mM)	47	97	96	97
Butanol (50mM)	87	99	101	82
Isoamyl alcohol (50mM)	23	91	75	99
DMSO (100mM)	98	101	97	101

Assays for O^6 MeGT, IDH, GSHT and UDPGT were as described in legends of Fig. 1 and Fig. 2. The DNA substrate contained 14.5 pmol O^6 MeG.

Chronic Ethanol Administration and Repair of AAF-adducts

Following the initial observation of the inhibition of repair of DMN-induced O^6MeG adducts in ethanol-fed rats, we sought to determine whether the ethanol-associated inhibition of DNA repair was specific for repair of alkylated DNA bases such as O^6MeG or whether ethanol consumption had a general effect on DNA repair.

To investigate this question we used another model carcinogen, acetylaminofluorene (AAF), which forms adducts that are repaired by the excision repair pathway (Kriek, 1972; Poirer et al., 1979). AAF requires metabolic activation to generate electrophilic intermediates which can form DNA adducts (Irving et al., 1967; Irving and Veazey, 1969). The mutagenic AAF-DNA adduct formed is the N-deoxyguanosine-8-yl AAF (C-8) adduct (Westra et al., 1976; Sakai et al., 1978). This adduct intercalates into the DNA helix and produces frameshift mutations unless repaired by the excision repair pathway (Levine et al., 1974). A minor adduct, N-2-deoxyguanosine-AAF (N-2), is also formed. This N-2 adduct has not been shown to be mutagenic and is not repaired as rapidly as C-8 adducts (Westra et al., 1976). Eighty percent of the AAF-DNA adducts produced after a single administration of AAF is in the C-8 configuration. Since this is the major adduct formed and is the adduct that

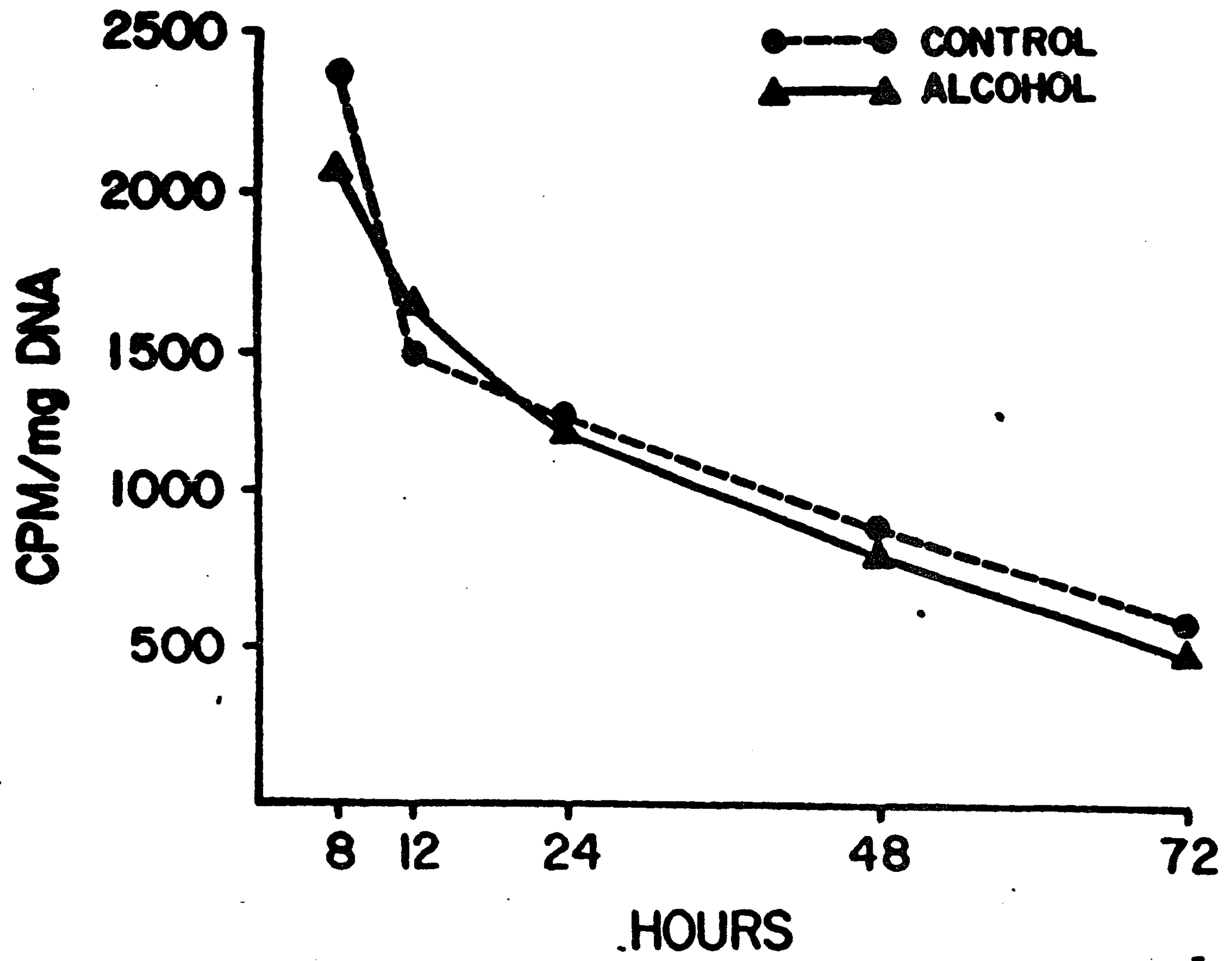
is enzymatically repaired, the loss of radioactivity from DNA labeled by administration of AAF can be used as a measure of DNA repair (via excision repair) of this adduct.

Male Sprague-Dawley rats weighing 125-150g were pair-fed nutritionally adequate, isocaloric liquid diets containing either dextrose or ethanol as 36% of their total caloric intake. The rats were kept on these diets for 4-5 weeks. Eight hours prior to AAF administration, the ethanol fed rats were given the carbohydrate control diet. Rats were injected with ^{3}H-AAF (8.2mCi/mmol) i.p. 30mg/kg and sacrificed over a 72 hour period.

DNA was isolated by CsCl gradient centrifugation and phenol extraction. The levels of AAF-DNA adducts were determined by scintillation counting. As shown in Figure 3, there was no difference in the initial levels of AAF-DNA adducts nor the repair of these adducts in ethanol-fed rats as compared to control-fed rats. The inhibitory effect of ethanol on DNA repair of O^{6}MeG did not appear, therefore, to extend to the repair of other carcinogen-damaged DNA.

Figure 3. Effect of Ethanol Consumption on the Formation and Loss of Acetylaminofluorene (AAF)-DNA Adducts in Nuclear DNA

Male Sprague-Dawley rats were pair-fed ethanol and control diets (Lieber and DeCarli, 1970). 8 hours prior to ethanol administration, the ethanol fed rats were given the control diets. ^{3}H-AAF (11.2mCi/mmol) at 30mg/kg was injected i.p. to each rat. A pair of animals were sacrificed at each time point. Livers were removed and DNA was isolated and radioactivity bound to DNA was determined by scintillation counting.


CONTROL
ALCOHOL
CPM/mg DNA
2500
2000
1500
1000
500
8
12
24
48
72
HOURS

Development of an HPLC Assay for O^6Methylguanine Transferase Activity.

During the course of this study, the assay procedure used to monitor O^6MeGT activity went through several changes as we acquired an HPLC system (which replaced the Sephadex G-10 chromatography step) and a fluorescence detector capable of detecting 1pmol of O^6MeG (which replaced the use of radiolabeled DNA substrate and scintillation counting of chromatography fractions).

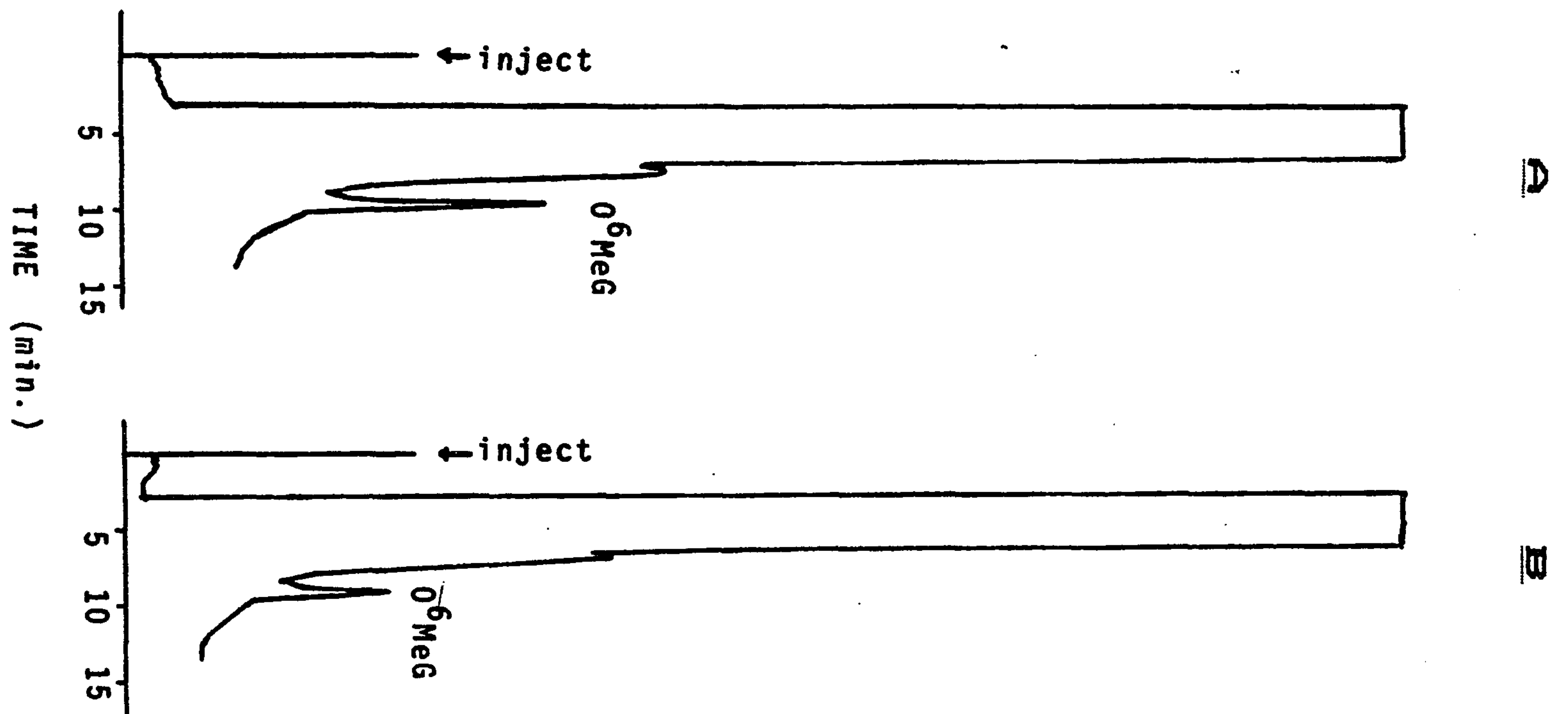
Figure 4 shows an HPLC profile for the separation of O^6MeG from a DNA hydrolysate assay mixture. Details of this HPLC assay was discussed in the previous chapter. O^6MeG elutes at 10.5 minutes and the data acquisition program (Appendix I) converts the analog signals of the fluorescence detector to digital units and calculates the peak area.

The program developed was designed to: a) collect data (lines 280-670); b) store data (1130-1420); c) retrieve data (5000-5014); d) calibrate signals (930-1120); e) calculate peaks (3000-3450); f) change calculation parameters (4000-4070) and g) bypass automatic calculations (6000-6250). Data collection was at a rate of one reading per second. To calculate the O^6MeG peak, the program required input of approximately where the O^6MeG eluted. The

default settings are such that calculation of O^6MeG peak areas start at fractions where rising slopes are greater than 100 units (on a 1-4095 scale) and end at fractions where falling slopes are less than 100 units. These slope default values can be changed i.e. decreasing them for small peaks and increasing them for large peaks. A baseline subtraction was included in the calculation sub-program that corrects for upward or downward drifts of the fluorescence signals during the HPLC run. The HPLC runs take less than 15 minutes per assay and thus are a marked improvement over the previous method of using radiolabeled DNA substrate and Sephadex G-10 chromatography.

Figure 4. HPLC Profile of Hydrolyzed DNA.

Assay conditions were as described in the previous chapter. 200ul of the hydrolyzed DNA mixture is applied through the HPLC system. Chromatogram on the left (panel A) is the control where no O^6MeGT was added. On the right (panel B) is the chromatogram of DNA hydrolysate that was reacted with O^6MeGT.


FLUORESCENCE (excit. 288nm; emmis. 375nm)
inject
5
10
15
TIME (min.)
O6MeG
A
inject
5
10
15
O6MeG
B

APPENDIX I

DATA ACQUISITION PROGRAM FOR THE APPLE II COMPUTER

```
5   DIM A(900),V(900),B(900)
10  D$ = CHR$ (4)
20  GOSUB 1620
30  GOSUB 2010
40  FOR II = 1 TO 2000: NEXT II
50  HOME
60  Z = PEEK (300)
70  ONERR  GOTO 90
80  GOTO 140
90  PRINT D$;"PR#0":HOME: FOR I = 1 TO 15 : CALL -198 :
    NEXT I: PRINT: PRINT
100 PRINT:PRINT:PRINT "AN ERROR WAS COMMITTED DURING
    OPERATION":PRINT:PRINT:PRINT
110 PRINT "          ERROR CODE = "; PEEK (222)
120 PRINT:PRINT: INVERSE : PRINT "    PRESS ANY KEY TO RESET
    PROGRAM     " : NORMAL : PRINT
130 GET ZZ$: GOTO 10
140 REM
150 Z1 = 3
160 AI13 = -16256 + Z1 * 16
170 GOSUB 1700
180 HOME
190 VTAB 2: PRINT "EXPERIMENT #: "; F
200 VTAB 4: INPUT "ENTER TREATMENT: "; TR$
205 IF MO <> 0 THEN 255
220 VTAB 6: INPUT "MONTH? "; MO
221 VTAB 22: PRINT "                        "
222 IF MO < 1 OR MO > 12 THEN VTAB 22: FLASH : PRINT "MONTH
    MUST BE 1 - 12": NORMAL : GOTO 220
224 VTAB 8: INPUT "DAY? "; DW
225 VTAB 22: PRINT "                         "
226 IF DW < 1 OR DW > 31 THEN VTAB 22: FLASH : PRINT "DAY
    MUST BE 1-31 ": NORMAL : GOTO 224
230 VTAB 10: INPUT "YEAR? "; DT
231 VTAB 22: PRINT "                         "
232 IF DT < 85 OR DT > 99 THEN VTAB 22: FLASH : PRINT "YEAR
    MUST BE 85-99 ": NORMAL : GOTO 230
255 PRINT : PRINT : PRINT
260 PRINT "ENTER 'Y' IF OK" : PRINT : PRINT "PRESS ANY OTHER
    KEY TO MAKE A CHANGE ";: GET Z$ : IF Z$ <> "Y" THEN 170
270 GOTO 710
280 HOME
285 IF FLAG = 1 THEN GOSUB 2010
286 GOSUB 2070
287 FLAG = 1
289 CT = CT * 60
290 VTAB 3:  HTAB 4:  INVERSE : PRINT "DATA ACQUISITION -
    ";MO;"/";DW;"/";DT: NORMAL
300 VTAB 5:  HTAB 2: PRINT "TIME"
310 VTAB 5:  HTAB 9: PRINT "SAMPLE"
```

```
320 VTAB 5:  HTAB 19: PRINT "ABS"
330 VTAB 5:  HTAB 28: PRINT "FLR"
340 VTAB 6:  PRINT "...................................."
350 VTAB 9:  PRINT "...................................."
360 FOR ZZ = 5 TO 9
370 VTAB ZZ: PRINT ":"
380 VTAB ZZ: HTAB 7: PRINT ":"
390 VTAB ZZ: HTAB 16: PRINT ":"
400 VTAB ZZ: HTAB 25: PRINT ":"
410 VTAB ZZ: HTAB 33: PRINT ":"
420 NEXT ZZ
430 KO = 0:TI = 1
435 VTAB 20: FLASH : PRINT "PRESS ANY KEY TO BEGIN";: NORMAL
    : GET Z$:VTAB
436 VTAB 20: PRINT
437 F$ = STR$ (MO) + STR$ (DW) + STR$ (DT) + "-" + STR (F)
438 VTAB 20: PRINT "RECORD NUMBER: ";: INVERSE : PRINT F$:
    NORMAL
440 FOR N = 1 TO CT
450 KO = KO + 1
460 IF KO = 60 THEN KO = 0:TI = TI + 1
470 TJ = INT (CT/60)
480 IF TI > TJ THEN TI = TJ
500 REM : SAMPLE CHAN #0 (ABS)
510 Y = 1 * 16 + 0
520 POKE AI13, Y
530 SM = PEEK (AI13 + 1) * 256 + PEEK (AI13)
540 A(N) = SM - CM
550 REM :SAMPLE CHAN #1 (FLR)
560 Y = 1 * 16 + 10
570 POKE AI13, Y
580 T = PEEK (AI13 + 1) * 256 + PEEK (AI13)
590 V(N) = T - CH
600 VTAB 8: PRINT
610 VTAB 8: HTAB 3: PRINT TI
620 VTAB 8: HTAB 10: PRINT N
630 VTAB 8: HTAB: PRINT A(N)
640 VTAB 8: HTAB 28: PRINT V(N)
650 IF PEEK ( - 16384) ) 127 THEN POKE - 16384, 0: GOTO 670
655 FOR 22 = 1 TO 335: NEXT ZZ
660 NEXT N
670 FOR II = 1 TO 5: CALL - 198: NEXT II: PRINT : PRINT :
    PRINT : PRINT
700 GOTO 1130
710 PRINT : PRINT : PRINT : PRINT
720 HOME
730 CALL - 198
740 VTAB 2: PRINT "*=*=*=*=*=*=*=*=*=*=*=*=*=*=*=*=*=*=*"
750 PRINT
760 PRINT "SELECTION"
780 PRINT "  A) DATA COLLECTION"
```

```
790 PRINT "  B) PRINT HARD COPY"
800 PRINT "  C) CALIBRATE"
805 PRINT "  D) CALCULATE PEAKS"
810 PRINT "  E) RE-CALCULATE PEAK RANGE"
815 PRINT "  F) CATALOG DATA DISK"
817 PRINT "  G) CHANGE PARAMETERS"
818 PRINT "  H) CHANGE CALCULATION PARAMETERS"
819 PRINT "  I) GO GET A DATA FILE"
820 PRINT "  J) QUICK SCAN RANGE"
822 PRINT "  K) END"
823 PRINT "  L) BYPASS CALCULATION"
825 PRINT : PRINT *=*=*=*=*=*=*=*=*=*=*=*=*=*=*=*=*=*=*=*=*=*"
830 VTAB 23: INPUT "    ENTER LETTER OF SELECTION: ";Z$
850 QE = 0
860 IF Z$ = "A" THEN 280
870 IF Z$ = "B" THEN 1440
880 IF Z$ = "C" THEN 920
885 IF Z$ = "D" THEN 3000
886 IF Z$ = "E" THEN 3600
890 IF Z$ = "K" THEN HOME : PRINT : PRINT : PRINT : PRINT
    : PRINT : PRINT : "SEE YOU LATER AND DON'T FORGET TO
    TURN OFF MY PUMPS" : FOR I = 1 TO 6000: NEXT : END
891 IF Z$ = "L" THEN 6000
895 IF Z$ = "F" THEN PRINT D$ "CATALOG, D"; DR: VTAB 23:
    GET Z$; GOTO 720
900 IF Z$ = "G" THEN 30
905 IF Z$ = "H" THEN 4000
906 IF Z$ = "I" THEN 5000
907 IF Z$ = "J" THEN 5015
919 GOTO 830
920 REM : CALIBRATION
930 HOME : PRINT "        CALIBRATION "
940 PRINT : PRINT "***********************************"
950 PRINT : PRINT "A) ABSORBANCE 254NM  (CHAN #0)"
960 PRINT
970 INPUT "     1) BASELINE READING  ";Z$
980 Y = 1 * 16 + 0
990 POKE AI13,Y
1000 CM = PEEK (AI13 +1) * 256 + PEEK (AI13)
1010 PRINT "                  " CM
1020 PRINT : PRINT : PRINT : PRINT
1030 PRINT "B) FLUORESCENCE (CHAN #10)"
1040 PRINT
1050 INPUT "     A) BASELINE READING  ";Z$
1060 Y = 1 * 16 + 10
1070 POKE AI13,Y
1080 I = PEEK (AI13 + 1) * 256 + PEEK (AI13)
1090 PRINT "            ";I
1100 CH = I
1110 PRINT : PRINT : PRINT: PRINT "PRESS ANY KEY "; GET Z$
1120 GOTO710
```

```
1130 REM : DISK STORAGE
1230 F$ = "DATA-" + F$
1240 HOME : VTAB 10 : PRINT "WRITING FILE NAMED: ";:INVERSE
     PRINT F$ : NORMAL : PRINT "   TO DISK ON DRIVE  "; DR
1250 PRINT D$;"OPEN";F$" ,D";DR
1260 PRINT D$; "WRITE";F$
1270 PRINT MO : PRINT DW :PRINT DT
1280 PRINT CT
1290 FOR Z = 1 TO 10 : PRINT Z: NEXT Z : REM  *** THESE ARE
     10 SPARE DATA LOCATIONS IN THE DISK FILE ***
1330 PRINT TR$
1350 FOR N = 1 TO CT
1370 PRINT A(N)
1380 PRINT V(N)
1400 NEXT N
1410 PRINT D$;"CLOSE";F$
1415 GOTO 3000
1420 PRINT D$:"LOCK";F$
1430 GOTO 710
1440 REM : HARD COPY
1442 Z$ = CHR$ (PEEK (-16386))
1450 PRINT : PRINT : INPUT "ENTER FILE NAME: ";F$
1454 PRINT D$; "OPEN ";F$;" ,D";DR
1455 PRINT D$; "READ ";F$
1456 INPUT MO : INPUT DW : INPUT DT : INPUT CT
1457 FOR Z = 1 TO 10 : INPUT Z : NEXT
1458 INPUT TR$
1459 FOR N = 1 TO CT
1460 INPUT A(N) : INPUT V(N)
1462 NEXT
1463 PRINT D$ "CLOSE"
1465 PRINT D$ "PR#1"
1470 PRINT "FILE NAME:    ";F$
1480 PRINT "CONDITION:    ";TR$
1490 PRINT "      DATE:    ";MO;"/";DW;"/";DT
1510 FOR I = 1 TO 38 : PRINT TAB (I);"-";: NEXT I
1520 PRINT : PRINT
1530 PRINT "SAMPLE": TAB (10):"ABS": TAB (19):"FLR":
     TAB (25) : "SAMPLE" :TAB (36);"ABS";TAB (45);"FLR"
1535 PRINT
1540 FOR N = 1 TO (CT/2)
1550 IF PEEK ( - 16384) > 127 THEN POKE - 16368,0: GOTO 1590
1560 M = N + (CT/2)
1570 PRINT TAB(3);N; TAB(8);A(N); TAB(17);V(N) TAB(27);M;
     TAB(34);A(M); TAB(43);V(M)
1580 NEXT N
1590 PRINT D$; "PR#0"
1600 GOTO 710
1620 TEXT : HOME
1630 NORMAL : PRINT "***************************************";
1640 INVERSE : PRINT "DATA ACQUISITION PROGRAM" :  NORMAL :
```

```
     PRINT "******************************************";
1650 PRINT "***************";: INVERSE : PRINT "BY";: NORMAL
     :PRINT "******************"
1660 PRINT "****" ;: INVERSE : PRINT " JOHN THORNBOROUGH ":
     NORMAL : PRINT "*********************";
1670 PRINT "****";: INVERSE : PRINT "AND";: NORMAL : PRINT
     "***********";
1680 PRINT "*****";: INVERSE : PRINT "NOEL ESPINA ";:
     NORMAL: PRINT "**********************";
1686 PRINT "**********************************************";
1687 PRINT : PRINT : PRINT THIS CALCULATION IS BASED ON
     RISING AND FALLING SLOPE OF 100.  THERE'S BACKGROUND
     SUBTRACTION AS WELL.
1688 FOR I = 1 TO 50 : NEXT
1690 RETURN
1700 TEXT : HOME
1705 IF CT = > 60 THEN CT = CT/60
1710 NORMAL : PRINT "=====================================";
1720 PRINT
1730 PRINT "               PROGRAM PARAMETERS "
1740 PRINT
1750 PRINT "============================================";
1760 PRINT  :PRINT
1770 PRINT "     A) DATA FILE DISK ON DRIVE:"
1780 PRINT
1790 PRINT "     B)   DATA FILE TO BE NAMED:"
1800 PRINT
1810 PRINT "     C)      DATA COLLECTED FOR:    MIN"
1820 PRINT
1830 PRINT "=============================================";
1840 PRINT : PRINT
1850 PRINT "ENTER LETTER TO CHANGE PARAMETER:"
1860 PRINT "     (PRESS RETURN TO SAVE)"
1870 PRINT
1880 PRINT "==============================================";
1890 VTAB 8: HTAB 32: INVERSE : PRINT DR
1900 VTAB 10: HTAB 32: PRINT F
1910 VTAB 12: HTAB 32: PRINT CT: NORMAL
1920 VTAB 17: HTAB 35: GET Z$
1930 Z$ = CHR$ (PEEK ( - 16384))
1940 IF PEEK( - 16384) = 13 THEN 2120
1945 IF PEEK ( - 16384) < 65 THEN 1920
1950 IF Z$ = "A" THEN VTAB 8: HTAB 32:
     INVERSE : INPUT " "; DR : NORMAL
1955 IF DR < 1 OR DR > 2 THEN VTAB 22: FLASH : PRINT
     "ENTER EITHER '1' OR '2'": NORMAL : GOTO 1950
1957 VTAB 22: PRINT "                         "
1960 IF Z$ = "B" THEN VTAB 10: HTAB 32:
     INVERSE : INPUT " ";F: NORMAL
1970 IF Z$ = "C" THEN VATB 12: HTAB 32:
     INVERSE : INPUT " ";CT: NORMAL
```

```
1980 VTAB 12 : HTAB 35 : PRINT "MIN"
1982 VTAB 22: PRINT "                              "
1984 IF CT < 1 THEN VTAB 22 : FLASH : PRINT "REENTER" :
     NORMAL : GOTO 1970
1985 IF CT > 15 THEN VTAB 22: FLASH: PRINT "15 MIN MAXIMUM -
     RE-ENTER" : NORMAL : GOTO 1970
1990 GOTO 1920
2000 RETURN
2010 PRINT D$"OPEN PARAM,D1"
2020 PRINT D$"READ PARAM"
2030 INPUT DR: INPUT CT: INPUT F
2040 PRINT D$"CLOSE"
2050 F = F + 1
2055 FLAG = 0
2060 RETURN
2070 PRINT D$"OPEN PARAM,D1"
2080 PRINT D$"WRITE PARAM"
2090 PRINT DR: PRINT CT: PRINT F
2100 PRINT D$"CLOSE"
2110 RETURN
2120 REM
2140 GOSUB 2070
2150 RETURN
3000 REM CALCULATION SECTION
3001 TEXT  :  HOME :  NORMAL :  PRINT "*********************
     *******************************************"
3002 PRINT : INVERSE : PRINT : "PEAK FINDER SUB-PROGRAM ";:
     NORMAL : PRINT : PRINT : PRINT "***********************
     *****************************************"
3003 IF F1 = 0 THEN PRINT "WHAT IS THE STARTING SAMPLE-?";:
     INPUT F1
3004 IF G = 0 THEN PRINT "WHAT IS THE ENDING SAMPLE-?";:
     INPUT G
3010 KP = 0 : B = 0 : R = 0 : FS = 0 : DF = 5 : ES = 0 :
     SS = 0 : SP = 0 : LP = 0 : EP = 0
3011 FOR N = F1 TO G
3066 K = V(N)
3070 IF V(N) > = V(N + 1) THEN K = V(N)
3080 IF K > KP THEN KP = K
3085 IF K = KP THEN LP = N
3092 K = K - 1
3198 NEXT N
3200 IF SL = 0 THEN SL = 100
3202 FOR N = F1 TO (LP - 5)
3205 NA = V(N) + V(N + 1) + V(N + 2)
3210 NB = V(N + 3) + V(N + 4) + V(N + 5)
3212 N = N + 2
3213 NC = NB - NA
3214 IF NC > SL THEN GOTO 3216
3215 NEXT N
3216 MV = V(N + 1) : LV = N + 1
```

```
3217 B = (LP - LV) : X = 1
3218 FOR N = (LP + 5) TO (LP + 40)
3219 NA = V(N) + V(N + 1) + V(N + 2)
3220 NB = V(N + 3) + V(N + 4) + V(N + 5)
3221 NC = NA - NB
3222 IF NC < SL THEN RV = N + 2 : GOTO 3226
3223 RW = N
3224 NEXT N
3225 IF RW = LP + 40 THEN RV = B + LP
3226 LS = INT ((V(LV) + V(LV - 1) + V(LV - 2)) / 3)
3227 RS = INT ((V(RV) + V(RV + 1) + V(RV + 2)) / 3)
3228 SS = ((RS - LS) / (RV - LV))
3229 IF RV = LP + 40 THEN GOTO 3233
3230 IF SS > 1 THEN RV = RV + 1 : GOTO 3227
3233 FOR N = LV TO RV
3235 B(N) = INT (SS * X + V(LV))
3240 FS = FS + V(N) - B(N)
3243 X = X + 1
3245 NEXT N
3296 PRINT :
3340 PRINT "THE FLOURESCENCE PEAK IS-" : KP
3345 PRINT " THE FLR PEAK FRACTION IS-" : LP
3360 ES = RV
3365 PRINT "THE FLR SUM FROM ";LV" TO ";ES" IS ";FS::
3400 PRINT : PRINT "DATA FILE NAME IS ";F$::
3450 GET Z$ : GOTO 710
3600 F1 = 0 : G = 0 : GOTO 3000
4000 TEXT : HOME
4002 NORMAL  : PRINT  "*************************************
     *********************************"
4004 PRINT  :  INVERSE  :  PRINT "CHANGE CALCULATION
     PARAMETERS";: NORMAL : :
4006 PRINT  :  PRINT  :  PRINT "*************************
     *********************************"
4010 PRINT "DATA FILE NAME: ";F$
4020 PRINT  :  PRINT "WHAT SLOPE WOULD YOU CHOOSE?"
4030 PRINT  :  PRINT "THE DEFAULT VALUE IS 100"
4040 PRINT  :  PRINT " CHOOSE SLOPES 50, 100, 150, 200, 250"
4050 PRINT  :  PRINT "ENTER ONE CHOICE OF ABOVE": INPUT SL
4055 FS = 0
4060 HOME
4065 PRINT  :  PRINT  :  INVERSE  :  PRINT "CHANGE
     CALCULATION PARAMETERS" : NORMAL  :  PRINT  :  PRINT
4070 GOTO 3200
5000 REM : QUICK SCAN
5002 Z$ = CHR$ ( PEEK ( - 16384))
5003 PRINT  :  PRINT  :  INPUT "ENTER FILE NAME: ";F$
5004 PRINT D$; "OPEN ";F$;", D";DR
5005 PRINT D$; "READ ";F$
5006 INPUT MO:  INPUT DW:  INPUT DT:  INPUT CT
5007 FOR Z = 1 TO 10: INPUT Z: NEXT
```

```
5008 INPUT TR$
5009 FOR N = 1 TO CT
5010 INPUT A(N) : INPUT V(N)
5011 NEXT
5012 PRINT D$"CLOSE"
5013 PRINT D$"PR#0"
5014 GOTO 710
5015 PRINT "      DATE:     ";MO;"/";DW;"/";DT
5016 PRINT "DATA FILE NAME: ";F$
5017 PRINT "SAMPLE"; TAB (10); "ABS"; TAB (19); "FLR"
5018 PRINT
5019 INPUT "STARTING SAMPLE NUMBER-";Q1
5020 INPUT "ENDING SAMPLE NUMBER-";Q2
5030 FOR N = Q1 TO Q2
5040 IF PEEK ( - 16384) > 129 THEN POKE - 16384, 0: GOTO
     5070
5050 PRINT TAB (3); N ; TAB (8) ; A(N) ; TAB (17) ; V(N)
5060 NEXT N
5070 PRINT D$;"PR#0"
5080 GET Z$: GOTO 710
6000 REM BYPASS CALCULATIONS
6005 HOME : PRINT : PRINT : PRINT
6006 PRINT : PRINT : INVERSE : PRINT "BYPASS CALCULATION
     SUB-PROGRAM": NORMAL : PRINT
6010 PRINT "WHAT IS THE STARTING FRACTION-";: INPUT LV
6020 PRINT "WHAT IS THE ENDING SAMPLE    -";: INPUT RV
6025 FS = 0 : X = 1 : KP = 0
6050 FOR N = LV TO RV
6055 K = V(N)
6060 IF V(N)) = V(N + 1) THEN K = V(N)
6070 IF K > KP THEN KP = K
6080 IF K = KP THEN LP = N
6090 K = K - 1
6100 NEXT N
6105 REM SET UP BASELINE SLOPE SUBTRACTION
6150 LS = INT ((V(LV) + V(LV - 1) + V(LV - 2))/3)
6155 RS = INT ((V(RV) + V(RV + 1) + V(RV + 2))/3)
6160 SS = ((RS - LS) / (RV - LV))
6170 FOR N = LV TO RV
6180 B(N) = INT (SS * X + V(LV))
6190 FS = FS + V(N) - B(N)
6195 X = X + 1
6200 NEXT N
6210 PRINT :
6220 PRINT " THE FLUORESENCE PEAK IS-";KP
6225 PRINT "THE FLR PEAK FRACTION IS-";LP
6230 PRINT :
6235 PRINT "SUM OF 06 PEAK ";LV" TO ";RV" IS ";FS
6240 PRINT : PRINT : PRINT "DATA FILE NAME IS ";F$:
6250 GET Z$: GOTO 710
```

DISCUSSION

Based on the data presented here we can conclude the following: 1) Chronic ethanol consumption results in a decreased capacity to repair DMN induced alkylation at the O^6 position of guanine in hepatic DNA. 2) This decreased capacity to repair alkylation at the O^6 position of guanine is associated with a decrease in O^6MeGT activity and this is seen after either chronic or acute ethanol administration. 3) The loss of O^6MeGT activity in ethanol-fed rats is due, for the most part, to an irreversible inhibition of the enzyme by acetaldehyde which is generated during ethanol metabolism. 4) Chronic ethanol consumption does not have a general inhibitory effect on DNA repair since it does not affect the repair of AAF-DNA adducts which are repaired via the excision repair pathway.

Properties of the O^6Methylguanine Transferase:

Over the past twenty years, many researchers have focused on O^6MeG as an important lesion involved in alkylation induced mutagenesis and carcinogenesis. Alkylation of the O^6 position of guanine disrupts base pairing with cytosine and after a replicative cycle can produce G-C to A-T transition mutations. This provides a

mechanism for the induction of somatic mutations that may act as initiating events in alkylation induced cancers.

The role of O^6MeGT in the repair of this adduct has been investigated in many different systems. O^6MeGT activity has been found in bacteria (Lawley and Orr, 1970; Schendel and Robins, 1978) as well as mammalian cells (Swann and Magee, 1968, 1971; Pegg and Perry, 1981a; Harris et al., 1983; Lefebvre and Laval, 1986). This strong evolutionary conservation of this particular DNA repair system suggests that the repair of O^6 alkylguanine is important for cell survival. In mammalian systems there is a strong correlation between the persistence of O^6MeG after treatment with alkylating agents and the appearance of tumors in different animal tissues (O'Connor et al., 1973; Eadie et al., 1984). There is also a strong correlation between the lack of O^6MeGT repair activity and susceptibility of various organs to alkylation induced carcinogenesis (Singer, 1979; Singer et al., 1981; Woodhead et al., 1985).

O^6MeGT removes alkyl groups from the O^6 position of guanine and transfers it to a cysteine on the enzyme itself. In *E. coli*, O^6MeGT only has one cysteine acceptor site (Demple et al., 1982; Lindahl et al., 1982) while in rat liver O^6MeGT, there may be two cysteine acceptor sites per O^6MeGT (Renard et al., 1983).

In bacteria, exposure to low levels of methyl-nitro-nitrosoguanidine (MNNG) results in what is termed the

adaptive response, whereby the cells exhibit resistance to mutagenesis at low levels of MNNG and exhibit increased survival at large doses of MNNG (Karran et al., 1979; Sedgwick and Robins, 1980; Sklar and Strauss, 1980). This adaptive response includes the induction of O^6MeGT and DNA glycosylases. In mammals, evidence for an adaptive response is not quite as clear as in the bacterial system. Various treatments such as partial hepatectomy, administration of alkylating agents such as DMN or non-alkylating agents such as AAF have been shown to increase O^6MeG repair in rats (Buckley et al., 1979; Pegg et al., 1981; Pegg and Perry 1981a). Other rodent species did not show this inductive effect on O^6MeGT (O'Conner et al., 1982). Mammalian cells in culture, however, have shown an 'adaptive'-like response. Chinese hamster ovary cells and rat hepatoma cells, when pretreated with alkylating agents, show increased resistance to cell killing and mutagenesis by MNNG (Samson and Schwartz, 1980; Laval and Laval, 1984). Human tumor cell lines have been designated as Mer^- or Mer^+ depending on their ability to reactivate MNNG-treated adenovirus 5 (MNNG-Ad 5) (Day and Ziolkowski, 1977; Day et al., 1980). The ability to reactivate MNNG-Ad 5 has been correlated with the presence of O^6MeGT activity. In some human tumors, O^6MeGT activity has been found to be significantly lower than in corresponding normal tissues (Myrnes et al., 1984). Whether

this lower activity resulted in higher risk for developing tumors or is part of the disease process is not clear.

Chronic Ethanol Consumption and Repair of DMN-induced O^6 Methylguanine Adducts:

Rats that were maintained on an ethanol-containing diet (36% of total calories as ethanol) for 4 weeks prior to DMN exposure, showed an increased persistence of DMN induced hepatic O^6MeG adducts relative to control rats (Farinati et al., 1984). As discussed in chapter 1, there have been two studies similar to our initial experiment on ethanol consumption and alkylation repair that failed to detect significant differences between control and ethanol fed rats in the persistence of O^6MeG DNA adducts (Belinsky et al., 1982; Schwarz et al., 1982). In one of these studies, no differences were observed in the extent of O^6MeG adducts between ethanol and control fed rats at four hours after a single injection of ^{14}C labeled DMN (Schwarz et al., 1982). This in fact is consistent with our data for that time point. At four hours we find no difference in the levels of N^7MeG and O^6MeG between control and ethanol fed rats. In the second study, following the administration of a DMN dose of 25mg/kg, the levels of O^6MeG declined at the same rate for ethanol fed and control rats over a 72 hour period

(Belinsky et al., 1982). This observation conflicts with our results but on close examination of the feeding protocols used in this study it appears that both the control and ethanol-fed rats were chronically underfed (maintained at approximately 50% of the caloric intake of our animals). This is consistent with the low weight gain for the animals used in the study, 10-20% over the four week feeding period whereas we consistently observe a 30-40% weight gain over a four week feeding period. Thus the lack of effect of the ethanol diet is likely due to the low levels of O^6MeGT in both diet groups as a result of undernutrition.

Chronic Ethanol Consumption and Repair of Acetylaminofluorene-DNA Adducts:

The repair of AAF-DNA adducts was also investigated in ethanol and control fed animals to determine whether chronic ethanol consumption had a general inhibitory effect on DNA repair. The results showed: 1) that the initial levels of AAF binding to hepatic DNA were not affected by ethanol pretreatment which is consistent with a study that demonstrated that chronic ethanol consumption does not enhance the mutagenic activation of AAF by hepatic microsomes (Smith and Gutmann, 1984) and 2) the subsequent

repair of AAF-DNA adducts in rat liver was not affected by chronic ethanol feeding. Thus it appeared that chronic ethanol consumption does not have a general inhibitory effect on DNA repair, and the increased persistence of O^6MeG adducts in ethanol-fed rats is relatively specific for that DNA repair pathway.

Effect of Chronic Ethanol Feeding on O^6Methylguanine Transferase Activity:

The initial observations whereby DMN induced O^6MeG persisted at higher levels and for longer periods of time in alcohol-fed rats naturally led us to the enzyme involved in the repair of this DNA adduct, O^6MeGT. We found that ethanol administration resulted in a decreased level of O^6MeGT activity in hepatic tissue and that this was true for both chronic ethanol feeding (36% of calories as ethanol for 4 weeks) or acute ethanol ingestion (3g/kg p.o.). In chronic ethanol-fed rats, the decreased levels of O^6MeGT activity persisted for at least 18 hours following the withdrawal of the alcohol diet. There thus appeared to be a persistent inhibitory effect on this repair pathway following chronic ethanol consumption.

When ethanol was administered by gastric intubation, we observed an inhibitory dose response on O^6MeGT at ethanol

doses of 0.03 to 3.0 g/kg. After an acute dose of ethanol (3g/kg p.o.), O^6MeGT was significantly depressed and required 24-48 hours to reach its basal levels of enzyme.

The ethanol associated inhibition of O^6MeGT, in vivo, could have been due to several mechanisms. It is known that O^6MeGT is inactivated when it catalyzes the transfer of an alkyl group onto its cysteine acceptor site. Re-synthesis of this enzyme is required in order for the cell to recover its activity. Ethanol consumption, both chronic and acute, may have lead to an inactivation of the O^6MeGT either by direct binding and denaturation by ethanol itself or by its reactive metabolite, acetaldehyde. Another possibility, which was initially considered, was that the decrease in O^6MeGT may have been due to an inhibition of O^6MeGT re-synthesis. Although chronic ethanol consumption does not have a general inhibitory effect on hepatic protein synthesis and in fact increases synthesis of some liver proteins (Baraona et al., 1977), ethanol has been shown to inhibit protein synthesis in vitro (Perin et al., 1974) and a differential effect of ethanol on protein synthesis could not be excluded.

In Vitro Inhibitory Effect of Ethanol on O⁶MeGT Activity:

As a first step in analysing these various possibilities we sought to determine whether ethanol at physiologically significant concentrations inhibited O^6MeGT activity. We found that in vitro, O^6MeGT activity was inhibited by ethanol over a 10-50mM concentration range (levels found in chronic alcohol abusers and animals on ethanol containing diets). The results show that at these concentrations of ethanol there is a dose response inhibition on O^6MeGT. This direct inhibitory effect of ethanol on O^6MeGT could not totally explain, however, the increased persistence of O^6MeG induced by DMN that was observed in our initial study. In that study, the ethanol diet was withdrawn 18 hours prior to DMN administration and animals were maintained on the control diet till the end of the experiment. In the 18 hours between withdrawal of ethanol and DMN injection, all of the ethanol should have been cleared from the circulation. Similarly, a direct inhibitory effect of ethanol on O^6MeGT could not explain the decreased levels of O^6MeGT observed in ethanol fed animals in a subsequent experiment as the livers in which O^6MeGT activity was determined were removed after the animals were fasted overnight without access to ethanol. Finally, the experiment that demonstrated that the in vitro inhibitory effect of 50mM ethanol was not reversed by dialysis of an

ethanol treated O^6MeGT preparation suggested that some form of covalent product was being formed as a consequence of exposure of the enzyme to the ethanol.

Inhibition of O^6MeGT activity due to ethanol feeding and its persistent inhibitory effect together with the in vitro experiment in which the inhibitory effect of ethanol was irreversible, led us to test the effect of acetaldehyde, the first metabolite of ethanol on O^6meGT. The data presented here demonstrate that acetaldehyde is a potent inhibitor of both rat and human liver O^6MeGT. Other soluble enzymes such as glutathione transferase and isocitrate dehydrogenase were not inhibited by acetaldehyde at concentrations that inhibited O^6MeGT. Acetaldehyde is a very reactive compound which has been shown to bind to sulfhydryl and amino groups on proteins and other substances. O^6MeGT has a reactive cysteine at its active site and this may be where acetaldehyde interacts with the enzyme. Some other enzymes with cysteine active sites such as yeast alcohol dehydrogenase and glyceraldehyde-3-phosphate dehydrogenase, however, were not inhibited at these concentrations of acetaldehyde. If the O^6MeGT cysteine active site is the point of acetaldehyde attack it must be particularly sensitive or available to acetaldehyde.

Further evidence that ethanol derived acetaldehyde is largely responsible for the loss of O^6MeGT activity in vivo was demonstrated by the use of the acetaldehyde

dehydrogenase inhibitor, disulfiram (DSF). DSF pretreatment has been shown to increase blood acetaldehyde levels after ethanol exposure (Ericksson, 1985). Rats pretreated with DSF and subsequently challenged with ethanol showed a further reduction in O^6MeGT activity as compared to rats that were treated with ethanol only.

The use of DSF together with alcohol produces a series of unpleasant symptoms in man which include increased heart rate, palpitation, flushing and hypotension (Hald and Jacobsen, 1948). This series of reactions has led to the use of DSF ("Antabuse") in the treatment of alcohol abuse. The results presented here, however, point to a potential danger in the use of DSF in the treatment of alcoholism. By increasing acetaldehyde levels in the circulation, the patient could be at higher risk for developing alkylation induced cancers as a result of the inhibition of repair of alkylation damaged DNA.

Ethanol itself may also exert a significant inhibitory effect on O^6MeGT in vivo. Distillation of ethanol to remove pre-existing acetaldehyde did not totally eliminate the inhibitory effect of ethanol when it was added to O^6MeGT assay mixtures. Although we can not eliminate the possibility that some acetaldehyde was generated from the ethanol by alcohol dehydrogenase (ADH) in the assay mixture, the possibility that ethanol may have a direct inhibitory effect is supported by the inhibition of O^6MeGT observed

with other alcohols such as methanol, isopropanol and isoamyl alcohol. These alcohols are not substrates for the ADH. Based on the limited number of alcohols tested here, it appears that the branched alcohols have a more inhibitory effect on O^6MeGT than the unbranched alcohols. Butanol, a longer chain alcohol may not fit in the active site of the enzyme while the other alcohols have either, a methyl ($-CH_3$) or ethyl ($-C_2H_5$) group that could "fit" into the active site thus competing with the natural substrate. A more detailed study using a wide array of related alcohols has to be done in order to determine whether branched alcohols have a more inhibitory effect on the O^6MeGT than unbranched alcohols. A comparison on the effects of propanol and butanol versus isopropanol and isobutanol would be valuable.

As noted above, it is possible that the apparent direct inhibitory effect of ethanol is due to acetaldehyde formed either from the spontaneous oxidation of ethanol or by trace levels of alcohol dehydrogenase activity in the O^6MeGT assay mixture. In order to separate the inhibitory effects of ethanol from that of acetaldehyde, a specific ADH inhibitor, 4-methylpyrazole, and an acetaldehyde scavenger, semicarbazone, could be used in the reaction mixture. These experiments have not been done.

There is at least one approach which may be used to address the question of whether there is also inhibition of O^6MeGT synthesis in ethanol treated rats. An immunological

probe i.e. antibody to O^6MeGT has to be obtained in order to quantify the amounts of O^6MeGT molecules present in the cell before and after ethanol treatment. The antibody to O^6MeGT has to be tested for its recognition of both active and inactivated O^6MeGT. By comparing the levels of enzyme and determining the recovery of O^6MeGT activity in ethanol and control animals, the question of whether ethanol reduces the resynthesis of enzyme can be answered.

In conclusion, the results presented here show that chronic consumption of ethanol leads to a loss of repair of O^6MeG in rat liver DNA. The basis for this inhibition appears to be due largely to the in vivo formation of acetaldehyde as a consequence of ethanol metabolism. Although other factors such as direct inhibitory effect of ethanol or an effect on O^6MeGT synthesis cannot be totally ruled out, it is clear that acetaldehyde, at concentrations that are present in the liver after ethanol ingestion inhibits both rat and human liver O^6MeGT. These observations indicate that ethanol consumption interferes with at least one type of DNA repair and that this effect may be a significant factor in alcohol abuse associated cancers.

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