MUTAGENICITY OF SELECTED ALCOHOLIC BEVERAGES

BY
SADHANA .P.

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Introduction

INTRODUCTION

Cancer in human beings is a complex disease characterized by uncontrolled multiplication and disorganised growth of the affected cells, it may arise in any of the body tissues. Cancer cells infiltrate and destroy adjacent tissues, eventually gain access to the circulatory system, are transported to distant parts of the body and ultimately destroy the host (Pinto R, 1993).

Genetists have known for decades that DNA does not replicate with hundred percent fidelity and that there is endogenous DNA damage. Thus, every time DNA replicates, there is a rare chance that a mistake might occur in a gene critical to the carcinogenic process (Cohen et al., 1991).

Carcinogenesis is multistep process in which cells accumulate multiple genetic alterations as they progress to a more malignant phenotype (Sugimura T, 1992).

Humans are exposed to a constantly changing chemical milieu, including a variety of chemicals that are hazardous to health. An agent can increase the

likelihood of DNA damage by either directly altering the DNA (genotoxicity) or by increasing the number of times DNA replicates (cell proliferation)

Major progress and understanding of complex causes of cancer stems from inquiries into etiological factors for each type (Weisburger J.H., 1991). missing in bibliography

Recent changes in cancer incidence patterns may reflect the trend towards a more western diet and life style. The majority of causes of cancer (such as tobacco, alcohol, animal fat, obesity, ultra violet light, highly salted foods) are associated with life style, that is with personal choices and not with the environment in general (Henderson et al., 1991) The mind-bending effects of alcohol begin soon after it hits the blood stream. The pathological consequences of alcohol overconsumption are related to ethanol or its metabolic products acetaldehyde and acetate (Nuutinen et al., 1985) Ethanol is a simple molecule, its affinity for water takes it everywhere in the body that water goes.

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Alcohol consumption has independent effects (as well as synergistic effects with tobacco) in increasing risk of cancers of the oral carity, pharynx,

larynx, liver, esophagus, colorectal, mouth and female breast cancer (Blot ., 1988).

The genetic risk of chronic alcoholism and DNA damage may be related to the mutagenic and carcinogenic activity of acetaldehyde (Topinka et al., 1991)

Acetaldehyde has been shown to be a clastogen, inducing chromosomal aberrations in human peripheral lymphocytes and other cells invitro and invivo as well as sister chromatid exchanges (Obe and Anderson, 1987).

This is the threshold of an era when many of the most prevalent human cancers can to a significant extent be prevented through life style changes or medical intervention. Better understanding of the possible impact of over exposure to alcohol is therefore necessary.

Chemical and physical carcinogens leave traces of their activities on DNA because of the specific patterns of base changes they induce. Infact many potent mutagens are also carcinogens (Reitz et al., 1985).

The identification of chromosome changes associated with carcinogen exposure of human cells is important because specific and consistent chromosome

alterations characteristic for several forms of cancer (Rowley J.D, 1984) appear responsible for modification in structure and expression of genes involved in cell growth regulation (Popescu et al., 1986).

Therefore it is imperative to check the mutagenic potential of the components present in alcoholic beverages. Since invitro mutagenicity tests can be used to indentify agents with high carcinogenic potential, the short term Ames Salmonella/microsome assay has been adopted to detect the mutagenicity of 3 alcoholic beverages - wine, beer and brandy collected from local shops in Coimbatore.

To evaluate the genotoxicety of the samples the residues from wine, beer and brandy were also tested for chromosomal aberrations and sister chromatid exchanges.

Review of Literature

REVIEW OF LITERATURE

The review of literature pertaining to the present study "Mutagenicity of selected Alcoholic Beverages" is discussed under the following headings.

1. Introduction
2. The Metabolism of Alcohol
   a. Alcohol Dehydrogenase pathway
   b. Microsomal ethanol oxidizing system (MEOS)
   c. H_2O_2 - Catalase system
3. Acute effects of alcohol abuse
4. Chronic effects of alcohol abuse
5. Chronic alcohol abuse : A contributary factor in cancer.
   a. Mutagenicity
   b. Molecular interpretations of the chemical reactions of acetaldehyde on the DNA bases.
   c. Creation of strange base pairs in replication.
6. Cancerogenicity
   a. Mechanisms which may be involved in alcohol mediated carcinogenesis.
   b. Induction of cross links, chromosomal aberrations and sister chromatid exchanges by acetaldehyde.

1. INTRODUCTION:

Alcohol is arguably the oldest drug used by man, dating back to the prehistoric times. Today, however its medical uses are limited and it is used primarily for self induced intoxication. Alcohol is consumed by man because it is a psychoactive drug and has sedative - hyprotic properties (Barar F.S.K., 1989).

Alcohol was almost certainly discovered accidentally in the pre agricultural stage of human development. Any mishmash of fruits or grains if left produces alcohol (Ray mills and Reg Passmore, 1988). In fact, alcohol is produced by fermentation of almost anything with a high carbohydrate content.

All alcoholic beverages arise from the process of fermentation. In the presence of water yeasts, are able to covert the sugar (glucose)of plants into alcohol according to the following reaction.

$$C_6H_{12}O_6 + \text{Yeast} \longrightarrow 2C_2H_5OH + 2CO_2$$

As alcohol is a toxin, fermentation is self limiting. Once alcohol concentration reaches about 14% or the sugar runs out, the multiplying yeasts die and fermentation ends. A stronger drink requires distillation (Gibbons B, 1992).

2. THE METABOLISM OF ALCOHOL:

Alcoholic beverages mainly contain ethanol or ethyl alcohol (concentration varies with the type of preparation) which is used for human consumption, although trace amounts of higher alcohols, methanol, aldehydes and esters are also present which lend different alcoholic beverages their distinctive tastes and aromas.

Alcohol does not require any digestion. It is absorbed from the buccal, oesophageal, gastric and intestinal mucosae, Approximately 80 % is absorbed from the small intestine.

Alcohol is rapidly absorbed from empty stomach but the presence of food in stomach, retards the rate of absorption. After absorption it is carried to different parts of the body but is primarily metabolized in the liver to its end products viz $Co_2 + H_2O$. During its metabolism it provides instantaneous energy and spares food constituents like carbohydrates and fats (Agarwal S.P, 1985).

Alcohol is metabolised by zero order kinetics, which means that a constant amount of alcohol is metabolized in a specific period of time (Lieber C.S, 1976).

This amounts to about 10 ml or about 1 ounce of liquor or about 10 ounces of beer in 1 hour. It is ultimately oxidized to CO_2 & H_2O with the release of 7 calories per gram of alcohol. The remainder is excreted unchanged in the breath, urine and sweat (Mezey. E, 1976).

The liver parenchymal cell (hepatocyte) contains 3 pathways for the oxidative metabolism of alcohol.

a. Alcohol Dehydrogenase pathyway
b. Microsomal ethanol oxidising pathway
c. H_2O_2 - Catalase system

a) ALCOHOL DEHYDROGENASE PATHWAY:

The major pathway involves the enzymes alcohol dehydrogenase (ADH) and acetaldehyde dehydrogenase (ALDH).

These two enzymes are dehydrogenases that oxidize alcohol via the intermediate acetadehyde to acetate with the concomitant reduction of the cofactor NAD to its reduced form NADH

REACTION I

$$C_2H_5OH + NAD^+ \xrightarrow{ADH} CH_3CHO + NADH + H^+$$

REACTION II

$$CH_3CHO + NAD^+ + H_2O \xrightarrow{ALDH} CH_3COOH + NADH + H^+$$

NET REACTION

$$CH_3CH_2OH + 2NAD^+ + H_2O \longrightarrow CH_3COOH + 2NADH + 2H^+$$

Acetate is readily metabolised in the body, the initial reaction in the pathway being its conversion to acetylcoA. ADH is present in the cell cytosol, whereas ALDH activity is found both in the cytosol and mitochondria. The coupled reduction of NAD^+ to NADH associated with alcohol metabolism via this pathway generates an excess of reducing equivalents as NADH in the cytosolic and mitochondrial compartments. This redox imbalance is one important route by which alcohol perturbs liver metabolism. (Smith. M, 1986).

b. MICROSOMAL ETHANOL OXIDISING SYSTEM (MEOS):

This system located on edoplasmic reticulum is dependent on a special cytochrome cyt p 450 and involves the cofactor NADPH and molecular O_2. This pathway also

generates acetaldehyde, the further oxidative metabolism of which involves ALDH.

$$C_2H_5OH + NADPH + H^+ + O_2 \xrightarrow{\text{MEOS}} CH_3CHO + NADPH^+ + 2H_2O$$

The MEOS is increased in activity by chronic exposure to alcohol and this induction is asociated with proliferation of the endoplasmic reticulum. Habitual heavy drinkers have an accelerated capacity to metabolize alcohol and this is largely a function of the induction of MEOS.

c) H_2O_2 - CATALASE SYSTEM:

The third albeit minor pathway of hepatic alcohol oxidation is associated with peroxisomes and involves the enzyme catalase in the presence of an independent oxidising system which generates H_2O_2.

$$C_2H_5OH + H_2O_2 \xrightarrow{\text{CATALASE}} CH_3CHO + 2H_2O$$

Metabolism via this route is limited by the rate of peroxisomal H_2O_2 generation and its contribution to total alcohol oxidation in vivo is unquestionably small. (Palmer T.N. 199; Khanna and Israel, 1989).

The acetate formed by the action of ALDH mixes with acetate derived from the biochemical reactions and enters the Kreb's tricarboxylic acid cycle producing energy. Many steps later it is converted to CO_2 and H_2O. Co is removed from the body in the exhaled air.

$$CH_3COOH \xrightarrow{ALDH} CH_3COASH \xrightarrow{\text{Krebs cycle}} CO_2 + H_2O.$$

3. ACUTE EFFECTS OF ALCOHOL ABUSE:

Many of the acute effects of alcohol are commonly presumed to be a consequence of the NADH generation secondary to alcohol metabolism via the ADH and ALDH reactions.

It has long been established that alcohol consumption may severely inhibit gluconeogenesis. In malnourished individuals whose liver contains little or no glycogen, this inhibition can have serious consequences. A dramatic albeit relatively rare, complication of acute alcohol abuse is a severe and potentially life threatening hypoglycemia which is the immediate consequence of this inhibition of gluconeogenic flux (Williams H.E, 1984).

Recent research suggests that acute alcohol abuse may lessen the liver's capacity to replenish its

glycogen stores following consumption of a carbohydrate containing meal, particularly when glycogen has been severely depleted by fasting or excercise. This diminition in liver glycogen storage may further compromise the liver's role in glucose homeostasis (Palmer T.N, 1988).

The NADH generation associated with alcohol metabolism decreases TCA cycle activity (several reactions of which are NAD^+ dependent) and suppresses fatty acid oxidation. As a consequence alcohol replaces fatty acid as the primary fuel of the liver and instead of being oxidized, fatty acid is deposited as neutral fat (triglyceride) in the liver cell, this deposition being exacerbated by stimulation of alcohol of denovo lipid synthesis in the liver (Palmer T.N, 1989)

The central nervous system is most sensitive to the depressant effects of ethyl alcohol. It is similar to barbiturates and other general anaesthetics. Contrary to common belief alcohol is not a stimulant, and the excited behaviour of the drinker is actually due to the depression of the brain areas which ordinarily exert inhibitary control over psychomotor activity and behaviour. In fact alcohol depresses nervous system so

much so that in high doses it may cause respiratory arrest leading even to death (Balasubramanian D, 1993).

In moderate doses alcohol causes cutaneous and gastric vasodilation, flushing of the skin and a feeling of warmth. Increased sweating can produce hypothermia. This effect is more pronounced when environment temperature is low. Although small doses of alcohol (0.2 to 1g/kg) may not have any adverse effects, heavy drinking in severe cold may lead to rapid loss of body heat and could be dangerous (Agarwal S.P, 1985)

Alcohol acts on the hypothalamohypophyseal system to decrease the release of antidiuretic hormone from the posterior pituitary resulting in diuresis.

Plasma amino acid and vitamin abnormalites and behavioural disorders are known to be associated with alcoholic patients (Rajurkar V. and Shastri N.V, 1990).

In humans the requirement for ascorbic acid is increased by ethanol, because the serum and tissue levels of ascorbic acid decrease (Fazio et al., 1981).

The metabolic changes in ascorbic acid, lipids and drug metabolising enzymes observed when xenobiotics such as PCB or DDT are given were also found when

ethanol was administered to rats in one large dose (Mochizuki et al., 1992).

4. CHRONIC EFFECTS OF ALCOHOL ABUSE:

Chronic use of alcohol, affects various organ systems including the central nervous system, gastrointestinal system, skeletal muscle, bone marrow, liver, purine and lipid metabolisms.

It has been suggested that ethanol exerts its effects on the central nervous system through its interaction with the neuronal membranes (Lalitha et al., 1987).

Chronic ethanol treatment results in membrane changes, primarily in the lipid fraction, that serve to increase rigidity of the membrance (chin et al., 1978). These changes include increase in membrane cholesterol content (Johnson et al., 1979) and in the saturation of fatty acids (Waring et al., 1981) both would serve to decrease the fluidity of the lipid fraction of the membrane. Ethanol could disrupt the function of membrane bound enzymes by acting directly on the enzymes themselves or by disrupting membrane lipid - protein interations (Collins et al., 1984).

Becasue of its simple chemical structure, ethanol appears to act on the membrane non specifically by physical means. Ethanol molecules may insert into the lipid membranes thereby altering the lateral mobility of membrane lipids or react with the hydrophobic part of the membrane proteins resulting in steric changes. (Topel, 1985).

These reactions may change the properties of the cell membranes (fluidity, permeability) and consequently may cause differences in intracellular concentrations of various compounds.

A number of investigators have examined the ethanol induced changes in membrane components. Increase in the levels of acidic phospholipids, phosphatidyl inositol and phosphatidic acid and phosphatidyl serine in synaptic plasma membranes have been reported after chronic ethanol administration (George.S. and Parekh L.J. 1990).

Chronic alcohol abuse clearly reduced the normal increase in total and LDL cholesterol as well as in LDL phospholipids, proteins, total LDL during the 24th to 40 th weeks of pregnant women. LDL changes were more pronounced in alcholic women who later produced

fetal alcholic syndrome infants. Alcohol abuse also increased HDL . Furthermore in alcohol users, normal increase in VLDL is accentuated especially in women with fetal alcohol syndrome infants and that of HDL was reduced. The significance of the changes found remains obscure, but they may be related to impairment function of the fetoplacental unit (Valiniaki et al., 1990).

An increased lipid peroxidation levels was found in the plasma of chronic alcoholics which is probably connected with the higher concentration of active O_2 species in the blood (Sram et al., 1990).

Alcohol in all probability, has a direct hepatotoxic effect, which implies that dietary supplements cannot effectively counter the effects on the liver and the only way to avoid this toxicity is complete abstinence. Approximately 10% of alcoholics develop cirrhosis of variable severity which might later lead to cancer.

5. CHRONIC ALCOHOL ABUSE: A CONTRIBUTARY FACTOR IN CANCER:

Much attention is being paid to the causative relation between intake of alcoholic beverages and the incidence of human cancer.

Consumption of alcohol entails an increased risk for cancer of the buccal cavity, pharynx, larynx, esophagus colon and liver (Tuyns, 1979). Nonvolatile residues of some alcoholic beverages increases the frequency of revertants in the Ames salmonella mutatest (Nagao et al., 1981). However ethanol itself remains unverified as either a carcinogen or mutagen by standard carcinogenicity and mutagenicity tests (Hayes S. 1985; and Soderman, 1982).

a. MUTAGENICITY:

Alcohol is mutagenic, cancerogenic and teratogenic in Man. The mutagenic activity of ethanol seems to be mediated via its proximal metabolite ie. acetaldehyde (Obe and Ristow, 1979). Methanol a contaminant of many alcoholic beverages is also mutagenic via its metabolite formaldehyde. In addition different indirect pathways may lead to mutations by alcohol. Ethanol and other alcohols as well as aldehydes inhibit RNA synthesis in cells and in cell free transcriptional systems. A reduction of cellular RNA synthesis may play an important role in the mutagenic, carcinogenic and teratogenic activity of alcohol.

Berryman et al., (1992), have reported that chronic treatments of male CF - 1 mice with ethanol at levels resulting in minimal fertility impairment, increases the frequency of dominant lethal mutations.

b. MOLECULAR INTERPRETATIONS OF THE CHEMICAL REACTIONS OF ACETALDEHYDE ON THE DNA BASES:

Acetaldehyde is a highly reactive chemical that is ubiquitous in the human environment. it occurs naturally in various food products (fruits, spices, beverages) it is present in tobacco smoke and exhaust gases and is used or formed in the manufacture of several industrial process. More over acetaldehyde is the first metabolite of ethanol oxidation. Altogether this reveals that human exposure to acetaldehyde is wide spread, but for obvious reasons the interest has largely been on its action in ethanol metabolism (Helander and Kiessling, 1991)

Acetaldehyde impairs hepatic oxygen utilization and forms protein adducts resulting in antibody production, enzyme inactivation and decreased DNA repair. It also causes alterations in microtubules, plasma membranes and mitochondria. It also enhances pyidoxine and perhaps folate degradation and stimulates collagen production by vitamin A storing cells

(lipocytes) and myofibroblasts (Lieber C.S, 1991 (a)). The hepatic retinoid depletion was found to be associated with lysosomal lesions and decreased detoxification of chemical carcinogens (Lieber C.S. 1991 (b)).

c. CREATION OF STRANGE BASE PARIS IN REPLICATION

A retrospect on the reactivities of acetaldehyde reveals it to be a perfect deaminator cum oxidative initiator, proton neutraliser, competent to bring about identical molecular changes in the DNA bases as were done by nitrous acid and hydroxylamine molecule and acetaldehyde could also rightly claim to be linear aliphatic molecule competent to deaminate and alter the chemical structural constituents of purine and pyrimidine bases in DNA to produce other cyclical structured residues that continued to remain firmly bonded to deoxy ribose residues of DNA (Nandi S.N, 1991).

6. CANCEROGENICITY

Epidemiological analysis have demonstrated a correlation between alcohol consumed and cancer of the mouth, Pharynx, larynx, esophagus, liver and bladder (Kulkarni.D, 1988).

a)MECHANISMS WHICH MAY BE INVOLVED IN ALCOHOL MEDIATED CARCINOGENESIS:

1) Ethanol may change the reactivity of the tissues by toxic effects, by malnutrition or by liver cirrhosis. Alcoholics often show nutritional deficiencies and their mineral metabolism is often imbalanced (Beard and Knott 1971; Flink E.B, 1971) Liver cirrhosis is frequently associated with alcoholism (Naccarato and Farinati, 1991).

2) Carcinogenic substances may be dissolved in alcoholic beverages. Alcoholic beverages are highly complex mixtures of different organic and inorganic compounds. One group of organic consitutents, the fusal oils, consist of alcohols other than ethanol and of aldehydes and is present is most alcoholic beverages (Barar F.S.K.1989, and Obe and Ristow,1979)

3) Alcohol may increase diffusion of carcinogenic substances into cells (Berg J.W, 1977,and Lieber C.S. 1991)

4) Alcohol inhibits salivation and this may lead to a higher concentration of carcinogens from tobacco smoke in the mouth region. This is of importance because most alcoholics are smokers as well (Gori. G.B, 1976).

In addition to these factors others may be of importance for the cancerogenic activity of ethanol. The increased incidence of cancer seen among alcoholics may be due to atleast in part to the enhanced capacity of these individuals to activate procarcinogens in the intestine. The same may be true for the enhanced drug metabolizing capacity of the liver in alcoholics.

A meta-analysis of studies of alcohol and breast cancer has shown a linear dose response relation between alcohol intake and breast cancer risk. Recent evidence that alcohol may increase oestrogen levels adds support to the epidemiological data (Hunter and Trichopoulos, 1992; singletary KW and MCNary, 1992)

One possible link between alcohol consumption and breast cancer may be the induction by ethanol of P450 a subfamily of enzymes that may potentiate other carcinogens (Davis et al., 1992)

Yamagiwa et al., (1991) have examined the effect of alcohol ingestion of hepatocarcinogenesis induced by oral administration of synthetic female hormones Ethynylestradiol (EE) and norethinindrone acetate (NA) They reported that alcohol affects the hepato carcino genesis by EE and NA, promoting not only

the change in kinetics of endoplasmic reticulum, but also DNA.

Experimental and epidemiological investigations in alcoholic and non alcoholic populations have suggested a role of alcohol in lung carcinogenesis (Bandera et al., 1992)

Alcohol consumption is reported to have an enhancing effect on rectosigmoidal colonic carcinogenesis (Niwa et al., 1991; Cope et al., 1991).

Jalovaara et al., (1986), have reported that alcohol and a high fat diet together might have a carcinogenic effect on pancreatic ductal epithelium in rats.

Hsu et al., 1991 have reported the interaction between alcohol and mutagens in carcinogenesis and suggest that alcohol may have co-carcinogenic properties.

b. INDUCTION OF CROSS LINKS, CHROMOSOMAL ABERRATIONS AND SISTER CHROMATID EXCHANGES BY ACE TALDEHYDE.

Alcoholics (Obe et al., 1980) as well as heavy cigarette smokers (Hopkin and Evans, 1980; Sorsa et al., 1982) have an elevated frequency of exchange aberrations of the chronosome type (dicentric chromosomes, ring

chromosomes) and of the chromatid type (chromatid interchanges) in their peripheral lymphocytes.

A synergistic effect of alcohol consumption and cigarette smoking has been reported with respect of cancer of the Oesophagus, pharynx, larynx and the oral cavity (Kulkarni. D, 1988, Tuyns, 1978). Chromosome analysis show that alcoholics who smoke have a higher frequency of chromosomal aberrations than alcoholics who do not smoke (Sorsa et al., 1982)

Acetaldehyde is produced mainly in the liver and has been found in the blood of alcoholics may be atleast in part responsible for the elevation of chromosomal aberrations found in alcoholics (Obe et al., 1980)

It has been shown that acetaldehyde caused a dose dependent linear increase in the frequency of sister chromatid exchanges in cultured human peripheral lymphocytes. The sister chromatid exchange frequency was on an average 2 fold higher when the cells were exposed to the acetaldehyde after 24 hours incubation instead of at the time of mitogen stimulation (Helander & Kiessling 1991).

Kucheria et al., (1986) have reported chromosomal aberrations and increased sister chromatid exchange frequency in male alcoholics.

It has been reported that acetaldehyde induces sister chromatid exchanges in chinese hamster ovary cells (Korte and Obe 1981, and Obe and Beek 1979).

Acetaldehyde also acts as a cross linking agent (Obe and Ristow, 1979) in isolated DNA.

There seems to be a relationship between the induction of sister chromatid exchanges and chromosomal aderrations. Most compounds that induce sister chromatid exchanges induce chromosomal aberrations as well as mutations (Wolff S. 1977) Because mutagenicity and carcinogenicity are positively correlated (Ashby and Tennant, 1988, Ashby et al., 1989, Gold et al., 1989; Rosenkranz & Ennever 1990, Lave et al., 1988) and induction of chromosomal aberrations by acetaldehyde in human lymphocytes invitro, chinese hamster ovary cells, and in mice after treatment with acetaldehyde or after prolonged consumption of ethanol support the suspected mutagenic / carcinogenic activity of alcohol in man.

Experimental Procedure

EXPERIMENTAL PROCEDURE

The experimental procedure pertaining to the present study "Mutagenicity of selected alcoholic beverages", involves the follwoing:

1. Source of alcoholic beverages
2. Preparation of residue from the samples
3. Bacterial tester strains
4. Induction of drug - metabolising enzymes in mouse.
5. Preparation of the S9 fraction
6. Preparation of the S9 mixture
7. Testing the mutagenicity of the residues either in the presence or absence of metabolic activation.
8. Induction of chromosomal aberrations and sister chromatid exchanges by invitro exposure of human lymphocytes to the residues.

1. Sources of alcoholic beverages:

Samples of wine, Beer and brandy were purchased from local shops in Coimbatore.

2. Preparation of residues from the samples:

The 3 samples wine, beer and brandy were evaported separately in a vacuum using a flash evapoator at 70° C. The alcohol portion was distilled and finally the residue which

remained in the flask was used for the mutagenic assay and also for chromosomal aberrations and sister chromated exchanges. The residues from wine, beer and brandy were dissolved in an organic solvent namely 100 % dimethyl sulfoxide (DMSO) and were used for the experiments.

3. **THE BACTERIAL TESTER STRAINS:**

A set of histidine - requiring strains is used for mutagenicity testing. Each tester strain contains a different type of mutation in the histidine operon. In addition to the histidine mutation, the standard tester strains contain other mutations that greatly increase their ability to detect mutagens. One mutation (rfa) causes partial loss of the lipopolysaccharide barrier that coats the surface of the bacteria and increase permeability to large molecules such as benzo (a) pyrene that do not penetrate the normal cell wall (Ames et al., 1973). The other mutation (uvr B) a deletion of a gene coding for the DNA excision repair system resulting in greatly increased sensitivity in detecting mutagens (Ames, 1973). For technical reasons, the deletion excising the uvr B gene extends through the bio gene and as a consequence these bacteria also require biotin for growth. TA 102 does not contain the uvr B mutation because it was constructed primarily for detecting

mutagens that require an intact excision repaire system. The standard tester strains TA 98, TA 100 and TA 102 contain the R factor plasmid PKM 101. TA 102 also contains the multicopy plasmid PAQ1 which carries the his G 428 mutation and a tetracycline resistance gene.

The bacterial strain of Salmonella typhimurium TA 98 was obtained from Amala cancer Research Institute Thrissur, TA 100 and TA 102 were obtained from prof B.N. Ames, Department of molecular and cell Biology, university of california, Berkeley, CA/94720.

MAINTENANCE OF TESTER STRAINS:

Upon receiving the strains, they were inoculated into nutrient broth (8 g of butrient broth, 5g Nacl 1000 ml of distilled water) and allowed to grow at 37 C. The genotypes of the tester strains were confirmed as described in Appendix III and the cultures streaked on nutrient agar plates (master plates)

SPONTANEOUS REVERSION:

Spontaneous reversion of the tester strains to histidine independence is measured routinely in mutagenicity experiments and is expressed on the number of spontaneous revertants / plate. Each tester strain

reverts spontaneously at a frequency that is characteristic of the strain.

Each mutagenicity assay conducted included control plates without the test compounds to assess the spontaneous revertant (SR) frequency. The SR frequency was approximately close to the number specified by Ames (Maron and Ames, 1983).

4. INDUCTION OF DRUG METABOLISING ENZYMES IN MOUSE

Upon entering the system the carcinogens and mutagens are either converted to active metabolites by the carcinogen metabolising enzymes or they are converted to less active forms. The enzymes are of 2 major types.

(1) The activating enzymes

(2) The detoxifying enzymes.

The components of the drug metabolising system include arlyhydrocarbon (benzo (a) pyrene) hydroxylase (AHH), cytochrome b5 and cytochrome p450, aminopyrene N-demethylase (APND), ethoxy resorufin - 0 de ethylase (ERRD), epoxide hydroxylase (EH), glutathione - S - transferase (GST) and glutathione reductase. Many chemicals are used to induce the drug metabolising enzymes. The most commonly used inducers include

phenobarbital and polychlorobiphenyl mixture (Arochlor 1254). In the present dissertation, phenobarbital was used according to the methods reported earlier (Kunz et al., 1987).

A male Swiss albino mouse of 8-10 weeks old and weighing about 23 g was taken and injected intraperitonealy with phenobarbital (1mg/day) for 3 consecutive days.

5. **PREPARATION OF S9 FRACTION:**

After phenobarbital administration for 3 consecutive days, on the fourth day, the animal was killed by cervical dislocation, following overnight fast. The liver was quickly excised using sterile surgical tools, washed with cold isotonic KCl, blotted dry between sterile filter paper folds and weighed. A 20 % homogenate was prepared in cold isotonic kCl. The homogenate was spun at 9,000 x g in a refrigerated centrifuge at 4 C for 15 minutes. The supernatant (S9 fraction) was distributed into sterile 2.0 ml vials after adjusting the protein concentration to 40 mg/ml protein estimation was carried out according to the method described by Lowry et al., (1951) (Appendix I) The S9 fraction was stored in deep freeze (-80 C)

6. PREPARATION OF S9 MIXTURE:

The S9 mixture was prepared fresh just prior to each assay by mixing S9 fraction and the cofactors such as $Mgcl_2$, Kcl, NADP and Glucose - 6 phosphate in 0.1 m phosphate buffer as given in Appendix II.

7. TESTING THE MUTAGENICITY OF THE SAMPLES:

At present the most extensively studied and utilised short term mutagenicity assay is the Ames Salmonella typhimurium test (Appendix III) developed by Ames and his co workers. This test detects a wide variety of mutagens including many that requires metabolic activation quickly and inexpensively. Other advantages are reduced space requirements ability to utilise live cells as potential assay systems and requirements of very small quantities of the test agent.

8. INDUCTION OF CHROMOSOMAL ABERRATIONS AND SISTER CHROMATED EXCHANGES BY INVITRO EXPOSURE OF HUMAN LYMPHOCYTES TO THE RESIDUES.

The ability of a chemical to produce chromosomal damage in human lymphocytes after an invitro exposure is the basis of this test system. The damage is usually induced after the stimulation of the

lymphocytes into division by compounds such as phytohaemagglutinin (PHA-M)

When cells are grown in the presence of bromo deoxyuridine (Brdu), it substitutes thymidine, thus getting incorporated in the DNA strand during semi conservative replication. By the end of the second cycle, one chromatid is bifilarly substituted with Brdu, while the other is unifilarly substituted. On further treatment with a fluorocent dye (Hoechst 33258) followed by exposure to light (sunlight or uv) and staining with Giemsa, the bifilarly substituted chromatid shows light staining, while the unifilarly substituted one shows dark staining. thus exchanges between sister chromotids can be visualised microscopically. The experimental procedure and the staining methods is given in Appendix IV.

STATISTICAL EVALUATION:

The students 't' test has been used to estimate the statistical significance of the mutagenicity expressed by the residues.

Results and Discussion

RESULTS AND DISCUSSION

Most alcoholic beverages contain a number of substances formed as by products during the process of fermentation, collectively termed as fusal oils and these are pharmacologically active and relatively more toxic than ethanol.

In the present study the evaporated residues obtained after separation of the ethanol fraction in a flash evaporator in vacuum at 70° C were used for the mutagenicity assay.

The evaporated residues obtained from three popular alcoholic beverages namely wine, beer and brandy were dissolved in an organic solvent namely 100% dimethyl sulfoxide (DMSO) and was tested using 3 tester strains of Salmonella typhimurium TA 98, TA 100 and TA 102.

TA 98 detects various frame shift mutagens, TA 100 detects mutagens that cause base pair substitutions, TA102 detects various oxidative mutagens.

The Ames test was carried out according to the revised methods for the Salmonella mutagenicity test (Maron & Ames, 1983). Initially the assay was carried

out without the preincubation step, since inconclusive results were obtained by the standard test, the preincubation step was included for all the 3 beverages with all the 3 tester strains.

MUTAGENICITY OF WINE:

The residues obtained after evaporation of wine were applied at doses of 1.0, 2.0, 3.0, 4.0 mg in 0.1 ml DMSO/ plate. 4 minimal glucose agar plates/ concentration were set up. The mutagenic potential of wine was demonstrated in all the 3 tester strains, TA 98, TA 100 and TA102. The number of revertant colonies obtained are tabulated in Table I. The results are expressed as mean ± standard deviation of the 4 plates.

A comparison was made between the number of histidine revertants at various dosage levels and the number of spontaneous revertants obtained for each tester strain. The mutagenicity of wine was demonstrated in all the 3 tester strains both in the presence and absence of S9 mix.

A look at the results obtained for TA 98 indicates a clear dose dependent increase in the number of revertant colonies obtained. It was found that the residue showed mutagenic effects even at the lowest concentration tested ie. 1.0 mg. All the four

concentrations tested showed statistically significant results in the absence of metabolic activation. On metabolic activation the number of histidine revertants were found to decrease considerably. The lowest concentration showed no significant difference.

Similarly the results obtained for TA 100 were analysed. Here also the number of Histidine revertants increased as the dosage increased . In the absence of S9 mix the revertant colony count obtained for the lowest concentration was not significant when compared to the control. However the higher concentrations exhibited increasing number of revertant colonies. This increase also was found to be statistically significant ($P<0.01$).

The results obtained in the presence of metabolic activation showed no statistical significance even at the highest concentration tested. This clearly shows that the enzymes in the S9 mixture might have metabolically inactivated the mutagens in wine.

The tester strain TA102 also demonstrated the mutagenic potential of the residue from wine. In the absence of S9 mix the colony count obtained for the lowest concentration ie. 1 mg did not show any

significant difference. The other three concentrations however showed statistically significant ($P<0.01$) revertant colony counts when compared with the control plates.

In the presence of S9 mix only the higher concentrations ie. 3.0 mg and 4.0 mg showed statistically significant reversion ($P<0.01$).

Lin and Tai (1980) have demonstrated a similar dose dependent increase in the mutagenicity of chinese wine treated with nitrite.

The results obtained show that the residue obtained from wine is directly mutagenic requiring no microsomal enzyme activation. The mutagen precursor might be a polar compound which is either present in the raw materials used to make wine or produced during fermentation.

TABLE I

MUTAGENICITY OF WINE IN *Salmonella typhimurium*

Dose mg/plate	No. of His revertants in TA 98		No.of His revertants in TA 100		No. of His revertants in TA 102	
	-S9	±S9	-S9	±S9	-S9	±S9
SR	49 ± 2.58	42 ± 2.58	172 ± 3.65	164 ± 5.88	282 ± 8.16	267 ± 3.162
1.0	*57 ± 2.58	44 ± 2.94	175 ± 4.96	165 ± 4.54	300 ± 4.32	265 ± 4.54
2.0	**69 ± 0.81	**53 ± 3.74	**189 ± 3.65	167 ± 2.1	**319 ± 4.16	268 ± 4.76
3.0	**77 ± 2.58	**56 ± 3.91	**202 ± 7.3	169 ± 6.21	**329 ± 4.76	**280 ± 2.94
4.0	**91 ± 2.58	**67 ± 7.14	**213 ± 4.7	174 ± 8.28	**354 ± 5.16	**291 ± 4.69

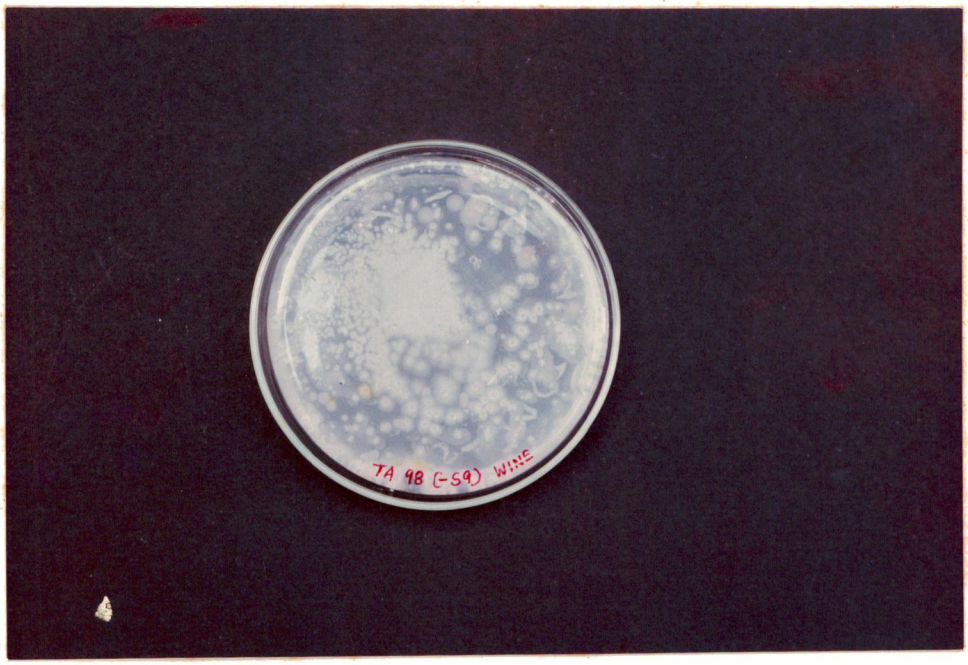
The values are mean ± S.D of four plates

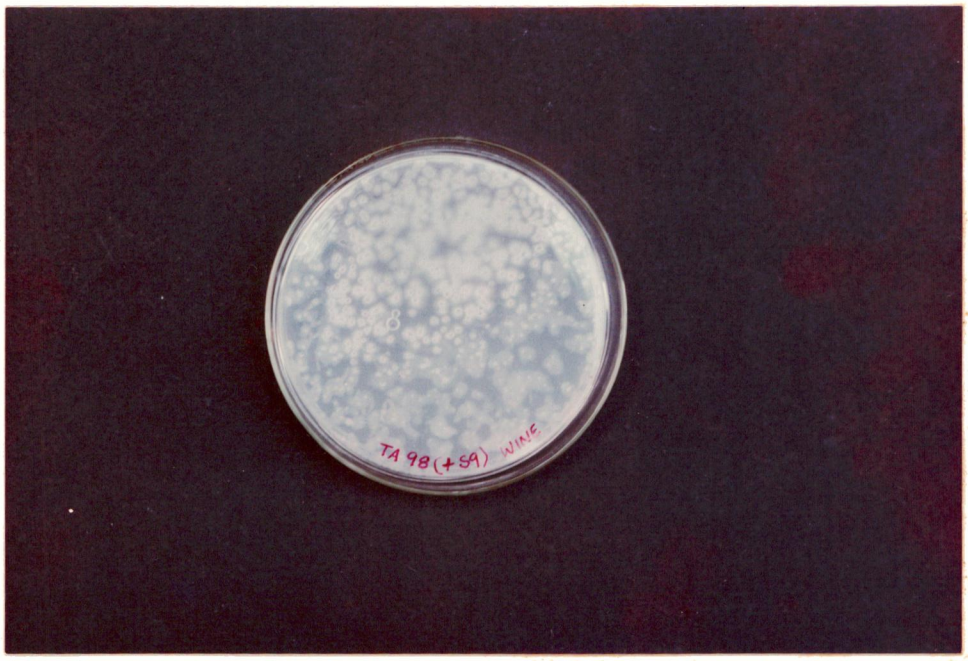
SR - Spontaneous revertants

** - $P < 0.01$

* - $P < 0.05$

MUTAGENICITY OF WINE IN TA 98





Mutagenicity of Beer:

The residues obtained after evaporation of beer were applied at doses of 1.0, 2.0, 3.0, 4.0mg in 0.1 ml DMSO/plate. 4 minimal glucose agar plates/ concentration were set up. The mutagenic activity of beer was demonstrated in all the 3 tester strains TA 98, TA 100 and TA102. The number of histidine revertants obtained are tabulated in Table II. The results are expressed as mean ± standard deviation of the 4 plates.

The mutagenicity of beer was demonstrated in all the tester strains used for the assay. A comparison was made between the number of Histidine revertants at various dosage levels and the number of spontaneous revertants for each tester strain.

The results obtained for TA 98 indicate a clear dose reponsive increase in the number of revertant colonies. The residue showed mutagenic effect even at the lowest concentrations both in the presence and absence of metabolic activation. A comparison between the number of revertant colonies obtained in the presence and absence of S9 showed a decrease in the number of revertant colonies in the presence of S9 indicating a role of microsomal enzymes in detoxifying

the mutagens. All the results obtained showed statistical significance ($P<0.01$).

The results obtained for TA 100 also demonstrated an increase in the number of Histidine revertants as the dosage increased. In the absence of S9 the lowest concentration did not show statistical significance.

All the other concentrations tested exhibited statistically significant difference when compared to the control plates. In the presence of S9 mix only the higher concentrations ie. 3.0 and 4.0 mg showed statistically significant revertants ($P<0.01$).

The tester strain TA102 also indicated the mutagenicity of the residue from beer. In the absence of metabolic activation the lowest concentration ie. 1 mg did not show statistically significant number of revertants. However the other three concentrations showed statistically significant number of revertants ($P<0.01$) when compared with the spontaneous revertant colony count.

In the presence of the mouse liver microsomal enzyme fraction only the highest dose ie. 4.0 mg exhibited statistically significant difference ($P<0.01$).

TABLE II

MUTAGENICITY OF BEER IN *Salmonella typhimurium*

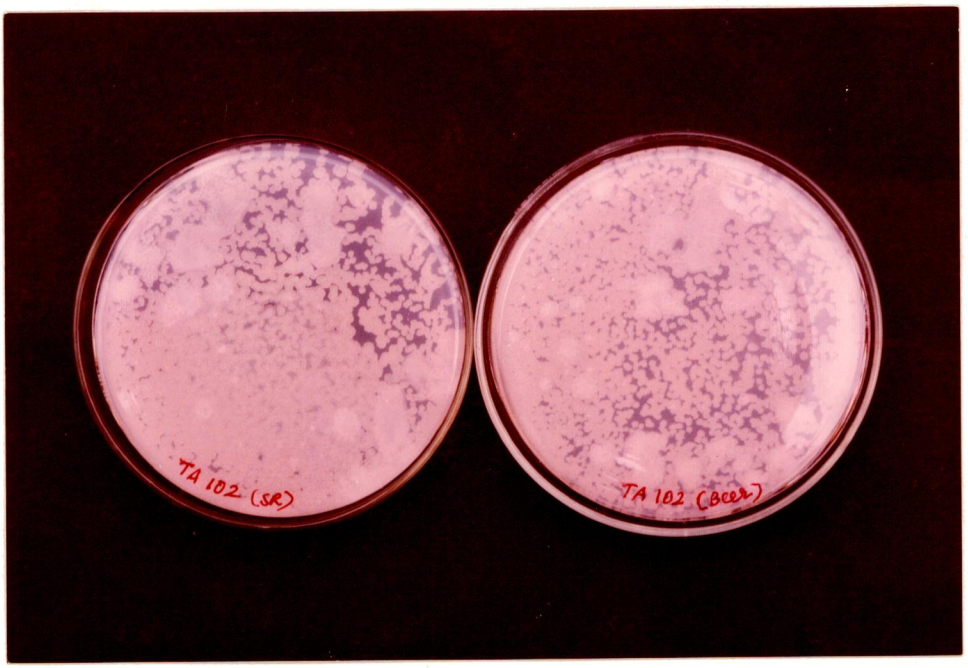
Dose mg/plate	No. of His revertants in TA 98		No.of His revertants in TA 100		No. of His revertants in TA 102	
	-S9	±S9	-S9	±S9	-S9	±S9
SR	49 ± 2.58	42 ± 2.58	172 ± 3.65	164 ± 5.88	282 ± 8.16	267 ± 3.162
1.0	**66 ± 6.976	**57 ± 3.65	175 ± 10.39	165 ± 2.94	286 ± 10.98	261 ± 2.58
2.0	**80 ± 5.1	**68 ± 5.29	**200 ± 6.58	170 ± 3.16	**305 ± 42.58	263 ± 5.16
3.0	**92 ± 8.6	**77 ± 5.59	**217 ± 7.18	**177 ± 5.29	**320 ± 2.45	275 ± 3.16
4.0	**109 ± 7.48	**81 ± 6.21	**231 ± 8.98	**181 ± 4.96	**328 ± 13.56	**288 ± 3.16

The values are mean ± S.D of four plates

SR - Spontaneous revertants

** - $P < 0.01$

MUTAGENICITY OF BEER IN TA 102 WITHOUT S9 MIX



MUTAGENICITY OF BRANDY:

The residue obtained after evaporating brandy were applied at doses of 100, 200, 300, 400 μg in 0.1 ml DMSO/plate. Here also 4 minimal glucose agar plates/ concentration were set up. Even at microgram levels the residue from brandy was found to be mutagenic in all the strains tested ie TA 98, TA 100 and TA 102. The results obtained are tabulated in Table III.

The results obtained for TA 98 show a clear increase in the number of Histidine revertants as the concentration of the residue increased. The residue showed mutagenic effects even at the lowest concentration ie. 100μg. Metabolic activation brought down the number of revertant colonies considerably. All the values obtained for TA 98 showed statistically significant difference ($P<0.01$).

A glimpse at the data obtained for TA 100 indicates a linear increase in the number of revertant colonies obtained as the dosage increased. All the 4 doses tested in the absence of S9 mix showed significant number of revertants. However none of the doses tested exhibited significant number of revertants in the presence of the S9 mixture. This mixture contains a number of detoxifying enzymes and cofactors which might

have detoxified the mutagens present in brandy to lesser toxic forms.

Similarly the results obtained for TA102 were analysed. They showed a dose responsive increase in the number of Histidine revertants. In the absence of metabolic activation all the counts obtained were statistically significant. Upon metabolic activation none of the doses tested showed a statistically significant increase in the number of Histidine revertants when compared to the control plate.

The results obtained were in accordance to that reported by Nagao et al., (1981), and Loquet et al., (1981), Nagao et al., (1981) have demonstrated the mutagenicities of 5 brands of brandy & 1 apple brandy. They have reported that addition of S9 mix decreased or abolished the mutagenicities of the residues.

Similarly Loquet et al., (1981) have demonstrated the mutagenic potential of the constituents of apple brandy and various alcoholic beverage collected in Western France.

Bhide and Zariwala (1992) have reported the mutagenicity of alcoholic beverages and their cancer causative effect in men and women.

Because the production of Whisky & Brandy involves distillation it is easily conceivable that mutagens may be produced during maturation in the barrel.

In all, the comparison of results obtained for each strain showed that the residue obtained from brandy was mutagenic in all the 3 tester strains in the absence of metabolic activation. However on metabolic activation it was found that the number of revertant colonies decreased considerably showing a role in the detoxification of the mutagens.

TABLE III

MUTAGENICITY OF BRANDY IN *Salmonella typhimurium*

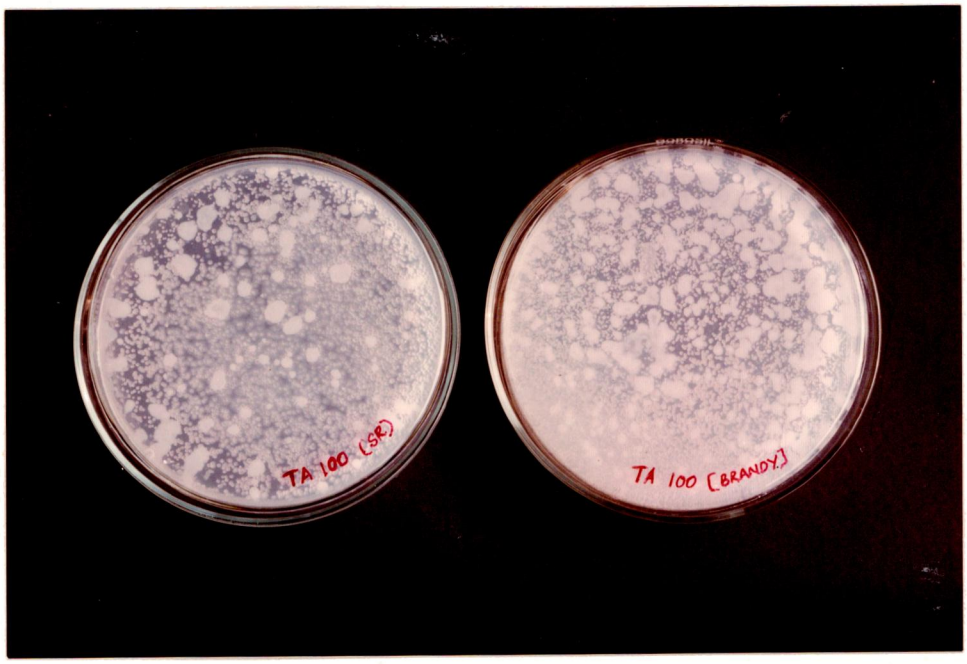
Dose µg/plate	No. of His revertants in TA 98		No.of His revertants in TA 100		No. of His revertants in TA 102	
	-S9	±S9	-S9	±S9	-S9	±S9
SR	49 ± 2.58	42 ± 2.58	172 ± 3.65	164 ± 5.88	282 ± 8.16	267 ± 3.162
100	**81 ± 4.76	**73 ± 2.94	**182 ± 2.94	169 ± 4.54	**308 ± 6.21	263 ± 3.55
200	**104 ± 6016	**81 ± 2.16	**198 ± 5.47	174 ± 2.82	**318 ± 4.08	266 ± 8.04
300	**107 ± 4.69	**90 ± 6.48	**210 ± 7.07	182 ± 5.88	**322 ± 5.35	270 ± 2.94
400	**128 ± 4.83	**99 ± 10.95	**217 ± 4.76	186 ± 8.64	**339 ± 3.91	274 ± 5.88

The values are mean ± S.D of four plates

SR - Spontaneous revertants

** - $P < 0.01$

MUTAGENICITY OF BRANDY IN TA 100 WITHOUT S9 MIX



Chromosomal aberrations and sister chromatid exchange analysis was also carried out using the blood sample obtained from a normal person. The peripheral blood lymphocytes were cultured and the test compounds ie. residues obtained from wine, beer and brandy were added at doses of 4.0 mg, 4.0 mg 400 μg respectively after 24 hours of setting up the cultures for chromosomal aberrations and 48 hours of setting up the cultures for sister chromatid exchanges. The doses selected were the highest concentration used in the Ames Salmonella mutagenicity assay.

In all 8 cultures were set up for chromosomal aberration study and 8 cultures for sister chromatid exchange analysis. The cultures set up included the residues from wine, beer, brandy separately, then wine + BP, beer + BP and brandy + BP one DMSO control culture and one benzo (a) pyrene (BP) culture. A control blood culture without adding any compound was also set up.

The cultures were arrested at metaphase using colchicine and after staining the metaphases were analysed. 100 complete well spread metaphases per culture were analysed.

On analyzing the metaphases for chromosomal aberrations it was found that the frequency of chromosomal aberrations fron wine, beer and brandy cultures and the control DMSO were almost nearer to that found in the control blood culture to which no compound had been added. This shows that the residues from wine, beer and brandy did not have any effect in increasing the frequency of chromosomal aberrations.

On analysing the metaphases from BP + wine, BP + beer, BP + brandy, and on comparing it with that obtained for Benzo (a) pyrene (BP) alone there was no significant increase in the number of chromosomal aberration frequency.

Similarly on analysing the metaphase plates for sister chromatid exchanges it was found that the residues from wine, beer and brandy did not exhibit any increase in the SCE frequency as compared to the control blood culture and DMSO control. Moreover the metaphases from BP + wine, BP + beer and BP + brandy also did not show any increase in the number of SCE's when compared to the Benzo (a) pyrene culture.

TABLE IV

CHROMOSOMAL ABERRATIONS PER 100 METAPHASES INDUCED BY THE RESIDUES FROM WINE, BEER AND BRANDY

SUBSTANCE	TOTAL ABERRATIONS
Untreated control	12
DMSO control	13
Wine	13
Beer	14
Brandy	16
Benzo (a) pyrene	20
Benzo (a) pyrene + Wine	24
Benzo (a) pyrene + Beer	25
Benzo (a) pyrene + Brandy	28

TABLE V

SISTER CHROMATID EXCHANGES PER 20 METAPHASES INDUCED BY THE RESIDUES FROM WINE, BEER AND BRANDY

SUBSTANCE	NUMBER OF SCE
Untreated control	2.5± 0.20
DMSO control	3.5± 0.25
Wine	3.61± 0.29
Beer	3.66± 0.45
Brandy	3.72± 0.65
Benzo (a) pyrene	8.1± 0.48
Benzo (a) pyrene + Wine	8.3± 0.52
Benzo (a) pyrene + Beer	8.33± 0.49
Benzo (a) pyrene + Brandy	8.35± 0.62

These results indicate that the residues from wine, beer and brandy did not cause any significant increase in the number of chromosomal aberrations and sister chromatid exchange frequency at this dose tested.

Summary and Conclusion

SUMMARY & CONCLUSION

Modern technology has produced a veritable chemical renaissance, allowing us to enjoy an unprecedented era of abundance of consumer goods. Little it is realised that these very chemical servants pose a threat to our genetic system, sine qua non of all that we are and all that we have. Now it has been established beyond doubt that our environment is beset with many deadly chemicals that cause deleterious heritable changes (mutations) without showing any immediate toxic effects. There also exists sufficient ground that these mutagens are the root cause of cancer. Additionally, when such mutations affect germ cells, children may be born with heriditary illness.

Apart from these, the majority of the causes of cancer are associated with a persons's life style that is with personal choices and not with the environment in general. Presently much attention is being paid to the causative relation between intake of alcoholic beverages and the incidence of human cancer.

Since human carcinogens are primarily mutagens (genotoxicants) in the present dissertation

"Mutagenicity of selected alcoholic beverages", the Ames Salmonella/microsome assay was carried out using the mutant strains of Salmonella typhimurium (TA 98, TA 100 and TA 102).

The residues obtained after evaporation of three alcoholic beverages wine, beer and brandy were dissolved in an organic solvent namely dimethyl sulfoxide and used for the assay. Initially the assay was carried out without the preincubation step. Since this provided inconclusive results the preincubation step was included for all the three beverages tested in all the 3 tester strains namely TA 98, TA 100 and TA 102.

The results obtained clearly indicate that all the three beverages (wine, beer and brandy) are mutagenic in all the three tester strains.

The residue obtained after evaporation of wine was tested in all the three tester strains at doses of 1.0, 2.0, 3.0, and 4.0 mg. All the three strains showed that the nutagens in wine can cause frame shift mutation, base pair mutation and oxidative damage to DNA.

Similarly the residue obtained after evaporation of beer was tested in TA 98, TA 100 and TA

102 at doses of 1.0, 2.0, 3.0, and 4.0 mg. The mutagenicity of the residue from beer was demonstrated in all the three tester strains of S. typhimurium.

The residue obtained from brandy was found to be more mutagenic than that obtained from wine and beer. When brandy was applied at doses of 1.0, 2.0, 3.0, and 4.0 mg the number of revertant colonies obtained were enormous making it difficult to count. So the dosage was reduced to 100, 200, 300 and 400 μg and applied in the subsequent assay in the presence and absence of metabolic activation. It was found that the residue obtained from brandy was mutagenic even at microgram concentrations.

To sum up all the three selected alcoholic beverages wine, beer and brandy were found to be mutagenic in all the three tester strains of S.typhimurium In the absence of metabolic activation mixture the residues from all the three alcoholic beverages wine, beer and brandy showed high mutagenicity. However addition of S9 mix (mouse liver microsomal enzymes plus cofactors) decreased the mutagenicities of the 3 residues in all the three tester strains.

The chromosomal aberration and sister chromatid exchange analysis did not provide conclusive results about the action of the residues in increasing the frequency of chromosomal aberration and sister chromatid exchanges. Since only one concentration (ie the highest concentration used for Ames assay) for each beverage was tested one cannot say with confidence that the residues from wine, beer and brandy do not have any effect in increasing the frequency of chromosomal aberration and sister chromatid exchanges.

Much more data regarding the cumulative effect of ethanol and also the metabolite of ethanol ie. acetaldehyde and the residues in increasing the frequency of chromosomal aberration and sister chromatid exchanges should be checked out.

In conclusion the finding that the residues from the alcoholic beverages wine, beer and brandy are mutagenic suggests that they may be initiators. Further the mutagenicity was demonstrated in all the 3 tester strains ie TA98, TA100 and TA102 indicating that frameshift mutagens, base pair substituting mutagens and oxidative mutagens are present in these beverages. However the residues should be checked out to see if they have any cumulative effect in increasing the

frequency of chromosomal aberrations and sister chromatid exchanges along with ethanol and its metabolite acetaldehyde. Of course the finding of mutagens in alcoholic beverages does not rule out the possibility that ethanol itself acts as an initiator/or promoter.

RECOMMENDATIONS

1. The urine from alcoholics can be collected and the urine mutagenicity can be checked using the Ames Salmonella Microsome Assay.

2. CA and SCE analysis can be carried out invitro as a dose dependant study along with ethanol and acetaldehyde.

3. A survey can be conducted among chronic alcoholics to find out the incidence of cancer and the types of cancers to which they are most susceptible.

4. Identification of the compound present in the alcoholic beverages which is responsible for the mutagenicity.

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Appendices

APPENDIX - I

PROTEIN ESTIMATION BY LOWRY METHOD

PRINCIPLE:

This method is based on the principle that different proteins contain different amounts of aromatic amino acid residues which react with Folin Ciocalteau reagent giving a blue color which is read in a colorimeter at 660 nm (red filter).

REAGENTS:

A. Alkaline sodium carbonate solution (2% Na_2Co_3 in 0.1 N NaOH)

B. Copper sulphate - Sodium potassium tartarate solution (0.5% $CuSO_4$ in 1% sodium potassiun tartarate).

C. Alkaline copper reagent: 50 ml of reagent A and 1 ml of reagent B.

D. Folin ciocalteau reagent: Diluted the reagent with equal volume of distilled water just before use.

E. 1 N NaOH.

PROCEDURE:

The protein sample was suspended in 1 ml of 1 N NaOH at 100° C for 4.5 minutes. 5 ml of reagent C was added to it and the mixture was allowed to stand at room

temperature for 10 minutes. 0.5 ml of Folin Ciocalteau reagent was added rapidly and the contents in the tube mixed thoroughly.

The amount of protein in the sample was calculated with a standard curve prepared using bovine serum albumin.

APPENDIX II

PREPARATION OF S9 MIX (MOUSE LIVER MICROSOMAL ENZYMES + COFACTORS).

INGREDIENT	PER 50 ML STANDARD S9 MIX
Mouse liver S9 (Phenobarbital induced)	2.0 ml (4%)
$MgCl_2$ - Kcl salts	1.0 ml
1 M glucose 6 phosphate	0.25 ml
0.1 M NADP	2.0 ml
0.2 M phosphate buffer, PH 7.4	25.0 ml
Sterile distilled water	19.75 ml

Salt solution (1.65 M Kcl + 0.4 M $Mg\ cl_2$)

INGREDIENT	PER 5 ML
Potassium chloride (Kcl)	0.615 g
Magnesium chloride ($Mgcl_2 . 6 H_2O$)	0.407 g
Distilled water	to a final volume of 5 ml.

1 M glucose 6 - phosphate

INGREDIENT	PER 1 ML
Glucose 6 - phosphate (G-6-P)	0.282 g
Sterile distilled water	1 ml

0.1 M NADP solution

INGREDIENT	PER 5 ML
NADP (F.W 765.4)	383 mg
Sterile distilled water	5 ml

0.2 M SODIUM PHOSPHATE BUFFER pH 7.4

INGREDIENT	PER 500 ML
0.2 M Sodium dihydrogen phosphate (13.8g / 500 ml) ($NaH_2PO_4 . H_2O$)	60 ml
0.2 M disodium hydrogen phosphate (14.2g / 500 ml) (Na_2HPO_4.)	440 ml

The stock solutions of the cofactors were prepared and stored as described by Maron and Ames (1983) The S9 mixture prepared fresh, was held on ice throughout the assay. The sterility was tested by plating the S9 mixture on the minimal glucose agar plate. The use of optimum amount of S9 mixture is important in testing the mutagenicity of a compound. In the present assay, 0.5 ml of S9 mix per plate was found to be optimum and hence this concentration was used throughout the assay.

APPENDIX III

Ames salmonella / microsome assay

(Maron and Ames, 1983)

PRINCIPLE:

A set of histidine requiring strains is used for mutagenicity testing. These strains are incapable of growth in the absence of histidine in the growth medium. When a mutagen is added to the culture, the strain is mutated back, thereby losing the histidine dependence for its growth. The number of revertant colonies resulting after the action of the mutagen depends on the potency of the compound.

The type of mutation in the histidine operon in the strains are different, thereby enabling the identification of frameshift mutagens (those which are mutagenic towards TA 98), base pair substituting mutagens (Those which are mutagenic towards TA 100), oxidative mutagens (those which are mutagenic towards TA 102).

CONFIRMING THE GENOTYPE OF THE TESTER STRAINS:

In addition to the histidine mutation, the standard tester strains contain other mutations that greatly increase the sensitivity in detecting the mutagens. The rfa mutation causes partial loss of the

lipopolysaccharide barrier that coats the surface of the bacteria, thus increasing the permeability of large molecules such as benzo (a) pyrene that do not penetrate the normal cell wall (Maron and Ames 1983). The other mutation (uvr B mutation) involves the deletion of a gene coding for the DNA excision repair system, resulting in greatly increased sensitivity in detecting many mutagens (Ames, 1971; 1973).

1. **HISTIDINE REQUIREMENTS:**

The His Character of the tester strain was confirmed by demonstrating the histidine requirements for growth on selective agar plates (Biotin is also required by all the standard tester strains [except TA 102]) because of the uvr B deletion which extends through bio gene. TA 102 does not require biotin as it has intact excision repair system.

Each plate (with 0.1 ml of 0.5 mM biotin and with or without 0.1 ml of 0.1 M histidine) were streaked with the strains, incubated overnight at 37° C and examined for growth. The histidine requirement was shown by growth observed only in the histidine/biotin plate, but not in the control plate with only biotin.

2. **rfa MUTATION:**

The presence of rfa mutation was checked by testing the permeability of large molecules. Crystal violet is used for this purpose. Sterile filter paper disc on to which 10 ml of crystal violet solution (1μg/ml) had been delivered was carefully plated on the solidified top agar to which the bacterial culture had been added. After overnight incubation at 37°C a clear zone of inhibition was observed around the disc, indicating the presence of rfa mutation, permitting large molecules like crystal violet to enter and kill the bacteria.

3. **uvr B MUTATION:**

The uvr B mutation is quite stable and can be confirmed by demonstrating uv sensitivity in strains containing this mutation. For this the cultures were streaked on the nutrient agar plates. A piece of cardboard was placed over the uncovered plate so that half of each bacterial streak is covered. The plates were then irradiated with a 15 - W germicidal uv lamp at a distance of 33cm for 8 seconds. A strain with wild type excision repair enzymes (eg TA 102) is tested on the same plate as a control for the uv dose. The irradiated plates were incubated overnight at 37° C

after which time it was observed that the strains with uvr B deletion (TA 98, TA 100) grow only on the un-irradiated side of the plate.

4. R - FACTOR:

The presence of R - factor should be tested routinely by the presence of ampicillin resistance, because the plasmid is somewhat unstable and can be lost from the bacteria (Mc Cann et al., 1975). For this, plates containing ampicillin (25 mg/ml) in the basal agar were prepared and the cultures were streaked on this. After 12-24 hours incubation at 37° C growth was observed along the streaks made with the strains, showing ampicillin resistance.

5. **pAQ Plasmid:**

The pAQ1 strain (TA 102) should be tested for both ampicillin and tetracycline on ampicillin/tetracycline plates.

PROCEDURE FOR THE MUTAGENESIS ASSAY

Overnight cultures of TA 98, TA 100 and TA 102 of Salmonella typhimurium, in nutrient broth were used for the mutation tests.

Mutagenicities were tested by a modification (Nagao et al., 1977) of the Ames method (Ames et al.,

1975). A mixture of the test substance in 0.1 ml of 100% DMSO, 0.5 ml of S9 mix or 0.2 M sodium phosphate buffer (pH 7.4) and 0.1 ml of a culture of the bacterial tester strain was incubated at 37°C for 20 minutes. Then 2.0 ml of molten top agar (45°C) was added and the contents were mixed gently and thoroughly and poured over the basal agar (Appendix III) and spread evenly. After the top agar had solidified, the plates were incubated inverted at 37°C for 48 hours. At the end of 48 hours the number of histidine - revertant colonies were counted.

Each assay included 5 sets of plates as follows.

1. The overnight growth cultures were serially diluted and known amounts of these cultures were plated on nutrient agar plates to determine the number of viable bacteria in the inoculum. The plates were incubated at 37° C overnight and the number of colonies were counted.

2. The spontaneous revertants in the inoculum were determined as above, with the exception that 0.1 ml of the culture was added to the top agar before pouring on to the plate.

3. The number of spontaneous revertants due to the additon of S9 mix was determined by adding 0.1 ml of culture and 0.5 ml of S9 mix.

4. The number of revertants induced by the test substance was determined by plating 0.1ml of the culture and 0.1 ml of appropriate concentration of the test compound on minimal glucose agar plates supplemented with trace amounts of histidine.

5. The number of revertants in the presence of the S9 mix were determined as in the above step with 0.5 ml of S9 mix.

After counting the number of viable bacteria in the inoculum, the number of induced revertants were finally converted per 2×10^8 bacterial cells and this was referred to as revertants per plate in subsequent results (Maron and Ames, 1983).

Each concentration of the test substance was tested in 4 plates and the results are expressed as Mean $\pm$ standard deviation of 4 plates.

PREPARATION OF SOLUTIONS FOR THE MUTAGENESIS ASSAY

1. Minimal Glucose (Basal Agar) plates:

For the mutagenesis assay, the minimal glucose agar medium was prepared as follows:

Agar	- 15 g
Distilled water	- 930 ml
50 x VB salt solution	- 20 ml
40% glucose	- 50 ml

The VB salt solution and glucose solution were autoclaved separately and mixed to the agar solution (also autoclaved) just before pouring on the petriplates.

2. Vogel Bonner ((VB) Salt solution (50 X):

Warm distilled water	- 67 ml
Magnesium sulphate ($MgSo_4$ 7 H_2O)	- 1.0 g
Citric acid monohydrate	- 10 g
Dipotassium hydrogen phosphate	- 50 g
Sodium ammonium phosphate	- 17.5 g

The salts were added in the order indicated, to warm water (45°C). Each salt was allowed to dissolve completely before adding the next. The volume was adjusted to 100 ml and the solution was autoclaved.

3. TOP AGAR

Agar	-	0.6 g
Sodium chloride	-	0.5 g
Distilled water	-	100 ml

4. HISTIDINE - BIOTIN SOLUTION:

This solution contained 0.5 mM biotin and 0.1 M histidine.

L - Histidine	-	0.7758 mg
D - Biotin	-	1.2215 mg
Distilled water	-	10 ml

The agar was dissolved by autoclaving. The solution was cooled to 45 degree celcius and Histidine - Biotin solution was added to it (10 ml to 90 ml of top agar solution) and 2.0 ml aliquots was distributed into sterile tubes for the assay.

APPENDIX IV

CHROMOSOMAL ABERRATION AND SISTER CHROMATID EXCHANGE ANALISIS INVITRO [Kulkarni et al., 1982]

SETTING UP OF LEUCOCYTE CULTURES

Blood from a healthy individual who was non alcoholic, non user of tobacco in any form, not on any drugs, antibiotics, analgestics or antihistamines and not exposed to excess radiations was drawn by venipuncture under aseptic conditions, using a heparinised syringe (which was heparinised using preservative free heparin just prior to blood collection) Sterile techniques should be used throughout the sampling & culture procedures:

Leucocyte cultures were set up for chromosomal aberration analysis and sister chromatid exchange (SCE) analysis together, along with control cultures for each. The cultures were set up using a modified method of Hungerford (1965). 5 ml of F-10 medium supplemented with 10% human AB serum and 0.3 ml of PHA-M were taken in sterile glass bottles. About 0.3 ml of blood was added to this and the bottles tightly corked and incubated at 37°C for 24 hours.

At the end of 24 hours, the test compounds (Wine, Beer and brandy at concentrations of 4.0 mg, 4.0 mg and 400 µg respectively per 0.1 ml DMSO) were added to the cultures used for scoring chromosomal aberration. Thecultures used forSCE analysis were supplemented with BrdU at a concentration of 5 µg / ml and all the bottles were further incubated at 37°C for 24 hours in complete darkness.

After 48 hours of setting up the cultures, the cultures used for the chromosomal aberration analysis were terminated while the cultures used for SCE analysis were treated with the test compounds (Wine, beer and brandy at concentrations of 4.0 mg, 4.0 mg and 400 µg 0.1 ml DNSO respectively) and incubated at 37° C for another 24 hours at the end of which they are terminated.

HARVESTING OF THE CULTURES:

Harvesting of the cultures was carried out at the end of 48 hours (for chromosomal aberration studies) or 72 hours (for SCE analysis). Colchicine at a concentration of 0.2 µg/ml was added to the cultures 90 minutes prior to harvesting, to arrest the cells at metaphase. 90 minutes after colchicine addition, the cultures were centrifuged for 10 min at 1000 rpm. the

At least 100 complete, well spread metaphases were scored for chromosomal aberrations from each culture.

SCE Analysis:

Phosphate buffered saline (PBS)_ (pH 7.0)

Kcl	-	40 mg
KH_2PO_4	-	20 mg
$Na_2HPO_4 2H_2O$	-	57.5 mg
Nacl	-	800 mg
Distilled water	-	100 ml

Stock Giemsa solution:

Giemsa powder	-	1 g
Glycerol	-	50 ml
Methanol	-	50 ml

Buffer for diluting Giemsa stain

$Na_2HPO_4 2H_2O$	-	0.445 g
KH_2PO_4	-	0.340 g
Distilled water	-	200 ml

Working solution of Giemsa

Stock Giemsa	-	2 ml
Buffer	-	45 ml
Methanol	-	5 ml

Saline salt citrate (SSC) solution

Nacl - 1.7535 g

Trisodium citrate - 0.8823 g

Distilled water - 100 ml

The slides were aged for al least 3 days prior to staining.

The slideswere stained in the dark with hoechst 33258 stain dissolved in PBS to give a concentration of 10 μg/ml, for 15 minutes. They were then rinsed with distilled water and arranged on a petridish.

Distilled water was added to the slides, so that a thin film of water was formed on the slides. The slides were then exposed to uv lamp (360 nm) at a height of 15 cm - 17 cm for 2 hours. After uv exposure, the slides were treated with SSC solution maintained at 60^{o} C for 2 hours. The slides were then washed throughly with distilled water, care being taken not to precipitate the salts. The slides were then counter-stained with diluted Giemsa for 15 minutes.

After staining, the slides were aged for 7 days. At least 20 well spread metaphases per culture were scored for the occurence of SCEs.