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Metabolic Activation of the Mutagens/Carcinogens 2-Aminofluorene and 2-Amino-3,4 Dimethyl-Imidazo[4,5-F]-Quinoline by Ethanol-Induced and Uninduced Rat Tongue Tissues in a Modified Salmonella Mutagenicity Test

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METABOLIC ACTIVATION OF THE MUTAGENS/CARCINOGENS 2-AMINOFLUORENE AND 2-AMINO-3,4 DIMETHYL-IMIDAZO[4,5-f]-QUINOLINE BY ETHANOL-INDUCED AND UNINDUCED RAT TONGUE TISSUES IN A MODIFIED SALMONELLA MUTAGENICITY TEST

by

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1990

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VITA

The author, Norman Lawrence Eskoz, is the son of Sidney and Greta Eskoz. He was born May 25, 1953, in Chicago, Illinois.

His elementary and high school education was obtained in the schools of Chicago, Illinois. In September of 1971, Mr. Eskoz entered Loyola University in Chicago, receiving the degree of Bachelor of Science in Biology in January, 1975. In September, 1975, Mr Eskoz entered Loyola University Dental School in Maywood, Illinois receiving his D.D.S. degree in May, 1979. From July 1979 to June 1980, he completed a general practice residency program at Hines Veterans Hospital.

Upon completion of the general practice residency program, Dr. Eskoz was employed as a staff dentist at Hines, and became a part time clinical instructor in the Department of Removable Prosthodontics at Loyola Dental School. In 1986 he received his fellowship in the Academy of General Dentistry.

In September of 1987 Dr. Eskoz became a part time student in the graduate school at Loyola University in Maywood, Illinois. In July, 1989, he entered into a full time two year Endodontic residency program at Loyola, which enabled him to complete the Master of Science in 1990.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Many agents are known to induce neoplastic transformations. These agents can be grouped into three broad catagories; radiant energy, certain viruses and chemical substances.

Radiation overexposure to ultraviolet light of the sun, appears to be responsible for most cancers of the skin including melanomas among light skinned individuals (Urbach et al., 1972). Backround ionizing radiations do not appear to be a major factor in the overall incidence of human cancers (Upton et al., 1977).

Viruses such as the human papilloma virus have been associated with anogenital cancers, particularly carcinoma of the uterine cervix (Bonfiglio and Stoler, 1988; Pfister, 1987). Epstein-Barr Virus, a member of the herpes family, has been implicated in the pathogenesis of Burkitt's lymphoma and undifferentiated nasopharyngeal carcinomas (Richtsmeier, 1987; Dillner et al., 1988). Hepatitis B virus is associated with liver cancer (Beasley, 1988). Human T-cell leukemia virus is associated with a form of T cell leukemia/lymphoma that is endemic in certain parts of Japan and the West Indies but is found sporadically elsewhere (Kim and Durack, 1988).

A wide variety of chemical carcinogens appear to be of major importance in the induction of human cancer. Chemical carcinogens are either direct acting or indirect acting (procarcinogens). Indirect carcinogens do not exhibit carcinogenicity until metabolically converted or activated in the host to the ultimate carcinogenic chemical species. Direct acting carciongens are innately reactive entities, usually electrophiles, and therefore do not require metabolic activation. Direct and indirect chemical carcinogens are classified according to similar functional groups. Direct acting ones are classified as either alkylating or acylating agents while indirect carcinogens may be classified as polycylic aromatic hydrocarbons, nitrosamines, aromatic amines or miscellaneous chemicals such as aflatoxin B_1.

Procarcinogens usually exert their most potent effects on the tissues that have the capacity to convert them into ultimate carcinogens. Most known procarcinogens are metabolized by cytochrome P-450 dependent mono-oxygenases located in the endoplasmic reticulum. Certain chemicals have been shown to enhance the amount and activity of these enzymes and consequently to promote tumorigenesis in experimental animals. For example alcohol has been shown to induce P-450 enzymes. The liver has the greatest concentration of P-450 and hence the greatest capacity for metabolic conversion. Virtually all tissues and organs tested also have the ability to activate different

procarcinogens e.g. the esophagus, intestines, lung and tracheal rings.

Many chemicals that are carcinogenic are also mutagens. A mutagen is a substance which can attack the DNA of a single cell and cause an inheritable change in the cell genome. A carcinogen is an agent that causes cancer in animals. The mutagenic potential of chemical carcinogens is commonly investigated by using the Ames test, which measures the ability of a chemical to induce mutations in strains of the bacterium Salmonella typhimurium. These specially genetically constructed strains are unable to synthesize histidine because of a mutation on the histidine operon. The tester strains are incubated with a putative mutagenic carcinogen along with liver homogenate to provide for the necessary enzymes for metabolic activation. If the metabolites of the test chemical are mutagenic, they cause reversion of the microorganisms to its histidine protrophic state so that the induced mutants can now grow on histidine-free culture medium. By testing a known P-450 metabolizable promutagen in the presence of a tissue enzyme preparation, the test can also be used to assess P-450 mediated metabolic activation of the test chemical by the tissue enzyme preparation.

The Ames test can be modified to increase the sensitivity. These modifications are based on two premises. First microorganisms are more sensitive to mutations when they are activily growing and secondly there is a point

where the concentrations of the reactants i.e., mutagen, P-450 and enzyme will react best. This modified Ames test can then be used to detect for minute quantities of p-450 in tissue enzyme preparations.

LITERATURE REVIEW

Chemical Carcinogenesis

One of the earliest correlations of exposure to an environmental agent and cancer came in 1775 when Sir Percival Pott noticed that there was a high incidence of scrotal cancer among chimney sweeps. He hypothesized that chronic exposure to soot was the casitive agent. Just by advocating cleaniness this scrotal cancer was successfully controlled (Pott, 1775). It wasn't until more than a century later that two investigators Yamagiwa and Ichikawa, (1915) showed that by repeatedly painting a rabbit's ear with coal tar, cancer could be induced. The chemical agent was later isolated and identified as a polycyclic aromatic hydrocarbon (Kennaway, 1925). Since then many pure chemicals and mixtures have been proven to be carcinogenic in experimental animals.

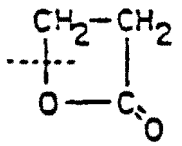
For the most part carcinogens interact with DNA, RNA, and proteins. Most chemical carcinogens are mutagens, and conversly most mutagens are carcinogenic (Miller, 1978). There is a growing belief that carcinogens ultimately affect two sets of genes: Proto-oncogenes, are converted to cancer genes (oncogenes), and suppressor genes (anti-oncogenes) are

inactivated. Proto-oncogenes and cancer suppressor genes are normal components of the animal genome, and their products are involved in the physiologic regulation of cell growth and differentiation (Bishop, 1987).

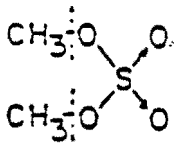
Direct Acting Carcinogens: A direct-acting carcinogen causes cancer on its own. Usually an electrophilic reagent, a direct-acting carcinogen does not require metabolic activation. Many times these carcinogens are inactivated when systemically given or taken orally and so may fail to reach key targets. However, when these are administered directly near a target, they readily induce neoplastic transformation (Miller and Miller 1971). Included in this category are a diverse group of alkyl and acylating compounds, such as B-propiolactone, nitrogen and sulfur mustards, and nitrosamides. Some direct acting carcinogens are shown in figure 1. These chemicals may bind directly to nucleopholic centers in DNA, RNA, and proteins. A typical example is the alkylation of the N-7 position of guanine. The alkylated guanine pairs with thymine rather than cytosine. Such mispairing leads to a point mutation (Singer, 1985) which is thought to represent a first step in the neoplastic induction by some alkylating agents.

Indirect Acting Carcinogens: Indirect carcinogens do not exhibit carcinogenicity until metabolically converted (activated) in the host to the ultimate carcinogenic form.

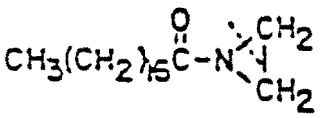
ALKYLATING AGENTS -



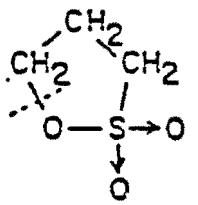
β-PROPIOLACTONE



DIMETHYL SULFATE



N-STEAROYL-
ETHYLENE IMINE



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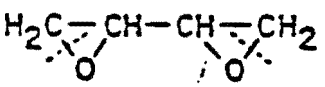
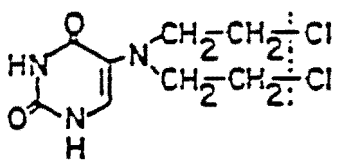
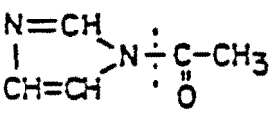


DIEPOXYBUTANE

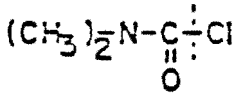


URACIL MUSTARD

ACYLATING AGENTS



1-ACETYL-IMIDAZOLE



DIMETHYLCARBAMYL
CHLORIDE

Fig.1 Some direct-acting chemical carcinogens.

These chemicals typically exert their effect on the tissues that have the capacity to convert them into ultimate carcinogens. The liver appears to be the organ that is subject to the effects of most indirect carcinogens since it has the greatest capacity for metabolic conversions. For instance when 2-acetylaminofluorene or dimethylnitrosamine is fed to susceptible animals, cancers develop in the liver (Miller, 1970). Many other tissues and organs have also demonstrated the ability to activate procarcinogens. The intestines, lung and tracheal rings are some examples (Seitz, 1981). Sometimes the activation occurs outside the animal cells as in the case with cycasin a constituent of the cycad nut. Cycasin is activated by microorganisms of the intestinal flora. It is a beta-glucoside which is not carcinogenic until the glucoside linkage is broken by these microorganisms. The aglycon, methylazoxymethanol, is the active form, which after it is absorbed systemically causes cancer in the liver and kidney (Robbins, 1989).

Metabolic Activation: Internal metabolism of foreign compounds (xenobiotics), including carcinogens and toxins does not always represent detoxification. The term biotransformation has come into use to denote both the detoxifying and activating actions of xenobiotic metabolizing enzymes. Because of the wide diversity of chemicals in the environment these enzymes by necessity have wide substrate specificity. These reactions include oxidation,

reduction, epoxidation, deamination, hydroxylation, sulfoxidation, desulfuration, dehalogenation, and conjugation with endogenous compounds (Hayes, 1989). Biotransformation can be divided into two distinct phases. Phase I reactions result in functionalization, i.e. the addition or the uncovering of specific functional groups. In phase II, the enzymes conjugate the functionalized xenobiotic with an endogenous compound. The common result of biotransformations is the conversion of usually hydrophobic xenobiotics into more polar compounds that are more easily excreted in the urine (Bartsch, et al. 1982). Phase II reactions not only result in greater water solubility, but in some cases the added chemical group is recognized by specific carrier proteins or proteins involved in facilitated diffusion or active transport. Most chemicals are detoxified by these reactions; however, there are some chemicals that are made more toxic than the parental compound. Conversion of a foreign compound to a more toxic metabolites by host enzymes has been termed metabolic activation (Weisburger, 1982).

Detoxification and metabolic activations occur concurrently. Thus, what determines the susceptibility of an organism to a particular chemical seems to depend, in many cases upon the delicate balance between these two opposing pathways. These xenobiotic metabolizing enzymes are sensitive to the influence of many endogenous and exogenous factors such as age, sex, physiologic state, diet and other

chemicals. The balance between activation and detoxification can differ among different individuals or even the same individual at different points in time (Miller and Miller, 1976).

Cytochrome P-450: Most of the known indirect carcinogens are metabolized by cytochrome P-450 dependent mono-oxygenases or mixed-function oxidases (Miller and Miller, 1981). It is the main enzymatic system for metabolism of many xenobiotics and catalizes the initial step or phase I type reactions (Weisburger, 1982). Cytochrome P-450 is located in the endoplasmic reticulum, but is also present in the nuclear membrane. Many environmental factors have been shown to affect the activity and/or synthesis of these enzymes and consequently modify carcinogenesis. Phenobarbital for example, influences tumorigenesis in experimental animals by increasing the levels of the cytochrome P-450 oxygenase system (McCann and Ames, 1978). Alcohol has similarily been shown to induce P-450 enzymes and is positively correlated with tumorigenesis (Coon and Koop, 1985).

The mixed-function oxidase system consists of three components: 1) reduced nicotinamide adenine dinucleotide phosphate cyctochrome-c reductase (NADPH-cytochrome-P450 reductase), 2) cytochrome P450 (a haemoprotein), and 3) a phospholipid (phosphatidycholine), (Lu, 1975). NADPH-cytochrome-P450 reductase uses reducing equivalents supplied by NADPH to reduce cytochrome P-450, the terminal

oxidase which binds the lipophilic substrate (e.g., drug, carcinogen ,steroid). The haemoprotein catalizes incorporation of one of the oxygen atoms into the substrate and the second atom of oxygen is reduced to water. The reaction stoicheiometry is: $RH + O_2 + NADPH + H^+ = ROH + H_2O + NADP^+$ (Hayes, 1989). Figure 2 summarizes the P-450 mediated xenobiotic oxygenation system. Figures 3 thru 6 show some of the P-450 catalyzed reactions of major indirect chemical carcinogens.

Chemical Classes of Indirect Carciongens: Polycyclic aromatic hydrocarbons were the first chemical carcinogens to be discovered. Cancers they induced include the scrotal cancer of chimney sweeps and the lung cancers of cigarette smokers (Doll and Peto 1981). These chemicals are also produced from animal fats in the process of broiling meats and are present in smoked meats and fish (Sugimura and Sato, 1983). They are very potent carcinogens . The final carcinogenic form of these agents are most likely the dihydrodiol epoxides which are strong electrophilic reactants that combine with nucleophilic sites in the target cells including DNA, RNA, and proteins. One representation from this group is benzo(a)pyrene. The dihydrodiol epoxides from benzo(a)pyrene are formed in a three-step reaction sequence shown in fig.3. Epoxidation catalyzed by the cytochrome P-450 system first occurs at the 7, 8-position. The resulting epoxide is very susceptible to hydrolysis by

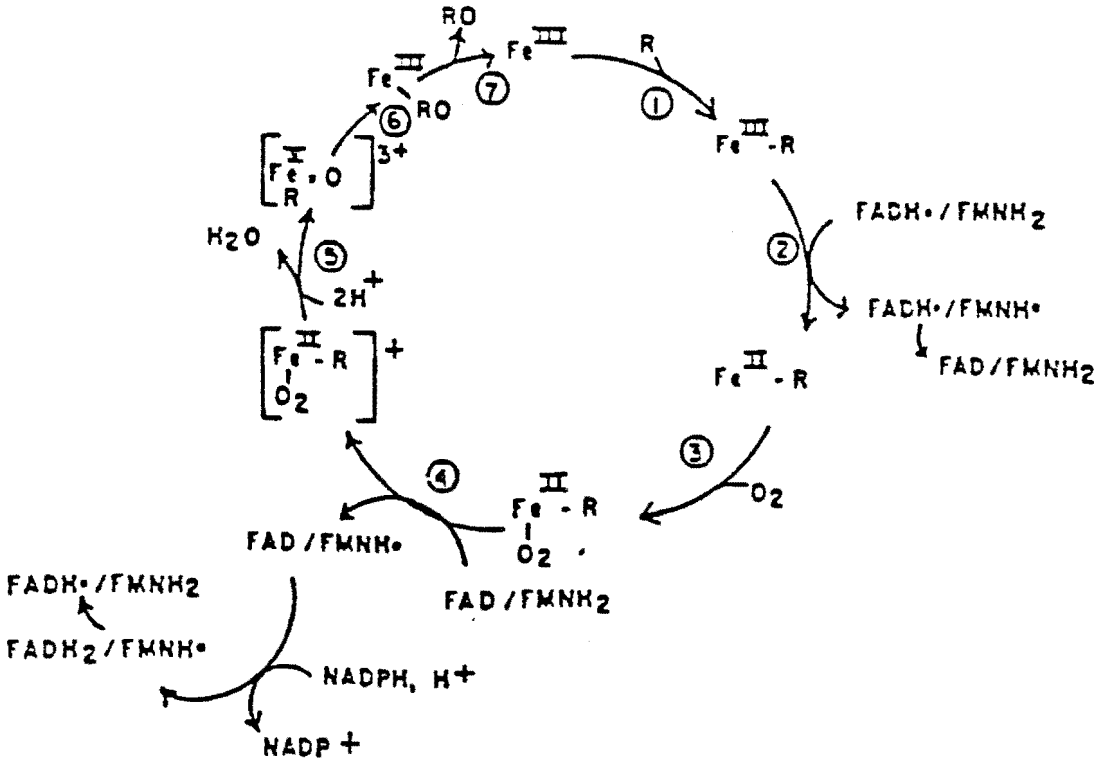


Fig.2 Working scheme for the catalytic mechanism of cytochrome P-450.

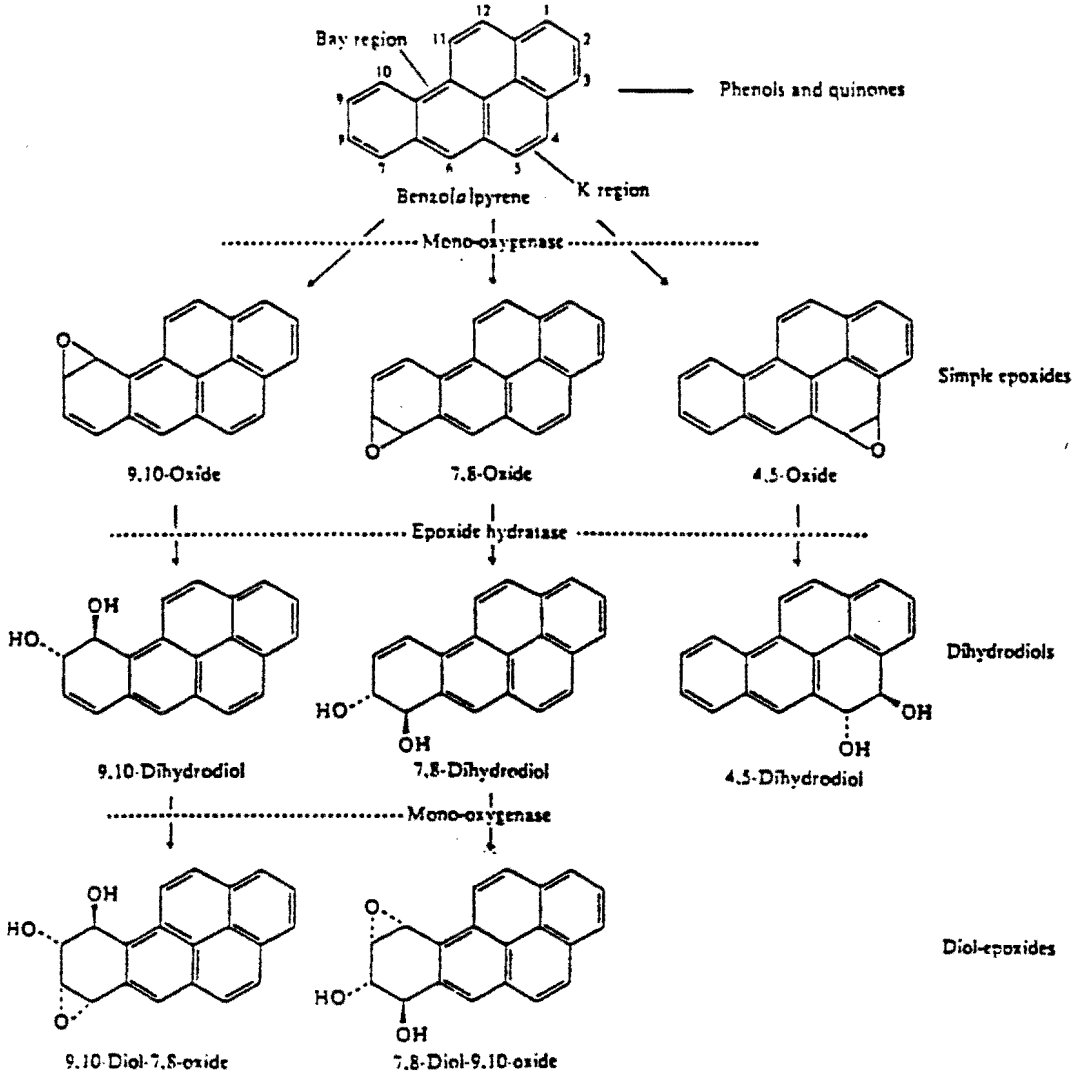


Fig.3 Stages in the metabolic activation of benzo[a]pyrene. The *anti* isomers of the diol-epoxides are shown.

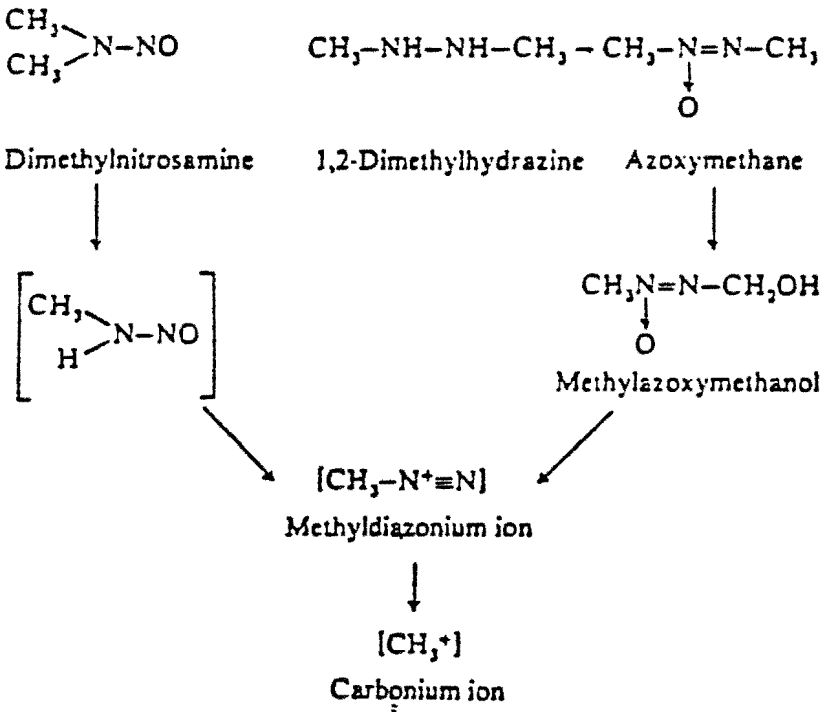


Fig.4 The metabolic action of Dimethylnitrosamine.

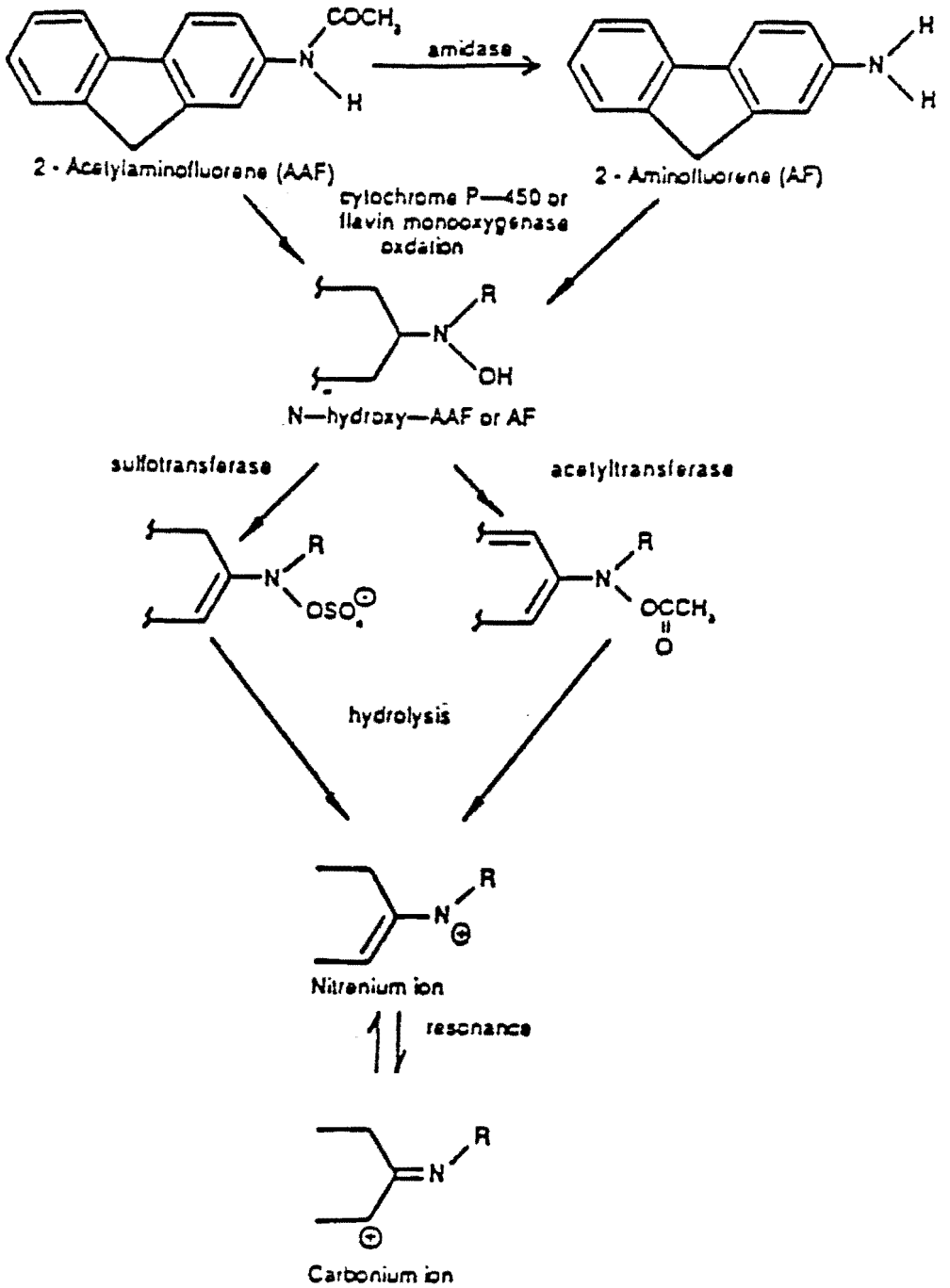


Fig.5 Metabolic activation of 2-acetylaminofluorene to a reactive metabolite capable of covalent modification of macromolecules.

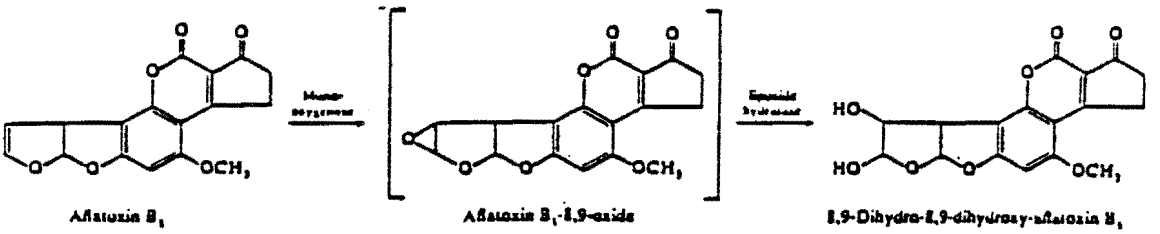


Fig.6 The metabolic activation of aflatoxin B_1.

the epoxide hydrase in the endoplasmic reticulum. The product or the hydrase reaction 7,8-dihydrodiol, is a substrate of P-450 for further epoxidation at the 9,10-position in a steroselective manner (Levin, et al. 1982).

Nitrosamines are ubiquitous environmental chemicals. They may be also formed internally in the mammalian acidic stomach from nitrites and secondary amines. They are also activated by the P-450 system (Miller and Miller 1981). The final activated compounds are alkyl diazonium ions that are strongly electrophilic (alkylating agents). For example, Dimethylnitrosamine is oxidatively dealkylated to yield the corresponding monomethlynitrosamine. This monoalkyl derivative after an internal rearrangement yields the electrophilic carbonium ion (Pegg, 1980) (fig.4).

Aromatic amines and azo dyes form another group that seems to exert most of it's carcinogenicity on the liver. Thus 2-acetylaminofluorene fed to rats induces hepatocellular carcinomas but not cancers of the gastrointestinal tract (Miller J.A. 1970). Some of the azo dyes were developed as food colors. The metabolic activation of 2-aminofluorene and 2-acetylamonofluorene (2-AcAF) is shown in figure 5. The first step in the activation of 2-AcAF is N-hydroxylation by the cytochrome P-450 system in the endoplasmic reticulum with the formation of

N-hydroxy-2-acetylaminofluorene. The carcinogens are further metabolized by soluble liver cytosolic sulfotransferase systems to form the corresponding 2-aminoflourene-N-sulfate and the ester 2-acetyl-aminofluorene-N-sulfate, which appear to be the principle ultimate carcinogenic metabolites in rat liver (Krick and Westra, 1979). The sulfuric acid ester is a very strong electrophile which can react with DNA, as evidenced by it's high mutagenic activity (McCann and Ames, 1976).

Naturally occurring carcinogens are also produced by plants and microorganisms. The potent carcinogen aflatoxin B1 for example is elaborated by certain strains of Aspergillus flavus. Aflatoxins have been implicated in the causation of hepatocellular carcinoma in Thailand, Singapore, Kenya, Swaziland, and Mozambique, where the incidence of this form of neoplasia is proportional to the aflatoxin content of the local diet (Shand, 1977). Aflatoxin requires metabolic activation by hepatic microsomal oxygenases to yield the 8,9-dihydrodiol epoxide as the ultimate carcinogen fig.6 (Essigmann et al., 1983).

2-aminofluorene (2AF) **and** ***2-amino-3,4-dimethylimidazo[4,5-f]quinoline (MeIQ)***: 2-aminofluorene is a hetrocyclic amine. It's relative compound 2-acetylaminofluorene was once used as an insecticide but when it's carcinogenic properties were discovered (Wilson, DeEds, and Cox 1941) a search for other fluorene compounds that showed insecticidal promise was

undertaken (Wilson et. al. 1947). One of these compounds was 2-aminonfluorene. This compound was also proved to be carcinogenic. 2-acetylaminofluorene is a good representative compound of the heterocyclic amines and its metabolism has been worked out quite extensively as shown in fig.5 (Miller 1970). 2-aminfluorene is metabolized the same way and is a good laboratory diagnostic mutagen. The activation of 2-aminofluorene is dependant upon the formation of the N-hydroxy derivative which is catalyzed by the P-450 monooxygenase system (fig.5). The N-hydroxy derivative can then form electrophilic species by conjugation reactions which could alkylate DNA (Miller, 1969; Sakai et al., 1978).

The heating of many foodstuffs has been shown to produce mutagenic heterocyclic amines. These amines have been isolated from pyrolysates of amino acids, proteins and a variety of cooked foods. They have been shown to be carcinogenic to mice and rats (Sugimura, 1985). Precursor foodstuffs include red meats (Sugimura and Nagao, 1982; Sugimura and Sato, 1983), fish (Kato et al., 1986; Kikugawa and Kato, 1987; Kikugawa et al., 1986),soybean proteins (Yoshida et al., 1978) and coffee beans (Kikugawa et al., 1989). One of the most potent bacterial mutagens is the carcinogen (fig.7) 2-amino-3,4- dimethylimidazo [4,5-f]quinoline (MeIQ) (Holme et al., 1987). This compound seems to be formed during cooking by reaction between creatine, which occurs naturally in meat, and Maillard reaction products generated from glucose and free amino

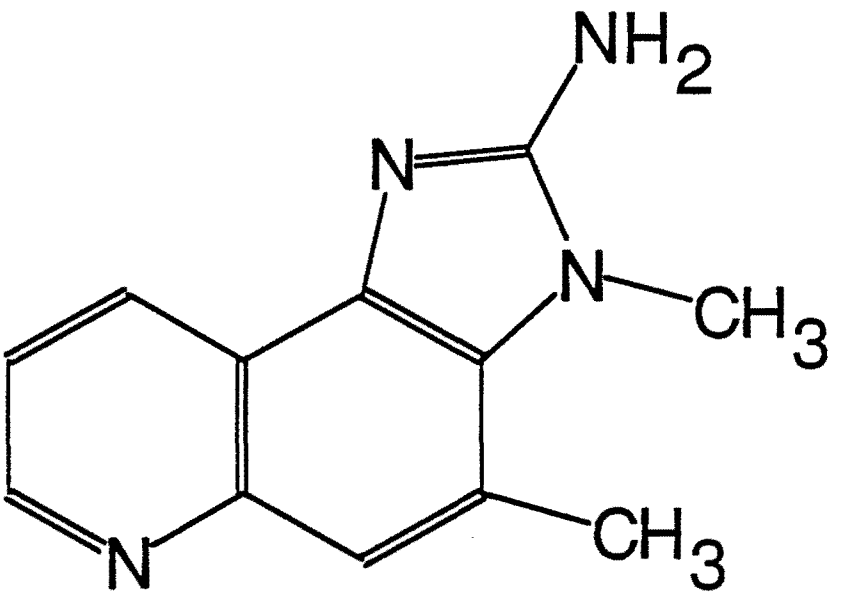


Fig.7 2-amino-3,4 dimethyl-imidazo[4,5-f]-quinoline.

acids (Jagerstad et al., 1983; Grivas and Jagerstad, 1984). Similarily like most heterocyclic amines, MeIQ requires metabolic activation through a cytochrome P-450 dependent N-hydroxylation (Okamoto, 1981; Kato et al., 1983; Yamaqoe et al., 1983) to exert its mutagenic effects. Grivas and Jagerstad (1984) have demonstrated that for MeIQ, the imidazole and 2-amino groups are essential for bacterial mutagenesis. Nagao et al. (1983) have produced evidence suggesting that the ultimate 14 mutagenic forms were sulphate esters of the N-hydroxyl derivatives. Alldrick, et al., (1986) has shown that methylation at the 4-position increases the reactivity of the active MeIQ metabolite and hence it's ability to exert a mutagenic effect. Kato et al. (1989) has shown that MeIQ induced tumors in various organs such as the zymbal gland, oral cavity, colon, skin and mammary gland when given systemically to rats.

Alcohol and Cancer

Alcohol by itself is not a carcinogen (Ketcham et al., 1963); however, when administered in combination with carcinogens, ethanol enhances carcinogenesis in some organs under certain experimental conditions (Seitz, 1985). Many epidemiological studies have shown a link between alcohol over-consumption and of cancer of the oropharynx (Wynder et al., 1957; Keller 1967,; McCoy 1978), larynx (Wynder et al.,1976; Gregoriades 1974) and esophagus (Wynder et el., 1961; Tuyns 1970; Schoftenfeld et el., 1974). Flamant et al., (1964) assessed both smoking and alcohol and found a strong association of alcohol intake with cancer of those sites that come most directly in contact with alcohol. In a series of studies (Wynder et el., 1956 & 1957) heavy drinkers were found to have roughly a 10-fold increase risk for developing cancer of the mouth. The risk of developing oral cancer for a heavy drinker who smokes was 6-15 times higher than for nondrinkers and nonsmokers (Feldmann et el., 1975). Women who drink and smoke heavily develop cancer of the tongue and buccal cavity 15 years earlier than do women who abstain from both alcohol and tobacco (Bross et al., 1976).

Induction of Metabolic Activation of Mutagens/Carcinogens By Alcohol: Although alcohol is not a carcinogen per se it seems to have a promoting effect on carcinogensis. It has been well established that one of the adaptive responses to chronic ethanol consumption in both experimental animals and man is the proliferation of liver smooth endoplasmic reticulum and marked increases in many of the enzymatic components of the mixed fucntion oxidase system (Coon and Koop, 1987). By increasing the amount or types of cytochrome, theoretically more of a procarcinogen would be converted into its carcinogenic form.

The effect of chronic alcohol consumption on the metabolism of various mutagens/carcinogens has been studied intensely. Seitz et el., (1978) reported that microsomes isolated from the intestines of rats that had consumed ethanol at 35% of their caloric intake for 3 to 4 weeks had three times the benzo(a)pyrene hydroxylase activity of pair-fed controls. In bacterial mutagenicity assays, the microsomes from the intestines of rats consuming ethanol produced more mutants with benzo(a)pyrene than did microsomes from control rats. Alcohol-inhanced activation of procarcinogens has also been reported for liver (Seitz et el., 1979) and the lung (Seitz et el., 1981), with benzo(a)pyrene. McCoy et al., (1979) also found that microsomes from the livers of hamsters fed chronically with ethanol showed enhanced mutagenic activation of N-nitrosopyrrolidine. In 1982 MCoy et al., found that

tracheal rings isolated from ethanol-consuming Syrian golden hamsters metabolized N-nitrosopyrrolidine at a higher rate that similar preparations from control animals.

In 1981, Seitz, Garro, and Lieber discovered that chronic ethanol consumption produced significant increases in pulmonary microsomal cytochrome P-450 and microsomal ethanol oxidation. The ethanol diet also enhanced the capacity of pulmonary microsomes to activate compoumds present in tobacco pyrolyzates to bacterial mutagens. Similarly Farinati, Zhou, Bella, Lieber, and Garro (1984) found that chronic ethanol exposure resulted in an increased capacity to activate nitrosopyrrolidine by rat microsomes derived from liver, lungs, and esophagus but not from stomach.

Koop et al., (1982) isolated a unique isozyme of P-450 from liver microsomes of ethanol-treated rabbits. This was referred to as P-450$_{ALC}$ to indicate that alcohol is both an inducer and substrate. This alcohol-oxidizing enzyme system is designated as APO for alcohol P-450-oxygenase. The term APO indicates that alcohols in general are substrates, not just ethanol.

A protein immunochemically related to P450$_{ALC}$ has been found in human liver microsomes and of a similar alcohol-inducible protein in the rat and in the normal and alcohol dehydrogenase-deficient deermouse (Yang, 1985). P-450$_{ALC}$ catalyzes a variety of xenobiotic chemicals such as acetaminophen, various nitrosamines, and carbon

tetrachloride and is therefore believed to play a pivotal role in the metabolic activation of these substances following alcohol administration (Coon and Koop 1987).

Correlation Between Mutagencity and Carcinogenicity

Mutagenicity tests are of practical significance because they permit rapid and inexpensive screening of chemicals for potential carcinogenicity bases on the high positive correlation between mutagenicity and cacinogenicity. The Ames Salmonella/mammalian microsomal assay system provides a simple screening method to assess mutagenicity and to predict carcinogenicity (Maron and Ames, 1983). The Ames test and other mutagenicity tests have shown there is a positive correlation between carcinogenicity and mutagenicity (McCann and Ames 1975).

Salmonella/Mammalian Microsome Mutagenicity Test (Ames Test)

In 1971 Malling first to described the use of a postmitochondrial supernatant (9000-20,000g) from mouse liver to cause in vitro mutagenesis. This supernatant was co-incubated with Salmonella typhimurium G-46 and dimethyl-nitrosamine (DMN) in liquid suspension. This strain of Salmonella could not normally synthesize it's own histidine or activate DMN to a mutagen. However after coincubation with postmitochrondrial supernatant and DMN, many colonies were formed on histidine-deficient plates, demonstrating a significant activation of the carcinogen DMN

to a mutagenic form and the general utility of cell free enzyme preparations. Ames and coworkers in 1973 led in the development of a standardized version of the plate assay for Salmonella reverse mutation to detect mutagens as a proxy for carcinogens. One of the primary reasons this test proved so useful was that the agar overlay contained the rat liver postmitochondrial supernatant or microsomal preparation which was used to activate carcinogens to mutagens.

The Ames test determines the ability of a test chemical to induce mutations in specially constructed (histidine operon mutant) strains of Salmonella typhimurium. A male rat is injected with a general inducer of the hepatic-mixed function oxidase system (P-450), such as phenobarbital. Several days later, microsomes are prepared from the liver by homogenizing the tissues and centrifuging for 10 minutes at 9000g. The supernatant, S9, is decanted and saved. The test chemical is mixed with the S9 liver fraction and cofactors and with a tester (mutant) strain of salmonella which cannot synthesize histidine (auxotrophic strain). The mixture is incorporated into a soft top agar and poured onto petri dishes containing histidine dificient medium. If the S9 activates the test chemical to a mutagenic form then, some of the bacterial cells will mutate. Mutations on the histidine operon would cause the bacteria to revert back to the histidine independent state (protrophy). That would give rise to visible colonies on the histidine deficient plates. A relationship exists, that is

the stronger the mutagen, the more colonies form (fig.8).

Bacterial tests for mutagenicity, such as the Ames test are now the most widely used screens for genotoxins. Numerous validation studies in the past have shown that such assays have a high sensitivity and specificity for the detection of genotoxic rodent carcinogens (Tennant et al., 1987). It must be stated that most, but not all, mutagens are carcinogenic. This close correlation between carcinogenicity and mutagenicity presumably reflects the fact that both damage DNA. Thus, while not infallible, the in vitro mutagenicity assay has proved to be a valuable tool in screening for the carcinogenic potential of chemicals. It is also useful in studying the metabolic activation capabilities of various tissues by testing their respective S9 against known mutagens/carcinogens (Seitz et al., 1981).

Ames Test Modifications: The Ames Test can be made more sensitive by changing several of its paramaters. This may be necessary when testing for minute quantities of mutagens or testing for the presence of low levels of P-450 against a known mutagen. Standardization of the Salmonella plate incorporation assay allows for greater control of inter-laboratory variation. Modification of the standard protocol can have a critical effect upon the test outcome. A minimum number of bacteria should be added per plate to allow for the detection of a significant mutagenic effect. To detect weakly mutagenic chemicals exposure to even larger

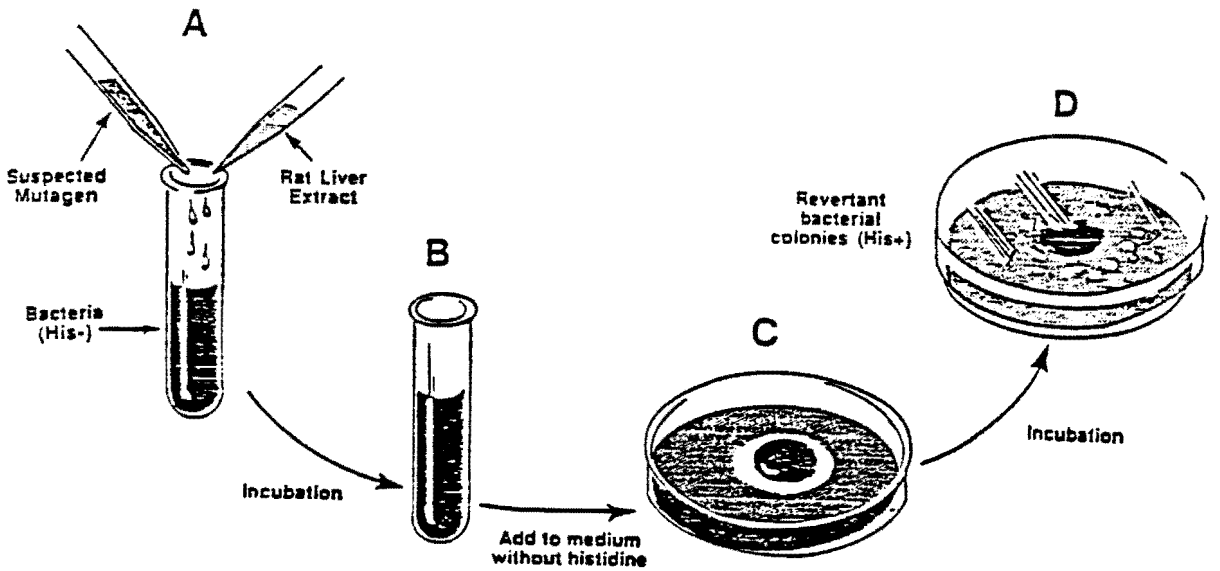


Fig.8 Liquid preincubation Protocol of the Standard Plate Incorporation Test.

numbers of bacteria is required. Kado et al., (1983) found a 13 to 29-fold increase in sensitivity of the assay when he exposed 10^8 cells to urine concentrates from mutagen-treated rodents rather than the usual 10^8 cells.

One of the features of the salmonella plate incorporation assay which enhances the test's overall sensitivity, is that the bacterial cells are actively growing in the presence of the mutagenic species. However, during the standard assay, only a relatively small number of cells exposed to the mutagen are actively growing during the peak of the metabolic activation. Exposing higher numbers of actively growing cells to mutagen effectively increases the sensitivity of the test. Booth et al. (1980), Gatehouse (1980), and Neudecker et al. (1981), have proven this independently by demonstating that bacterial cells are much more sensitive to genotoxins during the logarithemic phase of growth. Yahaghi et al. (1977) have advocated the use of a 20 to 30 minute preincubation period before plating. In 1983 Maron and Ames incorporated preincubation in their assay. Lefevre and Ashby (1981), Ashby (1986), and Gatehouse et al. (1985), have extended preincubation to 60 minutes. Neudecker and Henschler (1985) reported that the rat bladder carcinogen, allyl isothiocyanate was not detectably mutagenic using the standard 20 minute pre-incubation assay, but the compound's mutagenicity was readily detected when the pre-incubation time was extended to between 60-120 minutes at 37° C with aeration. Because these experiments

used overnight cultures which had been fully grown in rich nutrient broth, it is possible that the need for longer pre-incubation times was a reflection of an extended lag phase during mutagenesis.

Thus, harvesting the cells for the assay at the logarithemic phase of growth increases sensitivity. The bacteria should not be nutritionally shocked during any phase of the procedures, for example, by growing the cells in an enriched media prior to placing them in the minimal growth media during mutagenicity testing. Goggelmann and Grafe (1983) reported the mutagenicity of chlorodinitrobenzene, a compound yielding false negative results in standard assays, by just culturing the bacterial strains in minimal medium prior to inoculation of the minimal overlay agar during mutagenicity testing.

Modification of the standard protocol using the "delayed" plating protocol which allows more cells to enter the log phase made it more sensitive. Further modifications that allowed higher numbers of cells also resulted in increased sensitivity. Therfore, by increasing the inoculum size and utilizing the bacterium at the logarithmic phase of growth when in contact with the enzymes and mutagen a more sensitive Salmonella assay has been developed compared to the stssandard Ames assay.

PURPOSE OF THE STUDY

The purpose of this study was to determine 1) if rat tongue tissues can activate the promutagen/carcinogen 2-aminofluorene (2AF) and the dietary promutagen/procarcinogen 2-amino-3,4-dimethyl-imidazo[4,5-f]- quinoline (MeIQ) to mutagens and 2) if ethanol can increase this activation. Another major objective was also the modification of the salmonella mutagenicyty assay to adapt it to the particular needs of this study.

MATERIALS AND METHODS

Chemicals

Dimethylsulfoxide (DMSO) (spectrophotometric grade) was purchased from Fisher Scientific Co. Fair lawn, NJ. U.S.A., 4-nitro-O-phenylenediamine (NPD), benzo(a)pyrene (BaP), and 2-aminofluorene was from Aldrich, Milwaukee, WI. Lieber DeCarli rat chow was purchased from Bio-Serv (Frenchtown, NJ), and 190 proof ethanol from Publicker Chemical Corporation (Linfield, PA). Histidine assay media and agar were purchased from Difco Laboratories Detroit, Mi. and MeIQ from Toronto Chemicals Inc. Downsview Ontario, Canada. Nitrosopyrolidine, D-biotin, L-histidine HCl, nucleotide adenosine diphoshate, glucose, glucose-6-phosphate monosodium salt, glucose-6-phosphate dehydrogenase and sodium dithionite and salts were obtained from Sigma Chemical Co., St.Louis Mo.

Bacterial Tester Strains

Salmonella typhimurium tester strain TA98 was generously supplied by B.N. Ames and stored in cryogenic vials at -80° C. Cultures of this tester strain were stored at 4° C. on master plates as described by Maron and Ames 1983. These were made by applying one drop of thawed culture to the surface of a histidine/biotin plate with ampicillin and isolating single colonies by streaking the drop with a sterile platinum wire. That was repeated three times. TA98 containes the R-factor plasmid, pKM101 which increases chemical and spontaneous mutagenesis by enhancing an error-prone DNA repair system which is normally present. This plasmid is somewhat unstable and also carries an ampicillin resistant factor. Testing with ampicillin checks for the R-factor plasmid. That was repeated three times. The plate was subsequently stored in a dark incubator at 37° C. for 48 hours. Three times for each plate a sterile wire loop was used to isolate a single colony and place it in a phosphate buffered solution. A sterile cotton swab was then used to make 4 or 5 parallel streaks across the surface of the appropriate agar plate which was then placed in a dark incubator at 37° C. overnight. These were the master plates.

Animal Treatment

54 young male Sprague-Dawley rats (approx. 200g each) were used. 34 rats were caged separately and fed the liquid Lieber-DeCarli diet (fig.9) with 35% of their caloric intake substituted with ethanol (Seitz. et al., 1981). Another 20 rats were pair-fed the normal Lieber DeCarli diet with dextrose supplying the calories for controls in lieu of alcohol. The animals were weighed weekly for assurance that they were ingesting their food properly. After 4 weeks the rats were sacrificed by decapitation.

	Grams/liter
Casein	41.1
L-cystine	0.5
DL-methionine	0.3
Corn oil	8.5
Olive oil	28.4
Safflower Oil	2.7
Dextrin-maltose	115.2
Vitamin mix	2.5
Salt mix	8.75
Choline bitartrate	0.53
Fiber	10.0
Sodium carrageenate	2.0

Fig.9 Composition of control Lieber-Decarli Liquid Diet. In the ethanol formula there is 83.6 grams of dextrin-maltose and 50 grams of ethanol.

Enzyme Preparations (S9) For The Mutagenicity Tests

Liver S9: All steps in the preparation of S9 were carried out at 0-4° C using chilled sterile solutions and glassware. Only portions of the left lobe of each rat liver was used (the whole liver not being necessary). The freshly excised livers were pooled and placed in preweighed beakers. After weighing, the livers were washed several times in fresh chilled KCl, to remove excess hemomglobin which can inhibit the activity of the cytochrome P-450 enzymes. The washed livers were transferred to a beaker containing 3 volumes of 0.15M KCl (ie. 3ml/g wet liver), minced with a sterile scissors and homogenized in a Potter-Elvehjem apparatus with a glass teflon pestle homogenizer. The homogenate was centrifuged for 10 minutes at 9000g and the supernatant (S9) was decanted and saved (Garner et al., 1972) (fig.10). The protein concentrations for the control liver and ethanol liver were measured using the procedure described by Lowry et al. (1951). Protein concentration of both preparations was arbitarily adjusted to 23 mg/ml (equal to the preparation with the highest protein concentration). An aliquot portion of the S9 from both groups was tested spectrophotometrically for the amount of P-450 present (Omura and Sato, 1964). The rest of the freshly prepared S9

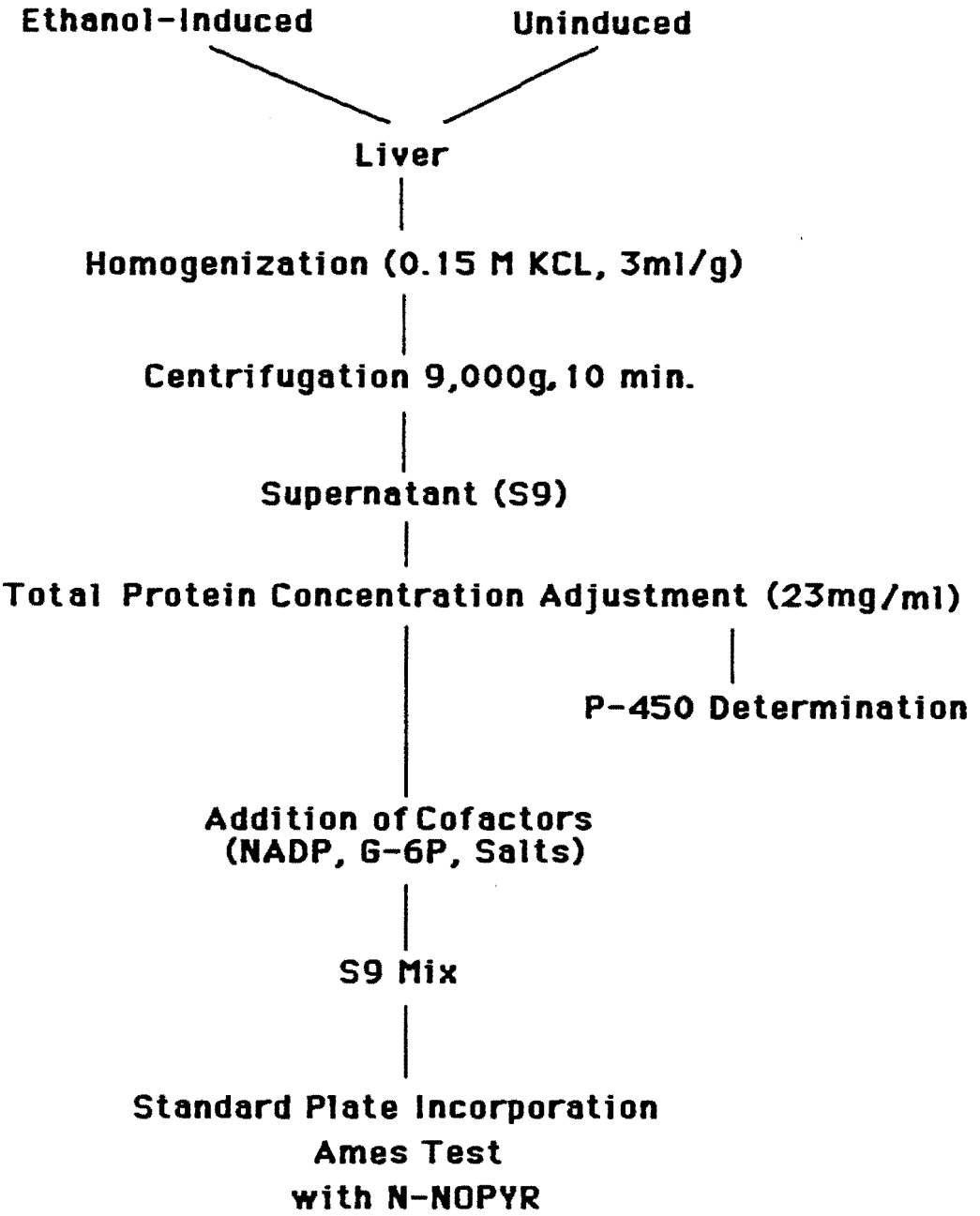


Fig.10 Liver Homogenate Preparation

fraction was distributed in 2 ml. portions in cryogenic vials, frozen quickly in a bed of crushed dry ice, and stored immediately at -80° C. To determine if the ethanol did have an effect on the rats of this study, S9 from the livers of the ethanol-induced and uninduced rats were tested for the level of activation of N- nitrosopyrrolidine in the standard plate incorporation of the Ames test (Maron and Ames 1983). Three different trials were performed with duplicate plates for three different concentrations of nitrosopyrrolidine per trial. Each trial consisted of growing the salmonella test strain overnight (7 hours) in oxoid nutrient broth. 100 microliters of this strain was then mixed with either 25, 50, or 100 millimoles of N-NOPYR and 500 microliters of ethanol-induced or non-induced liver S9 in 2ml of top agar. The S9 mixture consisted of the liver homogenate containing the S9, H_2O $MgCl_2$-KCl salts, NADP, and glucose 6 phosphate. To that 2ml of top agar containing only traces of histidine/biotin was added, mixed, and then poured onto the minimal agar plates. The plates were then placed in a dark incubator at 37° C for 48 hours. After that the plates were scored for revertant colonies (fig.10).

Tongue Epithelium and Tongue Muscle S9: The tongue of each rat was completely excised and placed in a petri dish with .1M tris-HCl buffer pH7.4. The ventral side of the tongue was incised and a glass slide was used to separate mechanically the epithelium from the underlying connective

Fig.11 Mechanical separation of tongue epithelium from the underlying muscle tissue.

tissue (figure 11). Tissue separation of epithelium from underlying connective and muscle tissue was not complete; however, separation of portions of pure muscle appeared almost complete. Tongue epithelium and muscle tissue were pooled separately in four groups. Four separate tissue homogenates were prepared: 1) ethanol-induced epithelium, 2) uninduced epithelium, 3) ethanol-induced muscle, 4) uninduced muscle. The term "induced" for a preparation refers to the tissue preparation from animals fed alcohol. For this the four groups of tissue were frozen quickly in a bed of dry ice and stored immediately at -80° centrigrade. One week later the solid frozen specimens were pulverized in a chilled mortar and resuspended in .01M tris-HCl buffer, pH 7.4, .4mM Na_4EDTA solution and then homoginized in a Potter-Elvehjem apparatus. The homogenate was centrifuged for 10 minutes at 9000g and the supernatant was decanted and saved (S9 fraction). The protein was measured for each group using the Lowry method and the final protein concentration for each group was adjusted to 5mg/ml. A sample of S9 from each group was assayed spectrophotometrically for P-450. The rest of the S9 was dispensed as 1 and 2 ml portions into cryogenic vial tubes then quickly frozen on a bed of dry ice and stored at -80° C (fig.12).

P-450 Content Determination of The Enzyme Preparations: The method originally devised by Omura and Sato 1964 was used to determine the P-450 concentration of the tissue enzymes

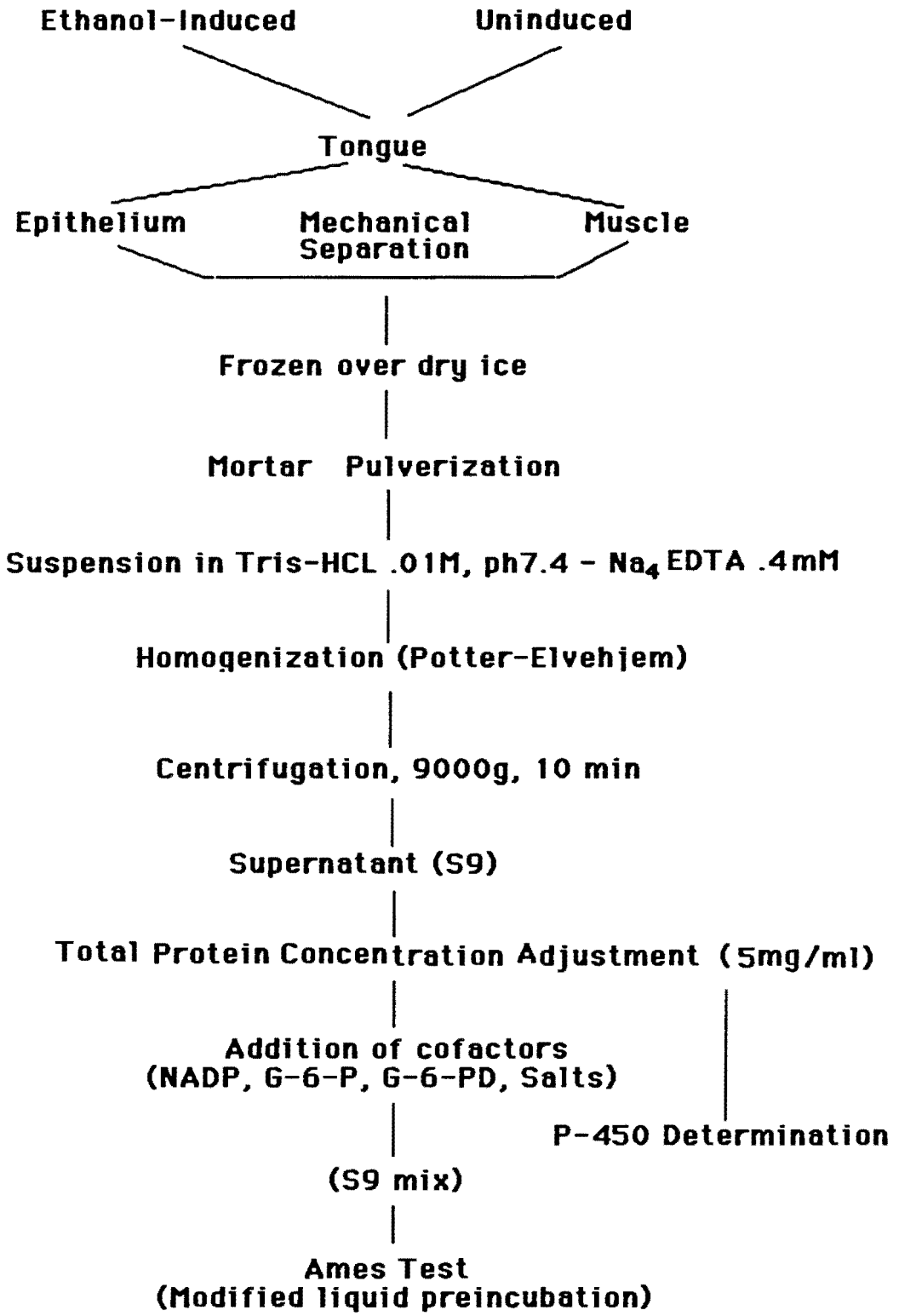


Fig.12 Tongue epithelium and muscle homogenate preparations

preparations. One milligram of total protein was then diluted with 0.1M KPO_4 pH 7.4 with 20% glycerol to make a volume of 2.99 ml. Ten microliters of 1.37M dithionite solution was subsequently added. After two minutes, the mixture was divided into two equal fractions; in one fraction carbon monoxide was bubbled in at a flow rate of 5 bubbles per second for 30 seconds. The fractions after seven minutes were placed in a dual beam spectrophotometer calibrated with DMSO and the differences in the spectra were recorded. The nanomoles of P-450 were calculated using the formula as devised by Omura and Sato 1964),

$[(A_{450-490})_{observed} - (A_{450-490})_{baseline}]/0.91$ = nonomoles cytochrome P-450 ml^{-1} .

Media, Salts, Reagents, and S9 Mixes

Growth Media: Four chemically defined minimal histidine growth media were tested to determine which one would provide for the fastest growth of the Samonella typhimurium. Bacteria were not grown in enriched media in order to keep the bacteria in a familiar nutrient environment throughout the mutagenicity tests, i.e. without nutritional shocking on transferring in the assay medium. Out of the four biotin supplemented media preparations two (A and B) had different concentrations of Histidine Assay medium (fig.13), which contains all the chemical nutrients needed for bacterial growth except for histidine and two (C and D) had two different concentrations of glucose, and no histidine assay media (fig.14). The extra histidine concentration was the same in all four media. Salmonella typhimurium tester strain TA98 was inoculated into 10 ml of each medium in Erleymeyer flasks. The flasks were then incubated in a dark 37° C. shaking incubator. Growth was monitored by measuring the optical density using a Klett-Summerson Colorimeter.

Top Agar: Top agar was prepared by dissolving 6 grams of Difacto agar and 5 grams of sodium chloride to one liter of distilled water in an autoclave for 7 minutes. Then 100

Bacto Dextrose	50g
Sodium acetate	40g
Amonium chloride	6g
Monopotassium phosphate	1.2g
Dipostassium phosphate	1.2g
Magnesium sulfate	0.4g
Ferrous sulfate	20mg
Magnganese sulfate	40mg
Sodium chloride	20mg
Adenine sulfate	20mg
Guanine hydrochloride	20mg
Uracil	20mg
Xanthine	20mg
Thiamine hydrochloride	1mg
Pyrodoxine hydrochloride	2mg
Pyridoxamine hydrochloride	800mg
Pyridoxal hydrochloride	600mg
Calcium pantothenate	1mg
Riboflavin	1mg
Nicotinic acid	2mg
p-Aminobenzoic acid	200ug
Bioton	2mg
Folic acid	20ug
Glycine	0.2g
DL-Alanine	0.4g
Bacto Asparagine	0.8g
L-Aspartic Acid	0.2g
L-Proline	0.2g
DL-Serine	0.1g
DL-Typtophane	80mg
L-Cystine	0.1g
L-Glutamic acid	0.6g
DL-Phenylalanene	0.2g
DL-Threonine	0.4g
L-Tyrosine	0.2g
DL-Yaline	0.5g
L-Lysine hydrochloride	0.5g
DL-Methionine	0.2g
DL-Isoleucine	0.5g
L-Arginine hydrochloride	0.484g
DL-leucine	0.5g
water	1 Liter

Final pH at 25^0C is 6.7 ± 0.2 for all formulations.

Fig.13 Histidine Assay Medium

INGREDIENTS	A (ml)	B (ml)	C (ml)	D (ml)
H20	8.5	5.5	9.0	9.5
HIST.ASSAY MEDIA	1.0	4.0	0	0
V.B. SALTS	0.2	0.2	0.2	0.2
HISTIDINE (.5mM)	0.1	0.1	0.1	0.1
BIOTIN (.5mM)	0.2	0.2	0.2	0.2
GLUCOSE (40%)	0	0	0.5	.06
FINAL GLUCOSE	0.5%	2%	2%	.25%

Fig.14 Ingredients of test media A, B, C, & D.

milliter aliquotes were transferred to 250 millliter glass bottles with screw caps and autoclaved for 20 minutes with loosened caps. After slow exhaust the agar cooled and the caps were tightened.

Minimal Glucose Plates: 15 grams of Difco agar was added to 930 milliliters of distilled water in a 2 liter flask. The solution was autoclaved for 20 minutes at 121° C using slow exhaust. When the solution cooled 20 milliters of sterile 50x Vogel Bonner salts and 50 milliters of sterile 40% glucose were added and mixed using a large magnetic stir bar which was added to the flask before autoclaving. The solution was thoroughly stirred and approximatly 30 milliliter portions measured and poured into petri dishes.

$MgCl_2$-KCl salts: 6.15 grams of potassium chloride (KCl) and 4.07grams of magnesium chloride ($MgCl_2 \cdot 6H_2O$) were dissolved in 50 milliters of distilled H_2O. It was subsequently autoclaved for 20 minutes at 121° C and then stored in a sterile glass bottle at 2° C.

1M Glucose-6-Phosphate: 2.82 grams of glucose-6-phosphate was mixed with 10 milliters of distilled H_2O. It was dispensed in 1 milliter portions in cryogenic vials and stored in the freezer at -10° C.

0.2M Sodium Phosphate Buffer pH 7.4: 60 milliters of .2M

sodium dihydrogen phosphate ($NaH_2PO_4 \cdot H2O$) (13.8g/500ml) were added to 440 milliters of 0.2M disodium hydrogen phosphate (Na_2HPO_4) (14.2 grams/500 milliters). Disodium hydrogen phosphate was added to adjust the pH to 7.4. the solution was then autoclaved for 20 minutes at 121° C.

0.5mM Histidine/Biotin Solution: This solution, of which 10 milliters was added to every 100 milliters of top agar was prepared by dissolving 248 milligrams D-Biotin(F.W.247.3) and 19.2 milligrams of L-histidine HCl(F.W.191.7) in 200ml. boiling water. It was then autoclaved for 20 minutes at 121° C stored in a glass bottle at 4° C.

0.5mM Biotin Solution: This solution which was added to the growth Media was prepared by dissolving 248 milligrams D-Biotin (F.W.247.3) in 200 milliters boiling water. It was stored in a glass bottle at 4° C.

Vogel-Bonner Medium E(50x): This solution which was used for the minimal agar plate was prepared as follows. Ten grams of magnesium sulfate ($MgSO_4 \cdot 7H_2O$), 100 grams of citric acid monohydrate, 500 grams potassium phosphate dibasic (anhydrous)(K_2HPO_4) and 175 grams of sodium ammonium phosphate ($NaHNH_4\ PO_4 \cdot 4H_2O$) were added to 679 milliters of warm distilled water (45°C) in a 2 liter beaker placed on a magnetic stirring hot plate. Each salt was allowed to dissolve completely before the next one was added. The

volume was then adjusted to one liter. It was then transferred to a glass bottle loosely capped and autoclaved for 20min at 122°C. After the solution cooled the cap was tightened.

.01M Tris-HCl, Na_4 EDTA 0.4mM Buffer: This was made by dissolving 12.1 grams of (Hydroxymethyl)amino-methane $NH_2C(CH_2OH)_3$ and 134.4 milligrams of Na_4EDTA with 100 milliliters of distilled water. HCl was added until the pH was adjusted to 7.4.

Rat Liver S9 Mix per 10 milliliters: The S9 mixture contained 3.75 milliliters of sterile distilled water, 5 milliliters 0.2M phosphate buffer pH 7.4, 200 microliters of MgCl2-KCl salt solution, 50 microliters of 1M glucose-6-phosphate, 31 milligrams of NADP and 1ML. of liver S9 mix. The solution which was kepted chilled was filter-sterilized using a .22um milipore filter. Unused S9 was discarded after each experiment. A new S9 mix was prepared for every experiment.

Aroclor 1254-Induced Rat Liver S9: This was obtained from Dr. Parisis who produced it according to Maron and Ames, 1983.

40% Glucose Solution: This was made by adding 40 grams of glucose to one hundred milliliters of water. This mixture was then filter sterilized.

***Tongue Epithelium and Muscle S9* Mixes:** The other tissue mixes contained the same compounds as the liver S9 mix except that glucose 6-phosphate dehydrogenase (extrahepatic tissues have little glucose 6-phosphate dehydrogenase) was added and the water eliminated to increase the amount of tissue S9 (fig.15). To 5 milliters of S9 mix the following were added; 4500 microliters contained either the tongue S9 homogenate or muscle S9 homogenate, 100 microliters of 1M glucose-6-phosphate, 100 microliters of $MgCl_2$-KCl salts, 0.2M phosphate buffer pH 7.4, 1M glucose-6-phosphate-dehydrogenase and 31 milligram NADP. The mix was kept chilled and filter sterilized using a .22um milipore filter. Unused S9 mix was discarded after each experiment, a new mix was prepared for every experiment.

LIVER S9 MIXES (10ml)

S9	1000 microliters
NADP	31 miligrams
G-6-P	50 microliters
MgCl2-KCl	200 microliters
Phosphate Buffer	5000 microliters
H2O	3750 microliters

TONGUE EPITHELIUM OR MUSCLE S9 Mixes (5ml)

Tongue or muscle S9	4500 microliters
NADP	32 miligrams
G-6-P	100 microliters
MgCl2-KCl	100 microliters
Phosphate Buffer	250 microliters
Glucose-6-Phosphate Dehydrogenase	50 microliters

Fig.15 S9 mixes from ethanol-induced and uninduced rat tissues.

Adaptation of Salmonella Mutagenicity Assay

Bacterial Cell Concentration and Mode of Incubation:
Determination of an optimal inoculum size and preincubation time was accomplished simultaneously. Three flasks, each with 10 ml. of medium A, were inoculated with Salmonella and placed in an shaking incubator at 37° C. At late exponential phase, when growth reached 7.5 x 10^8 cells/millileter corresponding to a cell turbity of 100 on the Klett spetrophotometer, the cultures were pooled and cells pelleted with a low-speed centrifugation of 2000 rpms. The cells were then resuspended aseptically in a medium similar to medium A but with five times the concentration of all components except for the histidine which was kept the same. Three different cell suspensions of 3.4 x 10^8, 7.5 x 10^8 and 1.6 x 10^8 cells/millileter respectively were prepared corresponding to cellular turbity reading on the Klett spectrograph of 50, 100, and 200. 0.1 milliter of each culture were added to 3 test tubes containing 0.4 milliter Aroclor-induced rat liver S9 mix and 2.5 micrograms of B(a)P in 0.01 milliter of DMSO each. Three other tubes were inoculated with 0.1 milliter of each culture, 0.4ml S9 mix and .01 milliter of DMSO as negative controls. 2 milliliters of top agar was either added to the tubes and immediately

poured onto the minimal glucose plates or the tubes were incubated in a 37° C shaking incubator for 30, 60, or 90, minutes after which they were mixed with top agar and then poured onto minimal glucose plates. The plates were placed in a 37° C dark incubator. After 48 hours. the revertant colonies were scored. This experiment was repeated three times.

Conditioning of the Bacterial Innoculum Suspension: In order to increase the sensitivity of the bacterial cells to mutagenic agents, the harvested and resuspended cells (1.6 x 10^8 cell/millliter) were incubated at 37° C just before adding them into the reaction mixture containing the mutagen (BaP) and Aroclor-induced rat liver S9 mix. Incubation times of 0, 30, 60, 90, and 120 minutes were tested. A 0.1 milliter inoculum from this was mixed with 0.4 milliter S9 mix and 2.5 micrograms BaP in 0.01 milliter of DMSO. Inoculi from the "conditioned" suspensions were used for each of the different conditioning times. Control plates with buffer instead of S9 mix were also included. Reaction mixtures were incubated for an additional 90 minutes before mixing with top agar and pouring onto minimal glucose plates (in triplicate). Three different experiments were carried out.

Mutagenic Activation Assay

The most sensitive test protocol as determined above was used to test the epithelial and muscle S9 for the activation of the promutagens 2-aminofluorene (2AF), and 2-amino-3, 4-dimethyl-imidazo[4,5-f]quinoline (MeIQ). Cultures were grown overnight in minimal medium (medium A) and harvested at the late-exponential phase of growth (approx.7.5 x 10^8 cell/milliliter). They were then resuspended at a concentration of 1.6 x 10^8 cells/milliliter in the same medium at 5x the strength except for histidine. The culture was subsequently placed in a covered shaking incubator for 30 minutes at 37° C. 100 microliters innoculum from this suspension was then mixed with different concentrations of the promutagens in 10 microliters of DMSO, and 400 microliters of ethanol-induced or uninduced tongue or muscle S9 mix (1:5 dilution). That was done in duplicate. As a control, 400 microliters of phosphate buffer was substituted for S9 mix. Incubation of the mixture for 90 minutes at 37° C in a covered shaking incubator preceded the addition of the top agar and the pouring onto the minimal glucose plates. Incubation of the plates took place in a dark incubator at 37° C for 48 hours after which the revertant colonies were scored. This experiment was repeated

three times (fig. 16). For 2AF, 25 and 50 nanomoles of mutagen per plate were used. For MeIQ, 100 and 200 nanomoles of mutagen were used.

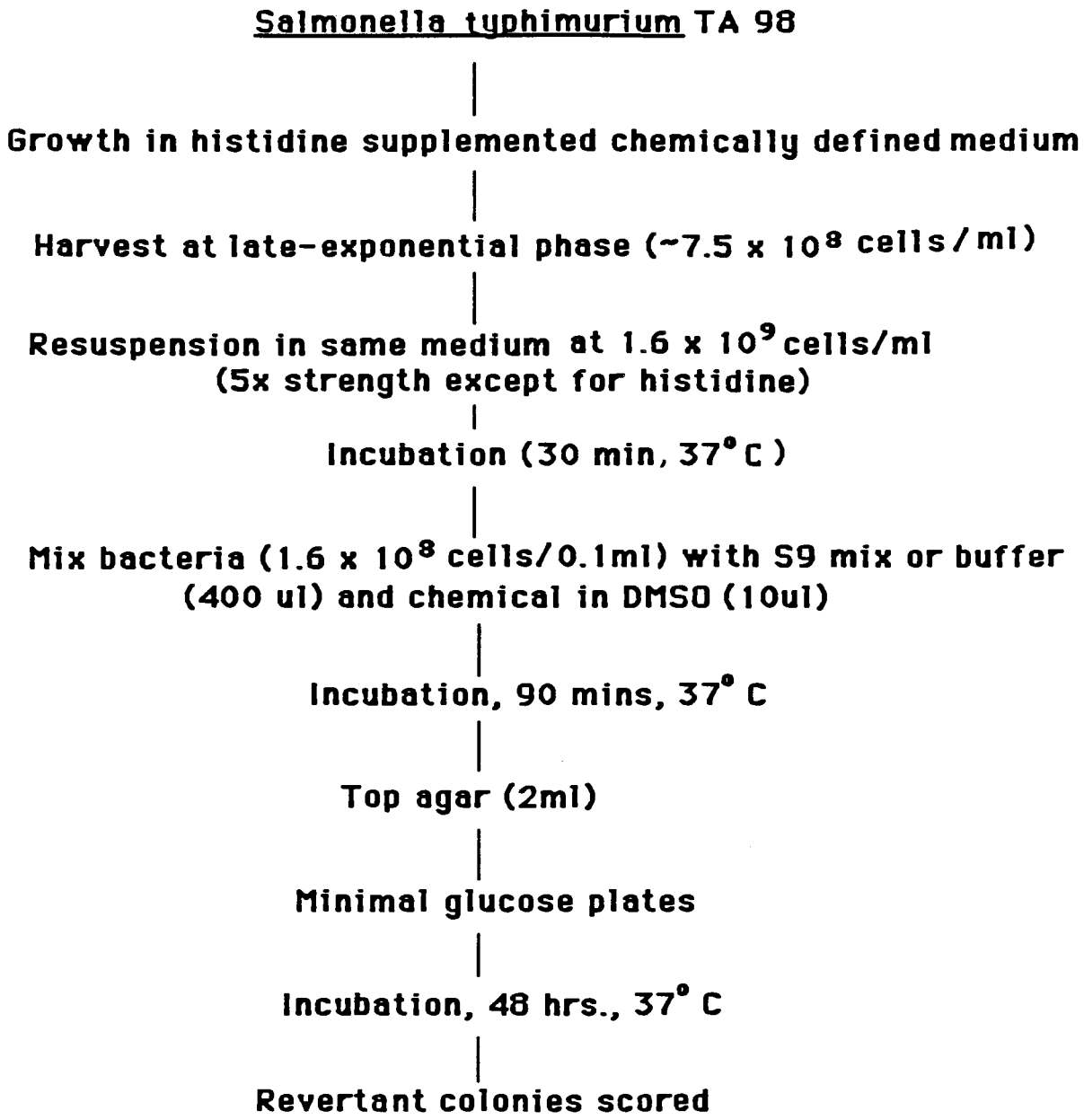


Fig.16 Modified liquid preincubation of the Ames Test.

Statistics

An analysis of variance (ANOVA) was performed to see how much variability existed amoung the treatment, specimens and the dose for the experiments using 2-aminofluorene and MeIQ.The model formula fit ways $Y = B_o + B_1X_1 + B_2X_2 + B_3X_3$ where X_1 refers to treatment (ethanol induced or non-induced), X_2 refers to specimen (muscle or epithelium and muscle), and X_3 refers to dose (25 and 50 nMoles for 2AF and 100 and 200 nMoles for MeIQ). The results are summarized in Table 1.

Table 1

ANOVA ANALYSIS OF TREATMENT, DOSE AND SPECIMEN

The model formula fit ways $y=B_0 + B_1X_1 + B_2X_2 + B_3X_3$

X_1: treatment (ethanol induced or non ethanol induced

X_2= specimen (muscle or epithelium)

X_3= dose (25 & 50 nMoles for 2AF and 100 & 200 nMoles for MeIQ)

For 2Aminoflourene the following data was obtained:

	sum of squares	F-value	P-value
treatment (X_1)	.15	1.59	.22
specimen (X_2)	5.85	61.14	.0001
dose (X_3)	.06	.65	.43

The only significant difference is between specimens (X_2).

For MeIQ the following data was obtained:

	sum of squares	F-value	P-value
treatment (X_1)	.37	1.17	.22
specimen (X_2)	3.99	18.52	.001
dose (X_3)	.003	.02	.89

The only significant difference is between specimens (X_2).

RESULTS

Bacterial Tester Strains And Animals

Genetic characteristics of the strains on the master plates were tested according to the method of Maron and Ames (1982). Tester strains that yielded the expected rates for spontaneous, NPD and BaP induced mutation were used in the experiments.

At the end of four weeks the ethanol fed rats averaged 280 grams and the control rats averaged 323 grams. This is consistant with the observations of Pirola and Lieber (1975), that animals fed drugs or ethanol that induce microsomal enzymes gain less weight then their pair fed controls. Figure 17 graphically illustrates the weight gain of the rats over 4 weeks.

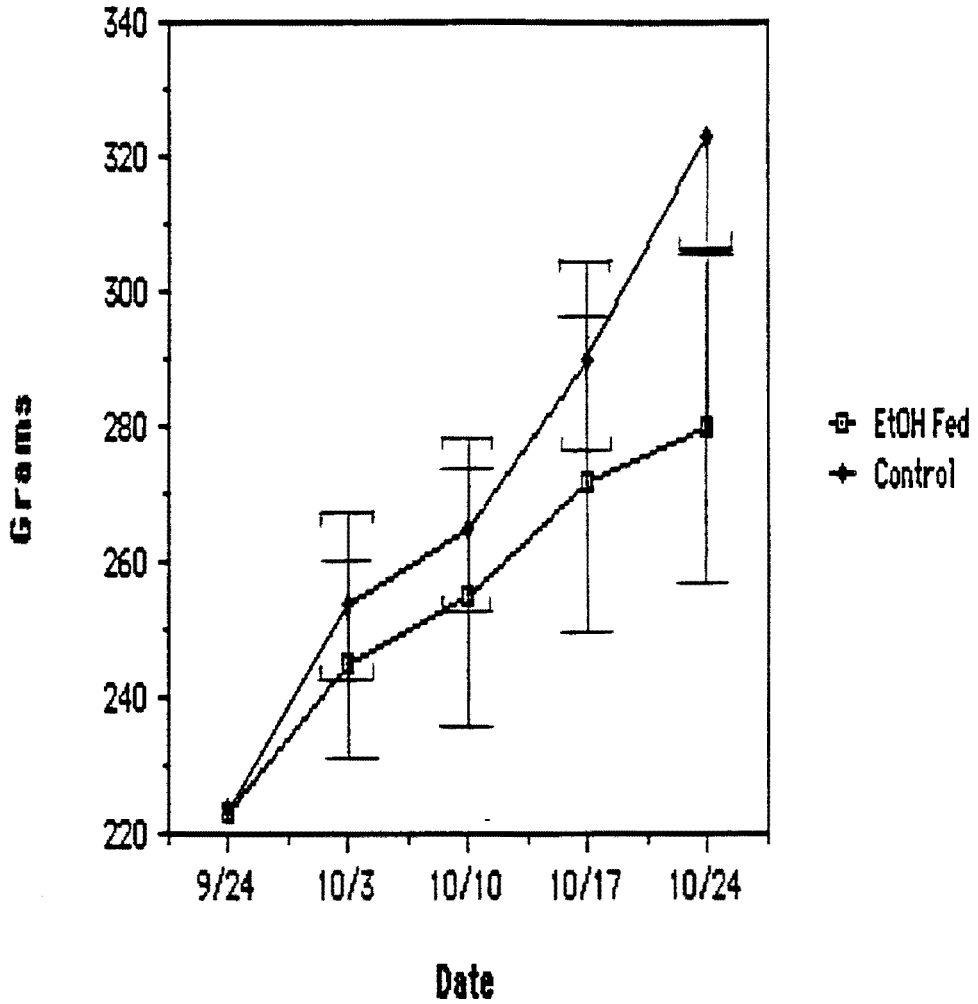


Fig.17 Weight gain of rats from 9/24/88 to 10/24/88.

Liver And Tongue S9

Liver S9-N-NOPD: The average number of revertants from two plates per each concentration of N-nitrosopyrrolidine in the presence of ethanol-induced liver S9 or uninduced liver S9 was divided by the corresponding average number of revertants in the presence of buffer to obtain a ratio. The mean ratios of three experiments and the standard deviations are shown in figure 18. In general the ethanol induced liver S9 was more active in converting the N-nitrosopyrrolidine to a mutagen than the uninduced preparation. Statistical significance was attained at 50 millimoles and 100 millimoles ($p<.05$).

Liver P-450: The results of the spectrtophotometric determinations of P450 were calculated (Omura and Sato 1964). The ethanol-induced liver showed almost twice as much P-450 as the uninduced liver, ie 0.330 nanomoles/milligram protein to 0.165 nanomoles/milligram protein respectivly (Table II).

Tongue P-450: The ethanol-induced tongue epithelium showed slightly more P-450 than the corresponding uninduced, ie 0.066 nanomoles/milligram protein vs 0.055

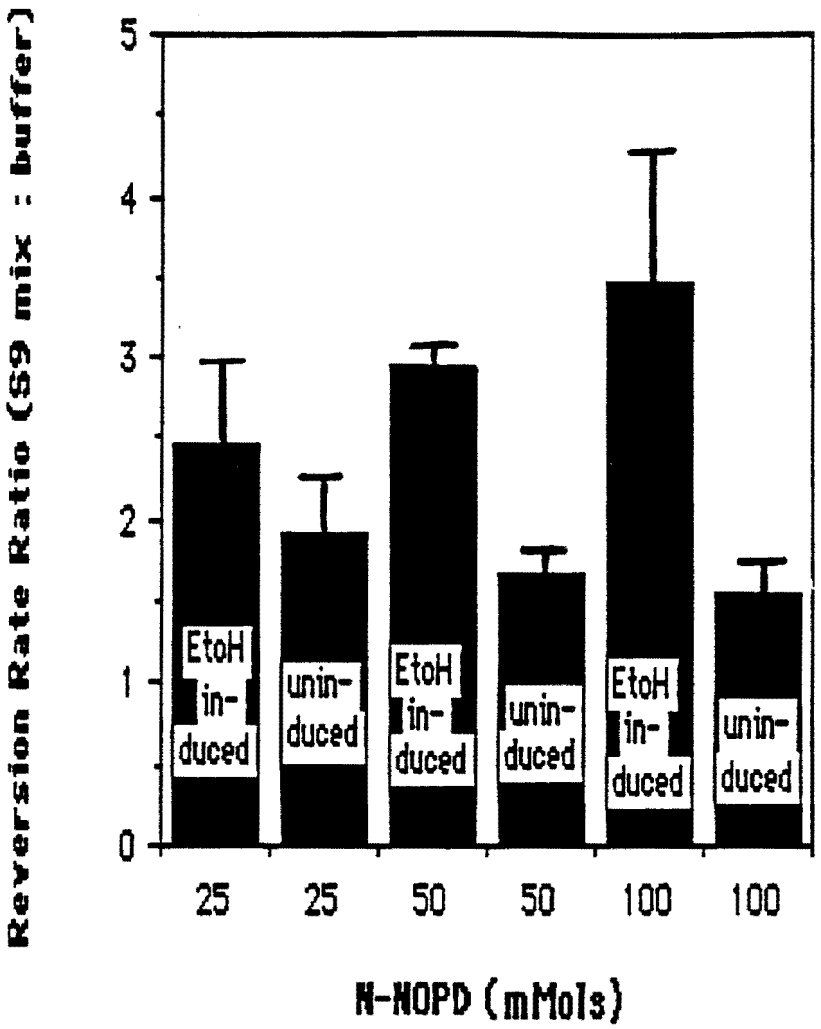


Fig.18 Reversion in the standard Ames Test using N-NOPD as the premutagen with ethanol-induced and uninduced rat liver S9. Results are shown as the mean of reversion rate ratios from three trials. Each ratio was obtained by dividing the afverage number of revertanets from two plates in the presence of liver S9 with the average number of revertants from two plates in the presence buffer.

TABLE II

P-450 Enzyme Concentrations In Various Preparations

	P-450 nmoles/mg protein	
	Uninduced	EtoH Induced
Liver	0.330	0.165
Epithelium	0.066	0.050
Muscle	0.132	0.000

nanomoles/milligram protein (Table II). The ethanol-induced muscle preparation showed considerably more P-450 than the uninduced one, 0.132 nanomoles/millgram protein to 0 nanomoles/milligram protein (Table II).

Adaptation Of The Salmonella Mutagenicity Assay

Media: As shown in figure 19, growth medium A proved to be the best chemically defined growth medium the four tested. It supported a good growth rate of the Salmonella comparable to the complex media. Although the others eventually supported growth it was rather the rate of growth that was the most important parameter for this experiment.

Innoculum Size and Preincubation Times: Inoculi from suspensions of 3.4×10^8, 7.5×10^8 and 1.6×10^9 cells/ml corresponding to cell turbity readings of 50, 100, and 200 were each tested in the reaction mixture with enzyme preparation and premutagen with preincubation times of 0, 30, 60, and 90 minutes. There were three plates per inoculum size in the presence of S9 mix and three plates in the presence of buffer (control). Ratios were obtained by dividing the average reversion rate of the three plates with S9 mix by the corresponding rate of the plates with buffer (ie. spontaneous reversions). Figure 20 shows the results as the mean of the ratios for the different inoculum sizes from three separate experiments carried out with a 90 minute preincubation. Figure 21 shows the results of reversion rates obtained at different preincubation times when an

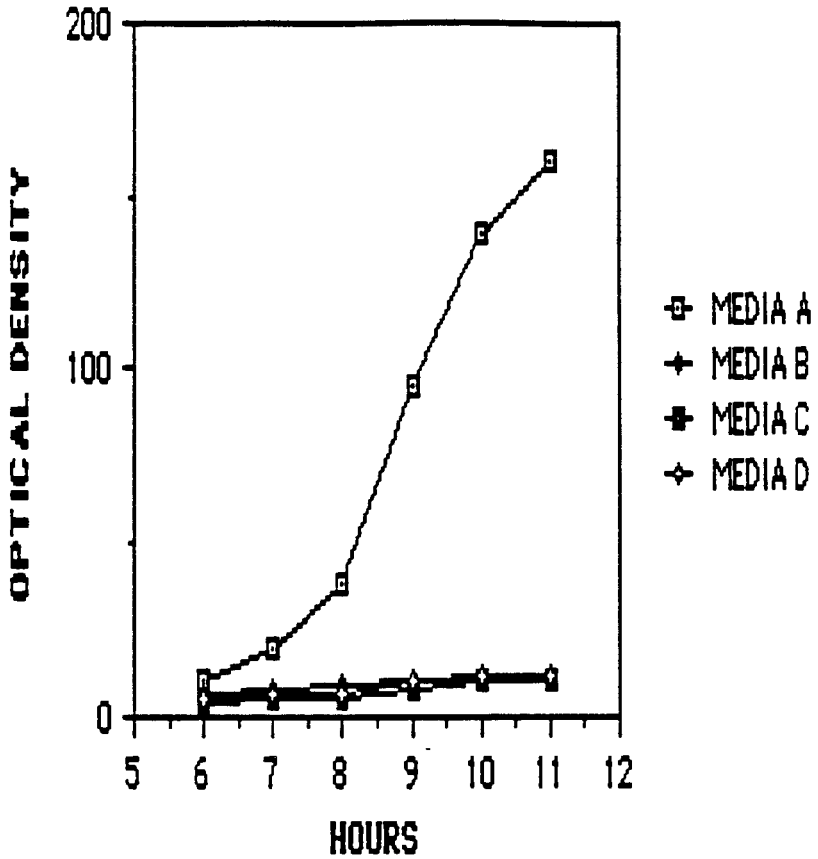


Fig.19 Growth of TA98 in test media A,B,C,&D.

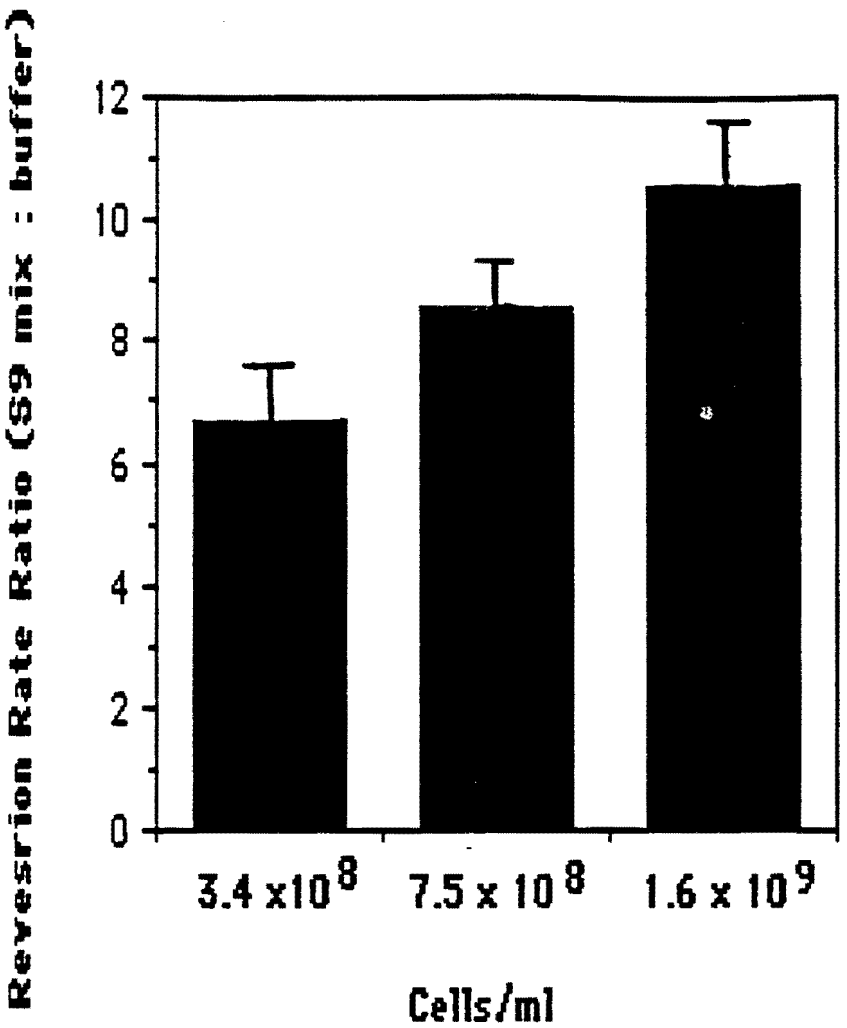


Fig.20 Reversions in the modified liquid preincubation assay. Reaction mixtures containing BaP, aroclor-induced rat liver S9 (or buffer) were incubated with different inoculum sizes (0.1ml per 0.5ml mutagenicity reaction mixture) and preincubated for 90 minutes. Results are shown as the mean of reversion rate ratios from three experiments. The ratios were obtained by dividing the average reversion rate of three plates with S9 mix by the corresponding reversion rate of the three plates with buffer.

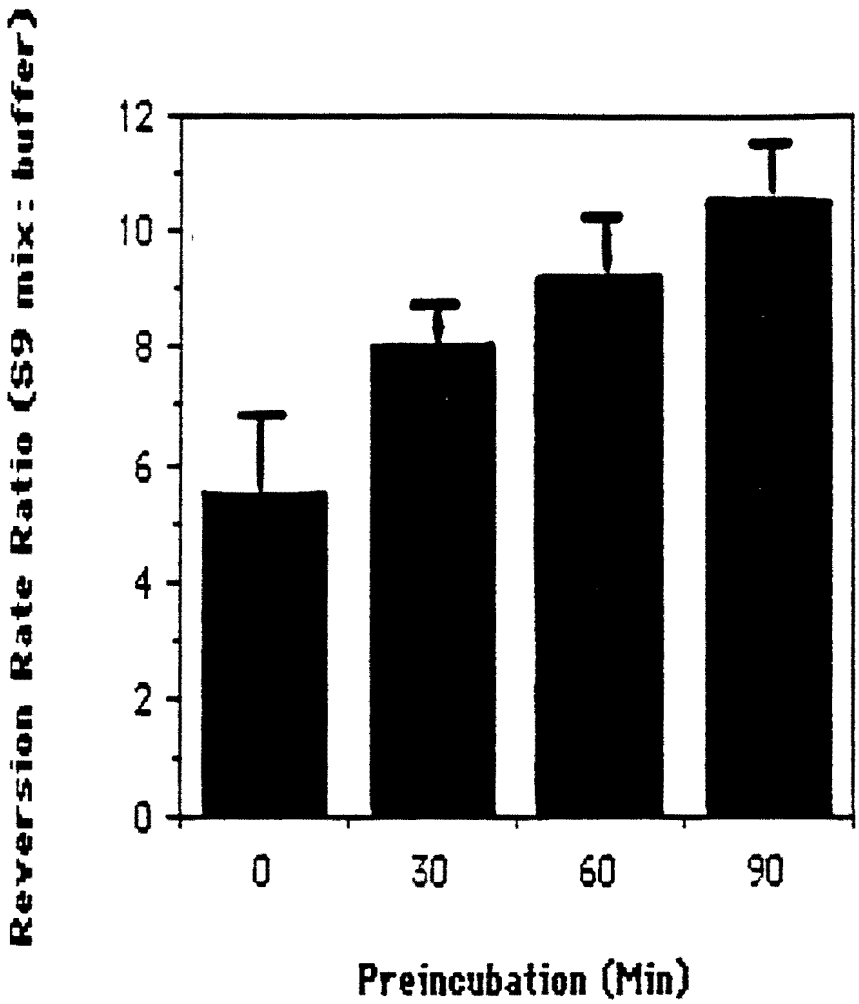


Fig.21 Reversions in the midified liquid preincubation assay. Cells (1.6 x 10^8/0.5 ml mutagenicity reaction mixture) were preincubated with the aroclor-induced rat liver S9 (or buffer) and the premutagen BaP at the indicated times before being plated. Results are shown as the mean of reversion rate ratios from three experiments. The ratios were obtained by dividing the average reversion rate of three plates with S9 mix by the corresponding reversion rate of the three plates with buffer.

inoculum was used from the suspension containing 1.6 x 10^8 cells/milliliter. The inoculum of 1.6 x 10^8 cells/milliliter proved to produce significantly ($p<.05$) more revertant colonies than inoculi from suspensions with of 3.4 x 10^8 and 7.5 x 10^8 cells/milliliter. The preincubation time of 90 minutes yielded significantly ($p<.05$) more revertants than the zero and 30 minute preincubation times. The results of the test which was run only once with an inoculum size of 3.4 x 10^8 cells/milliliter revealed only slightly higher reversion rate ratios than 1.6 x 10^8 cells/milliliter. Also the results of the tests run with 120 and 150 minutes of preincubation times showed only slightly higher revertant rate ratios. It is evident that increasing the inoculum size and preincubation time increases the number of revertant colonies to a point. Therefore, in the test to assess metabolic activation of 2AF and MeIQ the inoculum (0.1ml) of 1.6 x 10^8 cells/milliliter and a preincubation period of 90 minutes were chosen.

Conditioning of Cell Suspension: It involved an extra incubation step of the tester strain alone just before incorporation into the reaction mixture with S9 mix and the premutagen. "Conditioning" incubation times of zero, 30, 60, 90, and 120 minutes were tested at a cell concentration of 1.6 x 10^8 cells/milliliter and a second incubation of the reaction mixture for 90 minutes before mixing. Thirty minutes of "conditioning" incubation yielded more revertant

colonies ($p<.05$) than zero incubation time. Even though 60, 90, and 120 minutes also showed a significant difference over no incubation time, they were not significantly different from that of the 30 minutes (fig. 22).

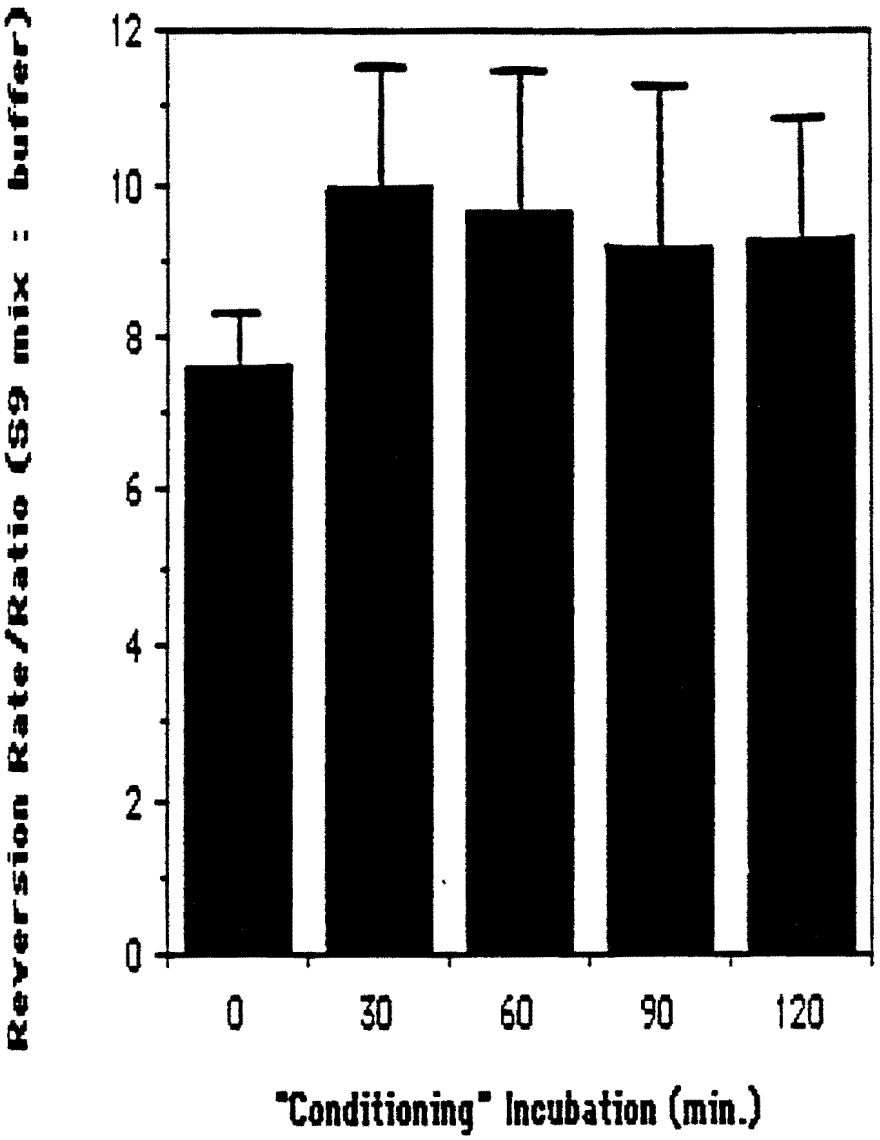


Fig.22 Reversion in the modified liquid preincubation assay, cells (1.6 x 10^9/ml) were "conditioned" by incubation at the indicated times just before mixing with liver S9 (or buffer) and the premutagen BaP and further incubation for 90 minutes before pouring onto plates. Results are shown as the mean of reversion rate ratios from three experiments. The ratios were obtained by dividing the average reversion rate of three plates with S9 mix by the corresponding reversion rate of the three plates with buffer.

Mutagenic Activation Assays

Two concentrations of 2-aminofluorene (25 and 50 nanomoles) and of MeIQ (100 and 200 nanomoles) were tested for activation with each of the four tissue preparations ie. ethanol-induced tongue epithelium, ethanol-induced tongue muscle, uninduced tongue epithelium and uninduced tongue muscle. Each concentration of either 2AF or MeIQ was tested in duplicate plates with each tissue preparation (S9 mix) or with buffer. Each experiment was performed three times. The average number (from 2 plates) of revertants (reversion rate) for each concentration of precarcinogen in the presence of S9 was divided by the corresponding number of revertants in the presence of buffer to obtain a reversion rate ratio per experiment. Results are expressed as the mean reversion rate ratio of three experiments.

Table 1 page 58 shows the results of ANOVA calculations for the treatment (ethanol-induced or uninduced), specimen (muscle or epithelium), and dose (25, 50, 100, and 200 nMoles of mutagens). The only significant difference was between the tissues.

Figure 23 shows the results of the experiments when S9 from ethanol-induced tongue epithelium and S9 from uninduced tongue epithelium were tested for their ability to

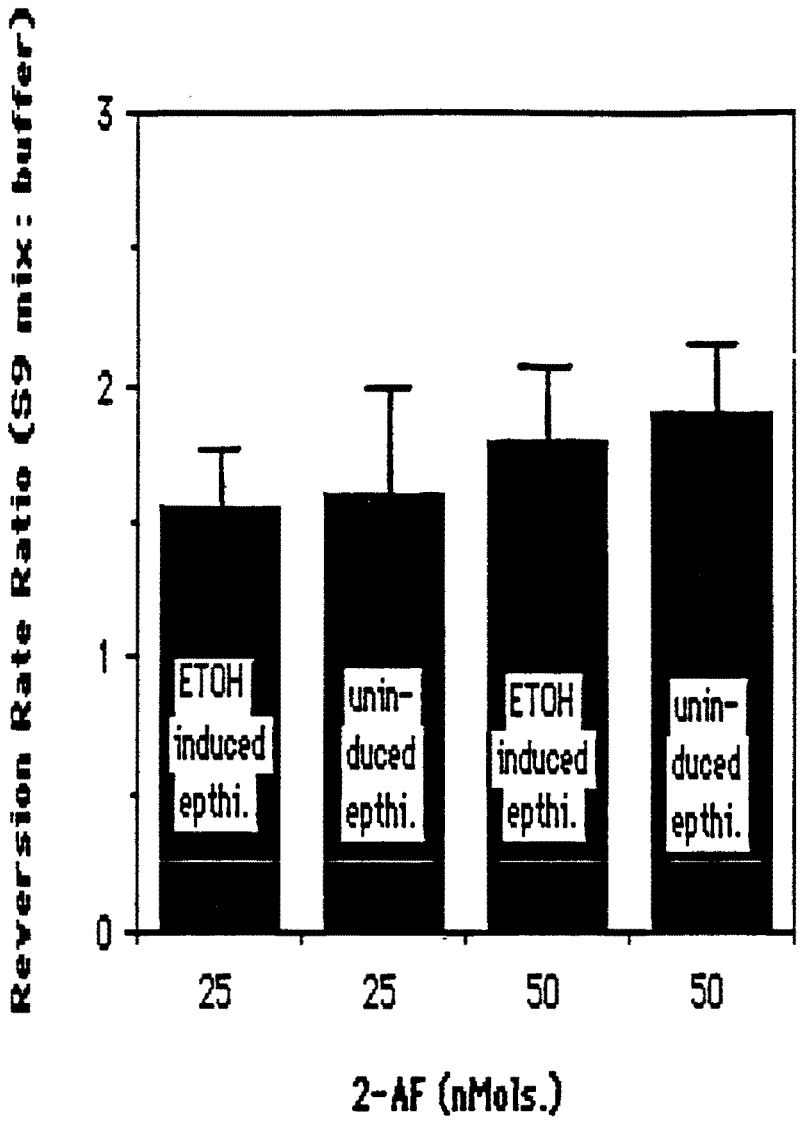


Fig.23 Reversions in the modified liquid preincubation assay when S9 from ethanol-induced tongue epithelium and S9 from uninduced tongue epithelium were tested with the premutagen 2-aminofluorene. Results are shown as the mean of reversion rate ratios from three experiments. The ratios were obtained by dividing the average reversion rate of two plates with S9 from tongue epithelium by the corresponding average of spontanious reversion rates of two plates with buffer.

activate the premutagen 2AF. Figure 24 shows the results for the premutagen MeIQ. The reversion rate ratios for both premutagens at all concentrations were significantly greater than one indicating that both ethanol-induced and uninduced tongue epithelium can activate the premutagens. In all cases, there was no significant difference of activation between the ethanol induced-epithelium and uninduced tongue epithelium. Figure 25 and 26 show the results of the experiments when 2AF and MeIQ respectively were tested for activation with the ethanol-induced and uninduced muscle S9. As with the case of the epithelium, there was no significant difference of revertant colonies formed between the ethanol-induced and the uninduced muscle S9. In both cases, the reversion rate ratios were not significantly greater than one, indicating that the muscle S9's could not activate the precarcinogens.

The results of the activation of the precarcinogen 2AF and MeIQ with the S9 from the ethanol-induced epithelium compared with the corresponding tests for the ethanol-induced muscle are shown in figures 27 and 28 respectively. For both 2AF and MeIQ, there was a significant difference ($p<.01$) between the amount of revertant colonies formed from the ethanol-induced epithelium S9 and from ethanol-induced muscle S9. Figure 31 shows the results of the activation of the precarcinogen 2AF by the S9 from uninduced epithelium compared with the same test for the uninduced muscle S9. Figure 30 shows the results for the

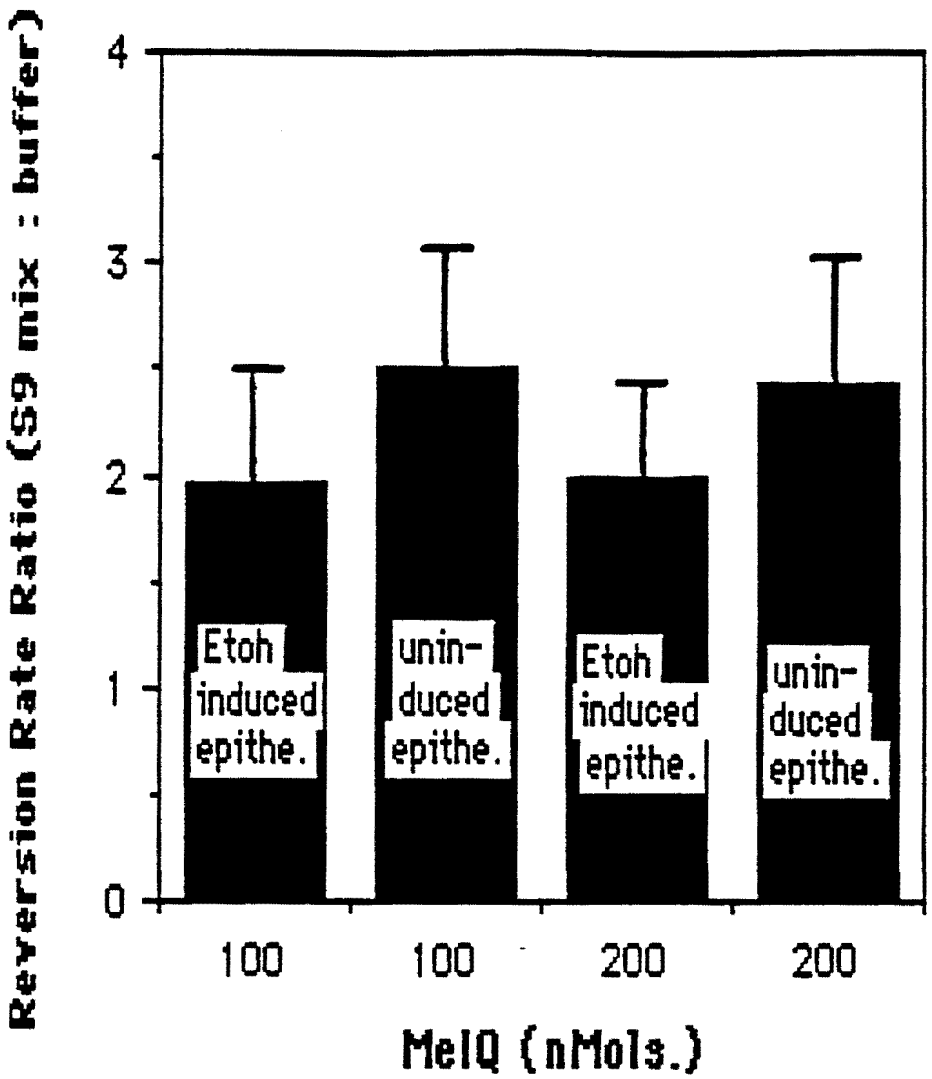


Fig.24 Reversions in the modified liquid preincubation assay when S9 from ethanol-induced tongue epithelium and S9 from uninduced tongue epithelium were tested with the premutagen MeIQ. Results are shown as the mean of reversion ratios from three experiments. The ratios were obtained by dividing the average reversion rate of two plates with S9 from tongue epithelium by the corresponding average of spontanious reversion rates of two plates with buffer.

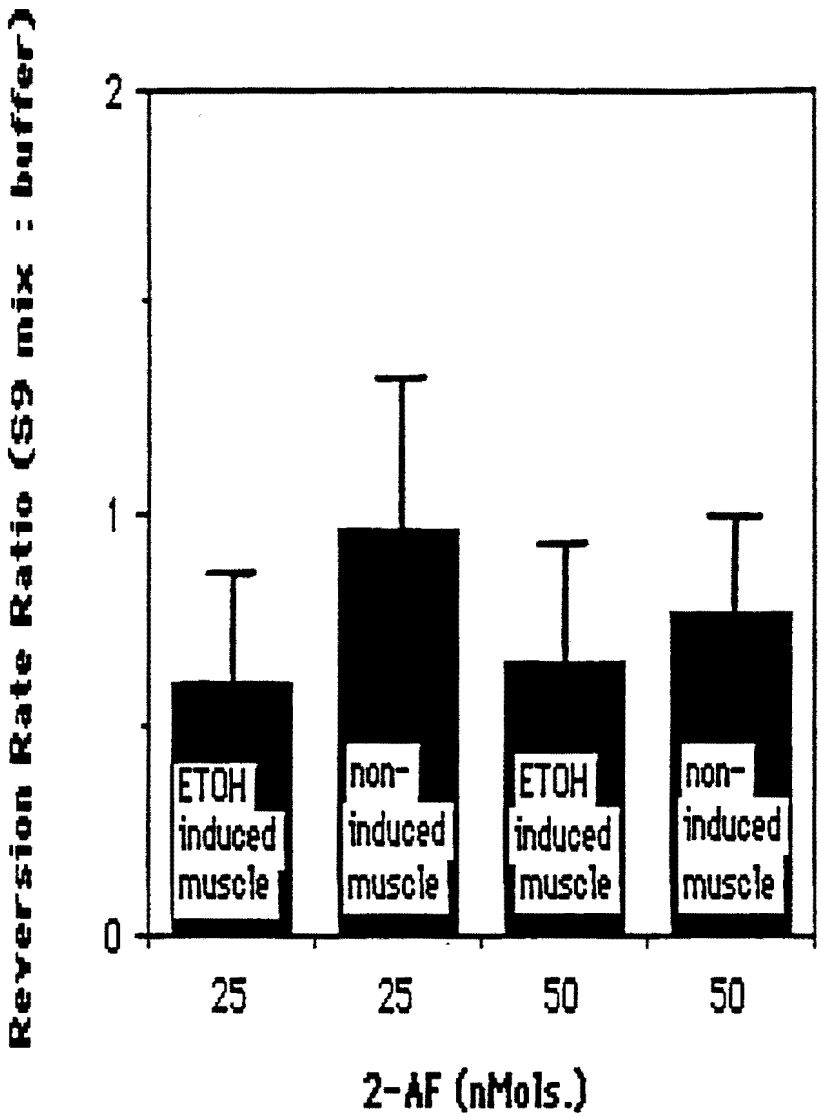


Fig.25 Reversions in the modified liquid preincubation assay when S9 from ethanol-induced tongue muscle and S9 from uninduced tongue muscle were tested with the premutagen 2-aminofluorene. Results are shown as the mean of reversion ratios from three experiments. The ratios were obtained by dividing the average reversion rate of two plates with S9 from tongue muscle by the corresponding average of spontanious reversion rates of two plates with buffer.

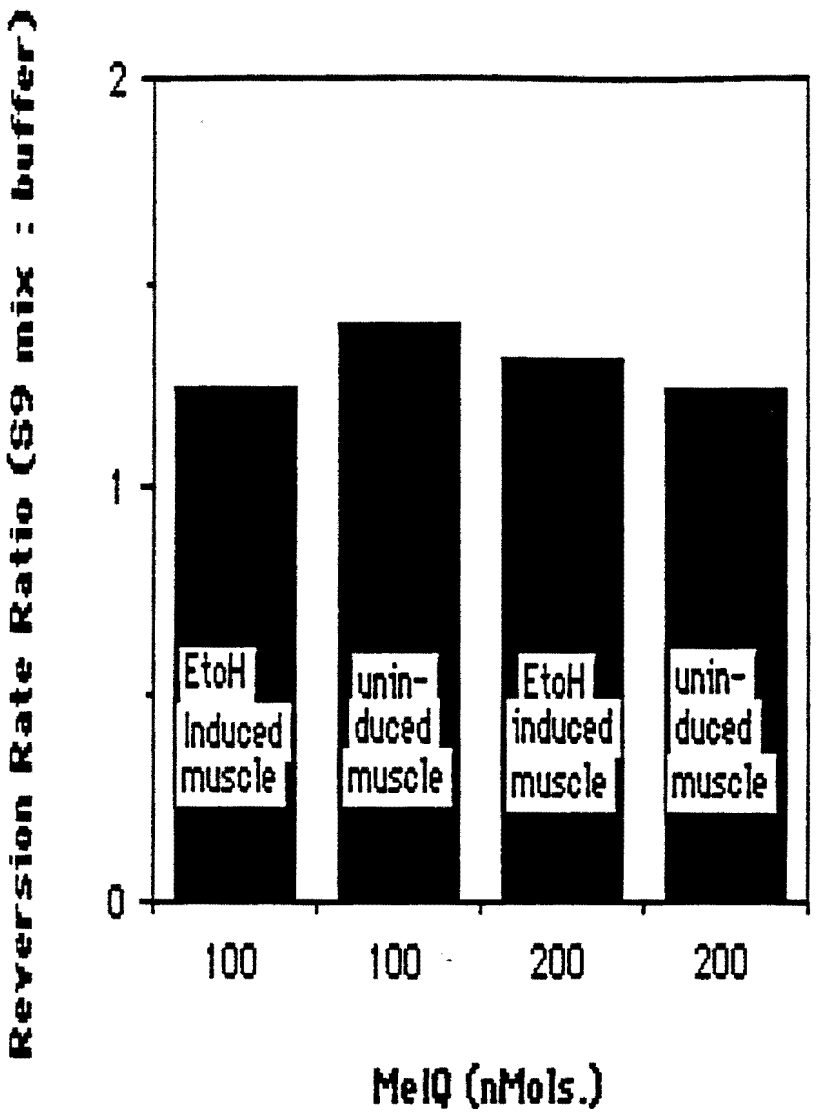


Fig.26 Reversions in the modified liquid preincubation assay when S9 from ethanol-induced tongue muscle and S9 from ininduced tongue muscle were tested with the premutagen MeIQ. Results are shown as the mean of reversion ratios from three experiments. The ratios were obtained by dividing the average reversion rate of two plates with S9 from tongue muscle by the corresponding average of spontanious reversion rates of two plates with buffer.

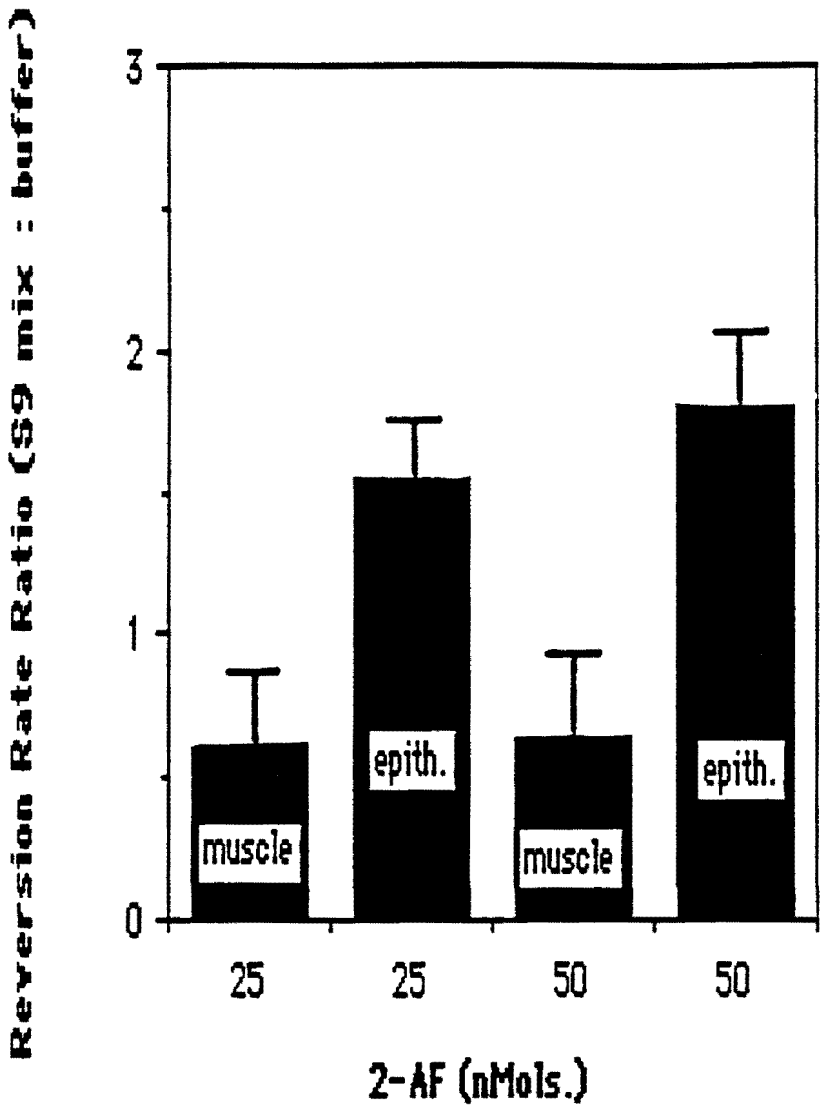


Fig. 27 Reversions in the modified liquid preincubation assay when S9 from ethanol-induced tongue epithelium and ethanol-induced tongue muscle were tested with the premutagen 2-aminofluorene. Results are shown as the mean of reversion ratios from three experiments. The ratios were obtained by dividing the average reversion rate of two plates with S9 from tongue epithelium or muscle by the corresponding average of spontanious reversion rates of two plates with buffer.

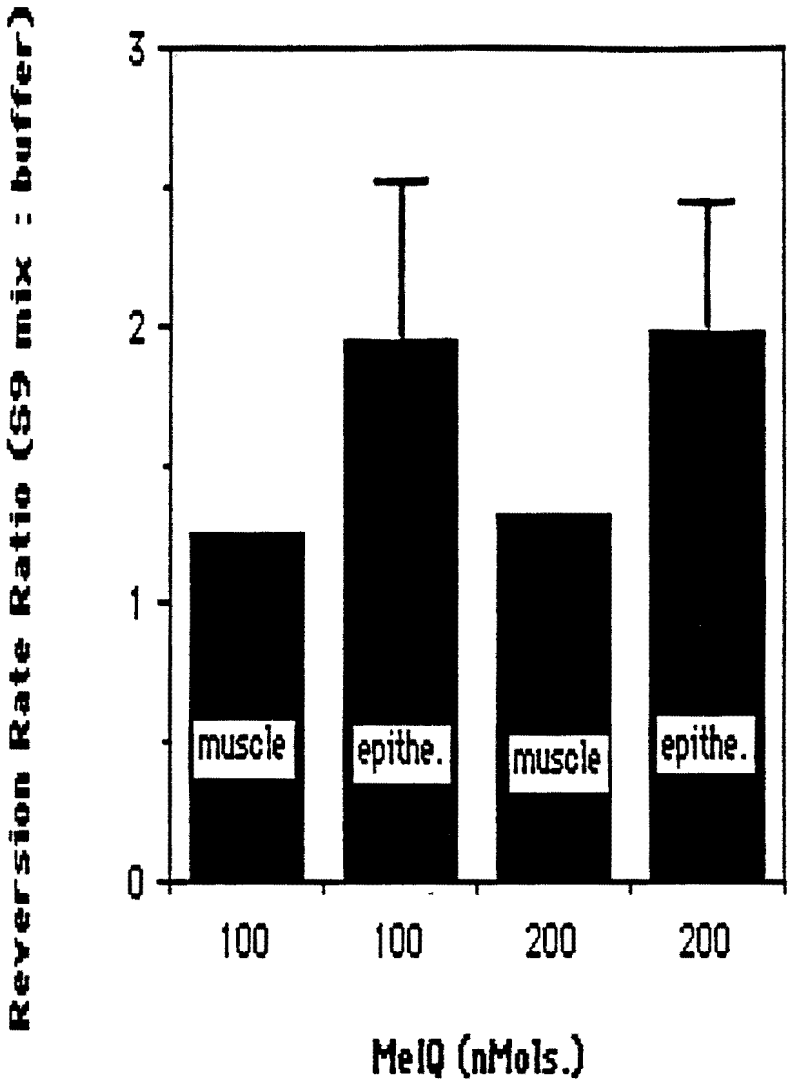


Fig.28 Reversions in the modified liquid preincubation assay when S9 from ethanol-induced tongue epithelium and ethanol-induced tongue muscle were tested with the premutagen MeIQ. Results are shown as the mean of revession ratios from three experiments. The ratios were obtained by dividing the average reversion rate of two plates with S9 from tongue epithelium or muscle by the corresponding average of sopntanious reversion rates of two plates with buffer.

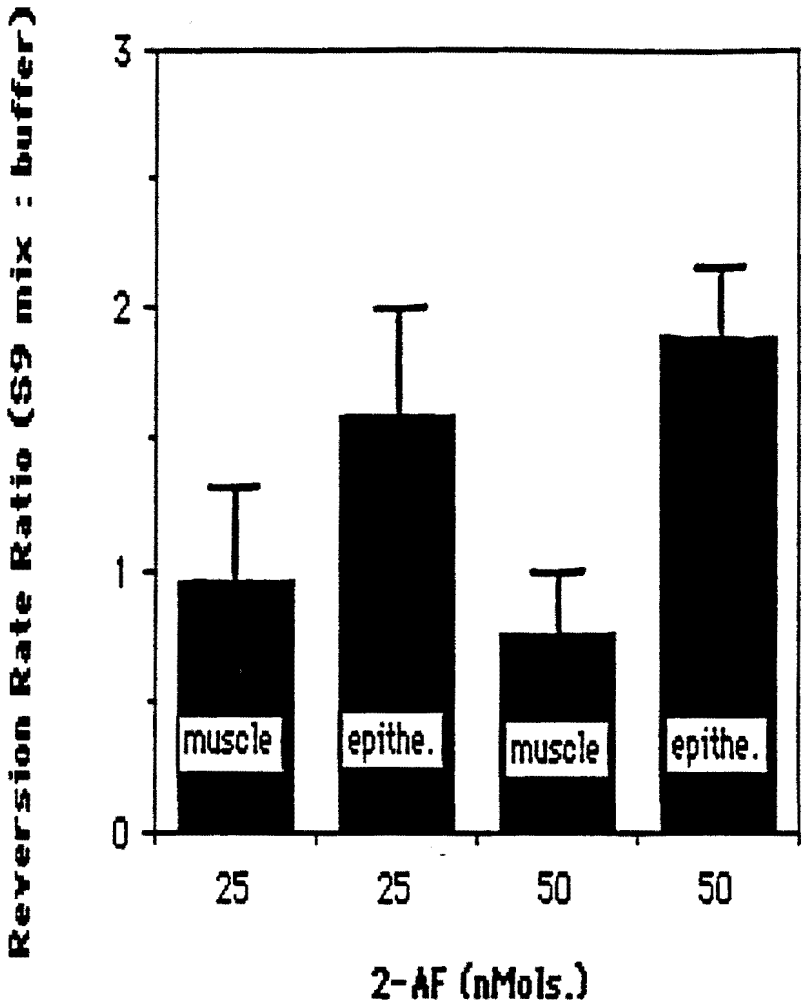


Fig.29 Reversions in the modified liquid preincubation assay when S9 from uninduced tongue epithelium and uninduced tongue muscle were tested with the premutagen 2-aminofluorene. Results are shown as the mean of reversion ratios from three experiments. The ratios were obtained by dividing the average reversion rate of two plates with S9 from tongue epithelium or muscle by the corresponding average of spontanious reversion rates of two plates with buffer.

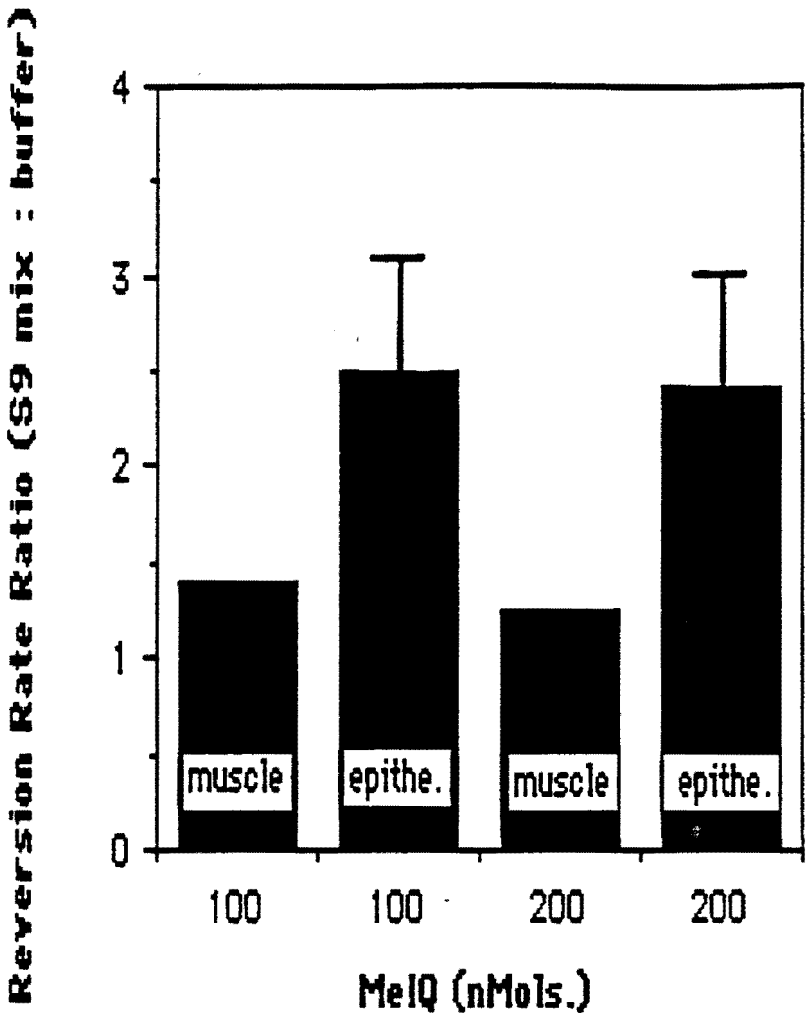


Fig.30 Reversions in the modified liquid preincubation assay when S9 from uninduced tongue epithelium and uninduced tongue muscle were tested with the premutagen MeIQ. Results are shown as the mean of reversion ratios from three experiments. The ratios were obtained by dividing the average reversion rate of two plates with S9 from tongue epithelium or muscle by the corresponding average of spontanious reversion rates of two plates with buffer.

precarcinogen MeIQ. As with the case of the ethanol-induced epithelium, there was a significant difference ($p<.01$) in the activation potential of epithelium over the muscle.

Discussion

This is the first study to demonstrate that rat oral epithelium is capable of activating premutagens/precarcinogens to bacterial mutagens. The fact that animal oral tissues can activate precarcinogens should not be surprissing, because oxidative metabolism takes place in gingival tissues. Futhermore, other investigators (Seitz et al., 1978, 1979, and 1981 and Seitz 1984). have demonstrated such metabolic activation in esophageal, intestinal, and lung epithelium.

Modification of the Ames test to increase it's sensitivity was necessary to demonstrate activation of premutagens by epithelial tissues. This may reflect the relatively small amounts of oxidative enzymes present in the rat tongue epithelium. Modifications of the test were based on the fact that mutation occurs more readily when the mutagen is present during DNA replication (Venitt et al., 1983). Also mutagenesis is enhanced when concentrations of bacteria, mutagen and enzyme are optimally increased. Others (Goggelman et al., 1983; Booth et al., 1980; and Yahagi et al., 1977), have demonstrated the importance of the physiologic state of the tester strain in the inoculum. no one has previously incorporated all these factors into one

test. Figure 16 illustrates the modified version of the Ames Test used in these experiments as compared to the standard version shown in Figure 8 (A, C, and D). The standard test procedure is to grow the bacteria overnight, in rich nutrient broth to the stationary phase. Then the mutagen, S9 mix, the bacteria and top agar are all mixed at the same time and poured immediately onto an agar plate. In the modified test, Salmonella are grown in a chemically defined medium, harvested at the late-exponential phase, resuspended in fresh medium of the same composition at higher cell concentrations, and incubated for 30 minutes; a cell aliquot (inoculum) with S9 mix and mutagen, is incubated for 90 minutes, and top agar is then added. Finally this mixture is poured onto the minimal glucose plates.

As shown in figure 21 growth medium A proved to be the best chemically defined growth medium of the four tested. It supported a full growth for Salmonella typhimurium TA98 in only 6 hours. Although the other media eventually supported growth the lag phase was inordinately long. By having the same compositioin of the media used in all the phases of the experiment the bacteria were not nutritionally shocked during mutagenesis. The bacteria were harvested during their late-exponential phase at a cellular concentration of 7.5×10^8 cell/milliliter, then centrifuged and resuspended in fresh A medium at a concentration determined as more sensitive for the mutagenesis protocol (fig.22).

Higher concentrations of the reactants i.e. bacterial cells, premutagen, and S9 ought to increase sensitivity. For this, different inoculum sizes were tested. In order for the cells to grow again after obtaining the inoculum size, different conditioning or incubating times were tested. The increase in sensitivity obtained after conditiioning for 30 minutes could also have been due to the increased number of cells. Possibly the reason that 60, 90, and 120 minutes did not increase the sensitivity more than 30 minutes of conditioning was due to medium depletion. Another factor which can increase sensitivity is the amount of time the different reactants are allowed to interact. Thus, different preincubation times were tested. Inoculum sizes of 3.4×10^8 and 7.5×10^8 and 1.6×10^9 cells/milliter were simultaneously tested with preincubation times of 0, 30, 60 and 90 minutes. As shown in figure 22 an inoculum size of 1.6×10^8 proved significantly better ($p<.05$) than other sizes and 90 minutes of preincubation time proved significantly better ($p<.05$) than zero and 30 minutes preinicubation (fig.21). An inoculum size of 3.6×10^8 cells/milliliter and princubation times of 120 and 150 minutes were each tested once. That inoculum size or princubation times did not signigicantly increase the sensitivity of the test, however, the number of revertant colonies formed was greater. That too, might have been due to the increase in numbers of microoragnisms and the depletion of the media. To keep the test practicle so it

could be completed in one day these other times were not investigated further.

One difficulty encountered was the scarcity of tongue epithelial and muscle tissue material for testing. The quantity of tissue S9 that had to be used of these tissues to cause detectable activation had to be increased to ten times over those for liver S9 figure 15. This indicates that although there is a definite epithelial activation, the levels of the metabolizing enzymes are very low as is evidenced also by the low reversion rate ratios.

Another major difficulty encountered was the separation of the epithelium from the underlying connective tissue and muscle. The muscle tissue samples tested were free of epithelium; however, the epithelium was slightly contaminated with connective tissue and muscle as it was visually assessed. This necessitated the inclusion of muscle tissue in the mutagenicity assays in addition to epithelium. Differences in activation trends between the contaminated epithelium and the pure muscle would consequently be traced to either tissue. As shown in figures 23 and 24 reversion rate ratios from the epithelial preparations were significantly greater than one. This means that the epithelial preparations activated the precarcinogens 2-AF and MeIQ. However, as shown in figures 25 and 26, no muscle preparation had reversion rate ratios significantly greater than one, i.e., they did not activate 2-AF and MeIQ. The same pattern is shown in

figures 27 through 30.

When ethanol-induced epithelum S9 and uninduced epithelium S9 were tested with either premutagen no significant differences in activation were noted (figs. 23 and 24). ANOVA (Table 1) suggests that the only significant difference ($p<.01$) lies between the epithelial tissue S9 preparation and the pure muscle tissue S9 preparation. This is consistant with the fact that the tongue epithelium and not tongue muscle activates these precarcinogens and that ethanol feeding does not increase this effect.

To prove that ethanol was appropriately administered, its well-known inducing effect on the liver for the enhanced activation of the premutagen N-nitrosopyrrolidine was tested. Ethanol-induced and uninduced rat liver S9 were used in the standard Ames test. The ethanol-induced liver preparation exhibited a significant ($p<.05$) increase in the amount of revertant colonies formed over the unninduced liver prep (fig. 18) (McCoy et al., 1979).

The significance of these results is that rat tongue epithelium is capable of activating precarcinogens, 2AF and MeIQ, to mutagenic metabolites. In contrast tongue muscle did not acitivate 2AF and MeIQ. There was also no significant difference between the ethanol-induced and uninduced tongue epithelium with respect to the magnitude of the mutagenic activation. This means that alcohol did not enhance the levels activity of the metabolizing enzymes for

the two chemicals tested.

In these experiments, only one group of chemicals, the heterocyclic aromatic amines, were investigated for activation by rat tongue tissues. There are other classes of precarcinogens e.g. hydrocarbons, azo and aminazocompounds, and the nitroso compounds which should be tested as well (Bartsch et al., 1982). The cytochrome P-450 oxidative system is really a mutilplicity of enzymes (Anthony et al., 1980). Even though the enzyme system activates more efficiently one class of precarcinogens it may not activate others to the same extent. Also the inductive effect of chemicals like ethanol could influence only one enzyme system that activates certain precarcinogens but not others. Future experiments should test more classes of precarcinogens and also the different effects of various inducing chemicals on oral tissues.

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Approval Sheet

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The final copies have been examined by the director of the thesis and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the thesis is now given final approval by the Committee with reference to content and form.

The thesis is therefore accepted in partial fulfillment of the requirements for the degree of Master of Science.

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