- NITROSAMINES AND OESOPHAGEAL CANCER -

A STUDY OF FACTORS INFLUENCING THE DISTRIBUTION AND METABOLISM OF NITROSAMINES AND THE ENZYMES RESPONSIBLE FOR THEIR ACTIVATION

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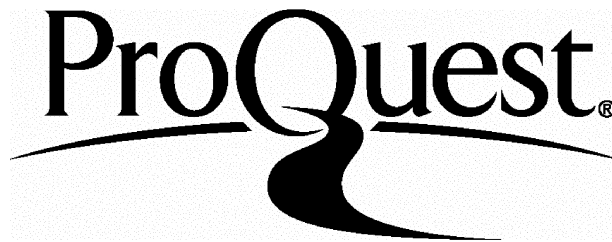
to my parents

ABSTRACT

Nitrosamines produce cancer in most species and possess remarkable organ specificity. Because they are the only carcinogens which can produce oesophageal tumours in experimental animals, and because all humans are exposed to them, it has been suggested that they produce oesophageal cancer in man. The organotropism of nitrosamines depends largely on the distribution of the nitrosamine and the P450s capable of metabolizing it in the animal body. Ethanol consumption, the main factor associated with oesophageal cancer in the West, changes the pharmacokinetics of nitrosamines, increasing the damage to extrahepatic organs, in particular to the oesophagus, and it has been suggested that these changes are responsible for the effect of ethanol on human oesophageal cancer.

The influence of two factors associated with a high incidence of oesophageal cancer, opium (and morphine the major alkaloid found in opium), and isoamyl alcohol, a contaminant of Calvados, on the pharmacokinetics of N-nitrosodimethylamine and N-nitrosodiethylamine was investigated through measurement of their influence on the organ to organ distribution of alkylation produced by these nitrosamines, inhibition of the metabolism of these nitrosamines *in vitro,* and with isoamyl alcohol, its influence on reactions metabolized by different P450s. Morphine and opium dramatically change the pharmacokinetics of NDMA and NDEA, with inhibition of first pass clearance of NDMA and a shift of the metabolism of NDEA from the liver to the oesophagus. Although the mechanisms of these changes has have not been completely elucidated, the results are very similar to those previously obtained with ethanol. However, although isoamyl alcohol inhibited the general metabolism of NDEA in the rat, it did not affected its organotropism, and affects a broader range of P450s than ethanol

The oesophageal monooxygenase system was investigated in depth and compared to the liver through administration of chemicals which induce P450s in the liver, spectrophotometric measurements of the components of the monooxygenase system, western blot analysis of these components and particular forms of P450s, the capacity of oesophageal microsomes to metabolize different compounds, and the influence of nitrosamines on the metabolism of these compounds. The presence of P450 1A1, but not 2E1 or 2B1 and 2B2 in the oesophagus together with evidence that P450 1A1 can participate in the metabolism of N-nitrosomethylbenzylamine, the most powerful oesophageal carcinogen in the rat, is suggested to be one of the factors contributing to the organotropism of some nitrosamines for that organ.

ACKNOWLEDGEMENTS

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ABBREVIATIONS

Ah	arylhydrocarbon hydroxylase
EDTA	ethylenediaminetetraacetic acid
EtOH	ethanol
HPLC	high performance liquid chromatography
IAA	isoamyl alcohol
N7EG	N7-ethylguanine
N7MG	N7-methylguanine
NADH	reduced nicotinamide adenine dinucleotide
NADP	nicotinamide dinucleotide phosphate
NADPH	reduced nicotinamide dinucleotide phosphate
NDEA	N-nitrosodiethylamine
NDMA	N-nitrosodimethylamine
NMAA	N-nitrosomethylamylamine
NMBA	N-nitrosomethylbutylamine
NMBzA	N-nitrosomethylbenzylamine
NNK	N-nitroso-4-(methyl)-1-(3-pyridyl)-1-butanone
NNN	N-nitrosonornicotine
O6EG	O^6-ethylguanine
O6MG	O^6-methylguanine
TEMED	N,N,N',N' - tetramethylethylenediamine
TSNA	Tobacco specific nitrosamines
S.C.	subcutaneously

CHAPTER 1

INTRODUCTION

GENERAL ASPECTS

The question underlying the work in this thesis is, do nitrosamines cause human oesophageal cancer ? This thesis concentrates on two aspects of this question. It is known that certain nitroso compounds are selective carcinogens for the oesophagus and that this is because the oesophagus contains enzymes of the P450 family which metabolically activate the nitrosamines. Work in this thesis tries to characterize this system better. The second part is based on the working hypothesis that factors influencing human oesophageal cancer incidence do so by altering the pharmacokinetics of nitrosamines. Plainly these questions cover almost every aspect of nitrosamine carcinogenesis. For this reason most aspects are covered briefly in this introduction, with the emphasis being on those aspects which are most relevant to the experiments which have been carried out.

I - NITROSAMINES

A - CARCINOGENICITY

The initial discovery by Magee and Barnes (Magee, 1956) that NDMA administered to rats induced liver tumours soon led to the investigation of other nitrosamines for their carcinogenic properties. Since then over 300 N-Nitroso compounds have been shown to be carcinogenic for one or more animal species, including several species of monkey, and no species has been found to be resistant so far (Bogovski, 1981), (Preussman, 1984b). Although the organotropism of nitrosamines is, in some cases, species-dependent, for some nitrosamines the organotropism can be correlated throughout a number of different species. For instance, NDEA has been shown to produce oesophageal tumours in rats, mice, hamsters and cats (recently reviewed by Craddock, 1993). However, most of the carcinogenesis studies have been carried out in the rat. 130 nitrosamines have been tested for carcinogenicity in the rat, with 66 (51%) of them producing tumours in the rat oesophagus, compared to 44% in liver and 38% in the nasal mucosa, the respective main target organs in this species (Preussmann, 1984); Lijinsky, 1992). Figure 1.1 shows the structure of some nitrosamines which induce tumours in the rat oesophagus. From these structures it can be seen that there is some relationship between the chemical structure of the nitrosamine and its organotropism for the oesophagus, as first noted by Druckrey (Druckrey, 1967). So Druckrey (Druckrey, 1967) showed that NDMA did not induce tumours of the oesophagus, but it produced tumours of the liver and kidney. NDMA also induces a small proportion of tumours in the lung and nasal epithelium (Lijinsky, 1992). Although its next homologue N-nitrosomethylethylamine also induces mainly liver tumours, it also produced a small proportion (35%) of oesophageal tumours when given to rats in the drinking water at relatively high doses (5 mmol/animal) (Lijinsky, 1992). The next higher homologues in the series of N-nitrosomethylalkylamines, N-nitrosomethylpropylamine, N-nitrosomethylbutylamine, N-nitrosomethylamylamine

and N-nitrosomethylhexylamine were all shown to induce predominantly oesophageal tumours, when given either in the drinking water, by gavage, or injected intravenously or subcutaneously (Druckrey, 1967; van Hofe, 1987; Lijinsky, 1992). There was no gender difference in the organotropism of these nitrosamines for the oesophagus, and a total dose between 0.1 and 0.3 nmol/animal for these nitrosamines given in the drinking water over 28 weeks produced 100% incidence of oesophageal tumours (Lijinsky, 1992). The next two homologues in this series, N-nitrosomethylheptylamine and N-nitrosomethyloctylamine, produced oesophageal tumour in rats only when given in the drinking water, but not by gavage (Lijinsky, 1992). Higher homologues in this series of asymmetric nitrosamines did not produce oesophageal tumours in rats. Additionally, NMBzA was shown to be the most powerful oesophageal carcinogen in rats, and also the most toxic, with a LD_{50} of 18 mg/kg (Druckrey, 1967). 1 ppm given in the diet over 1 year produced 100% carcinomas and 16 ppm over 12 weeks produced 50% carcinomas in the oesophagus of rats (Druckrey, 1967). As with N-nitrosomethylpropylamine, N-nitrosomethylbutylamine, N-nitrosomethylamylamine and N-nitrosomethylhexylamine, the route of administration of NMBzA did not influence its organotropism for the rat oesophagus (Stinson, 1978). With symmetric nitrosamines by contrast, NDEA is mainly an oesophageal carcinogen only when given to rats at small doses (0.05 mmol/animal given over 97 weeks or 1 mmol/animal given over 26 weeks in the drinking water), whereas higher doses of this nitrosamine given in a shorter time to rats causes mainly liver but also oesophageal tumours and occasionally kidney tumours (Lijinsky, 1992).

The striking organotropism of nitrosamines for different organs is the result of an intrinsic relationship between the distribution of the nitrosamine in the animal body, the distribution of the P450(s) capable of metabolizing the nitrosamine and the susceptibility of the particular organ to the carcinogen insult, which is reflected, in part, by its capacity to repair the DNA damage caused by the nitrosamine. These points and other factors affecting and changing carcinogenicity and organotropism will be

discussed in more detail below.

Figure 1.1: some nitrosamines carcinogenic for the rat oesophagus

N-nitrosodiethylamine (NDEA)
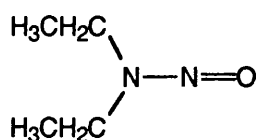
$$H_3CH_2C \diagdown$$
$$N-N{=}O$$
$$H_3CH_2C \diagup$$

N-nitrosodipropylamine (NDPA)
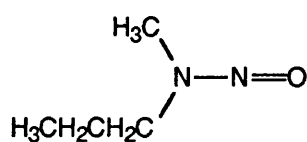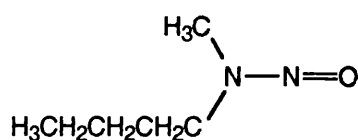
$$H_3CH_3CH_2C \diagdown$$
$$N-N{=}O$$
$$H_3CH_3CH_2C \diagup$$

N-nitrosomethylpropylamine (NMPA)

$$H_3C \diagdown$$
$$N-N{=}O$$
$$H_3CH_2CH_2C \diagup$$

N-nitrosomethylbutylamine (NMBA)

$$H_3C \diagdown$$
$$N-N{=}O$$
$$H_3CH_2CH_2CH_2C \diagup$$

N-nitrosomethylamylamine (NMAA)

$$H_3C \diagdown$$
$$N-N{=}O$$
$$H_3CH_2CH_2CH_2CH_2C \diagup$$

N-nitrosomethylhexylamine (NMHA)

$$H_3C \diagdown$$
$$N-N{=}O$$
$$H_3CH_2CH_2CH_2CH_2CH_2C \diagup$$
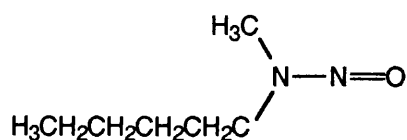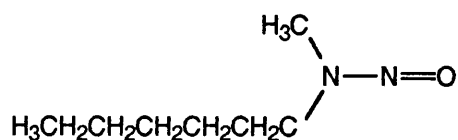
N-nitrosomethylbenzylamine (NMBzA)

$$H_3C \diagdown$$
$$N-N{=}O$$
$$-H_2C \diagup$$

16
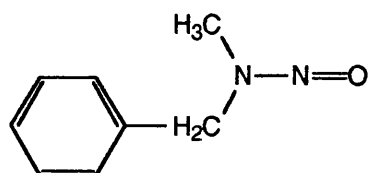
B - HUMAN EXPOSURE TO ENDOGENOUSLY FORMED NITROSAMINES

Human exposure to nitrosamines presents a particular characteristic for they are the only carcinogens which are, without doubt, formed inside the body. Formation of nitrosamines *in vivo* occurs by reaction of the nitrosating agent N_2O_3 with either secondary or tertiary amines as shown below (reviewed by Mirvish, 1975; Leaf, 1989).

$$NO_2^- + H^+ \rightleftharpoons HNO_2$$

$$2HNO_2 \rightleftharpoons N_2O_3 + H_2O$$

$$R_2NH + N_2O_3 \longrightarrow R_2NNO + HNO_2$$
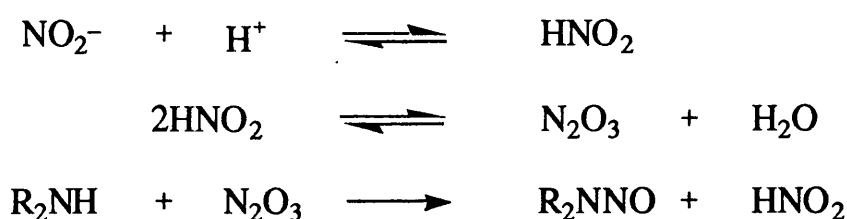
The nitrite can be derived from sodium or potassium nitrite in the diet, but the principal source is from oral bacterial reduction of the nitrate secreted in the saliva by the salivary glands. Nitrate is normally present in blood and like other anions is taken up by the salivary gland and excreted in the saliva at a much higher concentration than in the blood (Boyland, 1971). Some of these anions such as halide ions and thiocyanate can interact with the nitrite to form active nitrosating species (eg. thiocyanate which is found in significant amounts in the saliva of smokers forms nitrosyl thiocyanate - ON-NCS) and thus act as catalysts of the nitrosation reaction (Boyland, 1971). Thus, endogenous nitrosation takes place mainly in the stomach due to the formation of nitrous acid from ingested nitrite under acidic conditions (Mirvish, 1975). The reaction shows an optimum pH between 3.0 and 3.5 because the pKa of nitrous acid is 3.4. This low pH affects the ability of amines, particularly of strongly basic amines, to participate in this reaction because protonated amines are unreactive towards nitrous acid. The reaction rate is proportional to the non-protonated amine concentration, but second order to nitrite, thus being much more sensitive to slight increases in nitrite concentration. Apart

from these normal sources, nitrite can also be formed in the infected bladder and in the achlorhydric stomach by bacterial catalyzed reduction of nitrate (Camels, 1985). As far as nitrosamines are concerned, the capacity of bacteria to reduce nitrate to nitrite is not their only important property because they can also catalyze the nitrosation process itself (Camels, 1985).

Recently, it has been shown that a variety of cell types including vascular endothelial cells, neurons and stimulated macrophages can produce nitrite and nitrate from L-arginine via the intermediate formation of nitric oxide (Marletta, 1988). Stimulated macrophages can also carry out the N-nitrosation of secondary amines resulting in the formation of carcinogenic nitrosamines (Miwa, 1987). The nitrosating species N_2O_3 is, in this case, probably originated from the reaction of NO with molecular oxygen (Iyengar, 1987). The enzyme involved in catalyzing the formation of NO is called nitric oxide synthase and is present in a number of different cells including neutrophils, macrophages, endothelial cells, neurons, Kupffer cells and hepatocytes (Forstermann, 1991). Different forms of the enzyme are present in these different types of cells. In some of these cells the nitric oxide synthase is constitutive and not induced upon stimulation (Forstermann, 1991), but there is clear evidence for a markedly increase in the rate of endogenous nitrosation upon stimulation of nitric oxide synthase (Wu, 1993a), suggesting that some individuals with chronic inflammation or liver disorders may be subject to a significant increase in exposure to nitrosamines.

Amines are present in the normal diet as well as in a variety of drugs and have been quantitatively and qualitatively measured in different body fluids (Tricker, 1992). Dimethylamine, diethylamine, piperidine and pyrrolidine were the most common occurring amines present in biological fluids such as saliva, gastric juice, blood, urine and faeces. Their concentration in the gastric juice of individuals were variable, but contained as an average 0.87 and 0.05 µg/ml for dimethylamine and diethylamine, respectively (Tricker, 1992). Although secondary amines in general react with nitrite

more readily than do tertiary amines (reviewed by Leaf, 1989), this is not always the case. For example Lijinsky (Lijinsky, 1972) showed the very rapid formation of NDMA from the tertiary amine aminopyrene.

Although endogenous formation of nitrosamines had been demonstrated in 1969 by Sander and Burke (Sander, 1969), it was not until two major discoveries took place that this unusual route of exposure to these carcinogens could be better evaluated.

The first major advance was the introduction by Fine and Rounbehler (Fine, 1975) of the Thermal Energy Analyzer, a chemiluminescence detector specific for the N-nitroso group which can detect trace amounts of volatile nitrosamines. Usually the Analyzer is coupled to a gas chromatograph and thus allows identification of the particular nitrosamine through its retention time on the column. However this arrangement permits the detection of only volatile nitrosamines, which is a major drawback since recent estimates suggest that most of the endogenously formed nitroso compounds are nonvolatile (Licht, 1988). Nevertheless, the Thermal Energy Analyzer permits detection of nitrosamines in the order of $\mu g/kg$ levels and has been used to measure nitrosamine amounts in biological fluids as well as in environmental sources.

The second great advance was the development of the N-nitrosoproline test (NPRO) by Ohshima and Bartsch (Ohshima, 1981). This test is a simple noninvasive method for quantitative estimation of endogenous nitrosation. In the test, the person ingests a quantity of proline. Some of this is nitrosated *in vivo*. The amount nitrosated can be measured because NPRO is not metabolised and is excreted quantitatively in the urine (Chu, 1981), and can be detected together with other nitroso compounds formed from the nitrosation of amino acids and other natural substances using the Thermal Energy Analyzer after derivatization of the nitrosated compounds. NPRO and other nitrosamino acids excreted in the urine are determined as an index of the extent of endogenous nitrosation. The amount of NPRO formed is proportional to the dose of proline and

increases exponentially with the nitrate intake, particularly for intakes of more than 260 mg/day. Recently Tsuda *et al* (Tsuda, 1991) have shown that N-nitrosothiazolidine-4-carboxylic acid is probably a better marker for endogenous nitrosation. It is widely accepted that the NPRO test is considered a good index of human endogenous nitrosation, and its use has given substance to many of the theoretical points which had previously been assumed. For instance, it has been shown in a number of studies that ascorbate administration to individuals decreased the amount of NPRO excreted in their urine (recently reviewed by Bartsch, 1989), but does not decrease the background level of NPRO excretion (Wagner, 1985). The decrease in NPRO formation in individuals given ascorbate confirmed the studies *in vitro* which had shown that this vitamin is an inhibitor of nitrosation reactions due to its competition with the amine for the nitrosating agent (reviewed by Archer, 1984). So, in this respect, the involvement of nitrosamines in causing human oesophageal cancer is strengthened since a vast number of epidemiological studies have shown an inverse correlation between consumption of fresh vegetables and fruits (major sources of polyphenols, also inhibitors of nitrosation reactions, and vitamins such as ascorbate) and a risk to develop oesophageal cancer (Craddock, 1993)

Two important results of the test were to show that endogenous nitrosation is greater in smokers than in nonsmokers (Hoffmann, 1984) and that there is geographical variation in the amount of endogenous nitrosation (Wu, 1993b) with subjects living in high incidence areas of cancers suspected to be linked to nitrosamines having a higher rate of endogenous nitrosation. So, for instance when the test was applied in individuals living in high and low-risk areas for stomach cancer in Japan, Poland and Costa Rica, in all cases the individuals who lived in the high-incidence areas excreted substantially more NPRO in their urine then did individuals living in the low-incidence areas (reviewed by Bartsch, 1989) .

C - HUMAN EXPOSURE TO PREFORMED NITROSAMINES

Human exposure to nitrosamines from external sources can be divided into occupational exposure and exposure from diet, drinking, smoking etc, collectively described as life-style by Preussmann (Preussman, 1984b).

Certain occupational exposures, particularly of workers in the tyre and rubber industry, and in the metal working industries may represent the largest known human exposure to exogenously formed nitrosamines (Preussman, 1984a). Workers of the two first type of industries can be exposed to more than 50 µg/day of some nitrosamines, particularly N-nitrosomorpholine and NDMA (Fajen, 1979; Spiegelhalder, 1983; Spiegelhalder, 1984), whereas the exposure of workers in metal working industries to N-nitrosodiethanolamine contaminated cutting oils has been calculated to be 100 µg/day (recently reviewed by Tricker, 1989)

In contrast to the limited data on the presence of nitrosamines in the work place, most foods in developed countries have been analyzed for the presence of volatile nitrosamines. These analyses showed measurable amounts of nitrosamines in food, such as bacon, which are preserved with nitrite (Tricker, 1989) and in German beer due to the manufacturing process employed by german brewers (Preussman, 1984a). However human exposure to preformed nitrosamines from dietary sources is low when compared to other sources like tobacco and is decreasing in the west mainly due to improvements in beer production and in food preservation (Preussman, 1984a). Studies in Germany (Spiegelhalder, 1980), Holland (Stephaney, 1980), UK (Gough, 1978), United States (NAS (National Academy of Sciences, 1981), and Sweden (Osterdahl, 1988; Österdahl, 1991) have shown that NDMA and N-nitrosopyrrolidine are the most common contaminating nitrosamines, with an average daily intake between 0.5-1.0 µg/ person, mainly from beer and cured meats (specially fried bacon). Although the data regarding the presence of preformed nitrosamines in Asian foods is not so extensive,

some parts of Asia have been shown to present a somewhat different situation. For instance, in some parts of China, seafood seems to be the main source of human exposure to preformed nitrosamines with an unusual high frequency of NDEA being present (Song, 1988),) In fact, in another study (Gao, 1991) NDEA was found to be the most frequently present nitrosamine in chinese foods. In Kashmir, India, seafood and pickled vegetables seems to account for a higher than usual human exposure to preformed nitrosamines (Siddiqi, 1988), although in this particular case, the nitrosamines present were similar to those in western diet.

Apart from certain occupational exposures to nitrosamines, which occur in limited areas as discussed before, tobacco is the most important source of human exposure to preformed nitrosamines (Bartsch, 1984; Preussman, 1984b; Hoffmann, 1985). Tobacco use is causally related to human cancer at a number of different sites, inducing lung, pharynx, larynx, oesophagus, bladder, pelvis, pancreas, oral and nasal cavity tumours (reviewed by Hecht, 1989). Tobacco contains several carcinogens including volatile nitrosamines and the so called tobacco specific nitrosamines (TSNA). TSNA as a group are the most abundant and active carcinogens present in tobacco. They are formed during processing and burning of tobacco by nitrosation of the tobacco alkaloids nicotine (a tertiary amine), nornicotine, anabasine and anabatine (secondary amines) (figure 1.2). Seven TSNA have been identified so far, with N-nitrosonornicotine (NNN) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) being strong carcinogens (Hecht, 1989). NNN and NNK induce tumours in laboratory animals at a number of sites which have been epidemiologically implicated as tobacco related cancer in humans, notably lung, nasal and oral cavity, and oesophagus (Hecht, 1988), and human and rodent tissues metabolize these nitrosamines similarly, as will be discussed later.

The strongest association between nitrosamines and human cancer comes from the use of smokeless tobacco in the form of snuff dipping or the chewing of tobacco alone or in

betel quid and the epidemiologically related tumours of the oral cavity (IARC, 1986). These practices, which should not be confused with snuff inhalation as practiced in England, involves putting powdered tobacco, often in a packet similar to a tea bag, between the gum and the buccal cavity. The nicotine in the tobacco is absorbed directly into the blood stream without the first-pass clearance which would occur if it were swallowed. Nitrosamines are practically the only carcinogens found in these products and are present at extremely high concentrations, with typical values ranging from 1 to 100 µg/g (Hecht, 1989). Nitrosamines are readily extracted from snuff during chewing and can be absorbed from the saliva (Idris, 1992). In fact, recent reports suggest that snuff dippers are exposed to higher amounts of NNK in snuff tobacco than had been previously thought, which are actively extracted by the saliva (Prokopczyk, 1992). Furthermore, there is substantial formation of TSNA *in vivo* during chewing of tobacco due to the nitrosation of tobacco alkaloids in the oral cavity (Hoffmann, 1981). Although nitrosamines are not usually considered as topical carcinogens, tumours of the oral cavity in laboratory animals can be produced by repeated swabbing with a mixture of NNN and NNK (Hecht, 1986).

Figure 1.2: Formation of tobacco specific nitrosamines (TSNA)
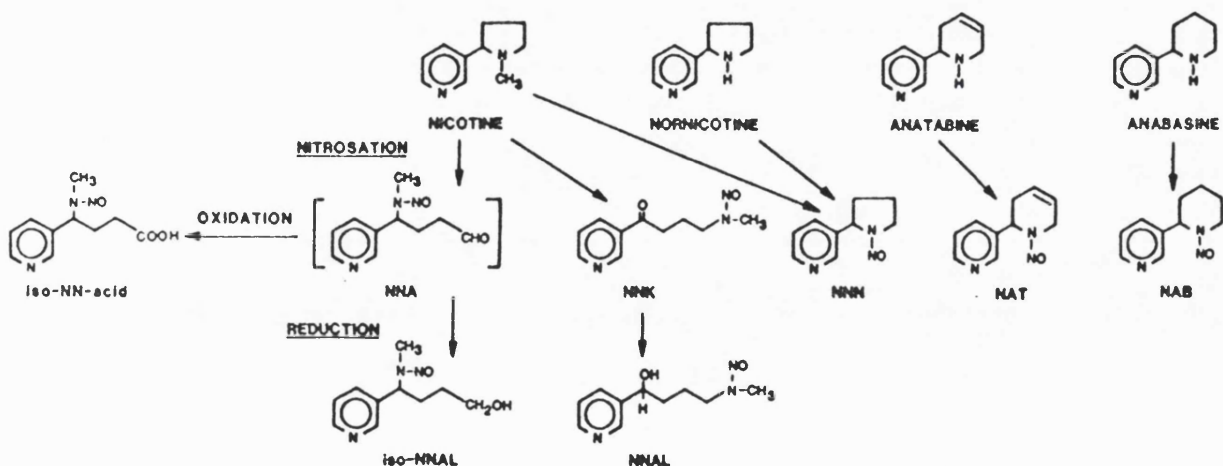
Taken from (Hecht, 1989)

The possible role of nitrosamines in smoking induced cancer is more difficult to

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establish due to the complex nature of tobacco smoke and the many physiological changes which smoking produces in the lung. Nevertheless, the estimated levels of TSNA, and in particular NNK, to which men are exposed to from tobacco smoke are in the same order of magnitude to the levels shown to produce tumours in laboratory animals (reviewed by Hoffmann, 1985; Hecht, 1988; Hecht, 1989). So, 30 mg/kg of NNK administered to rats in the drinking water (Hecht, 1989) or 6 mg/kg administered subcutaneously to hamsters (Hecht, 1983) caused malignant lung tumours, whereas a smoker of 40 cigarettes per day is exposed to about 3.6 mg/kg during a 40-year period. The striking organospecificity of NNK which produces lung tumours in rats, hamsters and mice, added to its high content in cigarette smoke (an average of 100 ng/cigarette, depending on the type of cigarette) supports its involvement in causing human respiratory tract tumours by cigarette smoking (Hecht, 1989). Furthermore, this high exposure must be added to the increased endogenous formation of nitrosamines in smokers as seen with the N-nitrosoproline test (Hoffmann, 1983). So the levels of NPRO excreted in the urine of smokers were 2-fold higher than in nonsmokers, which were kept in the same controlled diet as smokers. The elevated thiocyanate levels in the saliva of smokers is probably the main contributing factor for the higher endogenous nitrosation potential in these individuals. Administration of ascorbate to smokers reduced the levels of NPRO excreted in their urine (Hoffmann, 1983).

There has been some recent progress in identifying some biological markers as a measure of exposure to TSNA. Foiles *et al* have shown that 4-hydroxyl-1-(3-pyridyl)-1-butanone may be useful as a biochemical marker in humans for estimating the *in vivo* metabolic activation of NNK, the exposure to NNK and in clarifying its role in tobacco related carcinogenesis (Foiles, 1992). The metabolism of NNK produces a pyridyloxobutylating agent, which reacts with macromolecules such as haemoglobin and DNA. 4-hydroxyl-1-(3-pyridyl)-1-butanone can be released from haemoglobin by hydrolysis. Foiles *et al* (Foiles, 1992) have shown that higher levels of this compound are present in the haemoglobin of snuff dippers and smokers than non-smokers.

Metabolites of NNK have also been shown in the urine of smokers, but were undetected in the urine of nonsmokers (Carmella, 1993).

D - NITROSAMINES AND HUMAN OESOPHAGEAL CANCER

Since nitrosamines were discovered there has been speculation that they play a role in human cancer. As this thesis is concerned primarily with oesophageal cancer, this aspect will be considered below.

Since nitrosamines have been shown to be powerful carcinogens for the oesophagus in experimental animals and are the only active carcinogens in this organ, there has been speculation linking this class of carcinogens with human oesophageal cancer. Over 300 nitrosamines have been tested for carcinogenicity in different species and a great proportion of them have been shown to be carcinogenic for the oesophagus (Lijinsky, 1992). 130 nitrosamines have been tested for carcinogenesis in the rat, with 66 of them (51%) being carcinogenic for the oesophagus, in some cases exclusively (Lijinsky, 1992). For instance, NMBzA is an exclusive and powerful oesophageal carcinogen in the rat, inducing 100% of malignancies when given in the diet at 1 ppm over 1 year (Druckrey, 1967). Although other species have not been so extensively studied as the rat, some nitrosamines which are oesophageal carcinogens in the rat have also been shown to have the oesophagus as a target organ in other species. So, NDEA induces oesophageal cancer in the rat, but also in mouse, hamsters and cat; N-nitrosodipropylamine and N-nitrosodibutylamine induce oesophageal cancer in rat and mouse and NMBzA induces oesophageal tumours in rat, mouse and rabbits (Magee, 1976; Lijinsky, 1992; Craddock, 1993). However, the striking organotropism of asymmetric N-nitrosomethylalkylamines for the rat oesophagus is not seen in the hamster and has not been studied in other species(Lijinsky, 1992). In the hamster, nitrosamines seem to have a particular organotropism for the nasal epithelium (Magee,

1976; Lijinsky, 1992).

Oesophageal cancer is one of the most common type of cancers in the world and a peculiar characteristic of this disease is the sharp difference in incidence of these tumours within relatively small areas around the world. Different etiological factors have been related to the high incidence of the disease in different areas, and it has been suggested that nitrosamines may have a role in the development of oesophageal tumours in many of these areas (for a review see Craddock, 1993). For example in Kashmir, India, an area with relatively high incidence of oesophageal cancer, nitrosamines and precursors, including methylbenzylamine, have been detected at high levels in some locally consumed foodstuffs (Siddiqi, 1988; Siddiqi, 1991).

The problem has been most extensively studied in Lin-Xian, a county in Henan Province just north of the Yellow River in Northern China, where the inhabitants have one of the highest incidence of oesophageal cancer world-wide (the adjusted average incidence rate is 108/100000) (Yang, 1980) . Nitrosamines and a poor nutritional diet (particulary deficient in riboflavin, zinc, vitamin C and A) have been implicated as causative factors (Li, 1980; Yang, 1980; Lu, 1984). Nitrosamines, particulary NMBzA, have been found in high concentrations in some traditional foodstuffs consumed in that area including pickled vegetables and mouldy bread and grains (Singer, 1986; Lu, 1991) and nitrosamines have been detected in the gastric juice of Lin-Xian inhabitants (Lu, 1986).

The NPRO test has been applied in four studies relating to oesophageal cancer in Henan and other parts of China (Lu, 1986; Chen, 1987; Lu, 1987; Wu, 1993a). In the first study in which just 2 counties have been compared, inhabitants from Lin-Xian were shown to excrete more nitrosamino acids in their urine than did the inhabitants from Fan-Xian, a county about 150 kms away and with oesophageal cancer rates 5 to 7 times lower than those in Lin-Xian (Lu, 1986). In the second and third studies, the levels of

nitrosamino acids excreted in the urine of individuals living in different counties were positively correlated with oesophageal cancer incidence (Chen, 1987; Lu, 1987). The fourth and latest study is the biggest study involving the NPRO test executed so far. It involved 4000 male subjects living in all 69 counties in China, with variation of oesophageal cancer rates reaching 350-fold. The results showed that the levels of NPRO and N-nitrososarcosine excreted in the urine were positively and significantly correlated to oesophageal cancer in China (Wu, 1993a).

Another direct evidence for the role of nitrosamines in inducing oesophageal cancer comes from the study of Umbenhauer *et al* (Umbenhauer, 1985), who used monoclonal antibodies in a radio-immune assay to detect trace levels of the nitrosamine-induced promutagenic bases O^6-methylguanine and O^6-ethylguanine. DNA was extracted from patients undergoing oesophageal tumour operations in Lin-Xian (a high incidence area) and Europe (a low incidence area). 10 out of 37 oesophageal DNA samples from Lin-Xian had levels of O^6-methylguanine between 50-160 fmol/mg DNA, 10 samples showed levels between 15-50 fmol/mg DNA and in 10 samples O^6-methylguanine could not be detected. By contrast, 5 samples from Europe had levels of O^6-methylguanine between 15-45 fmol/mg DNA, and in the other 7 samples this promutagenic base could not be detected. No O^6-ethylguanine was detected in any DNA sample analyzed. Within an individual high levels of O^6-methylguanine in oesophageal DNA were not accompanied by high amounts of this promutagenic base in other tissues, suggesting that selective DNA methylation occurred only in the target organ. Although the higher level of O^6-methylguanine in oesophageal DNA of the individuals from Lin-Xian (ie 100 fmol/mg DNA which is approximately 100 nmol O^6-methylguanine/mol guanine) is 150 times less than is observed (14.6 μmol/mol guanine) after an oral dose of 2.5 mg/kg b.w. of NMBzA to rats (Wiestler, 1984), it is impossible to compare directly the level of this adduct obtained from the two experiments, because O^6-methylguanine can be rapidly removed by the DNA repair enzyme O^6-alkylguanine-DNA-transferase, and it was impossible to know how much

O^6-methylguanine had originally been present in the human DNA.

In other parts of the world there is a high incidence for oesophageal cancer for which a direct association between exposure to nitrosamines and oesophageal cancer has not yet been shown. One such area, with one of the highest incidence of oesophageal cancer in the world is situated along the Caspian Sea, in North East Iran. Both men and women are equally susceptible to develop oesophageal tumours and the contrasts in the incidence of the disease is sharply demarcated within this area (Kmet, 1972; Mahboubi, 1973). Epidemiological studies have associated the risk of developing oesophageal tumours in these areas with a high consumption of opium and poor nutrition (IARC, 1977; Ghadirian, 1985). This influence of opium has not been explained. Studies with the Ames test have shown the presence of mutagens in opium pyrolysate. These mutagens were shown to possess a hydroxyphenanthrene ring and were able to transform hamster cells (Friesen, 1985; Friesen, 1987), but they failed in producing tumours in rodents and a possible reason for their organotropism for the oesophagus has not been explained.

In the West, that is Europe and America, there is also no data showing a direct-relationship between exposure to nitrosamines and oesophageal cancer. In the West and in Africa, oesophageal cancer is associated with alcohol consumption, with cigarette smoking having a multiplicative effect on the risk (Wynder, 1961; Wynder, 1977; Tuyns, 1979a; Pollack, 1984). Although all alcoholic drinks seem to increase the risk of oesophageal cancer, epidemiological studies have demonstrated a strong correlation between tumour development and consumption of certain spirits, in particular Calvados (Tuyns, 1970; reviewed by Craddock, 1993). Craddock has suggested that the higher risk presented by some spirits maybe related to their high amounts of alcohols with longer alkyl chains than ethanol, which are formed by yeast during fermentation (Craddock, 1991; Craddock, 1992; Craddock, 1993).

Since neither ethanol nor alcoholic beverages are carcinogenic *per se* (IARC, 1988), they must act by increasing the effectiveness of some other carcinogen, and one might propose that opium acted in a similar way, also increasing the activation of some carcinogen active in the oesophagus. The hypothesis of Swann (Swann, 1982; Swann, 1984b) that ethanol induces oesophageal tumours by changing the pharmacokinetics and organotropism of nitrosamines provides a basis for the experiments done in chapter 2 and 3 of this thesis. The comparison between the effect of isoamyl alcohol, one of the highest contaminants of spirits, and ethanol on the *in vivo* and *in vitro* metabolism of NDEA is shown in Chapter 2, and the dramatic effect caused by morphine and opium on the distribution and metabolism of NDMA and NDEA is shown in Chapter 3.

E - METABOLISM

Since Barnes and Magee showed that NDMA was hepatotoxic (Barnes, 1954) and could induce liver tumours in rats (Magee, 1956), good knowledge about the general metabolism of nitrosamines as well as their mechanism of carcinogenesis has been gained by using NDMA as a model. Magee showed that when this nitrosamine was injected into rats and rabbits, it was quickly and uniformly distributed throughout the animal body water, and rapidly disappeared from the animal body so that only 30% of a dose of 50 mg/kg b. w. could be recovered after 6 hours (Magee, 1957). Total excretion of this nitrosamine in faeces and urine was minimal, and even with a ten times higher dose only 5-10% of the nitrosamine could be detected in the urine, leading to the conclusion that NDMA must be rapidly metabolized *in vivo*. By using hepatectomized rats, it was shown that the liver was the main organ responsible for the metabolism of NDMA(Magee, 1957). These studies argued against a preferential uptake of the nitrosamine by the liver and suggested that the damage is caused by a metabolite which is produced preferentially in the liver. Support for these conclusions came from the experiments of Dutton and Heath (Dutton, 1956) using [^{14}C]-NDMA. They showed

that after an injection of 50 mg/kg b.w. to rats, 40 % of the radioactivity was recovered

as $^{14}CO_2$ in the first 8 hours after the injection. The rest of the radioactivity was evenly

distributed throughout the body and 7% was excreted in the urine. Thus, the authors

concluded that NDMA must be rapidly demethylated *in vivo* .

Figure 1.3: mechanism of activation of nitrosamines
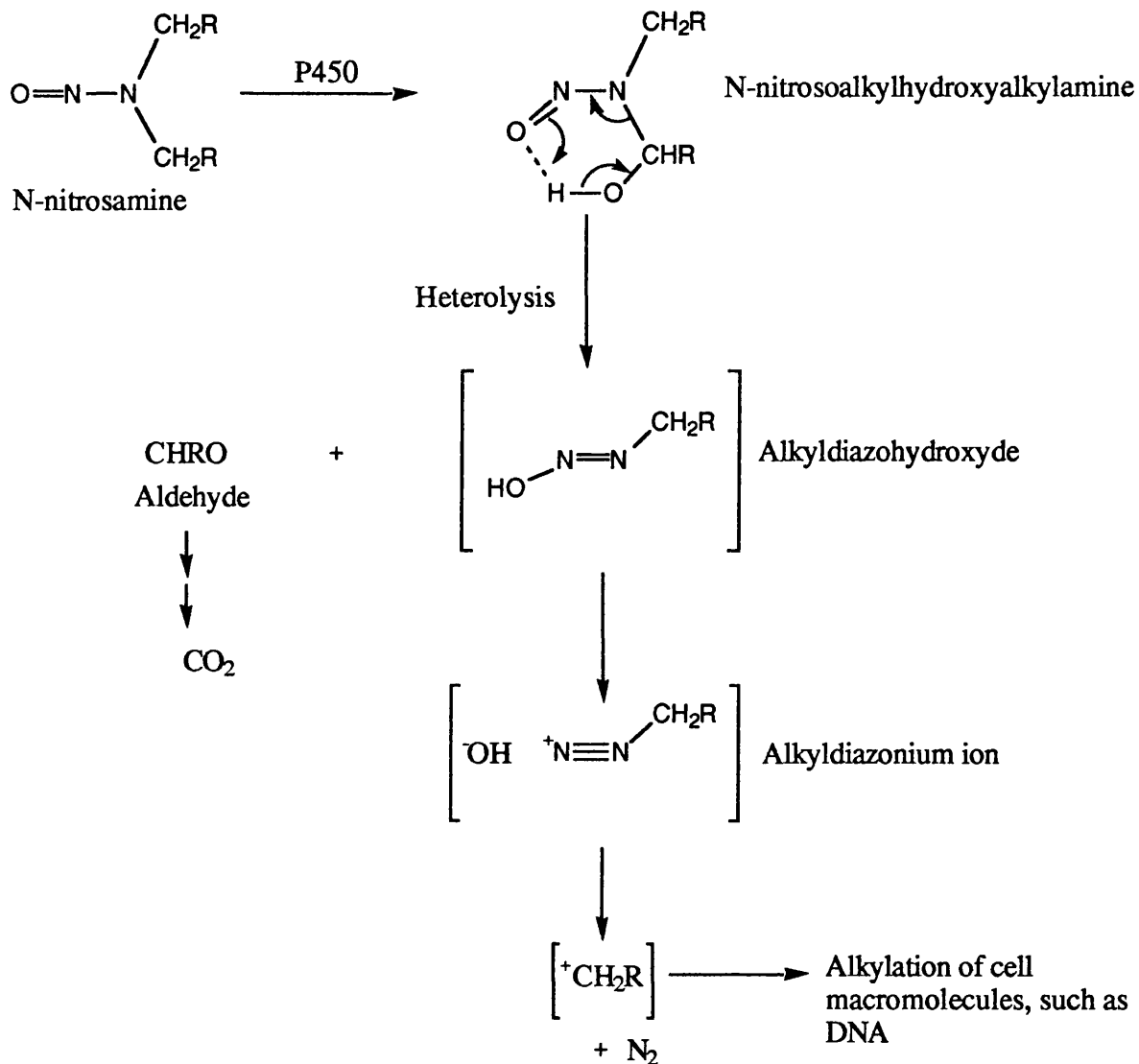
A large number of experiments on rates of metabolism of different

methylalkylnitrosamines and the effects of inhibitors on these rates were carried out by

Heath (Heath, 1962) in order to try to elucidate the nature of the toxic metabolite. The

importance of α-oxidation, leading to an alkylating agent, was shown by comparing the action of the isomeric nitrosamines N-nitrosomethylbutylamine and N-nitrosomethyl(tert)butylamine. N-nitrosomethylbutylamine caused centrilobular necrosis of the liver in rats given doses at around 100 mg/kg b.w., whereas rats which received the tert-butyl compound, which cannot undergo α-hydroxylation, did not show centrilobular necrosis of the liver (even with near lethal doses around 1500 mg/kg b.w.). The lack of metabolism of tert-butyl was confirmed by using N-nitrosomethyl(tert)butylamine with ^{14}C labelled in the tert-butyl group, only 1.3% of the radioactivity could be recovered as CO_2, indicating that this group is only poorly oxidized *in vivo* . An interesting observation by Heath, which is important to this thesis was that although NDEA was a competitive inhibitor of NDMA metabolism, NDMA only partially inhibited NDEA and N-nitrosomethylbutylamine metabolism, thus suggesting that at least two different enzymes are involved in the metabolism of NDEA and N-nitrosomethylbutylamine. Inhibitors of nitrosamine metabolism had no effect on the liver toxicity by NDMA. This is because NDMA is not significantly excreted unchanged and thus eventually has to be metabolised. The inhibitors decrease the rate of that metabolism but do not decrease the total nitrosamine eventually metabolised.

Magee and Vandekar (Magee, 1957) showed that liver slices could metabolize NDMA *in vitro* and that the metabolic activity was localized in the microsomal fraction. Microsomal metabolism required NAD and/or NADP and O_2, and was inactivated by boiling the slices, suggesting oxidative metabolism. They also detected some formaldehyde as a byproduct of NDMA metabolism. This suggested metabolism by cytochrome P450 but the metabolism was very slow and was not inhibited by SKF 525A, the most commonly used P450 inhibitor at that time. For this reason, the role of P450 was often disputed (see for instance (Lake, 1976). However Czygan *et al* (Czygan, 1973) showed that formaldehyde was produced from the hepatic oxidative microsomal metabolism of NDMA and the activation of NDMA to a bacterial mutagen

followed similar kinetics and were dependent on cytochrome P450 catalyzed metabolism and Jensen *et al* (Jensen, 1981) found, in an assay with liver microsomes, that DNA methylation by NDMA correlates well with the production of formaldehyde and that they were both dependent on P450-catalyzed NDMA metabolism. Finally the role of P450 was confirmed by Yang and his collaborators (reviewed by Yang, 1991b) who showed that most P450s can metabolize NDMA only at high substrate concentrations, but that the low Km form of NDMA demethylase was a P450 which was induced by ethanol, but not by phenobarbital or 3-methylcholanthrene and was not inhibited by SKF 525A (P450 2E1).

The metabolic activation of NDMA also takes place in human liver (Montesano, 1970) which is not surprising since human liver contains P450 2E1 with an aminoacid sequence which is 78% similar to the rat P450 2E1 (Song, 1986; Umeno, 1988).

When [14C]-NDMA was given to rats, methylation of proteins and nucleic acids of liver and to a smaller extent kidney was observed (Magee, 1962). The methylation product with nucleic acids was shown to be 14C labelled N7-methylguanine and the alkylation of nucleic acids, but mainly of DNA by nitrosamines was suggested to be the determining factor in nitrosamine carcinogenesis (Magee, 1962).

These facts lead to the widely accepted view that the metabolism of dialkylnitrosamines is carried out as stated in figure 1.3. P450-dependent hydroxylation at the methyl group adjacent to the N-nitroso group is the critical and rate limiting step in the biotransformation of nitrosamines. The alkyl(hydroxy)alkylnitrosamine formed is unstable and easily hydrolysed non-enzymatically to form formaldehyde and monomethylnitrosamine. Monomethylnitrosamine then undergoes a rearrangement to produce alkyldiazohydroxide. The alkyldiazohydroxide finally produces the potent electrophile the alkyl diazonium ion, which reacts either with water, resulting in an alcohol or with nucleophilic sites at biomolecules such as DNA. The main site for DNA

alkylation by nitrosamines is at the N7 position of guanine. This alkylated base probably does not play a significant role in the biological action of nitrosamines, but it is lost very slowly from DNA and measurement of the amount of N7-alkylguanine gives a good index of the time integrated metabolism of the nitrosamine in a particular organ (Swann, 1968a; Swann, 1971a).

Support for the formation of methyl(hydroxy)methylnitrosamine from NDMA comes from observation of DNA methylation and carcinogenicity by N-nitrosomethyl(acetoxymethyl)amine (Kleihues, 1979). Esterases which are ubiquitously distributed in tissues are responsible for the activation of α-acetoxynitrosamines *in vivo* so that it metabolises N-nitrosomethyl(acetoxymethyl)amine, resulting in N-nitrosomethyl(hydroxy)methylamine, which then decomposes to the methylating agent, thus resulting in the same metabolic product of NDMA, as shown in figure 1.4. Esterases are much more widely distributed than P450s, resulting in more organs being target for the carcinogenic effect of N-nitrosomethyl(acetoxymethyl)amine than with NDMA. Thus, unlike NDMA, the distribution of the N-nitrosomethyl(acetoxymethyl)amine is very important and for this reason tumour induction is dependent on the route of administration (Kleihues, 1979). The isolation and characterization of free α-hydroxynitrosamines is the best evidence for the activation mechanism (Mochizuki, 1980). In the presence of water, N-nitroso-N-hydroxymethyl(n-butyl)amine yielded formaldehyde and isomeric butanols, and, in the presence of the nucleophile thiophenol, butylated or methylated thiophenol was formed. Mochizuki also showed that the methyl(hydroxy)methylnitrosamine had a half-life of about 10 seconds at pH7. A small molecule will diffuse about 300 microns in this time thus although the active metabolite will not travel from organ to organ it can easily travel from the endoplasmic reticulum to the nucleus and between adjacent cells (Swann, 1982).

Figure 1.4: Activation of N-nitrosomethyl(acetoxy)methylamine and N-nitrosodimethylamine

N-nitrosomethyl(acetoxy)methylamine
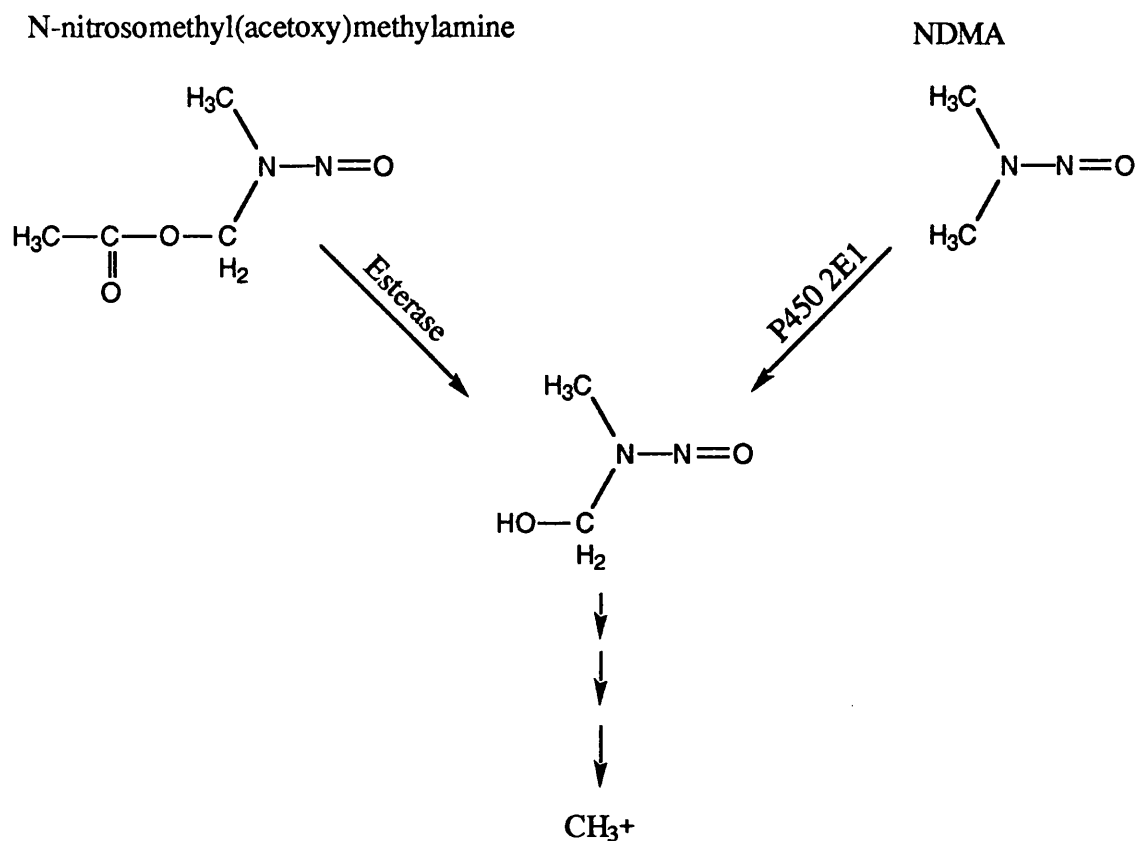
NDMA

Apart from the activation pathway which leads to the formation of an electrophile, nitrosamines can also be deactivated by metabolism. The best studied of these pathways, and the only one known for NDMA, is denitrosation. The nitrosamine is thought to go through an initial oxidative pathway that is closely related to α-hydroxylation, catalyzed probably by the same P450 enzyme responsible for metabolic activation (Lorr, 1982; Yoo, 1990), which leads to the formation of an intermediary imine, which hydrolyses to give methylamine and formaldehyde (Keefer, 1987). It is believed that the proportion of NDMA metabolised through the denitrosation pathway 10% that going through the activation route (Lorr, 1982; Keefer, 1987).

The overall picture for the metabolism of cyclic and long alkyl chain nitrosamines extremely complicated. They can be oxidised in a number of different positions (recently reviewed by(Lijinsky, 1992). Nevertheless, activation of nitrosamines by α-hydroxylation is essential for their carcinogenic effects.

The relative ability of different organs to metabolize a given nitrosamine is not always constant and can be influenced by a number of factors, including diet and chemicals which either induce or inhibit the P450(s) responsible for the metabolism of the nitrosamine.

Several nitrosamines, NMBzA, N-nitrosomethylamylamine, N-nitrosopiperidine and N-nitrosoethyl(hydroxy)ethylamine were shown to be cytotoxic for cultured rat oesophageal cells. The cytotoxicity decreased sharply as the time the cells had been in culture increased, probably due to the decline of the P450 content of the cells (Zucker, 1991). There is some inconclusive evidence that human oesophagus will metabolically activate NMBzA, NDEA and NDMA (Autrup, 1981). The report that NDMA is metabolized by the human oesophagus is surprising since NDMA is neither metabolised by rat oesophagus (van Hofe, 1987) nor it is an oesophageal carcinogen in this species (Druckrey, 1967).

F - THE MECHANISM OF THE CARCINOGENIC EFFECT OF NITROSAMINES: ALKYLATION, REPAIR AND ONCOGENES

Since the initial proposal by Magee and Farber (Magee, 1962) that alkylation of DNA by nitrosamines is responsible for their tumorigenic effect, much knowledge has been gained about the mechanisms by which alkylation of DNA can induce cancer.

Nitrosamines react with DNA most extensively at the N7 position of guanine and thus,

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this adduct was the first one to be systematically studied. However, studies trying to correlate production of N7-methylguanine by nitrosamines and methylating agents which did not contain the nitroso group with their respective carcinogenicity failed to show a causative role for N7-alkylation of guanine (Swann, 1968a) and this lack of correlation was confirmed by similar studies on the production of N7-ethylguanine (Swann, 1971a). However, Loveless (Loveless, 1969) then made the breakthrough proposal that alkylation at the 6-O position of guanine by nitrosamines, and subsequent misincorporation of thymine opposite this modified base during DNA replication was the important step in the production of tumours. Experiments *in vivo* showed a strong relationship between production of O^6-alkylguanine by different carcinogens with their potential of inducing tumours (Lawley, 1970; Pegg, 1983; Pegg, 1992).

O^6-alkylguanine is not the only promutagenic base formed by the action of nitrosamines. O^4-alkylthymine is also promutagenic because it causes T to C transition mutations. Although the formation of O^4-methylthymine by methylating agents is 1/100 of O^6-methylguanine, ethylating agents have a higher tendency of reacting with the oxygens of pyrimidines then do methylating agents and thus there is a relatively higher proportion of O^4-ethylguanine (Swenberg, 1991). Furthermore, O^4-ethylguanine accumulates to much higher levels than O^6-ethylguanine following multiple doses of NDEA, probably as a result of the poor repair of O^4-ethylguanine(Swenberg, 1991).

Apart from the positions already mentioned, nitrosamines can react with DNA at a number of different sites, including other ring nitrogen and exocyclic oxygen atoms of the bases and the oxygen atoms of the phosphate backbone (reviewed by Saffhill, 1985). The possible role of these other adducts in carcinogenesis and mutagenesis has been reviewed by Saffhill (Saffhill, 1985). These DNA damages can be repaired by a number of different mechanisms, depending on the type of adduct and the position in the DNA damaged (Pegg, 1983; Saffhill, 1985). Most of the O^6-alkylguanine is repaired *in vivo* by the action of the mammalian O^6-alkylguanine-DNA-alkyltransferase

(Pegg, 1992). The alkyltransferase repairs O^6-alkylguanine by transferring the 6-O-alkyl group to a cysteine acceptor residue in its active site, restoring the DNA back to its normal state. The cysteine acceptor site cannot be regenerated and thus the alkyltransferase becomes inactivated. The content of this enzyme in different organs varies and in the oesophagus its activity is much lower than in the liver (Craddock, 1986), a factor which may contribute to the susceptibility of the oesophagus to the carcinogenic effect of nitrosamines. The rate of repair of the O^6-alkylguanine decreases as the size of the alkyl group increases and for very large, bulky adducts the repair probably happens through other pathways. a notably exception for this is O^6-benzylguanine. This adduct has a much greater affinity for the mammalian alkyltransferase than does O^6-methylguanine either *in vitro* or *in vivo* (Dolan, 1991a; Dolan, 1991b).

The alkylation of the 6-O position of guanine in DNA by nitrosamine metabolites and the production by alkylation of other promutagenic bases such as O^4-alkylthymine is probably important because they can cause genetic changes which leads to the activation and/or suppression of genes involved in cell growth and differentiation. Perhaps the best studied proto-oncogene has been the ras-gene. The oncogenic form of the ras gene is usually activated as a result of point mutations in codon 12, 13 or 61 (Balmain, 1988). N-methyl-N-nitrosourea was shown to induce mammary tumours in the rat containing almost 90% of activated H-ras gene with G to A point mutations in codon 12 (Zarbl, 1985). These point mutations were probably caused by methylation of guanine at the O^6 position, leading to G to A transition.

Nitrosamines have been shown to induce tumours with different incidences of ras-activation. There is a 40% incidence of ras activation in NNK-induced liver tumours and a 13% incidence in NDMA-induced liver tumours (Belinsky, 1989). NMBzA have been shown to induce oesophageal papillomas in rats through the methylation of O^6 position of guanine in the oesophageal DNA with an incidence of 67% of mutation (G

to A transition) at the codon 12 of the ras gene (Barch, 1991). Alkylation by bulkier groups may result in other types of point mutations in the ras gene. Thus, pyridyloxobutylation can cause G to A transition as well as G to T transversion in the codon 12 of the ras gene (Ronai, 1993). O^6-benzylguanine has been shown to produce G to C and G to T transversions in addition to G to A transition in the codon 12 of the ras gene (Mitra, 1989).

Activated ras genes are seen in several types of human tumours (Bos, 1989), but no activated ras gene has been found in human oesophageal squamous cell carcinoma (Hollstein, 1988) or Barret's oesophagus adenocarcinoma (Meltzer, 1990). The most common genetic change known to occur in tumours in men is mutation of the tumour-suppressor gene p53 (Hollstein, 1991). This gene codes for a 53 KD protein involved in the control of cell proliferation and mutation in usually found in exons 5 though 8. The mutational spectrum differs among tumours of different organs (Hollstein, 1991). Oesophageal squamous cell carcinoma and Barret's adenocarcinoma were shown to carry p53 mutations in a number of different studies (Hollstein, 1990; Casson, 1991; Huang, 1993b). In one study carried out with patients from France, where ethanol and cigarette smoking are the main contributors to oesophageal cancer, oesophageal tumours were shown to carry mutations at the p53 gene which occurred at G-C base pair and which were either G to A transitions or G to T transversion (Hollstein, 1990). G to A transition is the predicted mutation following DNA alkylation at guanine and mispairing of O^6-methylguanine with thymine. Oesophageal tumour DNA from patients from the same area were shown to contain measurable levels of O^6-methylguanine as discussed previously. Furthermore, oesophageal tumours induced by N-nitroso compounds in rats contain G to A transition mutations at the p53 gene (Ohgaki, 1992).

G - FACTORS AFFECTING CARCINOGENICITY AND ORGANOSPECIFICITY

1. DNA Repair

The persistence of O^6-alkylguanine during cell replication leads to misincorporation of T, instead of C, opposite to the alkylated base and then leads to mutations which can activate protooncogenes (such as Ras) or inactivate tumour suppressor genes (such as p53). Thus the capacity of cells from a specific organ to repair this adduct is of vital importance in determining its susceptibility to tumours caused by the nitrosamine which originated the alkylating agent. The O^6-alkylguanine-DNA alkyltransferase content in the rat and other rodents has been shown to be highest in the liver and lowest in the brain, with kidney having a higher content than most of other tissues, but less than liver (Reviewed by Pegg, 83/92). Administration of N-nitroso-N-methylurea and N-nitroso-N-ethylurea to young rats always produce tumours in the brain, but never in the liver, despite the fact that alkylation in all organs occur to about the same extent. It has been shown (Goth, 1974; Margison, 1975) that the reason for this apparent discrepancy is that O^6-alkylguanine is repaired much more slowly from the brain of young rats than from the liver and other organs.

2. Metabolism

The importance of metabolism for the organotropism presented by nitrosamines can be highlighted from the comparison between nitrosamines and nitrosamides. Since the beginning of the studies involving N-nitroso compounds as carcinogens, it was clear that there were 2 kinds of compounds: nitrosamines, which presented a striking organotropism, usually independent of the route of administration; and nitrosamides, which have a much broader site action, which was highly dependent on the route of administration. Both compounds undergo similar pathways and generate the same alkylating agent, however nitrosamines are stable at physiological pH, and need to be metabolically activated (through P450-mediated α-hydroxylation) in order to generate an electrophile alkylating species, so that only the organs expressing the P450(s)

responsible for the metabolism of the nitrosamine will be susceptible to tumorigenesis. By contrast, nitrosamides are unstable at physiological pH and readily decomposes, producing the respective alkylating agent in every organ it can reach. So, NDMA can produce tumours only in liver, kidney, lung and nasal cavity of rats because these are the organs which contain the P450 which metabolically activates it, whereas N-nitroso-N-methylurea produces tumours in forestomach, brain, peripheral nervous system, kidney, intestine, jaw, skin, mammary tissue and lymphocytes because it requires no metabolic activation (reviewed by IARC, 1978; Lijinsky, 1992).

Different P450s can metabolise a particular nitrosamine with different affinities, and since the different organs express a spectrum of P450s which is quite unique, this has a marked effect on the organotropism of nitrosamines, which sometimes is influenced even by the amount of the nitrosamine present in the animal. So, NDEA administered to rats at relatively high doses (2 mmols/animal over 18 weeks) produces mainly liver and also kidney tumours, but when NDEA is given to rats at low doses (0.2 mmols/animal over 45 weeks), it induces predominantly oesophageal tumours (Druckrey, 1967; Lijinsky, 1992). It has been shown that the ethylation of the oesophagus by NDEA increases relative to that of the liver and kidney as the size of the dose of NDEA decreases (Swann, 1984a). When tissue slices were incubated with NDEA and the rate of production of CO_2 measured, it was shown that the Km for the oesophageal NDEA metabolism system (8 μM) was less than half of that of kidney (17 μM) and liver (20 μM) (Swann, 1984a). So that as the concentration of NDEA decreases the P450 in the oesophagus is working at a rate closer to its Vmax than the P450s in other organs. For this reason the balance of metabolism of shifts towards the oesophagus as the dose is decreased and this shift is accompanied by a parallel shift in the organotropism of the nitrosamine.

The importance of the organospecific metabolism of a nitrosamine in determining its organotropism as a carcinogen is very well exemplified in the case of NMBzA, an

exclusive oesophageal carcinogen in rats (Druckrey, 1967). Fong *et al* (Fong, 1979) compared the methylation of oesophagus and liver DNA *in vitro* by NDMA (a liver carcinogen) and NMBzA (an oesophageal carcinogen) and showed that the extent of formation of N7-methylguanine was higher when the nitrosamine was incubated with the susceptible organ. Following a single intravenous dose (2.5 mg/kg b.w.) to rats, NMBzA was distributed throughout the body and was metabolically cleared from serum following first-order kinetics with a half-life of 35 minutes (Hodgson, 1980). 49% of the total radioactivity was exhaled as $^{14}CO_2$ within 10 hours, and 5-10% was excreted in the urine or faeces. Ten minutes after the injection, 50% of the radioactivity present in oesophagus was metabolites, whereas in other tissues the proportion of metabolites varied between 10-20% (Hodgson, 1980). The highest extent of DNA methylation after 4 hours was found in the oesophagus, followed by liver, lung and forestomach. Whereas the level of N7-methylguanine in the oesophagus was 3 times that in the liver, the ratio for O6-methylguanine levels between these two organs was 10 (Hodgson, 1980). No doubt this reflects the higher alkyltransferase activity seen in the liver relative to the oesophagus (Craddock, 1986). Metabolic hydroxylation of both the methyl group and the methylene of the benzyl group have been observed (Labuc, 1982). *In vitro* studies with oesophageal and hepatic microsomes showed that oesophageal microsomes could metabolise NMBzA at the methylene only 5 times slower than liver microsomes (measured as nmol aldehyde/min/mg microsomal protein) but the oxidation of the methyl group was 60 times slower (Labuc, 1982). The metabolism of this nitrosamine at the methyl or at the methylene of the benzyl will produce different electrophilic species with different biological properties. Labuc's experiments emphasizes that one needs to know the rate at which different electrophilic species are generated as well as, or more than, the overall turnover rate for the metabolism of the nitrosamine. Following intraperitoneal administration of N-nitrosomethyl(^{14}C-methylene)amine, there was formation of benzylating species in various organs of rats as determined by autoradiography and densitometry (Kraft, 1980), although benzylation of DNA has never been detected in rats given NMBzA.

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3. The influence of distribution on carcinogenicity, in particular First Pass Clearance

One of the first observations on the carcinogenic N-nitroso compounds was that low levels of NDMA in the diet produce liver tumours, but a single (or a few) large dose produces only kidney tumours (Magee, 1959; Druckrey, 1967). The reason why the organotropism of NDMA was dependent on the schedule and route of administration was to remain subject to speculation until first pass clearance of NDMA was discovered by Diaz Gomes and colleagues while studying the dose-response for the alkylation of liver and kidney DNA by NDMA (Diaz Gomes, 1977). Following intravenous administration of NDMA to rats, the ratio between N7-methylguanine produced in the DNA of kidney and liver is 0.1, independently of the size of the dose given. This ratio is a reflection of the higher capacity of the liver in metabolising NDMA. When oral doses of this nitrosamine are given to rats in a range between 1 mg/kg and 10 mg/kg b.w., the ratio between the formation of N7-methylguanine in the kidney DNA to that of liver DNA is again 0.1. But when the size of the oral dose of NDMA given to rats is decreased below 1 mg/kg, the relative formation of N7-methylguanine in kidney DNA falls abruptly and when the oral dose of NDMA given to rats is 30 μg/kg b.w. the ratio between N7-methylguanine formed in the DNA of kidney and liver is only 0.01. Doses below 30 μg/kg b.w. NDMA given orally to rats produce no detectable methylation of kidney DNA ([Diaz Gomes, 1977; Pegg, 1981; Swann, 1984b).

The disproportional fall in the methylation of kidney DNA as the size of the oral dose decreases can be explained by a proportional increase in the amount of NDMA which is metabolized when it first passes through the liver as blood draining the gastro-intestinal tract enters the liver via the portal vein before reaching the general circulation. This phenomenon is called first-pass clearance and it has been suggested that it developed in order to prevent exposure of extrahepatic organs to this nitrosamine (Swann, 1982). This would serve to protect the animal, since liver is relatively more resistant to the carcinogenic insult produced by NDMA than the extrahepatic organs.

First-pass clearance of NDMA in the rat *in vivo* has been confirmed by experiments measuring blood clearance rates of small oral doses of this nitrosamine and a maximum NDMA clearance was calculated to be 90% (Mico, 1985). This phenomenon has also been confirmed in *in vitro* studies using perfused rat liver (Tomera, 1984; Graves, 1993). First-pass clearance of NDMA has also been shown to occur extensively in the rabbit (Swann, 1982), pig (Harrington, 1987) and mouse (Anderson, 1992a)), and there is some indication that first-pass clearance of NDMA also occurs in man (Mico, 1985). It is obviously impossible to measure directly the extent of first pass clearance in man, but it is possible to measure the Km and Vmax of the metabolic system using human liver *in vitro*. Clearance of xenobiotics by the liver has been described in analogous terms, that is Km (clearance) and Vmax (clearance) (Rowland, 1984). If these parameters for clearance could be predicted from the enzymic parameters Km and Vmax, it would be possible to predict clearance for any nitrosamine in man. This possibility has been studied by Graves *et al* (Graves, 1993) who measured Km and Vmax for the metabolism of NDMA and NDEA by rat liver, and also measured Km (clearance) and Vmax (clearance) for these two nitrosamines by the perfused rat liver. These two nitrosamines were chosen because there is almost total clearance of NDMA, but only limited clearance of NDEA (Swann, 1984b). They found that there was no discernible relationship between the parameters for clearance indicating that it will be impossible to predict clearance in man from the kinetic parameters for metabolism.

In contrast to NDMA, NDEA and NMBzA do not seem to be subjected to extensive first-pass clearance because when the route or size of the oral dose changes, the relative alkylation of the internal organs remains the same. The reason for this difference between such similar nitrosamines as NDMA and NDEA is not yet known. Graves and Swann (Graves, 1993) studied the clearance of NDMA and NDEA by the perfused rat liver and showed that the differences in clearance seen *in vivo* could be seen in the perfusion experiment. The Vmax for the clearance for these two nitrosamines was similar (11.2 nmol/g liver/min for NDMA against 8.9 nmol/g liver/min) but the Km (the

value of the logarithmic mean concentration at which there is half-maximal clearance) for NDEA clearance was 5-fold higher than that for NDMA (10.6 μM against 2.3 μM respectively). The reason for this is not clear because the Km and Vmax for the metabolism of both nitrosamines *in vitro* are in the same order of magnitude (Km NDMA = 20 μM, NDEA =37.8 μM; Vmax NDMA =1.5 nmol formaldehyde/min/mg protei,, NDEA =0.683 nmol acetaldehyde/min/mg) (Yoo, 1990).

4. Diet and other factors which influence the distribution and the relative organ to organ metabolism and organotropism of nitrosamines

In the sections above the influence of the relative organ to organ distribution of the enzymes which metabolically activate nitrosamines on the organ specificity of their carcinogenic activity was briefly discussed. The relative activity of these metabolizing enzymes can be dramatically altered, with a concomitant change in the organotropism of the carcinogen. Such change was first observed in diet (McLean, 1970). The outstanding example is the effect of a protein deficient/high carbohydrate diet on the carcinogenesis of NDMA (McLean, 1970). Rats kept on a protein-free/high carbohydrate diet were shown to have 100% incidence of kidney tumour after a single dose of 60 mg/kg b.w. was given against 35% kidney incidence tumours for rats receiving the same dose of NDMA, but kept on a normal diet (McLean, 1970)(Swann, 1968b). Metabolic studies showed that this had occurred because of the lower capacity of the liver of rats kept in the protein-free/high carbohydrate diet to metabolise NDMA. Thus rats kept on a protein-free/high carbohydrate diet for seven days metabolized NDMA at only 55% the rate of rats kept on a normal diet (Swann, 1971b). Experiments with tissue slices showed that liver slices from rats fed on the protein-free diet/high carbohydrate diet metabolized NDMA to CO_2 at less than half of the respective rate seen with liver slices of rats kept on the normal diet, but there was little difference in the rate of metabolism of NDMA by kidney slices prepared from rats kept in the different diets (Swann, 1971b). Consequently, there was an almost 3-fold increase in the methylation of kidney DNA of rats kept on the protein-free/high carbohydrate diet

due to the decrease in the capacity of the liver of these rats to metabolize NDMA (Swann, 1971b).

A second factor which is known to change nitrosamine metabolism is zinc deficiency. Zinc deficiency has been epidemiologically associated with a high incidence of oesophageal cancer (reviewed by Craddock, 1993) and has been shown to increase the incidence of NMBzA induced oesophageal tumour in rats (Fong, 1978; Fong, 1984). The oesophageal microsomal monooxygenase system has been shown to be stimulated in rats kept on a zinc-deficient diet (Barch, 1984), metabolizing NMBzA at higher rates and probably contributing to the carcinogenesis experiments related above

A third factor which has an effect on nitrosamine metabolism and distribution is ethanol. Ethanol, which has also been epidemiologically associated with oesophageal cancer in the west (reviewed by Craddock, 1993) has been shown to change the organotropism of a number of nitrosamines. Thus, oral administration of NDMA to mice in water produces only liver tumours, but when this nitrosamine is given in 40% ethanol there is a 36% increase in incidence of nasal cavity tumour (Griciute, 1981; Griciute, 1982). Coadministration of NDMA with ethanol (even at 1%) enhanced the incidence of lung tumours in mice by 4-fold (Anderson, 1992a). It was first shown by Swann *et al* (Swann, 1982; Swann, 1984b) that this effect of ethanol on NDMA carcinogenesis is the result of very strong inhibition by ethanol of NDMA metabolism and as a result of first pass clearance of NDMA, resulting in exposure of extrahepatic organs to this carcinogen. Normally the ratio of methylation of kidney to liver DNA falls dramatically as the size of the oral dose is reduced below 30 μg/kg b.w., because of the substantial first pass clearance of low oral doses of NDMA (Diaz Gomes, 1977; Pegg, 1981; Swann, 1984b), but the ratio of the methylation of the kidney to liver DNA produced by NDMA given in 1 ml 5% ethanol was the same for high and low doses of this carcinogen as a result of the inhibition of first pass clearance by the ethanol. As a result, coadministration of 1 ml 5% ethanol with NDMA (33 μg/kg b.w.) produced a

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6-fold increase in the methylation of kidney DNA, and a slight decrease in the methylation of liver DNA (Swann, 1982). Experiments carried out with liver slices (Swann, 1984b) and with the rat perfused liver (Tomera, 1984) showed that the inhibition of the first-pass clearance of NDMA by ethanol was the result of the competitive inhibition of the metabolism of NDMA by this alcohol. The inhibition was competitive (Ki = 0.5mM by liver slices (Swann, 1984b) or 0.31 mM by microsomes (Peng, 1982) because ethanol is a substrate for P450 2E1 (Peng, 1982), the P450 responsible for NDMA demethylase *in vivo* in all species tested so far, including rodents and humans (Yang, 1991b).

Inhibition by ethanol of first-pass clearance of NDMA has also been confirmed in mice using measurements of rates of disappearance of NDMA from the blood and organs of these animals (Anderson, 1992b; Anderson, 1994). Anderson *et al* (Anderson, 1992a) have also carried out experiments on the influence of different routes and schedules of administration of ethanol on NDMA carcinogenesis and concluded that inhibition by ethanol of first-pass clearance of NDMA in mice is responsible for the increase in extrahepatic tumour caused when ethanol is given with this nitrosamine. Anderson *et al* applied similar pharmacokinetic experiments to study the influence of ethanol on the metabolism of NDMA in patas monkey (Anderson, 1992b). Blood ethanol levels similar to that achieved with mice caused a similar 36-fold increase in the area under curve (AUC), and thus a similar increase in exposure of extrahepatic organs to the NDMA (Anderson, 1992b). A dose of isopropanol which produces a 5-fold induction of hepatic NDMA demethylase when measured *in vitro* in this species, resulted in a decrease of NDMA clearance instead of an increased clearance of this nitrosamine (Anderson, 1992b), probably due to the inhibitory effect of the residual isopropanol and the acetone which is a product of isopropanol metabolism and an inhibitor of P450 2E1.

The experiments reported above were done with NDMA but similar, though less

exclusive, experiments have been carried out with ethanol and other nitrosamines. Gibel showed that administration of NDEA in 30% ethanol to rats produces a 3-fold increase in incidence of oesophageal tumours (Gibel, 1967). Aze *et al* (Aze, 1993) also found that administration of NDEA in 10% ethanol to rats for 8 weeks significantly increased the incidence oesophageal tumours. The reason for the effect produced by ethanol on the carcinogenesis of NDEA is not inhibition of first pass clearance, since this nitrosamine is not subjected to extensive first pass clearance (Swann, 1984b; Graves, 1993). Instead, the effect of ethanol on NDEA carcinogenesis is caused by selective inhibition of NDEA metabolism in the liver. Thus, Swann *et al* (Swann, 1984b) showed that coadministration of NDEA with small doses of ethanol (1 ml 5% ethanol / rat, the equivalent of a man drinking a pint of beer) increased the ethylation of oesophageal DNA between 1.8 and 4.6 fold. Experiments with tissue slices showed that ethanol selectively inhibited the hepatic metabolism of NDEA (Ki of 1 mM) (Graves, 1993), whereas having little or no effect on the metabolism of this nitrosamine by oesophageal slices (Swann, 1984b). The authors showed that there was no need for the nitrosamine to be administered dissolved in ethanol and that a similar effect could be achieved if the nitrosamine and ethanol were given at the same time but by different routes. Furthermore, the administration of the nitrosamine with ethanol at the same time, rather than as a pretreatment, seemed to be essential to produce changes in the relative amounts of DNA ethylation in the rat organs. These experiments have been extended by Ludeke *et al* (Ludeke, 1991) have shown the effect of various modulators of nitrosamine metabolism on the *in vivo* metabolism of N-nitrosomethylbutylamine and N-nitrosoethylbutylamine. Ethanol (5% solution) showed an increase in oesophageal DNA alkylation by both nitrosamines.

Similarly, Yamada *et al* (Yamada, 1992) have shown that coadministration of ethanol (0-20% v/v) with NMBzA (2.5 mg/Kg b.w.) produced a dose-dependent increase in oesophageal DNA methylation with a maximum increase of over 4-fold compared to controls. Simultaneously, there was a tendency of decreasing levels of O6-

methylguanine in liver DNA produced by NMBzA coadministered with increasing levels of ethanol (Yamada, 1992). Yamada *et al* (Yamada, 1992) also compared the influence of coadministration of various alcoholic beverages diluted to either 4% or 20% ethanol content on oesophageal DNA methylation produced by NMBzA. Significant greater increases in DNA methylation were seen when commercially distilled Calvados and red wine (diluted to 4% ethanol), or farm made Calvados (diluted to 20% ethanol) were given with the nitrosamine, when compared to coadministration of aqueous ethanol at the same concentration.

The experiments reported above involved the concurrent administration of the nitrosamine with ethanol and reflect the ability of ethanol to inhibit metabolism of nitrosamines. By contrast, ethanol pretreatment of rats resulted in a increase of oesophageal (1.5- fold) and lung (2-fold) DNA methylation and a decrease (2.5-fold) in the liver DNA methylation by NMBzA (Kouros, 1983). However rats receiving chronic administration of ethanol together with NMBzA did not show an increase in oesophageal tumours, although when the ethanol was used as a promoter (after the NMBzA treatment), it increased the incidence of oesophageal tumours (Mufti, 1989). Teschke *et al* (Teschke, 1983) and Schwarz *et al* (Schwarz, 1984) found that pretreatment with ethanol did not have any effect on liver DNA methylation caused by NDMA nor change in organotropism for this nitrosamine. The failure of Teschke *et al* and Schwartz *et al* to observe any effect of chronic ethanol administration on the metabolism and carcinogenicity of NDMA is perhaps surprising, since P450 2E1 which is responsible for NDMA metabolism is induced by chronic ethanol administration (Yang, 1991a) and it has been shown that chronic ethanol consumption produces increased metabolism and carcinogenesis in the target organs of rats given N-nitrosopyrrolidine (Farinati, 1984) and N-nitrosonornocotine (Castonguay, 1984) and in Syrian Golden Hamsters given N-nitrosopyrrolidine (McCoy, 1979).

The experiments on diet, zinc deficiency and ethanol show the dramatic effect that

changes in nitrosamine metabolism can have on the organ specificity and carcinogenicity of nitrosamines. The effect of zinc deficiency and ethanol have been advanced as the explanation for the increase in human tumours which are epidemiologically associated with these factors. If this explanation was correct then one might expect that other differences on cancer incidence might have a similar mechanism. In this thesis two of these are considered. The first is isoamyl alcohol. This is studied because (as discussed in Chapter 2 and below) it is a common contaminant of Calvados - the alcoholic drink most strongly associated with an increase incidence of oesophageal cancer in man (Tuyns, 1970). The second is opium and its major alkaloid morphine because opium has been epidemiologically associated with oesophageal cancer in the high incidence area of Iran and Southern Russia (IARC, 1977; Ghadirian, 1985).

Isoamyl alcohol (3-methylbutan-1-ol) and 2-methylbutanol are alcohols which are normally present in spirits, originated during the process of fermentation, and have been shown to inhibit the *in vitro* metabolism of NMBzA by liver and oesophageal microsomes (Craddock, 1991). In this thesis it is shown that isoamyl alcohol inhibits NDEA metabolism in the rat *in vivo* and *in vitro*, but it had no influence on the distribution of change in alkylation of DNA which ethanol produces when given with NDEA. The results do not support the hypothesis that isoamyl alcohol plays a role in carcinogenesis through its effect on morphine metabolism because it decreased alkylation of DNA in all organs. The comparison in affinity between isoamyl alcohol and ethanol for different P450s help to provide a basis for the general inhibitory effect of isoamyl alcohol.

However this thesis shows that opium and its main alkaloid morphine produce a dramatic change in distribution of NDMA and NDEA. These compounds reduce first pass clearance of NDMA and significantly increases ethylation of oesophageal DNA by NDEA. Although the mechanisms have not been completely elucidated, it does not involve either induction or inhibition of the P450s responsible for the metabolism of

these nitrosamines, which makes morphine perhaps the first compound shown to dramatically change the distribution and metabolism of nitrosamines without affecting its metabolism at the enzyme level.

II - CYTOCHROME P450

A - GENERAL ASPECTS

As was discussed above, the metabolism of nitrosamines is of paramount importance. The carcinogenicity of nitrosamines depends absolutely upon the presence of enzymes which can metabolically activate them, and as was also discussed, the organ to organ distribution of the enzymes is a crucial factor in the organ specificity of these carcinogens. This metabolism is carried out by P450s and because they are at the heart of the work to be presented in this thesis, it is necessary to discuss then at some length.

Cytochromes P450 are a superfamily of hemeproteins involved in the metabolism of a number of chemicals such as ethanol, drugs such as antibiotics, endogenous compounds such as steroids and carcinogens such as nitrosamines. Over 200 different genes coding for these proteins have been identified in procaryotes and eukaryotes (Nelson, 1993). Because of the vast information regarding cytochromes P450, a detailed review of the field is out of the scope of this introduction. Instead, the factors regarding this enzyme with the metabolism of nitrosamines and its presence in the rat oesophagus will be presented.

The nomenclature and classification of cytochromes P450 and their genes is given in accord with similarity in their aminoacid sequence. Thus, P450s having sequence similarity $\geq$ 40% belong to the same family (indicated by an arabic number), whereas P450s having sequence similarity $\geq$ 60% belong to the same subfamily. (indicated by a capital letter). Each subfamily may have many individual numbers (indicated by an arabic number after the subfamily letter) (Nelson, 1993). Families 1-4 are mostly involved in the metabolism of xenobiotics and carcinogens, with family 2 having the greatest number of members, probably due to evolution related to animal-plant "warfare" (Nelson, 1993).

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Cytochromes P450 received their name due to the maximum absorbance at 450 nm for the CO-difference spectrum of sodium dithionite-reduced microsomes (Omura, 1964). In fact, they are not only cytochromes, but work as oxidases in the end of the monooxygenase system chain. The prosthetic group of this enzyme contains iron protoporphyrin IX (haem) bound in part by hydrophobic forces. The fifth or proximal ligand is a thiolate anion from a cysteine residue (located near the carboxy terminal and highly conserved in all members of the P450 superfamily), a factor which contribute for the unusual spectrum and catalytic activities seen with these enzymes and the sixth or distal ligand iron binds water, CO or molecular oxygen (reviewed by Paine, 1981; Black, 1987).

In mammals most P450s are embedded in the endoplasmic reticulum membrane. A minor part of P450s are present in mitochondria and are involved in the metabolism and/or biosynthesis of natural endogenous compounds such as steroids (reviewed by Paine, 1981; Black, 1987). The liver contains the greatest concentrations of the endoplasmic reticulum sited enzymes, followed by the nasal epithelium (Paine, 1981; Black, 1987), but several other organs including the oesophagus contain variable amounts of P450s.

B - REACTIONS AND MECHANISMS OF CATALYSIS

Cytochromes P450 are the main component of the monooxygenase system which is responsible for most of the phase I metabolism of xenobiotics. They require the presence of molecular oxygen, NADPH and the other components of the monooxygenase system in order to catalyze reaction. The reaction can be seen as the insertion of one atom of molecular oxygen into the substrate with consequent rearrangement and/or decomposition of the product which is usually more hydrophillic than the substrate (Gibson, 1994). However, sometimes the product formed

decomposes to a powerful electrophilic agent (as in the case of nitrosamines) which can react with DNA leading to mutation and tumour formation.

Although the mechanism by which P450s catalyze reactions has been reviewed many times (Paine, 1981; Black, 1987; Guengerich, 1990a; Guengerich, 1990b; Porter, 1991; Gibson, 1994), it is not fully understood. The probable mechanism is shown in figure 4.5. The substrate binds to the enzyme, facilitating the subsequent reduction of the ferric P450 by the NADPH P450 reductase. This flavin containing enzyme is unusual in that it contains a mole of flavin mononucleotide (FMN) and a mole of flavin adenine dinucleotide (FAD) per mole of apoprotein. The reason why the reductase has 2 flavin groups is that cytochrome P450 accepts one electron at a time, whereas NADPH + H is a 2 electron donor, and the reductase works then by sequentially transferring one electron at a time. It is thought that FAD is the electron acceptor flavin from NADPH + H and FMN is the donating electron flavin to cytochrome P450 in the electron transfer sequence. Next, molecular oxygen is bound to the ferrous P450, followed by transfer of the second electron. NADPH P450 reductase can donate this second electron, but it may also come from cytochrome b5 (NADH + H cytochrome b 5 reductase) depending on the particular P450 and substrate involved in the reaction. The subsequent steps are not well understood, but are thought to involve splitting of the oxygen-oxygen bond (which is helped by the trans thiolate ligand group) followed by hydrogen abstraction and "oxygen rebound". The product then dissociates to restore the ferricytochrome P450 as in the starting stage. Although different reactions catalyzed by different P450s may have different rate limiting steps, the chemistry in the different reactions is thought to be similar (since all the different P450s are thought to use the common intermediate - $(FeO)^{3+}$) and the key influence on the catalytic activity is the apoprotein.

The mitochondrial and cytoplasmic bacterial P450 monooxygenase system utilises NADPH and NADH respectively as the electron source. In both systems, there is an iron-sulphur protein (ferridoxin) in addition to the ferridoxin reductase which contains

only FAD as the prothetic group which have the same role as the P450 reductase containing the two flavins in the microsomal system (Paine, 1981; Black, 1987).

Figure 1.5: mechanism of catalysis of cytochrome P450

$$ROH \quad RH$$
$$Fe^{3+}$$
$$(ROH)Fe^{3+} \quad (RH)Fe^{3+}$$
$$e^-$$
$$RH(H)_2$$
$$(R\cdot)(Fe-OH)^{3+} \quad (RH)Fe^{2+}$$
$$e^-, 2H^+$$
$$2e^-, 2H^+$$
$$XOH \quad XOOH$$
$$H_2O \quad RH(Fe-O)^{3+} \quad O_2$$
$$H_2O \quad (RH)Fe^{2+}(O_2)$$
$$2H^+ \quad (RH)Fe^{3+}(O_2^-) \quad (RH)Fe^{3+}(O_2^-)$$
$$2H^+ \quad O_2^-$$
$$e^-$$
$$H_2O_2$$
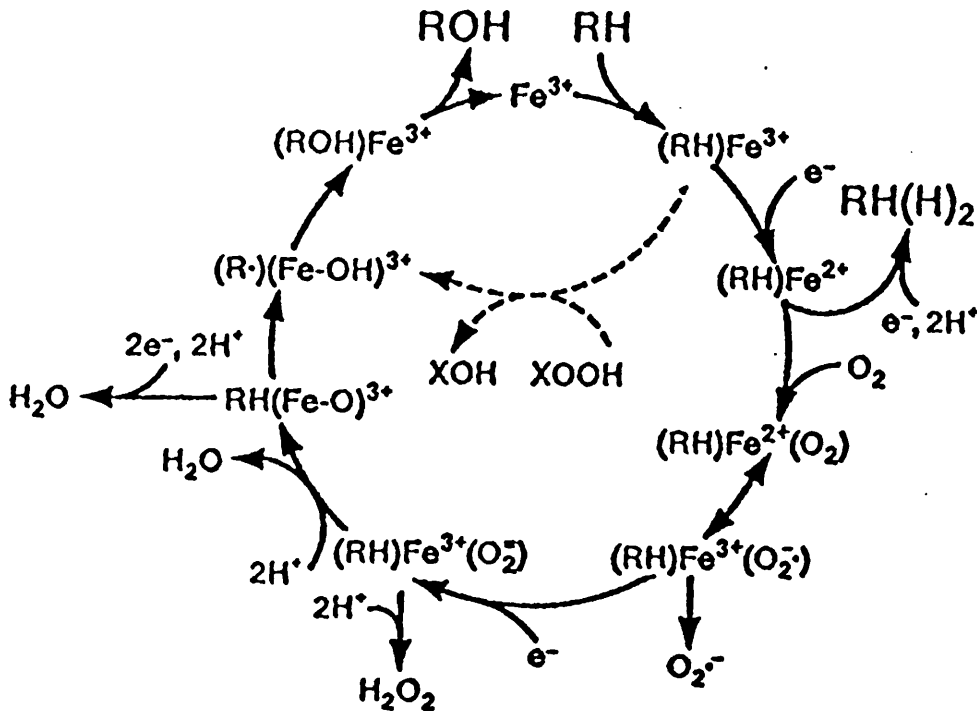
Taken from Porter and Coon (Porter, 1991)

C - EXPRESSION, INDUCTION AND INHIBITION OF P450S

The expression of each cytochrome P450 gene is dependent on a number of complex factors, including growth and sex hormones, dietary status, exposure to exogenous compounds and illness (Gibson, 1994). In rats the influence of gender in particular has been shown to dramatically affect the expression of certain constitutive P450s (for instance in rats P450 2C11 is expressed only in males and 2C12 in females) but not others such as P450 2E1. The inducible P450s such as 1A1 and 2B1 also seem to be less gender-dependent (Soucek, 1992a). In humans, no completely gender dependent P450s have yet been described and the differences between P450 activity in people

seems to be primarily a reflection of genetic polymorphism. In rats, most of P450s are maximally expressed at maturity but the rate of some P450 catalyzed reaction such as testosterone hydroxylation may show a dramatic reduction at older ages (Gibson, 1994).

Exposure of experimental animals to exogenous compound can induce a number of different P450s and the study of this was one of the initial factors which led to the idea of the multiplicity of cytochrome P450s (Lu, 1980). Most of these inducing agents are substrates for the P450(s) induced by them. The most extensively studied and understood mechanism of induction of cytochrome P450 is that by polycyclic aromatic hydrocarbons such as 3-methylcholanthrene. These compounds induce mainly P450 1A1 and to a smaller extent P450 1A2 in the rat liver. There is also a small induction of P450 2A1 in this organ. P450 1A1 can also be induced in a number of extrahepatic organs, including the oesophagus as will be seen in Chapter 4. The fact that some strains of mice did not respond to polycyclic aromatic hydrocarbon treatment, as seen by the lack on the induction of the aryl hydrocarbon hydroxylene activity in these mice led to be discovery of the Ah receptor (reviewed by Nebert, 1986) and the elucidation of the receptor - mediated induction of P450 1A1 by polycyclic aromatic hydrocarbons (recently reviewed by Whitlock, 1987; Nebert, 1989; Fujii-Kuriyama, 1992).

The gene for P450 1A1 (ie CYP 1A1) has between 3 to 5 of the so called xenobiotic response elements (XREs - also called drug regulatory elements or Ah regulatory elements) located in the DNA in the sequence on the 5'-side of the gene itself. The polycyclic aromatic hydrocarbon enters the cell and complexes to the cytosolic Ah receptor which is bound to the heat shock protein hsp 90. Upon the formation of this complex, the hsp 90 is displaced with a probable change in the configuration of the ligand-bound Ah receptor, allowing the subsequent binding of the Ah receptor nuclear translocator. This complex is then translocated into the nucleus of the cell and binds to the XREs. There also seems to be need of phosphorylation of the Ah receptor nuclear

translocator by protein kinase C in order to start transcription. This mechanism enhances the basal expression of this gene which is controlled through the binding of Sp1 (a ubiquitous transcription factor which binds to GC rich sequences) to the basal transcription element which is located just upstream of the transcription start site.

By contrast, the mechanisms of the induction of cytochromes P450 by barbiturates and related compounds has not been elucidated yet (recently reviewed by Waxman, 1992) although induction of 2B1 and 2B2 by phenobarbital is also at the transcriptional level. Phenobarbital induces mainly P450 2B1 and 2B2, but also 3A and 2C members in the rat liver. The induction of 2B1 levels in the rat liver by this compound can reach 100-fold and happens to a smaller extent in the intestine and adrenals. P450 2B1 is also expressed but not induced in lung and testis. Treatment of rats with phenobarbital produce an induction of other enzymes involved in drug metabolism such as aldehyde dehydrogenase, epoxide hydrolase, NADPH P450 reductase and glutathione S-transferase. It also promotes proliferation of smooth endoplasmic reticulum and stimulates increases in liver weight.

Another class of P450 inducers are pyrazole, acetone and ethanol. Although these compounds can induce P450s 2B1 and 2B2 to a small extent, they produce a marked increase in the levels of P450 2E1 in the rat liver although only slight increases in the total amount of P450 and NADPH P450 reductase (recently reviewed by Yang, 1991a). P450 2E1 is particularly important from the point of view of this thesis because it is the P450 responsible for the metabolic activation of NDMA. Factors such as diabetes (Dong, 1988), fasting (Tu, 1983b) and diet (Yoo, 1992) can also increase the amount of P450 2E1 in the rat liver and lung. The increase in P450 2E1 in the rat liver caused by fasting and diabetes happens through RNA stabilization (Hong, 1987a; Hong, 1987b; Dong, 1988; Song, 1989), whereas the increase produced by chemicals such as acetone and ethanol is mainly due to protein stabilization (Song, 1986; Song, 1989). There is evidence for similar mechanisms of induction of this isoenzyme in humans as

seen by the elevated levels of P450 2E1 in peripheral blood lymphocytes of diabetics who do not respond to insulin and in the liver of alcoholics (reviewed by Guengerich, 1993). P450 2E1 has been shown to be present at equimolar amounts in the liver of male and female rats (Thomas, 1987) and has been purified from the liver of rabbits (Koop, 1982), rat (Ryan, 1985) and human (Wrighton, 1986). Antibodies against P450 2E1 were shown to crossreact with human P450 2E1 (Robinson, 1989). By measuring the inhibition of NDMA demethylase with antibodies against rat P450 2E1, this enzyme was shown to be constitutively expressed in the liver of other rodents (Yang, 1985a). Apart from the liver, this enzyme is also expressed and induced in the kidney, lung, testis, ovaries, brain and nasal mucosa (Yang, 1991a). The CYP 2E1 gene from human and rats has a 78% amino acid similarity (Song, 1986; Umeno, 1988). P450 2E1 has been shown to be the low Km form of NDMA demethylase in the liver of rats and humans by studies *in vitro* using reconstituted purified P450s (Yang, 1985b), incubation of liver microsomes of animals treated with different inducers of P450 2E1 (Peng, 1982; Tu, 1983a), inhibition of this reaction with chemicals which are metabolized by P450 2E1 (Peng, 1982) and inhibition of NDMA demethylation by liver microsomes by inhibitory antibodies against P450 2E1 (Yang, 1985a). However results presented in this thesis (see Chapter 4) shows that this enzyme is neither constitutively expressed nor induced in the rat oesophagus, despite the fact that Shimizu *et al* have previously claimed to have detected its presence in ethanol treated rat oesophagus by immunohistochemistry (Shimizu, 1990).

Other classes of P450 inducers are the macrolide antibiotics (i.e. triacetyloleandomycin - induces mainly family 3), peroxisome proliferators (i.e. clofibrate - induce mainly family 4) and steroids (i.e. dexamethasone) (Gibson, 1994).

Although concurrent synthesis of haem is required for P450 synthesis, in all these cases the synthesis of the apoprotein is the main controlling factor in P450 synthesis, rather than synthesis of the haem (Gibson, 1994).

Just as a number of different compounds can induce different P450s through different mechanisms, so can they inhibit these enzymes. Because of the many steps in the P450 catalysis cycle and the number of different requirements for a monooxygenase reaction to occur, the inhibition of P450 catalyzed reactions can happen in a number of different ways (recently reviewed by Murray, 1990). The most common type of inhibition is competitive due to the many substrates which can usually compete for the active site of a P450 isoenzyme. Usually inhibitors are not specific for a particular P450 isoenzyme, but specific inhibition is very well illustrated in the case of the competitive inhibition of ethanol on the reaction catalyzed by P450 2E1, including NDMA demethylation (Peng, 1991). On the other hand, some chemicals such as certain hydrazines and alkylamine derivatives (Murray, 1990) produce a metabolite which forms a stable noncovalent complex with the P450 responsible for its metabolism, stopping further catalysis by the particular isoenzyme. Other chemicals, such as chloramphenicol and terminal olefins and acetylenic compounds and cyclopropylamides (Murray, 1990) can be converted to radical intermediates by the P450, which then alkylate either the haem or the apoprotein, inactivating the enzyme. These latter chemicals are also called suicide substrates due to the depletion inflicted by their metabolites on the P450s responsible for their metabolism.

D - NITROSAMINES, THEIR METABOLISM BY SPECIFIC P450S AND SPECIES SIMILARITIES.

Since organospecificity carcinogenic action of nitrosamines is greatly dependent on their metabolism in the affected organ by the locally expressed and/or induced P450(s), the extrapolation of experiments on the metabolism of nitrosamines in the rat, such as these in this thesis, to the human can only be done if the expression, induction and metabolic reactions of P450s in both species can be correlated.

One important difference between humans and rats is the polymorphism of P450s in humans due to "outbreeding". So, the levels of P450s expressed in the different human organs generally vary many fold among individuals, having a dramatic consequence on the final metabolism of drugs, such as debrisoquine, (a formerly used antihypertensive drug), which is metabolised by P450 2D6 in the human liver. In this case, about 10% of caucasians are "poor" metabolisers of this drug, being insensitive to the anti-hypertensive effects caused by this compound (recently reviewed by Gibson, 1994).

The simplest and best studied nitrosamine, NDMA is exclusively metabolised by P450 2E1 (Yang, 1991b). Cytochrome P450 2E1 is one of the best conserved P450s among different species, which suggests its importance in some basic physiological role such as the metabolism of ketone bodies (recently reviewed by Koop, 1992). Many of the initial failures to demonstrate the metabolism of NDMA in rat liver microsomes by P450s (reviewed by Lai, 1980; Yang, 1991b) was due to the use of the classic inducers 3-methylcholanthrene and phenobarbital which depress the activity of P450 2E1 in rat liver microsomes. The inhibitor used in these studies, SKF 525A, also failed to inhibit the metabolism of NDMA in the rat liver (Magee, 1957) because, although it is a suicide inhibitor of some P450s, it does not inhibit reactions catalyzed by P450 2E1 (Murray, 1990). By using the expression of the CYP 2E1 gene in different cell systems, reconstitution of the purified protein, immunoinhibition, and correlation studies of the effect of inhibitory chemicals and inducers of P450 2E1 on the metabolism of NDMA by rabbit, rat and human hepatic microsomes, it was definitely shown that P450 2E1 is the sole P450 responsible for the metabolism of this nitrosamine in the liver of these species (Peng, 1982; Yang, 1985a; Levin, 1986; Patten, 1986; Wrighton, 1986; Umeno, 1988; Yoo, 1988). Cytochrome b5 has been shown to enhance the metabolism of NDMA by P450 2E1 (Levin, 1986), probably contributing by donating the second electron of the monooxygenase cycle. Other P450s (as for instance 2B1 and 2B2) are able to metabolise NDMA *in vitro* only when extremely high concentrations of this nitrosamine is present (Yang, 1985b). Many low molecular weight compounds which

have been shown to be substrates in the rat are also substrates for the human 2E1, such as ethanol (Peng, 1982), diethyl ether (Brady, 1987) and NDMA (Yang, 1985b). So, not surprisingly, the inhibitory effect of ethanol on the metabolism of NDMA in the liver of rats seen *in vivo* and (Swann, 1984b) has been demonstrated *in vitro* with human liver slices (Swann, 1984b). P450 2E1 was also shown to be responsible for a significant part of the metabolism of NDEA in the rat liver, although it is clear that other P450 isoenzymes are involved in the metabolism of this nitrosamine (Yoo, 1990). Using liver microsomes prepared from untreated and acetone treated rats incubated with NDEA, it was shown that a low Km NDEA deethylase enzyme was induced several-fold by acetone treatment. However, antibodies (40 µg) against P450 2E1 caused only a 31% inhibition of the metabolism of NDEA (160 µM) by rat liver microsomes compared to a 73% inhibition of the metabolism of NDMA (Yoo, 1990). These results confirmed the study of Heath (Heath, 1962) who showed that NDEA must be metabolized by at least 2 enzymes, only one of which could metabolize NDMA, by showing that NDEA was a simple competitive inhibitor of the *in vivo* metabolism of NDMA, but that NDMA only partially inhibited the metabolism of NDEA in the rat.

There is evidence that one of the human P450s which metabolizes NDEA is P450 2A6. The CYP 2A6 gene has been cloned from human liver and characterized (Crespi, 1990; Miles, 1990; Yamano, 1990) and the expressed protein has been purified (Yun, 1991). This protein is usually present at 1% of the total P450 level in the human liver but shows a 100-fold variation among different individuals (Yun, 1991). Antibodies against the mouse liver P450 2A5 crossreact with the human P450 2A6 protein (Raunio, 1988) and both proteins are responsible for the high activity of the liver microsomes of these species in catalyzing the hydroxylation of coumarin (Raunio, 1988; Crespi, 1990; Miles, 1990; Yamano, 1990). In mice this activity can be markedly enhanced by pretreatment of mice with pyrazole and to a lower extent by phenobarbital (20 and 10-fold, respectively) (Raunio, 1988). In contrast to the mouse, rat liver has only a low but detectable activity in metabolizing coumarin, which can be induced only

2-fold by pyrazole pretreatment and 10-fold by phenobarbital pretreatment (Raunio, 1988). Antibodies against mice P450 2A5 did not inhibit the phenobarbital-induced coumarin 7-hydroxylation activity of rat liver microsomes (Raunio, 1988), so the coumarin metabolism is carried out by a P450 which is not closely related to mouse 2A5. Rat intestine and lung express P450 2A3, but this is not expressed in rat liver (Soucek, 1992b). Rat P450 2A3 has an 85% aminoacid similarity to human P450 2A6 (Gonzalez, 1992), but does not seem to have a high activity towards coumarin metabolism (Camus, 1993). Crespi *et al* (Crespi, 1990) expressed the human P450 2E1 and 2A6 genes in a human cell line and showed that, as expected P450 2E1 was the more efficient in activating NDMA and that P450 2A6 was the more efficient in activating NDEA to a mutagenic product. Camus *et al* (Camus, 1993) have incubated mice liver microsomes with coumarin and showed that NDEA was a much more effective inhibitor of this reaction than NDMA. Additionally, an antibody against P450 2A5 inhibited the mice liver microsomal metabolism of NDEA by 90%, whereas the metabolism of NDMA was only inhibited up to 40%. Furthermore, there was a particular good correlation between deethylation of NDEA and coumarin metabolism by human liver microsomes. All these experiments lead to the conclusion that in mouse liver NDEA is metabolised mainly by P450 2A5, and that humans expressing reasonable levels of P450 2A6 in their liver would metabolize NDEA predominantly by P450 2A6. Recently, Yamazaki *et al*, (Yamazaki, 1992) have confirmed these results by studying the metabolism of these nitrosamines by reconstituted purified proteins and immunoinhibition of their metabolism and have also shed light in the importance of P450 2E1 and 2A6 on the human metabolism of not only the two previously mentioned nitrosamines, but also on the metabolism of tobacco specific nitrosamines.

P450 2E1 can usually also demethylate some methyl alkylnitrosamines efficiently, though having no affinity for the longer or bulkier alkyl chains of nitrosamines (Yang, 1991b) which can be understood, at least in part, by steric hindrance due to the preference of this isoenzyme for small molecular weight substrates. So, for instance the

depentylation of N-nitrosomethylamylamine and debutylation of N-nitrosomethylbutylamine in rat liver microsomes is thought to be preferentially catalyzed by P450 2B1 and 2C11, whereas P450 2E1 shows relatively high rates in the demethylation of these nitrosamines (Ji, 1989; Mirvish, 1991a). It was shown that the rat and human oesophagus have similar activities in the demethylation of N-nitrosomethylamylamine which have higher rates than the demethylation of NDMA, whereas liver microsomes showed preferential demethylation of NDMA over demethylation of N-nitrosomethylamylamine (Huang, 1992). The authors suggested that the similar pattern of metabolism by these microsomes may be the result of the presence of homologous oesophagus specific expressed P450s in humans and rats (Huang, 1992).

The metabolism of the most potent oesophageal cancer inducing nitrosamine NMBzA in the liver seems to be quite complex, although most studies done so far agree that P450 1A1 may have a marked activity in the debenzylation of this nitrosamine, because 3-methylcholanthrene induced liver microsomes have an increased rate of NMBzA debenzylation (Kawanashi, 1983; Kawanashi, 1985) and, that antibodies against P450 1A1 could inhibit NMBzA debenzylation (Lin, 1990). P450 1A1 is one of the best conserved P450s. The human and rat protein have 78% sequence homology and are able to catalyze similar reactions with similar activities (Soucek, 1992a). In rats, P450 1A1 may be considered an extrahepatic enzyme because it is only expressed at very low levels in the liver ($\leq$ 0.5 pmol/mg protein) (Boobis, 1990; Soucek, 1992a; Funea, 1993) , although it is dramatically induced upon administration of polycyclic aromatic hydrocarbons. It is constitutively expressed and similarly induced in a number of different extrahepatic organs, including kidney, lungs, small intestine (recently reviewed (Soucek, 1992a), and throughout the rat alimentary tract (Traber, 1992), including the oesophagus as shown in this thesis. In humans also, P450 1A1 is expressed at only very low levels in the liver and in higher levels in extrahepatic tissues such as placenta, lungs and lymphocytes and should be considered essentially as an

extrahepatic enzyme. It is induced in these extrahepatic tissues and cultured cells but not

in the liver by cigarette smoking (Guengerich, 1989b; Guengerich, 1991; Gonzalez,

1992). Omeprazole, a H^+/K^+ adenosine triphosphatase inhibitor, has been shown by

the use of PCR in some individuals, but not in others, to induce P450 1A1 throughout

the alimentary tract (McDonnell, 1992).

E - P450S IN EXTRA-HEPATIC ORGANS

Although drug metabolism happens mainly in the liver, the capacity of extrahepatic

organs to metabolise drugs and carcinogens can have a marked local effect on toxicity

and carcinogenesis

The capacity of extrahepatic organs to carry out P450 dependent metabolism,as well as

the P450 isoforms present, has been well studied in the olfactory and respiratory tract

(recently reviewed by Dahl, 1993)). The olfactory tract of rodents such as hamsters,

mice and rats has the capacity to catalyze some reactions at rates superior than the liver,

such as 7-ethoxycoumarin deethylase and 7-ethoxyresorufin deethylase (Reed, 1986).

Rat and rabbit nasal epithelium, but not liver or lung S9 fractions were also able to

activate nitrosamines such as N-nitrosopyrrolidine to promutagenic products in TA100

strain of S. typhimurium (Dahl, 1985). The olfactory epithelium of cattle and humans

have also shown to activate NDMA and NDEA at higher rates than the respective livers

(Gervasi, 1990; Longo, 1991). Reed et al (Reed, 1986) studied the difference between

hamster olfactory epithelium and liver in rates of metabolism of some substrates. They

showed that 7-ethoxycoumarin and 7-ethoxyresorufin deethylation, aniline

hydroxylation and hexobarbitone oxidation were all P450 dependent in both tissues, but

the rates in the olfactory epithelium were higher than in liver. Spectroscopic

measurement of the kinetics of the binding of the substrates with P450s in microsomes

from both tissues were similar, thus suggesting a similar extent of binding. However

the NADPH P450 reductase activity, and the amount of flavoprotein, in the olfactory epithelium of hamster, mice and rats were higher than in the respective liver microsomes. Thus, whereas the reductase : P450 ratio in the liver of rodents is between 1 : 15 and 1 : 10, the ratio in the olfactory epithelium is 1 : 2. This was suggested as being one reason for the high activity of olfactory epithelium in the metabolism of substrates, since the rate-limiting factor in the metabolism of a number of substrates by P450s is the donation of the first electron by the reductase (Guengerich, 1990b). In this regard, it is important to emphasize that the hamster is particularly susceptible to nasal epithelium tumours produced by N-nitroso compounds (Lijinsky, 1992).

Immunohistochemistry has shown the presence of P450s 1A1, 2B1 and 3A2 in the different cells of the olfactory and respiratory tract of rats. In contrast to liver P450 1A1 was induced by arochlor 1254 in these tissues, but not by 3-methylcholanthrene (Baron, 1988). P450 2E1 is also expressed at low levels in the nasal mucosa and seems to be induced by acetone, however it contributes only weakly to the metabolism of NDMA and NDEA in that organ as seen with immunoinhibition and induction studies (Hong, 1991). Acetone pretreatment of rats had no effect on the metabolism of NDMA by nasal mucosa microsomes, but decreased the rate of metabolism of NDEA and NNK (Hong, 1991). Recently, two P450s have been purified from rabbit nasal epithelium (Ding, 1988). NMa was shown to be very active in the metabolism of NDEA, whereas NMb was shown to be very active in the metabolism of testosterone (Ding, 1988). NMa seems to be expressed at low levels in the rabbit liver (about 3% of the total P450 content) but is one of the main P450s in the respiratory and olfactory epithelium, whereas NMb is only expressed in the olfactory epithelium. No other tissues including brain, oesophagus, heart, intestinal mucosa, kidney and lung were shown to express either P450 (Ding, 1988). P450 NMb was shown to be homologous to the specific rat nasal epithelium expressed P450 2G1 which gene has been cloned and characterised (Nef, 1990; Zupko, 1991).

In contrast to liver, lung and nasal epithelium, no positive identification of any P450 isoenzyme at the protein level has been shown in the rat oesophagus, even though the capacity of this organ to activate some nitrosamines such as NMBzA and N-nitrosomethylamylamine is comparable or greater than the liver and has been shown to be P450-dependent (Labuc, 1982). The high levels of activation of some nitrosamines by the oesophageal P450 system has led to the suggestion that some specific oesophageal expressed P450(s) are responsible for this metabolism (Labuc, 1982). So, for instance, upon administration of NMBzA to rats, the levels of N7-methylguanine (resulting from the P450-mediated hydroxylation at the methylene with a subsequent generation of the methylating species which methylated the DNA at the N7 position of guanine) were shown to be highest in the oesophagus, followed by liver (Hodgson, 1980). NMBzA produced a higher methylation of the oesophageal DNA than of the liver DNA, in contrast to NDMA which methylated the liver DNA, but gave no measurable methylation in the oesophagus (Fong, 1979).

Van Hofe et al (van Hofe, 1987) have administrated single oral doses of 0.1 mmol/kg b.w. of a series of methylalkylnitrosamines, ranging from NDMA to N-nitrosomethyldodecylamine, to rats and quantified the levels of N7-methylguanine produced in the DNA of various organs of rats by these nitrosamines after 6 hours. The most active of these towards oesophagus was N-nitrosomethylbutylamine which produced a higher level of N7-methylguanine in oesophageal DNA (800 µmol N7-methylguanine/mol guanine) than in the liver (500 µmol N7-methylguanine/mol guanine). Levels in the oesophagus of this adduct after N-nitrosomethylamylamine (370 µmol N7-methylguanine/mol guanine) were also high, but about the same as in the liver. N-nitrosomethylpropylamine also produced detectable levels of N7-methylguanine in oesophageal DNA, but no detectable N7-methylguanine could be measured in oesophageal DNA after administration of the other asymmetric methylalkylnitrosamines. However methylation of liver DNA was detected after all the nitrosamines but the levels of N7-methylguanine fell with increasing length chain (from

3290 μmol N7-methylguanine/mol guanine with NDMA to 165 μmol N7-methylguanine/mol guanine with N-nitrosomethyldodecylamine). The selective capacity of the oesophagus to activate the asymmetric methylalkylnitrosamines containing 3 to 5 carbons in the larger alkyl chain (through α-hydroxylation of the methylene carbon) at rates comparable or higher than the oesophagus was suggested to be due to specific oesophageal expressed P450(s) (van Hofe, 1987). A similar conclusion was reached by Ji *et al* (Ji, 1991) who carried out a similar experiment as to the one mentioned above, except that the nitrosamines administered to rats were N-nitrosomethylamylamine and 6 of its isomers. In all cases (except where the α-carbon was blocked as in N-nitrosomethyl(1-methylbutyl)amine, N-nitrosomethyl(1,2-dimethylpropyl)amine and N-nitroso(1-ethylpropyl)amine) about 4-fold higher levels of N7-methylguanine and O^6-methylguanine were produced in the oesophageal DNA than in the liver DNA. Another difference between the liver and oesophageal P450 activation of nitrosamines was shown in the formation of benzaldehyde (formed from hydroxylation at the methylene carbon) and formaldehyde (formed from hydroxylation at the methyl carbon) produced by the *in vitro* incubation of NMBzA (5 mM) with oesophageal and liver microsomes (Labuc, 1982). The results need to be treated with caution because they showed that the oesophageal microsomes were less active than those from liver, which is plainly inconsistent with the data from the whole rat. However, they did show that while microsomes from both organs hydroxylated the methylene carbon more rapidly than the methyl carbon, the ratio in liver was 10 : 1 but in the oesophagus 100 : 1. Further evidence indicating that the P450(s) responsible for the metabolism of nitrosamines in oesophagus and liver are different comes from studies of the effectiveness of inhibitors. Thus, ethanol is a strong inhibitor of the metabolism of NDEA in the liver, but not in the oesophagus. This, taken with previous work showing that the oesophagus is unable to metabolize NDMA (Swann, 1984b; van Hofe, 1987), shows that the oesophagus does not contain P450 2E1 and that the metabolism of NDEA and other nitrosamines by the oesophagus must be carried out by some other

P450. In fact, P450 2E1 has not been detected in the oesophagus of either untreated or ethanol treated rats (Dewaziers, 1989; Shimizu, 1990; Huang, 1992) or humans (Dewaziers, 1989), although Shimizu *et al* (Shimizu, 1990) claim to have detected this isoenzyme in the oesophagus but only by immunohistochemistry in ethanol treated rats. P450 3A4, the predominant P450 in the liver of adult humans, has been detected at very low levels (2.5% of those levels in the liver) in the human oesophagus by immunoblotting (Dewaziers, 1989), although this isoenzyme does not seem to be involved in the metabolism of nitrosamines in humans (Guengerich, 1989b; Guengerich, 1993). Additionally, P450 1A1 mRNA has been shown, by use of the polymerase chain reaction, to be expressed and be induced in the oesophagus of rats upon administration of β-naphthoflavone, but was not detected by Northern blot (Traber, 1992). Detectable levels of 7-ethoxyresorufin metabolism could only be seen with oesophageal microsomes prepared from β-naphthoflavone treated rats (about 5 mmol/min/mg protein) (Traber, 1992). P450 1A2 mRNA was also detected at very low levels in the oesophagus of untreated and β-naphthoflavone treated rats by the use of the polymerase chain reaction. Additionally, P450 1A1 mRNA has been shown, by the use of the polymerase chain reaction in the oesophagus of 2 individuals treated with omeprazole, an inducer of this isoenzyme (McDonnell, 1992).

In Chapter 4 of this thesis, the absence of the P450 2E1 in the oesophagus of untreated or ethanol treated rats is shown, as well as the absence of P450 2B1 and 2B2 in the oesophagus of untreated or phenobarbital treated rats. However, the presence of P450 1A1 in the oesophagus of untreated rats and its inducibility (about 12-fold) upon administration of 3-methylcholanthrene to rats is demonstrated. Oesophageal microsomes can also metabolise 7-ethoxycoumarin and coumarin to umbelliferone and the former seems to be catalyzed by P450 1A1. It is also shown that P450 1A1 may be involved in the metabolism of NMBzA, but not of NDEA in the oesophagus.

CHAPTER 2

THE EFFECT OF ISOAMYL ALCOHOL ON THE METABOLISM AND DISTRIBUTION OF NDEA

INTRODUCTION

The previous work discussed in the introduction has shown that the most effective modulators of the carcinogenic activity of N-nitroso compounds are agents which change the pharmacokinetics of the nitrosamines, a protein deficient diet and ethanol being outstanding examples. Ethanol inhibits first pass clearance of simple nitrosamines, and it also selectively inhibits the P450, probably 2E1, mainly responsible for nitrosamine metabolism in the liver without affecting nitrosamine metabolism in the oesophagus. These two effects act in concert, the inhibition of first pass clearance increases the exposure of the oesophagus and all other extrahepatic organs, the selective inhibition of nitrosamine metabolism in the liver shifts metabolic activation from liver to the oesophagus. It has been suspected that these effects might explain the connection between alcohol consumption and oesophageal cancer incidence which has been observed in France (Tuyns, 1979a), the United States (Wynder, 1961), Europe and Africa (recently reviewed by Craddock, 1993).

The consumption of a locally produced alcoholic beverage and its relationship to oesophageal cancer incidence has been most intensively studied in Normandy, France, where the locally produced apple brandy, Calvados, is the most commonly consumed alcoholic drink. France has the highest incidence of the disease in the West (age-standardized mortality rate for oesophageal cancer is around 12/100000 for males, 20 times higher than for females (Craddock, 1993). The rate of incidence for the disease was shown to be much higher in Brittany and Normandy than elsewhere in the country and that the distribution of this rate throughout France correlates well with the mortality due to "alcoholism" (Tuyns, 1970). The author suggested that "alcoholism" is more

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probably caused by heavy consumption of strong spirits and liquors whereas cirrhosis is caused by heavy consumption of beer and wine (Tuyns, 1970). Due to the difficulty in finding people who consumed only one type of drink, a relationship between consumption of a particular type of alcoholic beverage and oesophageal cancer was established by comparing people consuming the same amount of ethanol but who never consumed one specific type of alcoholic drink. The hazard presented by a mixture of other alcoholic beverages could then be measured. Using this method it was found that consumption of apple brandy presented the greatest risk, followed by cider, wine and beer (Tuyns, 1979b), however the interpretation of this data has to be taken with caution because a number of studies carried out in other countries have found that the most popular drink consumed locally always appears to present an increased risk for the incidence of oesophageal cancer when compared in terms of absolute ethanol intake (Barra, 1990).

One characteristic of Calvados is that it contains an unusually high concentration of long chain and branched chain alcohols derived from amino acids such as isoleucine (Postel, 1981). Among the higher alcohols, isoamyl alcohol is present at the highest concentration in Calvados (around 300 mg/100 ml ie 0.16 mM) (Postel, 1981). Craddock and Henderson (Craddock, 1993) have suggested that the presence of higher alcohols at extremely high concentrations in Calvados is responsible for the high incidence of oesophageal cancer associated with its consumption (Tuyns, 1979b), although no viable mechanism was shown. Their work, which has been reported only in a meeting report showed that isoamyl alcohol will inhibit the metabolism of NMBzA but the significance of this work is difficult to evaluate because in their experiments they measured the effect of concentrations between 11.3 and 500 mM of isoamyl alcohol on the metabolism of 5 mM NMBzA. The concentration of the alcohol is greater than one might obtain from drinking Calvados, and the concentration of the nitrosamine probably 33 times the concentration produced by the median lethal dose (18 mg/kg b.w.) of the nitrosamine.

Since ethanol has been suggested to influence the development of human oesophageal cancer through changes in the metabolism and the distribution of nitrosamines which are ubiquitously spread in the environment (Swann, 1982), it seemed reasonable to check the effect of isoamyl alcohol on the pharmacokinetics of nitrosamines and compare it with ethanol.

MATERIALS AND METHODS

NITROSAMINES

N-nitrosodi[1-^{14}C]ethylamine was synthesized from di[1-^{14}C]ethylamine (53 mCi/mmol, Amersham) by Dr. P.F. Swann using the method of Dutton and Heath (Dutton, 1956) and N-nitrosodi[2-^{3}H]ethylamine was synthesized from sodium [^{3}H]-acetate (Amersham, 2.28 Ci/mmol) by Dr. P.F. Swann as previously described (Swann, 1984b). The purity of the radioactive nitrosamines was checked by HPLC using a ZORBAX ODS C18 column (Dupont) eluted at 1ml/min with 25% methanol : water. One minute fractions were collected and the absorbance at 230 nm measured in a spectrophotometer. 4 ml of ECOSCINT A (National Diagnostics) scintillation cocktail added to each fraction and counted for 10 minutes on a ^{14}C programme ([1-^{14}C]-NDEA) or on a ^{3}H programme ([2-^{3}H]-NDEA) in a Packard scintillation counter. 98% and 95% respectively of the radioactivity eluted as a single peak coincident with unlabelled NDEA (Sigma). The recovery of radioactivity from the column was 100% and 93% respectively.

IN VIVO EXPERIMENT

Male Sprague Dawley rats (200 g) were obtained from Charles River plc, Manston, Kent. Rats were allowed water but not food overnight. They were divided into 4 groups each of 20 rats and were given [2-^{3}H]-NDEA (3 mg/kg b.w.) orally by a tube passing deep into the oesophagus in either 1 ml water (group 1), 1 ml 10% ethanol (group 2), 1 ml 2.8% isoamyl alcohol (Sigma) (group 3), or 1 ml 10% ethanol / 2.8% isoamyl alcohol (group 4). 4 hours later the rats were killed by CO_2 and livers, kidneys and oesophagus were removed to ice-cold saline sodium citrate (SSC, 0.15 M NaCl / 0.015 M Tri-sodium citrate pH 7) and frozen in liquid nitrogen.

PREPARATION OF DNA FROM LIVER AND KIDNEYS

DNA was prepared from livers (1 g of the medium lobe of each liver pooled together) and kidneys as described originally by Kirby (Kirby, 1956) and modified by Nicoll et al (Nicoll, 1977). 10 g of liver or 20 g of kidney were homogenized in 7 ml SSC/g tissue using a Polytron homogenizer at full speed with 7 passes. The homogenate was transferred to glass centrifuge tubes and centrifuged at 2500 rpm for 20 minutes at 5 °C (Mistral 3000 centrifuge). The supernatant was discarded and the pellet was washed with 5 ml SSC/g original tissue by vortex mixing and centrifugation as before. The pellet was then transferred to a Dounce glass tissue grinder (Wheaton, USA) and suspended in 10 ml 1 M NaCl/g tissue by using a hand glass pestle. The suspension was transferred to a 500 ml stoppered conical flask, 1 ml 10% SDS (sodium dodecyl sulphate)/g tissue and 10 ml Kirby phenol reagent (500 g phenol / 77 ml m-cresol / 55 ml water / 0.5 g 8-hydroxyquinoline)/g tissue added and the mixture was shaken for 30 minutes in a mechanical shaker. Following centrifugation for 30 minutes at 12000 rpm 4 °C (Sorvall RC-5B centrifuge, SS-34 rotor) the top layer was carefully aspirated and put in a clean 500 ml stoppered conical flask and 5 ml Kirby phenol reagent/g tissue added, the mixture was left to shake for 15 minutes and centrifuged as before. The supernatant was again carefully aspirated and transferred to a 250 ml stoppered conical flask and the DNA precipitated with an equal volume of ethanol. The DNA was quickly drained, dissolved in 20 ml water and sodium acetate (5 % w/v) and ribonuclease A (Sigma) (1 mg/10 ml solution from a 10 mg/ml stock solution) added and the mixture was incubated for 20 minutes. The DNA was precipitated again with an equal volume of ethanol and then drained quickly and redissolved in 12 ml water. An equal volume of 2.5 M Na_2HPO_4 pH 7.3 and methoxy-ethanol were added, the suspension was well mixed and centrifuged at 2500 rpm 5 °C for 30 minutes (Mistral 300 centrifuge). The supernatant was then carefully aspirated and the DNA precipitated with an excess of 1% CETAB (cetyltrimethyl-ammonium bromide). The DNA was washed twice with water and left to stand for 15 minutes in 2% sodium acetate : 70% ethanol (w/v). The DNA

was finally washed in ethanol, then ethanol : diethyl ether (1:1), and finally in diethyl ether and placed in a desiccator and dried under vacuum. Typical yield of DNA was 1.5 mg/g tissue.

PREPARATION OF DNA FROM THE OESOPHAGUS

Oesophageal DNA was prepared based on the method previously described (Swann, 1984b). Oesophagus, 5 g were homogenized in 10 ml 50 mM tris pH 8, 20 mM EDTA, 20 mM NaCl/g tissue in a Polytron homogenizer at full speed with 10 passes. SDS (10 % w/v; 50 µl/ml homogenate) was added and the mixture shaken vigorously. Protease K (0.125 mg/ml homogenate, BDH) which had been predigested at 37 °C for 15 minutes, was added and the mixture incubated at 37 °C for 45 minutes with occasional gentle swirling. Then sodium 4-aminosalicylate (40 mg/ml homogenate) and 10 ml Kirby phenol reagent (500 g phenol / 77 ml m-cresol / 55 ml water / 0.5 g 8-hydroxyquinoline)/g tissue were added and the mixture shaken in a mechanical shaker for 30 minutes.

The procedure then followed as for the extraction of DNA from liver and kidney. Typical yield of DNA extracted from the oesophagus was 2 mg DNA/g wet tissue.

ANALYSIS OF DNA FOR THE PRESENCE OF ALKYLATED BASES

DNA (7-9 mg from liver; 40-50 mg from kidney; 8-10 mg from oesophagus) was placed in a centrifuge tube, 2 ml of 0.5 N HCl (1 ml for oesophageal DNA) was added and the mixture was heated at 100 °C for 30 minutes. The hydrolysate was chromatographed on a strong cation exchange resin (Aminex A9, 11-12 µm, 4.6 x 150 mm, Bio-Rad) using ammonium formate as eluent (0.4 M, pH 4.3, 1 ml/min, 60 °C). 1

ml fractions were collected, 0.1 ml concentrated HCl added to tubes where the eluent containing guanine peak was expected to be collected in order to avoid precipitation of guanine, the absorbance at 260 nm and the volume of fractions determined. 4 ml of scintillation cocktail added to each fraction and the radioactivity counted on a ^{3}H program for 30 minutes. For kidney hydrolysate, the sample was divided, injected successively and the fractions were pooled together. The amount of guanine and adenine was calculated from the absorbance at 260 nm of the respective peaks by taking E_{260} for adenine in neutral solution to be 13000, and the value for guanine in acid to be 8000. The amount of N7-ethylguanine could be calculated from the radioactivity because the specific radioactivity of the nitrosamine administered to the animal was known.

PREPARATION OF LIVER MICROSOMES

Rat liver microsomes were prepared by the method of Labuc *et al* (Labuc, 1982) with some modifications. Male Sprague Dawley rats (200 g) were starved overnight and killed the next morning by cervical dislocation. The liver was perfused with 20 ml 1.15% ice-cold KCl and removed to ice-cold homogenising buffer (50 mM tris / 1.15% KCl pH 7.4)

All subsequent steps were performed at 4 °C. The liver was chopped and homogenized in 4 ml of homogenizing buffer/g tissue with 5 passes of a motor-driven teflon/glass homogenizer (Thomas, USA). The homogenate was centrifuged at 10000 x g for 30 minutes in 26.3 ml polycarbonate bottles with noryl cap assembly (Beckman) in a Beckman L7 ultracentrifuge using a 50.2 Ti rotor. The supernatant was filtered through cheesecloth and centrifuged at 105000 x g for 60 minutes. The pellet was suspended in 2 ml of homogenizing buffer/g tissue and centrifuged at 105000 x g for 60 minutes. The final microsomal pellet was resuspended in 1 ml of 50 mM tris / 20% glycerol pH

7.4/g tissue and frozen in small aliquots at -70 °C. The protein concentration in the final microsomal suspension was 10 mg/ml.

METABOLISM OF NDEA BY HEPATIC MICROSOMES

The deethylation of NDEA by liver microsomes was assayed as described by Yoo *et al* (Yoo, 1990). The reactions were run in duplicates in capped borosilicate glass centrifuge tubes. The assay mixture contained 50 mM tris pH 7.4, 10 mM $MgCl_2$, 150 mM KCl, 0.4 mg microsomal protein, 0.4 mM NADP (sodium salt, Sigma), 10 mM glucose-6-phosphate (monosodium salt, Sigma), 0.2 unit glucose-6-phosphate dehydrogenase (type XV, from Bakers yeast, Sigma) and NDEA at concentrations ranging from 10 μM to 300 μM. Blanks contained boiled microsomes, and standards contained different amounts of acetaldehyde and no NDEA. The mixture was incubated at 37 °C for 2 minutes with shaking and the reactions were started by the addition of NADPH-generating system. After 10 minutes the reactions were stopped by the addition of 100 μl 25% $ZnSO_4$, 0.55 mM semicarbazide which serves to trap the acetaldehyde produced by the microsomal deethylation of NDEA. 100 μl of ice-cold saturated $Ba(OH)_2$ was added and the mixture was centrifuged at 2000 x g for 15 minutes at 4 °C. 0.35 ml of the supernatant was added to a clean glass centrifuge tube containing 100 μl 0.25% 2,4- dinitrophenylhydrazine in 6 N HCl; and 1 ml H_2O and 1.5 ml hexane which had been saturated with each other. The tubes were left shaking on a roller shaker for 1 hour at 4 °C. 1 ml of the hexane layer was then added to an eppendorf tube containing 0.35 ml acetonitrile (HPLC grade, Rathburn, Scotland) and vortex mixed for 30 seconds.

The acetaldehyde was measured as the 2,4-dinitrophenylhydrazone derivative by HPLC based on the original method of Farrelly (Farrelly, 1980) as modified by Yoo *et al* (Yoo, 1990). 50 μl of the acetonitrile layer was injected into the HPLC system (Varian,

9010, Australia) and the acetaldehyde derivative separated with the use of a Novapak C_{18} column 4 μM (8 x 100 mm, Waters, U.K.) using 65% acetonitrile as the eluent (1.1 ml/minute) and monitoring the absorbance at 340 nm. The output of the UV detector of the HPLC was fed through an analog/digital board (Strawberry Tree, California) to be captured as a digital input on an Apple Macintosh SE. The area of the peak of the acetaldehyde-derivative was calculated using an Analog Connection Chrom software (Strawberry Tree, California) and the amount compared against the area of the peaks of different amounts of standards.

METABOLISM OF [1-^{14}C]NDEA BY OESOPHAGEAL EPITHELIUM

The metabolism of N-nitrosodi[1-^{14}C]ethylamine to $^{14}CO_2$ by oesophageal epithelium was measured as described before (Swann, 1984b). Male Sprague Dawley rats (200 g) were starved overnight, killed next morning by cervical dislocation and the oesophagus removed to ice-cold Krebs-Ringer phosphate buffer. The mucosa was obtained by physically stripping the outer muscle-submucosa layers away from the mucosa with the use of forceps. By holding firmly one pair of forceps in the middle of the oesophagus, another pair was gently moved towards one end of the tissue and the process was repeated at the other end. The mucosas were cut into small pieces. Pieces of mucosa were blotted dry onto Whatman 54 paper, weighed and some pieces (60 mg) placed into the annulus of each 2 ml Warburg flask, containing 1.8 ml cold krebs-Ringer buffer and 2 mg/ml glucose. A Whatman 1 paper wick in the centre well of the flask was soaked with 0.15 ml 2 M NaOH. The NDEA, 0.2 ml in either water, or aqueous isoamyl alcohol to a final concentration of 1.6 mM, was placed in the sidearm. NDEA solutions with concentrations ranging from 1 μM to 30 μM were prepared by mixing [1-^{14}C]-NDEA (250000 dpm) and unlabelled NDEA. Blanks had 0.2 ml 1.2 M $HClO_4$ added to the side arm before tipping the NDEA to the main well of the Warburg flask. Concentrations of the nitrosamine were checked by measuring the absorbance at 230

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nm, taking the molar extinction coefficient for NDEA to be 7244.

Flasks were then placed in a shaking water bath at 37 °C, gassed with oxygen for 5 minutes and the closed flasks were equilibrated for a further 5 minutes. Reactions were started by tipping the NDEA into the flask at 30 second intervals. After 30 minutes the reaction was stopped by injecting 0.2 ml 1.2 M perchloric acid through the rubber cap into the sealed flask. The flasks were removed from the water bath and left to equilibrate at room temperature for 1 hour. The paper wick in each flask was removed into a 15 ml centrifuge tube, and the centre well of the Warburg flask washed twice with 0.5 ml 0.2 M Na_2CO_3. The washings were transferred to the centrifuge tube, 4 ml of water added and the tubes were vortex mixed for 30 seconds. The paper wicks were removed from the tubes, then 0.2 ml 0.4 M $BaCl_2$ was added and the tubes heated to 100 °C for 15 minutes. The tubes were then centrifuged at 1200 rpm for 2 minutes, the supernatant discarded and the precipitated $BaCO_3$ washed in 4 ml water. The washing was repeated twice and the final precipitate suspended in 2 ml water and transferred to a glass 15 ml scintillation vial. Any $BaCO_3$ remaining in the tube was washed into the vial with 2 ml H_2O and twice with 1 ml scintillation cocktail. More cocktail was added to the scintillation vials which were counted for 5 minutes on a ^{14}C programme.

In one experiment the proportion of NDEA metabolized to CO_2 was determined by removing 1 ml of incubation medium into a capped centrifuge tube containing 2 ml CH_2Cl_2. The tubes were vortex mixed for 15 seconds and centrifuged for 2 minutes at 1200 rpm. 1 ml of the organic layer was removed and the radioactivity measured. 100 μl of the respective NDEA stock solution in 1 ml buffer was similarly extracted with 2 ml CH_2Cl_2. The amount of NDEA which had been metabolized was estimated from the difference in radioactivity measured from the CH_2Cl_2 extracts of the incubated flasks and the respective NDEA stock solution. Recovery of NDEA from the stock solution was 70%.

PROTEIN CONCENTRATION DETERMINATION

Protein concentration was determined by the method of Lowry *et al* (Lowry, 1951). The sample (0.2 ml) was mixed with 1 ml of freshly prepared solution A (0.5 ml 2% potassium sodium tartrate + 0.5 ml 1% copper sulphate + 50 ml 2% sodium carbonate in 0.1 N NaOH) and after 20 minutes 0.1 ml solution A (1 normal Folin Ciocalteau reagent, Sigma) was added, mixed well and after 45 minutes the absorbance of the fractions at 700 nm were read against a blank containing only 0.2 ml buffer. Protein standards were prepared by adding different amounts of a bovine serum albumin (BSA) stock solution in the same buffer as microsomes were resuspended.

7-ETHOXYCOUMARIN-O-DEETHYLATION

The method used to measure the rate of the deethylation of 7-ethoxycoumarin by hepatic microsomes was based on the methods of Greenlee and Poland (Greenlee, 1978) and Guengerich (Guengerich, 1989a) with some modifications. Incubations were carried out in borosilicate glass centrifuge tubes in a total volume of 1 ml. The reaction mixture contained 50 mM tris pH 7.4, 10 mM $MgCl_2$, 10 mM glucose-6-phosphate, 0.5 mM β-NADP, 1 unit of glucose-6-phosphate dehydrogenase, 0.5 mg of liver microsomal protein and 300 μM of 7-ethoxycoumarin (from a 30 mM solution in methanol). The volume of methanol added was 1% of the volume of the reaction mixture and Greenlee and Poland (Greenlee, 1978) had shown that this does not interfere in the rates of the reaction. Blanks contained all the components mentioned above, except 7-ethoxycoumarin, which was added after the reaction had been stopped. Standards had different amounts of umbelliferone added from a stock solution prepared freshly in 0.01N NaOH / 1M NaCl. 7-ethoxycoumarin was also added to standards after the reaction had been stopped. The tubes were pre-incubated at 37 °C with shaking for 3 minutes and reactions were started at 30 second intervals by addition of the glucose-6-

phosphate. Reactions were allowed to proceed for 10 minutes and were stopped by the addition of 0.1 ml 2N HCl and 2 ml chloroform consecutively. The tubes were vortex mixed for 10 seconds and centrifuged at 1000 x g for 5 minutes. One ml of the organic phase was then added to 1.5 ml 0.01N NaOH / 1M NaCl, the tubes were vortex mixed for another 10 seconds and centrifuged as before. Fluorescence was read from the alkaline phase using a spectrofluorimeter with excitation at 368 nm and emission at 465 mm, with a 2.5 nm band width in both cases. Under these conditions, the production of umbelliferone was linear for at least 15 minutes and had a linearity dependence on protein concentration up to 0.8 mg/ml.

COUMARIN 7-HYDROXYLATION

The hydroxylation of coumarin at the 7-position by liver microsomes was done essentially as described for the deethylation of 7-ethoxycoumarin except the microsomal protein concentration used was 1 mg/ml, the concentration of coumarin used was 10mM (added from a 0.75 M coumarin stock solution in methanol) and the reaction was incubated for 15 minutes. The production of umbelliferone by hepatic microsomes from coumarin was linear under the conditions used.

AMINOPYRENE-N-DEMETHYLATION

The demethylation of aminopyrene (Sigma) was measured as described for NDMA demethylation except the concentration of hepatic microsomal protein used was 0.5 mg/tube, the concentration of aminopyrene was 5 mM and the reaction proceeded for 10 minutes. These conditions were used to assure the linearity of the rates of production of formaldehyde from the N-demethylation of aminopyrene by liver microsomes.

NDMA DEMETHYLATION

The demethylation of NDMA by hepatic microsomes was analyzed by measuring the rates of formation of formaldehyde essentially as described by Yang *et al* (Yang, 1991a). The reaction was carried out in borosilicate glass centrifuge tubes in a total volume of 0.5 ml. The reaction mixture contained 50 mM tris pH 7.4, 0.4 mM β-NADP, 10 mM glucose-6-phosphate, 0.2 units glucose-6-phosphate dehydrogenase, 1 mg of microsomal protein and 200 mM NDMA. Standards contained different amounts of formaldehyde but no NDMA, and the blank containing all the components of the reaction mixture except that boiled liver microsomes were used. After 2 minutes of pre-incubation at 37 °C with shaking the reaction was started by the addition of the NADPH-generating system (βNADP + glucose-6-phosphate + glucose-6-phosphate dehydrogenase) and the reactions were allowed to proceed for 20 minutes. It was then terminated by the addition of 50 μl 25% $ZnSO_4$ and 50 μl of saturated $Ba(OH)_2$. Following centrifugation for 15 minutes at 4 °C, 0.35 ml of the supernatant was mixed with 0.15 ml of a freshly prepared concentrated Nash reagent (5 g ammonium sulphate and 0.1 ml acetylacetone in 6 ml 3% acetic acid). The solution was heated at 50 °C for 30 minutes then, after cooling, the absorbance at 412 mm of the samples was read against the background and compared to the standards.

ETHOXYRESORUFIN-O-DEETHYLATION

The *O*-deethylation of ethoxyresorufin by liver microsomes was measured essentially as described by Burke *et al* (Burke, 1985). The reaction was carried out in a fluorimeter cells kept at 37 °C with the continuous monitoring of the formation of resorufin in a spectrofluorimeter with the excitation set at 530 mm and emission set at 585 mm and the band width 5 nm in both cases. The total reaction mixture (2 ml) contained 0.1 M

KH$_2$PO$_4$ pH 7.6, 0.2-0.4 mg microsomal suspension, 0.25 mM NADPH and 5 μM of

ethoxyresorufin (from a 2 mM stock solution in dimethyl sulfoxide). After 2 minutes of

pre-incubation at 37 °C, the reaction was started by the addition of NADPH and after 5

minutes 10 μl of a 10 μM resorufin stock solution was added to the reaction mixture in

order to calibrate the formation of resorufin. The reaction was usually linear for 5

minutes under these conditions.

PENTOXYRESORUFIN-0-DEPENTYLATION

The *0*-depentylation of pentoxyresorufin was measured exactly as described for the *0*-

deethylation of ethoxyresorufin, except the concentration of pentoxyresorufin used was

5 μM (10μl from a 1 mM stock solution in dimethylsulfoxide).

RESULTS

The effect *in vivo* of a 10% ethanol solution, a 2.8% isoamyl alcohol solution or a 10% ethanol / 2.8% isoamyl alcohol solution on the metabolism and distribution of a 3 mg/kg b.w.single oral dose of [2-^{3}H]-NDEA was measured after 4 hours by quantitating the amount of N7-ethylguanine produced by the ethylation of DNA of liver, kidney and oesophagus. The rate of metabolism of NDEA in the rat is 6 mg/kg b. w./hour, thus the dose of [2-^{3}H]-NDEA given to the rats would be expected to have been completely metabolized after 4 hours. N7-ethylguanine is the main DNA product of the ethylation of DNA by NDEA and since this ethylated base is only slowly lost or repaired from DNA, it gives a good measurement of the total amount of NDEA metabolized in a particular organ.

[2-^{3}H]-NDEA (3 mg/kg b.w.) was given orally by tube in 1 ml water (group 1, control); 1 ml 10% ethanol (group 2); 1 ml 2.8% isoamyl alcohol (group 3); or 1 ml 10% ethanol: 2.8% isoamyl alcohol (group 4). The rats were killed 4 hours later, liver, kidneys and oesophagus removed and DNA extracted from these organs. The DNA was then subjected to mild acid hydrolysis in order to liberate ethylated and non-ethylated purines and chromatographed on a strong cation exchange resin as shown in figures 2.1 A, B and C. Under these conditions, good separation of purines and ethylated guanine was obtained using up to 30 mg hydrolyzed DNA. However with the kidney, higher amounts of DNA hydrolysate (30 - 60 mg) had to be injected into the HPLC in order to detect the low amounts of N7-ethylguanine produced by NDEA in that organ. The procedure used to chromatograph DNA hydrolysate from kidney was thus to divide the kidney DNA hydrolysate into 2 parts and inject them successively onto the cation exchange column. The respective fractions from the two injections were pooled together and then analyzed as with DNA hydrolysate from liver and oesophagus. Figure 2 shows the chromatographic profile of the fractions of the two successive injections pooled together of DNA hydrolysate from kidneys of animals

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which had received the nitrosamine in water (control group)

The extent of alkylation is measured as the ratio between the amount of N7-alkylguanine and the amount of guanine. Guanine is very insoluble and can easily precipitate. If any guanine has been lost by precipitation on the column, a spuriously high level of alkylation will be calculated. To guard against this error the amount of adenine was also measured and the guanine/adenine ratio calculated. If there had been loss by precipitation of guanine this will be shown by a low guanine/adenine ratio.

Figure 2.1: Chromatography elution profile of DNA hydrolysate from liver, kidney and oesophagus.

2.1A - Chromatography of liver DNA hydrolysate of rats which received [2-^{3}H]-NDEA in water.
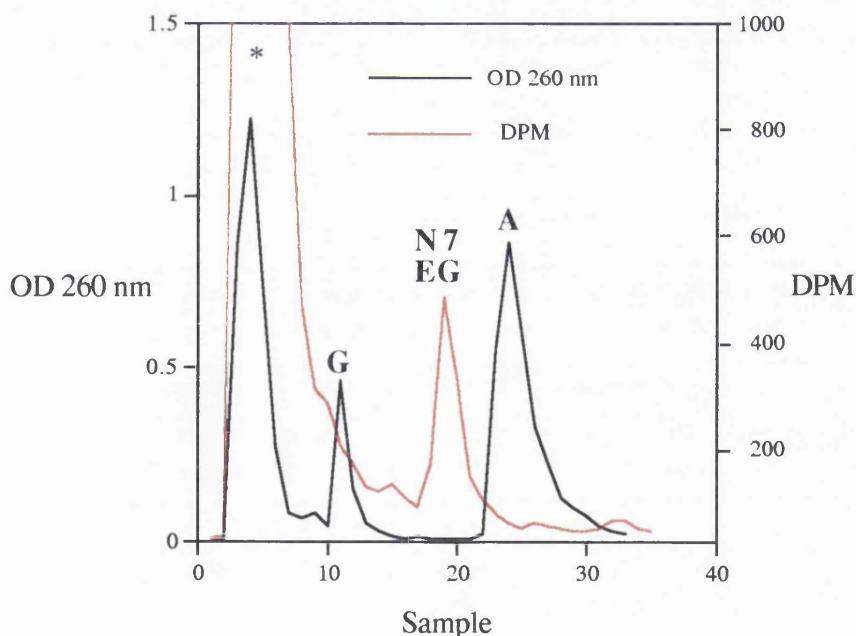
* maximum value for DPM in this peak was 4623. **G** is Guanine, **A** is Adenine and **N7EG** is N7-ethylguanine.

2.1B - Chromatography of kidney DNA hydrolysate of rats which received $[2\text{-}^3\text{H}]$-NDEA in water.
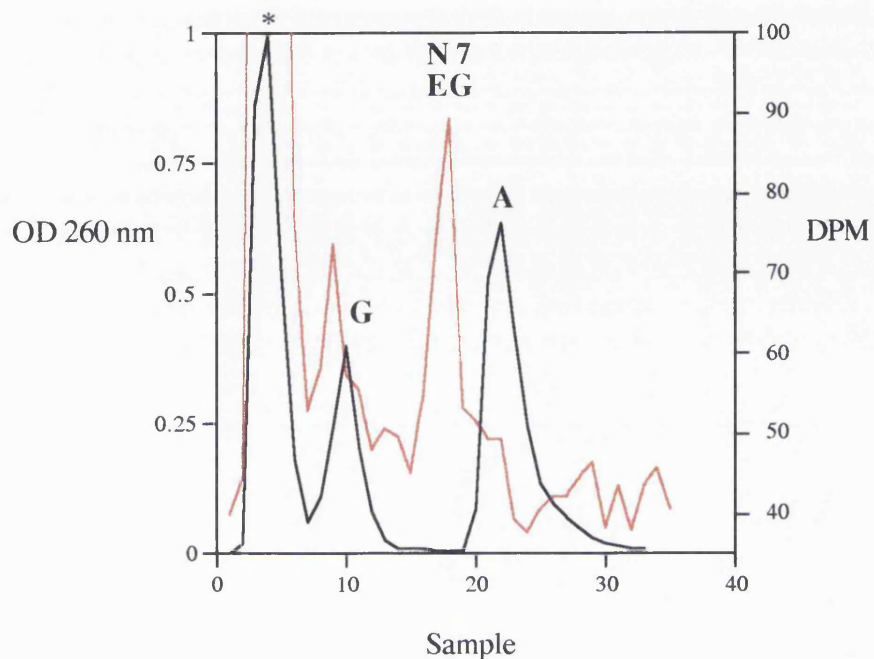
* maximum value for DPM in this peak was 383. **G** is guanine, **A** is adenine and **N7EG** is N7-ethylguanine.

2.1C - Chromatography of oesophageal DNA hydrolysate of rats which received $[2\text{-}^3\text{H}]$-NDEA in 1 ml 2.8% isoamyl alcohol / 10% ethanol.
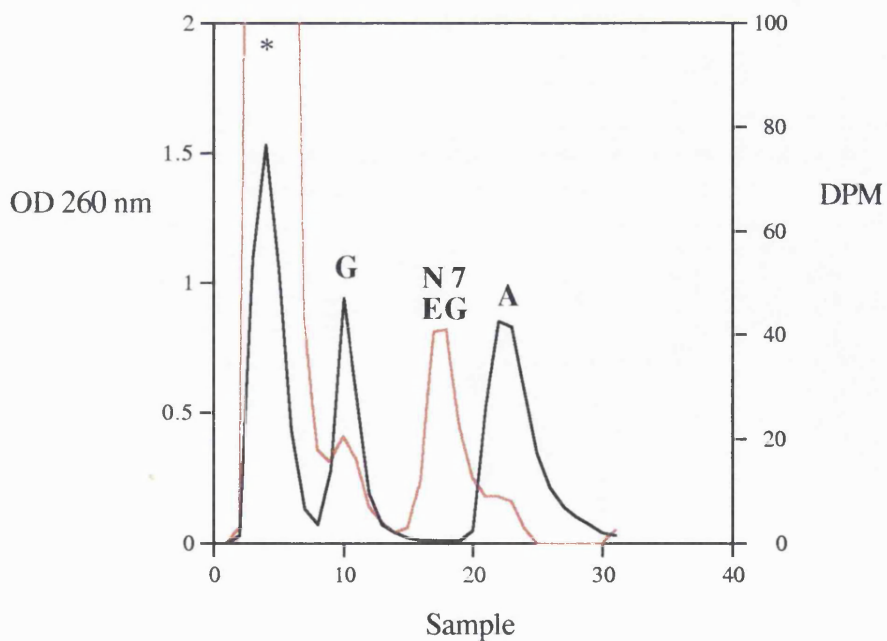
* maximum value for DPM in this peak was 697. **G** is guanine, **A** is adenine and **N7EG** is N7-ethylguanine.

Because there was plenty of DNA purified from the livers of animals which received the nitrosamine in water (control) two different samples were hydrolysed and chromatographed and the results are shown in table 2.1A. The results of the 2 analyses are very similar, showing that experimental conditions of analysis produce negligible variations in the results. The first injection was taken as the standard control to which the others were compared. The chromatography of DNA purified from liver of animals which received the nitrosamine in 2.8% isoamyl alcohol was also repeated and the results were again very similar.

The effect of 2.8% isoamyl alcohol and 10% ethanol, either alone or together (2.8% isoamyl alcohol / 10% ethanol (v/v), on the alkylation of the DNA of liver, kidney and oesophagus by NDEA (3 mg/kg b.w.) is summarised in table 2.1, with the raw data in table 2.1A. This shows that co-administration of ethanol decreased the levels of N7-ethylguanine in DNA of liver (66% of control) and kidney (67% of control), but increased the levels of N7-ethylguanine in the oesophageal DNA by 1.77-fold. Co-administration of isoamyl alcohol decreased the levels of N7-ethylguanine in liver DNA (29%), kidney DNA (39%) and oesophageal DNA (25%). The 2 alcohols together produced a decrease in the extent of alkylation in the DNA of liver and kidney which were indistinguishable from the decrease produced by either alcohol alone, but produced an increase in the oesophagus (1.87-fold) which was slightly greater than the increase produced by ethanol alone. One had not expected that co-administration of isoamyl alcohol with ethanol would produce a greater increase in the alkylation of the oesophageal DNA than ethanol alone because isoamyl alcohol alone decreased the alkylation of the oesophageal DNA. A possible explanation is that isoamyl alcohol has a greater affinity for alcohol-dehydrogenase than ethanol (Reviewed by Craddock, 1993), thus a greater proportion of ethanol will be metabolized by P450 2E1, with a consequent increase in the amount of NDEA reaching the oesophagus, but this was not tested directly.

Table 2.1: Summary of the influence of ethanol and isoamyl alcohol on the levels of N7-ethylguanine (N7EG) produced by [2-^{3}H]-NDEA (3 mg/kg b.w.) in the DNA of liver, kidney and oesophagus.

	Alkylation of DNA μmoles N7EG / mol G			Ratio of treated to control		
TREATMENT	LIVER	KIDNEY	OESOPHAGUS	LIVER	KIDNEY	OESOPHAGUS
H$_2$O (Control)	9.2	0.37	0.71	—	—	—
2.8% IAA	6.5	0.23	0.53	0.71	0.61	0.75
10% Ethanol	6.1	0.25	1.26	0.66	0.67	1.77
2.8% IAA / 10% Ethanol	6.5	0.28	1.33	0.71	0.76	1.87

Table 2.1A: Raw data showing the effect of ethanol and isoamyl alcohol (IAA) on the alkylation by NDEA (3 mg/kg b.w., orally) of the DNA of liver, kidney and oesophagus.

DNA sample + treatment	μmoles N7EG / mol G	ratio treated / control	DNA mg	Guanine OD peak	Adenine OD peak	Guanine μmoles	Adenine μmoles	A/G ratio	N7EG dpm	N7EG pmoles
Liver H$_2$O (control)	9.2	___	8.5	26.88	57.16	3.98	4.74	1.19	1170	36.57
Liver H$_2$O	10.1	___	7.8	25.92	58.88	3.75	4.79	1.28	1214	37.94
Liver 2.8% IAA	6.9	0.75	5.9	21.14	47.28	3.13	3.82	1.22	689	21.50
Liver 2.8% IAA	6.2	0.67	7.7	29.44	65.48	4.24	5.29	1.25	841	26.28
Liver 10% Ethanol	6.1	0.66	8.0	24.26	51.7	3.59	4.24	1.18	706	22.06
Liver 2.8% IAA / 10% Ethanol	6.5	0.71	7.3	26.12	55.1	3.83	4.52	1.18	795	24.84
Kidney H$_2$O (control)	0.37	___	27.1	42.56	93.24	12.57	15.17	1.21	148	4.59

DNA sample + treatment	μmoles N7EG / mol G	ratio treated / control	DNA mg	Guanine OD peak	Adenine OD peak	Guanine μmoles	Adenine μmoles	A/G ratio	N7EG dpm	N7EG pmoles
Kidney 2.8% IAA	0.23	0.61	45.8	72.96	155.56	21.51	25.46	1.18	155	4.84
Kidney 10% Ethanol	0.25	0.67	51	71.4	157.24	21.03	25.86	1.23	167	5.22
Kidney 2.8% IAA / 10% Ethanol	0.28	0.76	53.26	80.4	159.24	23.69	26.02	1.1	211	6.59
Oesophagus H$_2$O (control)	0.71	——	3	9.46	16.56	1.37	1.35	0.98	31	0.97
Oesophagus 2.8% IAA	0.53	0.75	4.9	17.3	29.8	2.5	2.41	0.96	43	1.34
Oesophagus 10% Ethanol	1.26	1.77	6.72	21.7	38.3	3.17	3.11	0.98	128	4
Oesophagus 2.8% IAA / 10% Ethanol	1.33	1.87	6.65	21.3	37.5	3.1	3.09	1.0	132	4.13

The effect of ethanol on the distribution and metabolism of NDEA *in vivo* and *in vitro* in well documented (Swann, 1984b). The increase in N7-ethylguanine levels in oesophageal DNA when ethanol is co-administered with NDEA is the result of the selective inhibition of ethanol on the metabolism of NDEA in the rat liver and kidney. Ethanol has previously been shown to inhibit the metabolism of NDEA by liver slices with a Ki of 1 mM (Graves, 1993), whereas concentrations of ethanol up to 1 mM produced little or no inhibition on the metabolism of this nitrosamine by oesophageal slices(Swann, 1984b).

In order to see if the decrease in the level of N7-ethylguanine in the DNA of liver and oesophagus produced by the co-administration of isoamyl alcohol with NDEA is the result of the inhibition of the metabolism of NDEA in the two organs, the effect of isoamyl alcohol on the rates of the metabolism of NDEA by liver and oesophagus *in vitro* was examined. The concentration of isoamyl alcohol to be used in the *in vitro* experiment (1.6 mM) was calculated to be similar to that which would have occurred *in vivo* if there had been an even distribution of isoamyl alcohol in the animal body.

Liver microsomes were prepared from male Sprague Dawley rats and 0.4 mg microsomal protein were incubated with cofactors and NDEA in a range 10 - 300 μM with or without 1.6 mm isoamyl alcohol for 10 minutes. After the reaction was stopped and the mixture deproteinized, the acetaldehyde produced by the deethylation of NDEA was trapped by semicarbazide and allowed to react with 2,4-dinitrophenylhydrazine. The 2,4-dinitrophenylhydrazone acetaldehyde derivative was then selectively extracted into hexane and acetonitrile and injected into a HPLC system. It was separated by reverse-phase chromatography and quantitated by monitoring the absorbance at 340 nm. The integrated peak-area was then plotted against peak-areas obtained from known standard amounts of acetaldehyde added to the incubation mixture. All areas were subtracted from the areas obtained from the blanks (containing all components of the normal incubation mixture, except that boiled microsomes were used).

Table 2.2: Influence of isoamyl alcohol (IAA) on the deethylation of NDEA by liver microsomes (the results from the duplicates of each experiment are shown).

Concentration of NDEA	Area of CH₃CHO peak	Mean rate ($\pm$ SD) (nmol CH₃CHO/min/mg protein)
10 µM	9060, 10608, 14095, 11822	**0.167** (± 0.015)
10 µM + 1.6 mM IAA	2138, 1998, 3588, 2727	**0.035** (± 0.004)
20 µM	7028, 12680, 15825, 15220	**0.184** (± 0.038)
20 µM + 1.6 mM IAA	2653, 4758, 4275, 4910	**0.059** (± 0.014)
50 µM	20898, 23720, 24053	**0.330** (± 0.065)
50 µM + 1.6 mM IAA	6653, 4988, 6115, 8745	**0.096** (± 0.017)
100 µM	32053, 27868, 35525, 29828	**0.472** (± 0.064)
100 µM + 1.6 mM IAA	5563, 7790, 9302, 9305	**0.116** (± 0.014)
200 µM	41890, 48998	**0.632** (± 0.046)
200 µM + 1.6 mM IAA	14895, 12963	**0.177** (± 0.013)
300 µM	58516, 48096, 55512	**0.861** (± 0.127)
300 µM + 1.6 mM IAA	15905, 22097, 17830	**0.262** (± 0.025)

Figure 2.2: Lineweaver-Burke plot for the deethylation of NDEA by liver microsomes and its inhibition by isoamyl alcohol (IAA).
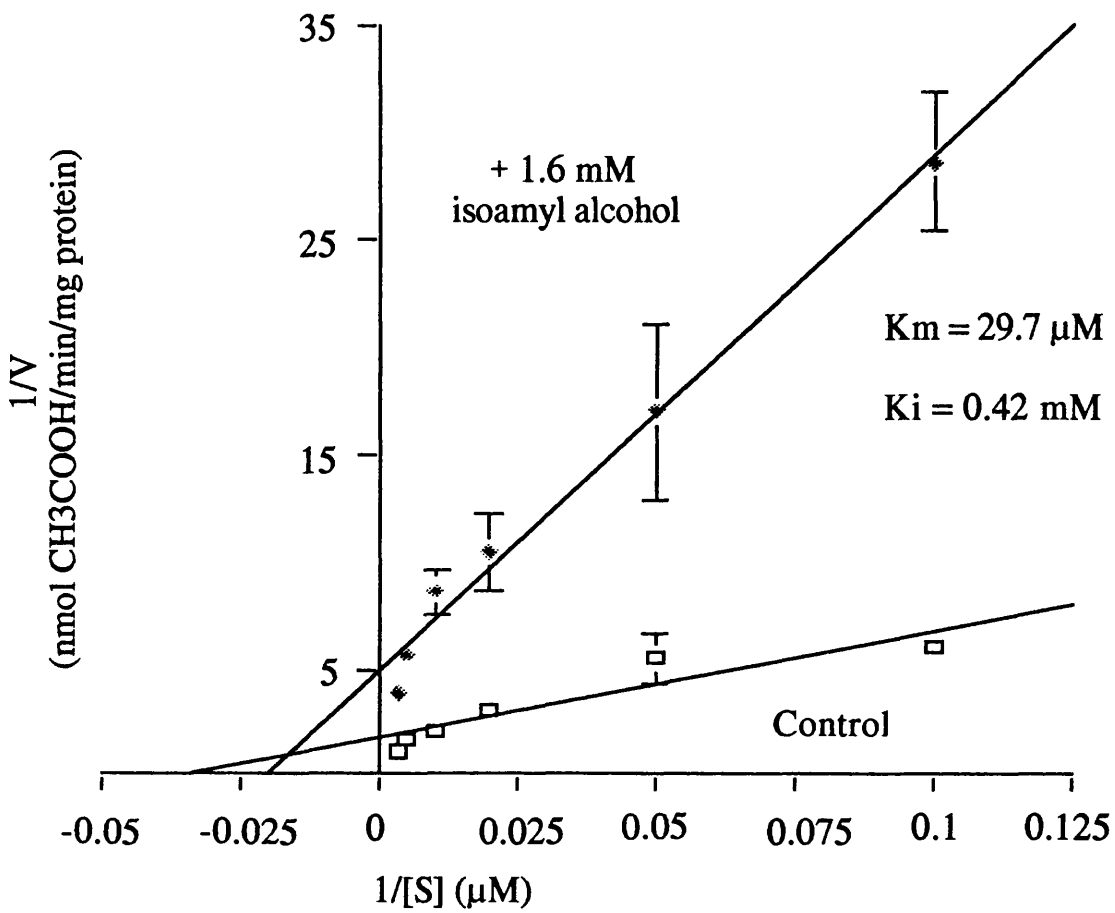
There are no standard deviation bars shown for most of the control points because these were too close to the mean for them to be printed.

The experiment was done twice and in duplicate. Table 2.2 gives the summary of the inhibition of isoamyl alcohol on the hepatic microsomal metabolism of NDEA. The standard deviation of the rates of production of acetaldehyde from the hepatic microsomal deethylation of NDEA for a given concentration of this nitrosamine was usually around 10 -15%. 1.6 mM isoamyl alcohol strongly inhibits the rate of NDEA deethylation by hepatic microsomes even when the concentration of NDEA was 300 μM. The Lineweaver-Burke plot for the metabolism of NDEA by liver microsomes and its inhibition by isoamyl alcohol is shown in figure 2.2. The data gives a good fit on the Lineweaver-Burke plot. For the control data, the plot gave a Km (29.7 μM) and Vmax (0.596 nmol acetaldehyde/minute/mg microsomal protein) close to the previously published value for the these two parameters (km = 37.8 μm Vmax = 0.683 nmol acetaldehyde/minute/mg (Yoo, 1990). Isoamyl alcohol clearly inhibited the metabolism of NDEA by liver microsomes, although the type of inhibition, competitive or non-competitive, cannot be concluded from the graph. The calculated Ki for the inhibition of NDEA metabolism by liver microsomes was 0.42 mM, a value which is less than half of the reported Ki (1 mM) (Graves, 1993) for the competitive inhibition by ethanol of NDEA metabolism by liver slices.

The decrease in the ethylation of oesophageal DNA seen when NDEA was given together with 2.8% isoamyl alcohol was probably the result of inhibition by this alcohol on the oesophageal metabolism of NDEA. In order to confirm this observation, the effect of isoamyl alcohol on the oesophageal metabolism of NDEA was measured *in vitro*. [14C]-NDEA was incubated with *oesophageal mucosa*, the rate of formation of $14CO_2$ measured and the influence of isoamyl alcohol on this rate analyzed. Although CO_2 is not a primary product of the metabolism of the metabolism of NDEA, it can be used to measure nitrosamine metabolism, as seen in many studies (Heath, 1962; Swann, 1971b; Swann, 1984b; Graves, 1993). By contrast, the metabolism of NDEA by oesophageal microsomes misrepresents completely the situation seen *in vivo* because

it can only be measured when extremely high concentrations of NDEA and protein are used (Labuc, 1982; Craddock, 1991).

Figure 2.3 gives the Lineweaver-Burke plot of the data in table 2.3 for the metabolism of NDEA to CO_2 by oesophageal mucosa and its inhibition by isoamyl alcohol. The data showed that, as with liver microsomes, isoamyl alcohol was a strong inhibitor of the deethylation of NDEA by oesophageal mucosa, although the type of inhibition could not be determined from analysis of the graph. The Km (18 μM) and Vmax (0.762 nmol CO_2/30 min/100 mg tissue) were similar to the results previously published (Swann, 1984b). To check that the evolution of CO_2 was a true measure of the amount metabolized, the amount of NDEA remaining in the flask at the end of the incubation was measured. This result (table 2.4) showed that the effect of isoamyl alcohol on CO_2 exactly corresponded to its effect on the disappearance of NDEA. The calculated Ki for the isoamyl alcohol inhibition of NDEA metabolism by oesophageal mucosa slices was 0.52 mM, a value which is very close to the Ki for the inhibition of isoamyl alcohol on the NDEA metabolism by liver microsomes. This contrasts with previous published results with ethanol which showed that 1 mM ethanol had little or no effect on the metabolism of NDEA by oesophageal mucosa, although it can inhibit the metabolism of NDEA in the liver and kidney (Swann, 1984b; Graves, 1993). Although NDEA is metabolised in the rat liver by P450 2E1 to a great extent (Yoo, 1990), this is not the only P450 to be involved in the metabolism of this nitrosamine in the liver (Heath, 1962; Yoo, 1990). The identity of other P450s which may metabolise NDEA in the rat liver is not known at the moment. In the oesophagus, it is clear that P450 2E1 is not responsible for any metabolism of NDEA because ethanol does not inhibit the metabolism of this nitrosamine in the oesophagus and this particular isoenzyme is not present in the oesophageal mucosa, as will be discussed in Chapter 4.

In an attempt to clarify the differences between ethanol and isoamyl alcohol, the inhibition by these two alcohols on a number of reactions catalyzed by different P450s

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Table 2.3: Rate of formation of $^{14}CO_2$ from [^{14}C]-NDEA by oesophageal epithelium and its inhibition by 1.6 mM isoamyl alcohol (IAA).

Concentration of NDEA	$^{14}CO_2$ produced dpm/100 mg tissue	$^{14}CO_2$ produced nmol/30min/100mg tissue
2.04	2707, 2370	0.091, 0.080
3.09	2528, 2587	0.106, 0.109
3.88	2336, 2498	0.136, 0.146
4	1689, 1726, 1647, 1406	0.164, 0.167, 0.160, 0.136
4.47	3163, 2191	0.207, 0.143
5.57	1644, 2089	0.168, 0.213
9.93	2315, 2397	0.366, 0.380
10.78	1182, 2089	0.306, 0.260
11.86	1625, 2048	0.276, 0.348
19.94	936.92, 798.35	0.306, 0.260
25.08	1656, 1430	0.605, 0.523
1.78 + 1.6 mM IAA	414, 394	0.012, 0.012
2.4 + 1.6 mM IAA	685, 870	0.025, 0.032
3.78 + 1.6 mM IAA	380, 471	0.023, 0.027
4 + 1.6 mM IAA	619, 680, 588	0.042, 0.046, 0.040
5.64 + 1.6 mM IAA	1025, 837	0.079, 0.065
6.34 + 1.6 mM IAA	580, 529, 613	0.056, 0.051, 0.059
10.46 + 1.6 mM IAA	372, 423	0.059, 0.068
13.27 + 1.6 mM IA	993.91	0.183
19.58 + 1.6 mM IAA	383, 432	0.129, 0.146
23.14 + 1.6 mM IAA	614.44	0.2

Figure 2.3 : Lineweaver-Burke plot of the production of $^{14}CO_2$ from [^{14}C]-NDEA by oesophageal epithelium and its inhibition by isoamyl alcohol (IAA).
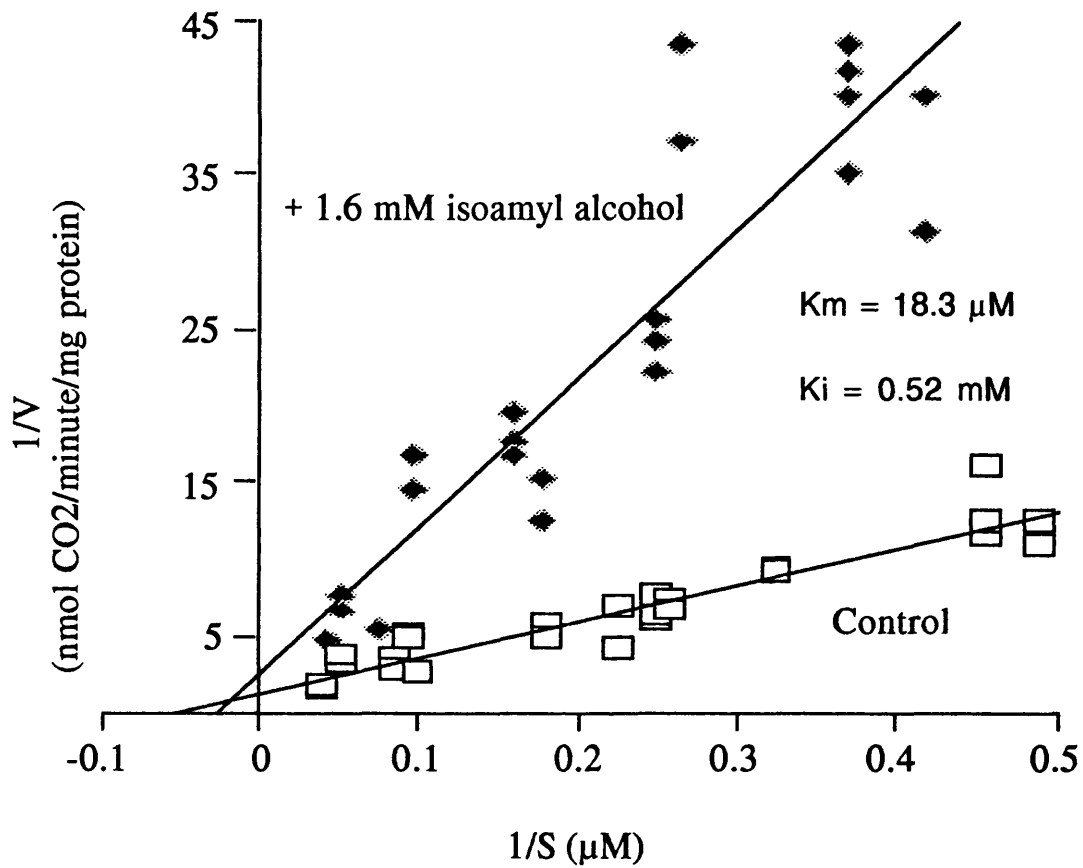
Table 2.4: Comparison between the amount of NDEA metabolized by oesophageal epithelium, in the presence or absence of 1.6 mM isoamyl alcohol (IAA) measured as the difference in the amount in the flask at the beggining and end of the incubation, and the amount metabolized estimated from the evolution of $^{14}CO2$ from $[^{14}C]$-NDEA. The initial concentration of NDEA was 4 μM and 240575 and 259875 dpm for NDEA and NDEA + 1.6 mM IAA, respectively.

	$^{14}CO_2$ recovered			
	incubated sample	blank (boiled epithelium)	difference	% converted to CO_2
NDEA alone	1617 (± 125)	92 (± 14)	1525	0.6
NDEA + IAA	629 (± 38)	168 (± 66)	461	0.2
	NDEA disappearance			
	incubated sample	blank (boiled epithelium)	difference	% disappeared
NDEA alone	151336 (± 3655)	164501 (± 3883)	13165	5.5
NDEA + IAA	168924 (± 1861)	173527 (± 4874)	4603	1.8

This table shows that 11 ± 0.1% of the nitrosamine which is metabolized, and thus disappears from the flask, is collected as $^{14}CO_2$. The effect on metabolism, ie NDEA + IAA / NDEA alone, measured as disappearance, 3.06, is statistically indistinguishable from the ratio measured by CO2 production, 3.0.

was measured.

Table 2.5: The effect of 1.6 mM isoamyl alcohol (IAA) or 1.6 mM ethanol (EtOH) on the demethylation of NDMA (0.2 mM) by liver microsomes (rates given as nmol formaldehyde / min / mg protein - mean ($\pm$ SD) of triplicates of 2 experiments).

Control I	+ IAA	Control II	+ EtOH
0.87	**0.28**	**0.69**	**0.28**
($\pm$ 0.14)	($\pm$ 0.11)	($\pm$ 0.16)	($\pm$ 0.15)
	(32% of Control I)		(40% of Control II)

Table 2.5 shows the influence of isoamyl alcohol and ethanol on the demethylation of NDMA by hepatic microsomes. The experiment was done twice for each inhibitor and respective control and in triplicates, using different preparations of liver microsomes. The difference seen in the rate of formation between the two controls is probably due to the different microsomal preparations used. At this concentration of NDMA (200 μM), this reaction is catalyzed exclusively by P450 2E1 (Yang, 1991b). Isoamyl alcohol (1.6 mM) and ethanol (1.6 mM) inhibit the demethylation of NDMA to a similar extent (68% and 60% respectively). Ethanol was previously shown to strongly inhibit the metabolism of NDMA to CO_2 by liver slices (Swann, 1984b)with a Ki of 0.5 mM and the metabolism of NDMA to formaldehyde with a Ki of 0.31 mM (Peng, 1982). Other higher alcohols were also shown to inhibit the demethylation of NDMA by liver microsomes (Peng, 1982), although the concentrations used were much higher than that used for isoamyl alcohol in this thesis. So, n-propanol (134 mM) and n-butanol (109 mM) inhibited the demethylation of NDMA (4 mM) by 92.9% and 95.1%, respectively. Thus, it is not surprising that isoamyl alcohol also inhibits reactions catalyzed by this P450.

Aminopyrene N-demethylation on the other hand can be catalyzed by a number of different P450s, including 2C11, 2B1 and 2B2 (Guengerich, 1982) and can also be catalyzed by other enzymes such as prostaglandin synthase (Gibson, 1994). The effect of 1.6 mM isoamyl alcohol or 1.6 mM ethanol on the demethylation of aminopyrene (5 mM) by liver microsomes is shown on table 2.6. The experiment was done twice, in duplicates for each inhibitor and respective control, with different microsomal preparations. Neither alcohol significantly inhibited this reaction.

Table 2.6: The effect of 1.6 mM isoamyl alcohol (IAA) or 1.6 mM ethanol (EtOH) on the demethylation of aminopyrene (5 mM) by liver microsomes (rates are given as nmol formaldehyde / min / mg protein - mean ($\pm$ SD) of duplicates of 2 experiments).

Control I	+ IAA	Control II	+ EtOH
4.31	**4.27**	**3.14**	**2.73**
($\pm$ 1.14)	($\pm$ 0.51)	($\pm$ 0.44)	($\pm$ 0.53)

7-Ethoxycoumarin deethylation can also be metabolized by a number of different P450s in liver microsomes from male rats, including 1A1 and 1A2, 2B1, 2A1, 2E1, 2C11 (Guengerich, 1982; Funea, 1993). Table 2.7 shows the influence of 1.6 mM isoamyl alcohol or 1.6 mM ethanol on the deethylation of 7-ethoxycoumarin (300 μM) by liver microsomes. This experiment was done twice and in duplicate for each inhibitor and respective control and with different microsomal preparations. Isoamyl alcohol (1.6 mM) significantly inhibited 7-ethoxycoumarin deethylation by 37%. By contrast, the same concentration of ethanol did not significantly inhibit this reaction, showing that P450 2E1 can make little contribution towards this reaction in liver microsomes prepared from untreated animals. These results (tables 2.5 and 2.7) show that isoamyl alcohol can inhibit both P450 2E1 catalyzed reactions and reactions such as

7-ethoxycoumarin deethylation which involve other P450 isoenzymes.

Table 2.7: The effect of 1.6 mM isoamyl alcohol (IAA) or 1.6 mM ethanol (EtOH) on the deethylation of 7-ethoxycoumarin (0.3 mM) by liver microsomes (rates are given in nmol umbelliferone / min / mg protein - mean ($\pm$ SD) of duplicates of 2 experiments).

Control I	+ IAA	Control II	+ EtOH
0.66	0.41	1.06	0.96
($\pm$ 0.14)	($\pm$ 0.05)	($\pm$ 0.07)	($\pm$ 0.08)
	(63% of Control I)		

It has been reported that 7-ethoxyresorufin deethylation is catalyzed exclusively by P450 1A1 in rat liver microsomes (Burke, 1985). Table 2.8 shows the effect of 45.7 mM isoamyl alcohol or 86.6 mM ethanol on the deethylation of 7-ethoxyresorufin (5 μM) catalyzed by liver microsomes. Such high concentrations of these alcohols produce a decrease in the rate of formation of umbelliferone when compared to control, probably due to the "solvent effects" of these alcohols on the microsomal membrane. A concentration 10 times lower of either alcohol did not produce any significant inhibition on this reaction. Thus, neither alcohol can effectively inhibit P450 1A1.

Table 2.8: The effect of 45.7 mM isoamyl alcohol (IAA) or 86.6 mM ethanol (EtOH) on the deethylation of 7-ethoxyresorufin (5 μM) by liver microsomes (rates are given as pmol resorufin / min / mg protein - mean ($\pm$ SD) from 3 determinations).

Control	+ IAA	+ EtOH
156.9	116.7	74.1
($\pm$ 6.7)	($\pm$ 17.8)	($\pm$ 14.6)

The effect of these two alcohols on the dealkylation of pentoxyresorufin derivative by liver microsomes was studied (table 2.9). It has been reported that this reaction is catalyzed exclusively by P450 2B1 (Burke, 1985). Isoamyl alcohol (457 μM) significantly inhibited this reaction (47%), whereas a 2 times higher ethanol concentration (866 μM) produced only a slight inhibition (13%). So, from this experiment it seems that isoamyl alcohol can effectively inhibit reactions catalyzed by P450 2B1, whereas ethanol has a low affinity for this isoenzyme.

Table 2.9: The effect of 457 μM isoamyl alcohol (IAA) or 866 μM ethanol (EtOH) on the depentylation of pentoxyresorufin by liver microsomes (rates are given as pmol resorufin / min / mg protein - mean ($\pm$ SD) of 3 determinations).

Control	+ IAA	+ EtOH
43.15	**22.74**	**37.74**
($\pm$ 3.22)	($\pm$ 4.17)	($\pm$ 1.02)
	(53% of Control)	

DISCUSSION

The effect of ethanol on the organ to organ distribution of alkylation of DNA by NDEA was confirmed in this study. When NDEA was given orally to rats in 1 ml 10% ethanol, it increased the N7-ethylation of guanine of the oesophageal DNA by 1.77-fold. This result is similar to a previously published study which showed that NDEA given orally in 1 ml 5% ethanol increased the levels of N7-ethylguanine in oesophageal DNA by 1.8-fold when compared to the respective control. In the study reported now (table 2.1) the levels of N7-ethylguanine in kidney and liver of rats which received NDEA in 1 ml 10% ethanol were reduced by about 30% when compared to rats which received the nitrosamine in 1 ml water. Previous studies *in vitro* measuring the rate of production of CO_2 by tissue slices incubated with NDEA with or without ethanol showed that the effect on alkylation produced by ethanol was the result of the influence of ethanol on the distribution of NDEA caused by selective competitive inhibition of the metabolism of NDEA in liver (Ki = 1 mM) and kidney (Ki = 0.06 mM). By contrast to the effect on the liver and kidney, 1 mM ethanol had a negligible effect on the metabolism of NDEA by oesophageal mucosa (Swann, 1984b).

In this study, by contrast, administration of NDEA in 2.8% isoamyl alcohol produced a decrease in the levels of N7-ethylguanine in the oesophagus DNA (25% decrease) as well as in liver DNA (29%), and kidney DNA (39%) when compared to rats which received the nitrosamine in water. This suggests that isoamyl alcohol, like ethanol, is a strong inhibitor of NDEA metabolism in the liver and kidney, but unlike ethanol, it is also an inhibitor of the metabolism of this nitrosamine in the oesophagus. Surprisingly however, administration of isoamyl alcohol and ethanol together increased rather than decreased the levels of N7-ethylguanine in the DNA of the oesophagus over those produced by ethanol alone. In the rat liver 80% of the ethanol ingested is metabolised by the cytoplasmic alcohol-dehydrogenase and 20% by P450 2E1 (reviewed by Craddock, 1993). The liver alcohol-dehydrogenase has a broad substrate specificity and

its affinity for higher alcohols increases with increasing chain length. Thus the probable explanation for the *in vivo* experiment is that in the presence of isoamyl alcohol the concentration of ethanol does not decrease so quickly and there is a greater inhibition of metabolism of NDEA by P450 2E1, leaving a larger proportion of NDEA to be metabolised in the oesophagus.

In vitro studies confirmed that isoamyl alcohol would inhibit the metabolism of NDEA in the liver. Isoamyl alcohol (1.6 mM - the expected *in vivo* concentration in the rat after administration of 1 ml 2.8% (v/v) isoamyl alcohol) strongly inhibited the metabolism of NDEA by liver microsomes. The inhibition could not be defined as either competitive or non-competitive, probably because the inhibition is of the different P450s capable of metabolising this nitrosamine in the rat liver rather than on one single P450, as will be discussed below. Nevertheless, the Ki for the inhibition was calculated to be 0.42 mM. By comparison ethanol has been shown to inhibit the metabolism of NDEA by liver slices with a Ki of 1 mM (Graves, 1993).

In order to assess the effect of isoamyl alcohol on the metabolism of NDEA by the oesophagus *in vitro*, the incubation had to be carried out with the stripped mucosa cut into small pieces and the end product of the metabolism of the radiolabeled nitrosamine, $^{14}CO_2$, measured. The reason for using this technique is that the metabolism of nitrosamines by oesophageal microsomes is very slow which makes it difficult to measure the rate of production of the aldehyde. Furthermore, the high amounts of microsomal protein required, make such experiment impracticable due to the low amount of tissue (about 70 mg of oesophageal mucosa) obtained from each animal. Another factor which hampers experiments with oesophageal microsomes is that much higher concentrations of nitrosamine are needed for the *in vitro* experiments than one gets when the nitrosamine is given to the animal. Thus, Labuc and Archer (Labuc, 1982) incubated oesophageal microsomes with 5 mM NMBzA for 30 minutes and measured the production of 0.55 nmol benzaldehyde/minute/mg protein and 5 pmol

formaldehyde/minute/mg protein. 5 mM is probably 33 times greater than the peak blood concentration achieved after a median lethal dose (18 mg/kg b.w.) (Druckrey, 1967) of NMBzA in rats. The importance of using physiological concentrations of nitrosamines in the *in vitro* studies, at least with the rat liver, has been demonstrated most clearly with NDMA (Lai, 1980; Yang, 1991b) and is due to the existence of multiple enzymes which can metabolize NDMA with different Kms, although only one (P450 2E1 the low Km NDMA demethylase) is responsible for the metabolism of this nitrosamine *in vivo*.

By contrast CO_2 production from the metabolism of nitrosamines by tissue slices has been used many times as a measurement of metabolism *in vitro* (Heath, 1962; Swann, 1971b; Swann, 1984b; Graves, 1993) and was repeated again in this study using oesophageal mucosa incubated with [^{14}C]-NDEA. Isoamyl alcohol strongly inhibited the rate of formation of CO_2 produced from the deethylation of NDEA by oesophageal mucosa slices with a Ki (0.52 mM) similar to that seen for the inhibition by this alcohol on the production of acetaldehyde from the deethylation of NDEA by liver microsomes. This inhibition is consistent with the small decrease in the formation of N7-ethylguanine in the oesophageal DNA of rats which received the nitrosamine in 2.8% isoamyl alcohol when compared to rats which received the nitrosamine in water. There is therefore a clear difference between isoamyl alcohol and ethanol in their effect on the metabolism of NDEA in the oesophagus, since 1 mM ethanol produced no detectable inhibition of the metabolism of NDEA by oesophageal slices (Swann, 1984b). This contrasts with the effect of both alcohols on the metabolism of NDEA in the liver, which was inhibited by either alcohol, even though the Ki for isoamyl alcohol inhibition was about half of that for ethanol inhibition of the above mentioned metabolism. Thus, isoamyl alcohol may have a greater affinity than ethanol for the P450s which could metabolize NDEA in the liver. Additionally, it also seems to have a strong affinity for the NDEA-metabolizing oesophageal P450(s).

Previous studies of the effect of isoamyl alcohol and 2-methyl-butan-1-ol in the *in vitro* metabolism of NMBzA by liver and oesophageal microsomes were made by Craddock and Henderson (Craddock, 1991). The interpretation of their results must be viewed with caution since the concentration of the alcohols (500 mM ethanol, 0.35-500 mM 2-methylbutanol, and 11.3-500 mM isoamyl alcohol) and the concentration of NMBzA (usually 5 mM) were much higher than are found in *in vivo* conditions. They found that isoamyl alcohol and the other higher alcohols inhibited the debenzylation of this nitrosamine by oesophageal microsomes to a much greater extent than did ethanol. They also observed that the debenzylation of NMBzA by rat liver microsomes was inhibited to a higher extent by 2-methylbutanol than by ethanol, although ethanol was more effective in inhibiting the metabolism of this nitrosamine by liver microsomes than by oesophageal microsomes.

Thus, it seems that isoamyl alcohol has a broader affinity for P450s than does ethanol, and that these P450(s) include those expressed in the oesophagus and capable of metabolizing nitrosamines. To better understand the difference between ethanol and isoamyl alcohol on different P450s, the effect of these alcohols on a number of different reactions catalyzed by different P450s was assessed. Not surprisingly, both alcohols could inhibit NDMA demethylase to a similar extent, since this reaction has been shown to be exclusively metabolized by P450 2E1 (Yang, 1991b), one of the main P450s involved in the metabolism of NDEA in the rat liver (Yoo, 1990). By contrast, neither alcohol inhibited the demethylation of aminopyrene, a reaction catalyzed in rat liver microsomes mainly by P450 2C11, but also by P450 2B1 and 2B2, 1A1 anʃ 1A2, and ⊲(others (Guengerich, 1982). This reaction can also be carried out by prostaglandin synthase (Gibson, 1994). Isoamyl alcohol, unlike ethanol, effectively inhibited the deethylation of 7-ethoxycoumarin in rat liver microsomes. This reaction can be catalyzed by a number of different P450s, including 1A1 and 1A2, 2B1, 2A1, 2E1 and 2C11 (Guengerich, 1982; Funea, 1993). The lack of inhibition of this reaction by ethanol is probably a consequence of the minor contribution provided by P450 2E1

towards the metabolism of 7-ethoxycoumarin. Additionally, the lack of inhibition by either alcohol on the demethylation of aminopyrene argues against the possibility that the inhibition of 7-ethoxycoumarin deethylase by isoamyl alcohol is the result of an inhibition of P450 2C11. Isoamyl alcohol selectively inhibited the depentylation of pentoxyresorufin, a reaction catalyzed exclusively by P450 2B1 (Burke, 1985), but neither alcohol inhibited the deethylation of 7-ethoxyresorufin, a reaction catalyzed exclusively by P450 1A1 (Burke, 1985). Thus, it seems that the isoamyl alcohol inhibition of the deethylation of 7-ethoxycoumarin is due, to a great extent, to the affinity of this alcohol for P450 2B1. The deethylation of NDEA has been shown to be induced by phenobarbital treatment of rats (Lai, 1980), which is a known powerful inducer of P450 2B1 in the rat liver. Furthermore, isoamyl alcohol has been shown to induce P450s 2H1/H2 (the P450s expressed in chicken liver homologous to P450 2B1/B2) and 2E1 in cultured chick hepatocytes, and to increase the induction caused by ethanol of P450 2B1/B2 in cultured rat hepatocytes (Louis, 1993). This suggests the possibility that the liver and oesophageal metabolism of nitrosamines, which are inhibited by isoamyl alcohol is carried out, at least in part, by P450 2B1. However, Western blot analyses reported in Chapter 4 show that P450 2B1 is not expressed in the oesophagus so it cannot be responsible for nitrosamine metabolism in that organ.

The results presented here and those published by Craddock and Henderson (Craddock, 1991) on the inhibition of NMBzA metabolism in liver and oesophagus by isoamyl alcohol, do not support the view that the isoamyl alcohol in Calvados enhances the incidence of oesophageal cancer through an effect on the nitrosamines to which man is exposed. However, some other chemical present in apple brandy may influence the incidence of oesophageal cancer. In support of this view is the study of Yamada et al (Yamada, 1992). They showed that among several alcoholic beverages diluted to a 4% ethanol concentration, only branded Calvados and red burgundy wine produced a statistically significant increase in the levels of O^6-methylguanine produced by NMBzA (2.5 mg/kg b.w. oral) in oesophageal DNA over that in rats which received the

nitrosamine in 4% aqueous ethanol. Additionally, farm-made Calvados diluted to a 20%

ethanol concentration produced a significantly higher increase in the levels of

O^6-methylguanine in oesophageal DNA than did aqueous ethanol.

CHAPTER 3

THE INFLUENCE OF MORPHINE AND OPIUM ON THE DISTRIBUTION AND METABOLISM OF NITROSAMINES

INTRODUCTION

A particular characteristic of oesophageal cancer is that great differences in the incidence of the disease can occur close together with a sharp demarcation between the areas of high and of low incidence (Craddock, 1993). One of the highest rates of incidence of oesophageal cancer in the world is in Northeast Iran, inland from the Caspian sea. In this area there is a 30-fold variation in the rates among women and a 10-fold variation among men across the region. (Mahboubi, 1973). The highest incidence is seen on the Northeast part of the region. (109 cases / 100 000 for men; 174 cases / 100 000 for women) (Kmet, 1972). Moving down the coast of the Caspian Sea the incidence rates decline and the disease becomes more prevalent in men than women (Mahboubi, 1973). Epidemiological studies have associated the high-incidence of oesophageal cancer with a poor diet, profoundly deficient in vitamin C, riboflavin and vitamin A (IARC, 1977). Nutritional deficiency is known to contribute to moderately high rates of oesophageal cancer, without gender discrimination and is commonly associated with increased exposure to endogenously formed nitrosamines (Craddock, 1993). Another factor which showed a striking correlation with the incidence rates of the disease along the region was opium consumption (IARC, 1977). Because it is difficult to assess opium consumption through the answer to questionnaires, it was assessed by measuring morphine metabolites in the urine. Over 55% of men and 30% of woman over 50 years old living in high incidence areas of the disease had levels of morphine metabolites ≥ 1 µg/ml urine compared to 13.5% for men and 11.6% for women living in low-incidence areas (Ghadirian, 1985). For people under 50 years old, 6 times more people had ≥ 1 µg/ml morphine metabolites in their urine in the very high and high incidence areas, compared to those living in low incidence areas (Ghadirian, 1985). People living in

houses with a case of oesophageal cancer had a higher chance of having morphine metabolites in their urine than did people living in control houses in the same village (Ghadirian, 1985). The official daily ration for addicts in the area is 3 g of opium/day/person (Hewer, 1978).

No exposure to any particular carcinogen was found in the region, but the only nitrosamines studied were NDMA and NDEA, the content of which in the diet was measured by gas-chromatography (Hewer, 1978). A more accurate and sensitive procedure would have been to have measured the levels of not only NDMA and NDEA, but also of other volatile nitrosamines using the Thermal Energy Analyzer (Fine, 1975). Furthermore since some particular populations with a high incidence of oesophageal cancer have been shown to be exposed to higher amounts of endogenously formed nitrosamines (reviewed by Bartsch, 1989), it is a pity that the NPRO test (see Introduction) has not been applied in this part of Iran to provide a more complete assessment of exposure to nitrosamines.

Dichloromethane extracts from opium pyrolysate, but not from crude opium, were shown to be mutagenic to *Salmonella typhimurium* 98 upon metabolic activation by rat S9 liver fraction (Hewer, 1978). These mutagens were shown to be formed during the pyrolysis of opium from its main alkaloid, morphine (Malaveille, 1982). Nine mutagens were isolated from morphine pyrolysate and characterised. They all contain a hydroxyphenanthrene ring (Friesen, 1985; Friesen, 1987). One of the mutagens was able to be nitrosated *in vitro*, forming a N-nitroso compound which had a 4-fold higher mutagenic activity than its parent compound (Friesen, 1985). Opium and morphine pyrolysates were shown to be effective in transforming Syrian hamster embryo cells, but not other cell cultures (Friesen, 1985). They were also not effective in producing tumours either when injected into or fed to rodents (Friesen, 1985). They also failed to produce tumours when applied to the mouse skin (Friesen, 1985). These experiments suggest that these compounds have biological activity but they give no clear reason for

the organotropism of the opium pyrolysate mutagens for the oesophagus.

The main factor associated with high rates of oesophageal cancer in the West, ethanol, has been shown to produce a dramatic effect on the distribution and metabolism of many nitrosamines, often with an increase in exposure of extrahepatic organs (Swann, 1987). This effect has been suggested as the mechanism for the influence of ethanol on oesophageal cancer and it seemed possible that the apparent association of opium and oesophageal cancer might have a similar mechanism. For this reason the influence of opium on the distribution and metabolism of NDMA and NDEA was studied.

Initially the effect on the pharmacokinetics of orally administered [^{14}C]-NDMA (30 μg/kg b.w.) were studied. At this dose, the methylation of guanine at the N7 position in kidney DNA, is at the limit of detection due to extensive first pass clearance (Diaz Gomes, 1977; Swann, 1984b). This nitrosamine is neither carcinogenic towards (Druckrey, 1967), nor metabolized by, the rat oesophagus (van Hofe, 1987), although it is well metabolized by human oesophageal cultured cells (Autrup, 1982). However, it is easily synthesized and its pharmacokinetic parameters as well as the enzymology of its metabolism are well known when compared to other nitrosamines. Following these experiments, experiments with NDEA, which is an oesophageal carcinogen to the rat, were carried out.

Because morphine sulphate is easier to use than opium and because most of the properties of opium are due to the pharmacological effects of morphine, which is the main alkaloid in opium and represents about 20% of the dry weight, morphine was used in most of the experiments. The oral route was avoided since this alkaloid is subjected to extensive first-pass clearance and as morphine is rapidly absorbed and distributed when it is injected subcutaneously, this route of administration was used.

The distribution, metabolism and excretion of morphine has been reviewed a number of

times (Miller, 1954; Way, 1960; Misra, 1978; Autrup, 1982). Following subcutaneous or intravenous injections, morphine is rapidly absorbed and distributed, being mainly concentrated in parenchymatous tissues such as kidney, liver , lung and spleen. Brain, skeletal muscle and other tissues can also concentrate this alkaloid, but to a much smaller extent than the tissues previously mentioned. The kidney has a particular capacity to concentrate the alkaloid. Plasma levels of this alkaloid administered by oral route are about 5 times lower than when administered intravenously, due to the extensive first-pass clearance to which this drug is subjected but the plasma half-life of morphine is not altered by the route of administration. The development of tolerance in rats also does not alter the half-life. Renal and biliary excretion are the most important routes of elimination. Within 89 hours about 65% of the morphine administered to rats is excreted in the urine as conjugates, with a minor part being excreted as free morphine. There is substantial excretion in the bile but only 20% of the morphine dose is excreted in the faeces due to the enterohepatic circulation. Most of morphine is excreted as its 3-glucuronide. Morphine can also be metabolized by a number of other routes, producing various metabolites, some with pharmacological activities such as its 6-glucuronide. 15% of the dose is N-demethylated by the monooxygenase system.

MATERIALS AND METHODS

NITROSAMINES

[^{14}C]-NDMA was synthesized from di[^{14}C]methylamine (57 mCi/mmol, Amersham) by Dr. Peter F. Swann using the method of Dutton and Heath (Dutton, 1956). The purity of the radioactive nitrosamine was checked by HPLC using a ZORBAX ODS C18 column (Dupont) eluted at 1 ml/min with water. 1 minute fractions were collected and the absorbance at 230 nm of the fractions measured in a spectrophotometer (Hitachi 1100). 4 ml of scintillation cocktail added to each fraction and counted for 1 minute on a ^{14}C programme. 95% of the radioactivity eluted as a single peak coincident with unlabelled NDMA (Eastman Kodak). The recovery of the radioactivity from the column was 89%. The concentration of [^{14}C]-NDMA to be used in the *in vivo* experiments were achieved by mixing the correct proportions of the labelled and unlabelled nitrosamine. [2-^{3}H]-NDEA was synthesized and its purity checked as described in the previous chapter.

EFFECT OF MORPHINE ON THE RELATIVE ALKYLATION OF THE DNA OF LIVER AND OF THE EXTRAHEPATIC ORGANS, THE KIDNEY AND LUNG BY [^{14}C]-NDMA.

In order to see the influence of different doses of morphine sulphate (Sigma) on the distribution and metabolism of [^{14}C]-NDMA in the rat, the amount of N7[^{14}C]-methylguanine produced from the metabolism of [^{14}C]-NDMA was measured in the DNA of different organs of rats in the experiments described below. In all the experiments the rats were killed 4 hours after the dose of NDMA. As the rate of metabolism of NDMA is 6 mg/kg/hour (Heath, 1962) the dose of NDMA given to rats would have been completely metabolized after 4 hours.

Experiment 1 - Male Sprague Dawley rats (200 ± 20 g) were starved overnight and the following morning six rats were given morphine sulphate (143 µg/kg b.w.) subcutaneously (injected in the femur) in 200 µl water, and six rats were subcutaneously administered water. This dose of morphine is approximately equivalent to the therapeutic dose of 10 mg to a 70 kg man. After 45 minutes, 30 µg [^{14}C]-NDMA/kg b.w. (5.8 µCi/rat) in 1 ml water was given orally by a tube passing deep down the oesophagus. After 4 hours the rats were killed by CO_2 asphyxiation, dissected and had their livers, kidneys and lungs removed and frozen as described in the previous chapter.

Experiment 2 - Experiment 2 was done exactly as experiment 1, except the dose of morphine sulphate was 30 mg/kg b.w.

Experiment 3 - Experiment 3 was done exactly as experiment 1, except 3 different doses of morphine sulphate were given, ie 6 mg/kg; 18 mg/kg; and 30 mg/kg b.w. respectively.

In all 3 experiments, DNA was extracted from the organs and analyzed as described in the previous chapter. DNA was extracted from lungs by the same method of extraction as for oesophagus. Yields of DNA extracted from the organs were similar as in the previous chapter. The yield of DNA extracted from lungs was 3 mg DNA/g tissue.

EFFECT OF MORPHINE AND OPIUM ON THE RELATIVE ALKYLATION OF DNA OF LIVER, KIDNEY AND OESOPHAGUS BY [2-^{3}H]-NDEA

The effect of morphine sulphate and opium extract on the amount of N7[^{3}H]-ethylguanine produced from the metabolism of [2-^{3}H]NDEA was quantitated in the DNA of different organs of the rat in the following experiments:

Experiment 1 - Male Sprague Dawley rats (200 ± 20 g) were starved overnight and the following morning divided in 4 groups each of 16 rats.

Group 1 (Control): rats were injected with 200 μl water subcutaneously and 45 minutes later they received [2-³H]NDEA (3 mg/kg b.w.; 149 μCi/rat) in 1 ml water orally by a tube passing deep down into the oesophagus. 4 hours later the rats were killed by CO_2 asphyxiation and the livers, kidneys and oesophagus removed and frozen as previously described. The DNA was extracted from these organs and analyzed as previously mentioned. Yields of DNA extracted from the organs were similar as previously mentioned.

Group 2: rats were given morphine sulphate (20 mg/kg b.w.) delivered in 200 μl water and injected subcutaneously in the femur. 45 minutes later they received [2-³H]-NDEA similarly as in group 1 and the experiment completed as in group 1.

Group 3: rats were injected morphine sulphate (20 mg/kg b.w.) subcutaneously in the femur in 200 μl water on 8 successive days. 45 minutes after the last injection of morphine sulphate, rats were given the same dose of [2-³H]-NDEA as group 1 and the experiment completed as before.

Group 4: rats were injected with morphine sulphate (20 mg/kg b.w.) subcutaneously in the femur in 200 μl water for 8 successive days. 24 hours after the last injection of morphine sulphate, rats were given the same dose of [2-³H]-NDEA as for group 1 and the experiment completed as before.

Experiment 2 - Male Sprague Dawley rats were starved overnight and the following morning divided into 5 groups each of 19 rats.

Group 1: rats were injected with morphine sulphate (20 mg/kg b.w.) in 200 μl water subcutaneously in the femur;

Group 2: rats were injected with morphine sulphate (10 mg/kg b.w.) in 200 μl water;

Group 3: rats were injected with morphine sulphate (5 mg/kg b.w.) in 200 μl water;

Group 4: rats were injected with 200 μl opium extract;

Group 5 (control), injected with 200 μl water.

45 minutes later the rats received [2-³H]-NDEA (3 mg/kg b.w., 214 μCi/rat) in 1 ml water, orally by a tube passing deep down into the oesophagus. 4 hours later the rats

were killed by CO_2 asphyxiation, the organs were removed and frozen and DNA was then extracted, hydrolysed and analyzed as previously described.

EFFECT OF MORPHINE SULPHATE ON THE HEPATIC METABOLISM OF NDEA *IN VITRO*

NDEA was incubated with liver microsomes (prepared as in Chapter 2) and the production of acetaldehyde was measured exactly as described in the previous chapter, except that different concentrations of the substrate (NDEA) (see table 3.6) and a different inhibitor (morphine sulphate) were used.

In a separate experiment, male Sprague Dawley rats (200 g) were starved overnight and the following morning injected with either morphine sulphate (20 mg/kg b.w.) subcutaneously in the femur in 200 µl water, or with 200 µl water. 1 hour later the rats were killed by cervical dislocation, the livers perfused with ice-cold 1.15% KCl (w/v) and hepatic microsomes prepared as described in the previous chapter. The hepatic microsomes of the morphine sulphate treated, and of the untreated rats were then incubated with different concentrations of NDEA and the production of acetaldehyde was measured exactly as described in the previous chapter.

PREPARATION OF OPIUM EXTRACT

An opium extract was prepared for injection into rats. 250 mg of crude opium (obtained from Iran through The International Agency For Research on Cancer) was mixed with 4.5 ml 0.1 M acetic acid and vigorously shaken. The mixture was centrifuged at 1000 x g for 2 minutes and the supernatant transferred to a clean test tube and neutralized with 0.45 ml 1 N NaOH.

ANALYSIS OF THE MORPHINE CONTENT OF THE OPIUM EXTRACT

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The morphine content of opium was measured by HPLC using as standards a 5 mg/ml; 10 mg/ml; and 20 mg/ml solution of morphine sulphate. 20 µl of the opium extract or 20 µl of one of the standard solutions, was injected into an HPLC system (Varian 3000) and the morphine separated by the use of a Novapak C18 column (4 µm; 8 x 100 mm; Waters U.K.) using 35% acetonitrile : 0.75% ammonium acetate as the eluent (1 ml/min) and monitoring the absorbance at 280 nm. The morphine eluted as a single peak and the peak area was integrated and quantitated by the use of the chromatography integrator system described in the previous chapter for the integration of the peak of the acetaldehyde derivative produced in that experiment. The amount of morphine in the opium extract was calculated by comparing the peak area with the peak area of the standards.

Three experiments were done in order to study the effect of morphine sulphate on the distribution and metabolism of NDMA *in vivo* in the rat.

Table 3.1 shows the result of the analysis of DNA hydrolysates of liver, kidney and lung of rats which were injected with 200 µl water (control) or morphine sulphate (143 µg/kg b.w. - the equivalent of a therapeutic dose of 10 mg to a 70 kg man - subcutaneously in 200 µl water followed 45 minutes later by oral administration of [^{14}C]-NDMA (30 µg/ kg b.w.) in 1 ml water. The results shown on table 3.1 show that there was a small decrease (15%) of N7-methylguanine levels in the liver DNA of rats which were injected with the morphine sulphate solution when compared to control. By contrast, no difference in the levels of N7-methylguanine was seen in the kidney or lung DNA of rats which were pretreated with morphine sulphate when compared to rats which were injected with water before NDMA. As was discussed in chapter 2 the amount of both adenine and guanine eluted from the column was measured. This showed (table 3.1) a greater sample to sample variation than one would have hoped for. The reason for that is not known, however it does not alter the conclusion that this dose (143 µg/kg b.w.) of morphine sulphate has no significant effect on this dose (30 µg/kg b.w.) of NDMA.

In experiment 2, rats were injected 0.2 ml water (control) or morphine sulphate (30 mg/kg b.w.) subcutaneously in 0.2 ml water followed 45 minutes later by administration of [^{14}C]-NDMA (30 mg/kg b.w.) in 1 ml water. This dose of morphine sulphate has been previously used to study the effect of this alkaloid on the capacity of the rat liver to N-demethylate drugs (Wladislawa, 1986). Rats given the morphine sulphate were visibly affected by this compound, displaying all the effects mentioned in the literature, such as walking in circles, muscle contraction and extensive torpor. When they were killed, their bladder was extremely dilated and full of urine. 1 rat out of 6

Table 3.1: Experiment 1: effect of 143 µg morphine sulphate/kg b.w. (S.C.) on the methylation of DNA of liver, kidney, and lung by 30 µg [^{14}C]-NDMA/kg (oral)

DNA sample + treatment	µmoles N7MG / mol G	Ratio to control	Guanine OD peak	Adenine OD peak	Guanine µmoles	Adenine µmoles	A/G ratio	N7MG dpm	N7MG pmoles
Liver Control	9.17	——	23.96	42.05	3.24	3.17	0.98	1877.26	29.72
Liver + morphine	7.84	0.85	25.52	47.52	3.45	3.58	1.04	1703.4	27.04
Kidney Control	0.29	——	39.8	101.5	5.38	7.65	1.42	97.84	1.55
Kidney + morphine	0.28	0.97	44.06	106.5	5.95	8.03	1.35	104.3	1.66
Lung Control	0.39	——	50.53	99.27	7.42	8.21	1.11	183.97	2.92
Lung + morphine	0.39	1.0	37.40	85.21	5.5	6.94	1.26	133.75	2.12

which received the morphine sulphate solution died, probably due to respiratory problems caused by the morphine. By the time they were killed, the rats seemed to have completely recovered from the torpor caused by the alkaloid. Table 3.2 shows the result of the analysis of the DNA of liver, kidney and lung. There was a clear reduction in the levels of N7-methylguanine in the liver DNA of rats which were injected with morphine sulphate when compared to rats treated with water (31% reduction). By contrast, the levels of N7-methylguanine in the kidney DNA of rats pretreated with morphine sulphate were 3.3-fold higher when compared to the respective control. The levels of N7-methylguanine in lung DNA of rats pretreated with morphine sulphate were 2.32-fold higher than the respective control. Again, the adenine : guanine ratios (table 3.2) were similar for liver and kidneys but not for lungs, suggesting the possibility that the amount of guanine may have been underestimated, a factor which may lead to overestimation of the methylation of lung DNA of the different treated groups of animals. However even if the same ratio of adenine : guanine is applied to the lung DNA of both groups of rats, the levels of N7-methylguanine in the lung DNA of rats pretreated with morphine sulphate is still 1.6-fold higher than the respective levels in the controls.

To study the dose-response relationship between the dose of morphine and the increase in methylation of kidney DNA caused by NDMA, experiment 3 was carried out. Rats were injected with 0.2 ml water or morphine sulphate (5; 18 or 30 mg/kg b.w.) subcutaneously in 0.2 ml water followed 45 minutes later by administration of [^{14}C]-NDMA (30 µg/kg b.w.) orally in 1 ml water. Table 3.3 shows the results of the analysis of the DNA from the liver and kidney of rats which received the different pretreatments. There was a clear, dose related, *reduction* in the levels of N7-methylguanine in the liver DNA of rats which received increasing doses of morphine sulphate (ie with 6 mg morphine/kg, methylation = 99% of control; with 18 mg/kg, methylation = 80%; with 30 mg/kg methylation = 59%). This was accompanied by an *increase* in the levels of N7-methylguanine in kidney DNA of rats receiving increasing

Table 3.2: Experiment 2: effect of 30 mg morphine sulphate/kg b.w. (S.C.) on the methylation of DNA of liver, kidney, and lung by 30 μg [^{14}C]-NDMA/kg (oral)

DNA sample + treatment	μmoles N7MG / mol G	Ratio to control	Guanine OD peak	Adenine OD peak	Guanine μmoles	Adenine μmoles	A/G ratio	N7MG dpm	N7MG pmoles
Liver Control	7.27	____	29.95	41.39	4.11	3.21	0.78	1900.26	29.93
Liver + morphine	4.98	0.69	26.83	48.50	3.98	3.84	0.96	1259.09	19.83
Kidney Control	0.24	____	49.14	103.81	7.29	8.45	1.16	110.7	1.74
Kidney + morphine	0.79	3.29	49.54	108.79	7.22	8.62	1.19	363.81	5.73
Lung Control	0.31	____	33.20	67.18	4.73	5.0	1.06	93.44	1.47
Lung + morphine	0.72	2.32	20.95	58.25	2.95	4.54	1.54	137.5	2.14

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Table 3.3: Experiment 3: Relationship between dose of morphine sulphate (6, 18, 30 mg/kg b.w.) and the effect on DNA methylation by NDMA (30 µg/kg b.w.) in liver and kidney

DNA sample + treatment	µmoles N7MG / mol G	Ratio to control	Guanine OD peak	Adenine OD peak	Guanine µmoles	Adenine µmoles	A/G ratio	N7MG dpm	N7MG pmoles
Liver Control	12.26	___	19.46	43.99	2.74	3.42	1.25	2165.85	33.58
Liver + 6 mg/kg morphine sulphate	12.09	0.99	27.13	58.80	3.85	4.61	1.2	2999.47	46.50
Liver + 18 mg/kg morphine sulphate	9.81	0.80	27.56	58.31	3.82	4.57	1.2	2415.82	37.45
Liver + 30 mg/kg morphine sulphate	7.21	0.59	25.23	57.69	3.59	4.55	1.27	1670.36	25.89
Kidney Control	0.17	___	54.83	163.78	7.83	12.86	1.64	86.99	1.35
Kidney + 6 mg/kg morphine sulphate	0.36	2.1	48.39	133.58	6.85	10.40	1.52	158.05	2.45

DNA sample + treatment	μmoles N7MG / mol G	Ratio to control	Guanine OD peak	Adenine OD peak	Guanine μmoles	Adenine μmoles	A/G ratio	N7MG dpm	N7MG pmoles
Kidney + 18 mg/kg morphine sulphate	0.62	3.6	63.10	137.43	8.69	10.15	1.17	348.07	5.40
Kidney + 30 mg/kg morphine sulphate	0.72	4.2	60.89	148.07	8.83	11.69	1.35	409.84	6.35

doses of morphine sulphate (ie with 6 mg morphine/kg b.w., methylation = 2.1 times control; with 18 mg/kg, methylation = 3.6 times; and with 30 mg/kg, methylation = 4.2 times control).

The experiments reported above show that morphine substantially alters the pharmacokinetics of NDMA in the rat. However this nitrosamine is not metabolized by the rat oesophagus, so this experiment gave no information on the possible influence of this alkaloid on nitrosamines which are oesophageal carcinogens. For this reason the influence of morphine on the distribution and metabolism of [2-^{3}H]-NDEA (a nitrosamine carcinogenic for rat oesophagus) in the rat was studied by measuring the end product N7-ethylguanine in the DNA of liver, kidney and oesophagus as mentioned in Chapter 2.

The first experiment was designed to study the influence of acute and subacute pretreatment with morphine sulphate (20 mg/kg b.w.) Rats received either water, one dose of morphine sulphate or eight doses of morphine sulphate (each dose - 20 mg/kg b.w./day) subcutaneously in 0.2 ml water. These rats were given [2-^{3}H]-NDEA (3 mg/kg b.w.) orally in 1 ml water 45 minutes after the single dose of morphine sulphate, 45 minutes after the last of the eight doses of morphine sulphate, or 1 day after the last of the eight doses of morphine sulphate, and in all cases were killed four hours later. A similar dose schedule of morphine sulphate has been previously used in order to study the effect of this alkaloid on the N-demethylation of drugs in the rat liver (Wladislawa, 1986). The rats receiving daily doses of morphine sulphate had become more resistant to the effects of the alkaloid on their behaviour by the time the last doses of morphine were given, and showed agitated behaviour instead of the torpor observed after a single dose of this alkaloid.

Table 3.4 shows the analysis of the DNA hydrolysate from liver, kidney and oesophagus of rats which received different pretreatments of morphine followed by oral

Table3.4: effect of single and repeated doses of morphine sulphate (20 mg/kg b.w. - S.C.) on alkylation produced by NDEA (3 mg/kg b.w. - oral) in DNA of liver, kidney, and oesophagus

Group 1: one subcutaneously injection of morphine sulphate (20 mg/kg b.w.) 45 minutes before administration of NDEA.

Group 2: 8 subcutaneous doses of morphine sulphate (20 mg/kg b.w.) on 8 days, with NDEA given 45 minutes after the last dose.

Group 3: 8 subcutaneous doses of morphine sulphate (20 mg/kg b.w.) on 8 days, with NDEA given 1 day after the last dose.

DNA sample	μmoles N7EG / mol G	Ratio to control	DNA mg	Guanine OD peak	Adenine OD peak	Guanine μmoles	Adenine μmoles	A/G ratio	N7EG dpm	N7EG pmoles
Liver - Control 1	9.2	____	8.5	26.88	57.16	3.98	4.74	1.19	1170	36.57
Liver - Control 2	10.1	____	7.8	25.92	58.88	3.75	4.79	1.28	1214	37.94
Liver - group 1	5.0	0.52	7.5	24.46	53.42	3.60	4.33	1.20	578	18.06
Liver - group 1	5.3	0.55	7.3	30	66.54	4.31	5.42	1.26	724	22.63
Liver - group 2	6.8	0.70	7	25.34	54	3.77	4.44	1.19	815	25.47
Liver - group 3	8.7	0.90	7.6	28.98	62.08	4.26	5.10	1.20	1181	36.92

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DNA sample	μmoles N7EG / mol G	Ratio to control	DNA mg	Guanine OD peak	Adenine OD peak	Guanine μmoles	Adenine μmoles	A/G ratio	N7EG dpm	N7EG pmoles
Kidney - Control	0.37	——	27	42.56	93.24	12.57	15.17	1.21	147.77	4.61
Kidney - group 1	0.17	0.47	50	65.08	156.52	18.95	25.57	1.34	104.38	3.26
Kidney - group 2	0.23	0.63	23	37.8	84.06	12.06	13.7	1.14	89.97	2.81
Kidney - group 3	0.31	0.85	55	71	152.76	20.96	24.96	1.19	210.19	6.57
Oesoph-Control	0.71	——	3	9.46	16.56	1.37	1.35	0.98	31.3	0.98
Oesoph - group 1	2.0	2.82	4.16	14.09	25.63	2.04	2.08	1.02	130.54	4.08
Oesoph - group 2	1.01	1.42	4.46	15.93	28.21	2.3	2.3	1.0	74.39	2.32
Oesoph - group 3	0.86	1.21	5.39	18.21	33.21	2.65	2.68	1.01	72.58	2.27

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administration of [2-^{3}H]-NDEA. This experiment was carried out in parallel with the *in vivo* experiment in Chapter 2, so the controls presented are the same. As sufficient liver DNA of rats which received a single dose of morphine sulphate as pretreatment was available, the DNA was hydrolysed and chromatographed twice. Table 3.4 also shows the influence of the morphine pretreatment on the N7-ethylguanine levels of in the DNA of liver, kidney and oesophagus. Rats which received a single dose of morphine sulphate had a 2.8-fold increase in the levels of N7-ethylguanine in their oesophageal DNA when compared to controls, and a 43 % and 53 % reduction in the levels of N7-ethylguanine in the liver and kidney DNA respectively. Rats which received 8 daily doses of morphine sulphate and NDEA 45 minutes after the last dose had a smaller increase (1.4-fold) in the level of N7-ethylguanine in their oesophageal DNA than with the previous treatment. This was accompanied by a 36% and 37% reduction in the levels of N7-ethylguanine in liver and kidney DNA respectively. By contrast when the nitrosamine was given one day after the last of the 8 daily doses of morphine sulphate, there was an insignificant increase in the levels of N7-ethylguanine in oesophageal DNA (only 1.2-fold increase) with an almost undetectable decrease in the levels of N7-ethylguanine in the liver and kidney DNA (5% and 15% decrease, respectively).

These results (tables 3.2 and 3.4) show that a single dose of morphine sulphate (20 mg/kg b.w.) changes the pharmacokinetics of NDMA and NDEA quite drastically. However, repeated administration of this alkaloid before administration of NDEA produced a smaller effect on the distribution and metabolism of NDEA than one single dose. The difference between the effect of 8 doses of morphine on NDEA when it was given 45 minutes after the last dose and the effect of 8 doses of morphine when NDEA was given 24 hours after the last dose show that the presence of morphine in the animal body is probably essential for the changes in NDEA pharmacokinetics.

Subsequently, an experiment was done in order to study the effect of *different doses* of morphine on the alkylation of the DNA of different organs by NDEA. An opium extract

Table 3.5: effect of opium (50 mg/kg b.w.) or morphine sulphate (5, 10, 20 mg/kg b.w.) on the ethylation of liver, kidney and oesophagus DNA by [2-³H]-NDEA (3 mg/kg b.w. - oral)

DNA sample + treatment	μmoles N7EG / mol G	Ratio to control	DNA mg	Guanine OD peak	Adenine OD peak	Guanine μmoles	Adenine μmoles	A/G ratio	N7EG dpm	N7EG pmoles
Liver Control	14.89	___	9.6	39.76	83.07	5.8	6.78	1.17	2677	86.35
Liver Control	15.33	___	6.7	31.6	69.14	4.51	5.59	1.24	2143	69.13
Liver + 5 mg morphine/kg	13.57	0.90	7.2	34.06	70.80	. 5.05	5.80	1.15	2125	68.55
Liver + 10 mg morphine/kg	14.24	0.94	6.6	30.72	62.99	4.52	5.18	1.15	1995	64.35
Liver + 20 mg morphine/kg	11.79	0.78	11.6	51.48	113.02	8.12	9.30	1.15	2970	95.81

DNA sample + treatment	μmoles N7EG / mol G	Ratio to control	DNA mg	Guanine OD peak	Adenine OD peak	Guanine μmoles	Adenine μmoles	A/G ratio	N7EG dpm	N7EG pmoles
Liver + 20 mg morphine/kg	12.17	0.81	7.26	33.81	73.41	4.97	5.98	1.20	1874	60.45
Liver + Opium	15.05	1.0	5.25	24.42	55.49	3.59	4.52	1.26	1675	54.03
Kidney Control	0.71	——	40	85.55	180.35	25.42	29.66	1.17	557.5	17.98
Kidney + 5 mg morphine/kg	0.65	0.92	41	87.11	183.98	25.60	30.13	1.18	513.9	16.58
Kidney + 10 mg morphine/kg	0.48	0.68	50	106.69	218.32	31.50	35.99	1.14	472.8	15.25
Kidney + 20 mg morphine/kg	0.64	0.90	63	112.94	229.87	32.36	37.84	1.17	642.26	20.72

DNA sample + treatment	μmoles N7EG / mol G	Ratio to control	DNA mg	Guanine OD peak	Adenine OD peak	Guanine μmoles	Adenine μmoles	A/G ratio	N7EG dpm	N7EG pmoles
Kidney + Opium	0.69	0.97	35	82.03	171.39	24.22	28.16	1.16	518.9	16.74
Oesophagus Control	1.39	——		23.39	38.02	3.45	3.10	0.89	148.18	4.78
Oesophag. + 5 mg morphine/kg	2.61	1.88	7.6	22.86	35.30	3.38	2.81	0.88	197.47	6.37
Oesophag. + 10 mg morphine/kg	2.67	1.92	8.8	24.43	38.23	3.60	3.12	0.87	298.17	9.62
Oesophag. + 20 mg morphine/kg	3.06	2.2	6.2	25.29	39.02	3.74	3.20	0.86	354.72	11.44
Oesophag. + Opium	2.0	1.44	5.12	21.61	34.08	3.19	2.81	0.88	197.47	6.37

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was also injected into rats and its effect on the pharmacokinetics of NDEA compared to that of different doses of morphine. The dose of the opium extract prepared and given to rats (50 mg/kg b.w.) was calculated by assuming its morphine content as 20%. The extract was prepared by suspending an amount of opium in mild acid solution, centrifuging and neutralising the supernatant as described in materials and methods. The morphine content in the opium extract was analyzed by injecting 20 μl of the extract into a HPLC system, separating the morphine peak by reverse-phase chromatography, and relating the peak area to morphine sulphate standards. Figure 3.1 shows the elution profile of the opium extract and of 100 μg morphine sulphate (20 μl from 5 mg morphine sulphate/ml). The 100, 200 and 400 μg morphine standards gave a linear standard curve for the morphine peak area and the peak area in the opium extract was calculated to correspond to 183.6 μg (9.18 mg/ml) morphine (653 units-peak area for the opium extract). The experiment was then executed as follows: rats were injected with water, morphine sulphate (5, 10 or 20 mg/kg b.w.), or opium extract (containing morphine equivalent to a dose of 9.18 mg morphine sulphate/kg b.w.) subcutaneously in a volume of 0.2 ml. 45 minutes later the rats were given [2-^{3}H]-NDEA (3 mg/kg b.w.) orally in 1 ml water and killed 4 hours later. The procedure of DNA extraction, hydrolysis and chromatography followed as before. Table 3.5 shows the analysis of the DNA hydrolysate from liver, kidney and oesophagus of the animals. Since there was sufficient DNA from the liver of animals which received water or morphine sulphate as pretreatment, 2 DNA samples were hydrolysed and chromatographed. There was little difference between these duplicates. Table 3.5 also shows the influence of different doses of morphine or opium extract on the levels of N7-ethylguanine in the DNA of liver, kidney and oesophagus produced by NDEA. There was a marked *increase* in the levels of N7-ethylguanine in the oesophageal DNA as the morphine sulphate dose was increased, when compared to controls (88% for a dose of 5 mg/kg ; 92% for a dose of 10 mg/kg ; 120% for a dose of 20 mg/kg). The opium extract clearly produced a significant increase in the levels of N7-ethylguanine in the DNA of oesophagus (44%). However, the increase in the levels of N7-ethylguanine produced in oesophageal DNA

by the opium extract was smaller than that produced by a dose of 5 mg/kg morphine

sulphate. This is rather puzzling because the opium extract contained morphine

equivalent to 9.18 mg/kg b.w. and it suggests that the effect of opium is complex and

that components other than morphine may play a possibly competing role. 5 mg/kg

morphine sulphate did not produce any significant decrease in the levels of N7-

ethylguanine either in the liver or kidney DNA. 10 mg/kg morphine sulphate also did

not produce any significant decrease in the levels of N7-ethylguanine in liver DNA, but

it produced a 32% decrease in the levels of N7-ethylguanine in kidney DNA. 20 mg/kg

of morphine sulphate produced a 21% decrease in the levels of N7-ethylguanine in liver

DNA but failed to produce any significant decrease in the levels of N7-ethylguanine in

kidney DNA.

Figure 3.1: HPLC of 20 μl opium extract or 100 μg morphine sulphate on Novapack
C18 (100 x 4 mm) eluted with 35% acetonitrile : 0.75% ammonium acetate (1ml/min).
Peaks were detected by absorbance at 280 nm.

Opium extract   100 μg morphine sulphate
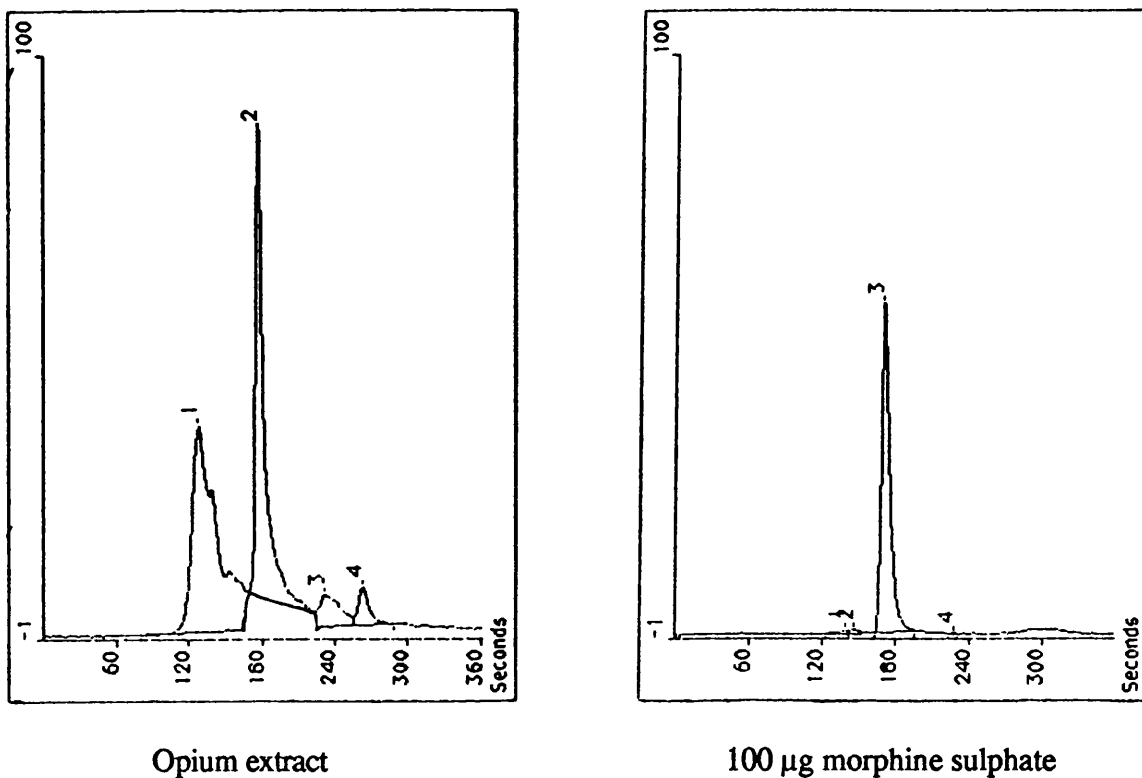
These results contrast sharply with those produced by a single dose of morphine

sulphate (20 mg/kg b.w.) in the previous experiment, which showed a marked decrease

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in the levels of N7-ethylguanine in the DNA of the liver (43% decrease) and kidney (53% decrease) compared to control rats. Additionally, opium also failed to produce any detectable decrease in the N7-ethylguanine levels in the DNA of either liver or kidney

The increase in the levels of N7-ethylguanine in the DNA of oesophagus were similar in both experiments (compare table 3.4 and 3.5) and these results confirm that there is a clear increase in the alkylation of guanine in the oesophageal DNA of rats which received morphine prior to administration of NDEA. This increase is somewhat related to the dose of morphine sulphate given to the animals, but small doses of morphine sulphate had a relatively more pronounced effect in increasing the levels of N7-ethylguanine in oesophageal DNA than did large doses of this alkaloid. It was also shown that an opium extract can have a similar effect on the distribution and metabolism of NDEA to that of morphine but that this effect can be only partially correlated to the amount of morphine present in the opium extract.

To find out whether the increase in exposure of the oesophagus to NDEA following morphine sulphate administration was due to an inhibition of the metabolism of this nitrosamine by the alkaloid in the rat liver, NDEA was incubated with liver microsomes and the rate of deethylation of this nitrosamine measured. The concentration of NDEA used was 50 μM which although higher than the expected *in vivo* concentration after administration of 3 mg/kg b.w. orally (7.5 μM), is still a low concentration in terms of *in vitro* studies, and allows a better measurement of the acetaldehyde produced from its deethylation. Three different concentrations of morphine sulphate were used: 0.132 mM, 0.66 mM and 1.32 mM. The dose of morphine sulphate given to the rat (20 mg/kg b.w.) would produce a maximum concentration in the liver of 0.63 mM (if all the compound was concentrated in the liver) or 0.031 mM (if the compound was evenly distributed in the animal). Morphine given subcutaneously disappears quickly from the blood, being concentrated in highly parenchymatous tissues such as liver, kidney and lung, although the concentration in kidneys is about 3 times higher that of the liver

(Miller, 1954).

Table 3.6 shows that there was no significant inhibition of the rate of deethylation of NDEA by any of the different concentrations of morphine sulphate used, thus showing that the mechanism by which morphine affects alkylation *in vivo* cannot be ascribed to an inhibition of NDEA metabolism in the liver by morphine itself. To investigate this further, the metabolism of NDEA (10 μM or 50 μM) by liver microsomes, prepared from either untreated rats or rats injected with morphine sulphate (20 mg/kg b.w.) subcutaneously in 0.2 ml water 75 minutes before being killed, was investigated. Table 3.7 shows the deethylation of NDEA (10 or 50 μM) by liver microsomes prepared from either untreated rats or morphine sulphate treated rats as described above. There was no significant difference between the metabolism of 10 μM NDEA by liver microsomes prepared from untreated rats and by microsomes from morphine treated animals. By contrast, when the deethylation of 50 μM NDEA was assessed there was a significant reduction (P = 0.05) in the metabolism of NDEA by liver microsomes prepared from morphine treated rats when compared to this metabolism by liver microsomes from untreated rats. The reason why a significant difference between the metabolism of NDEA by liver microsomes prepared from the two different groups of animals could be seen at 50 μM NDEA but not at 10 μM NDEA is probably because it is easier to measure the greater amount of acetaldehyde produced from the deethylation of 50 μM NDEA. The standard deviation in the rates of formation of acetaldehyde from the deethylation of 10 μM NDEA was about 58% (63% from untreated rat microsomes and 53 % from morphine sulphate microsomes), but the standard deviation for the rates of formation of acetaldehyde from the deethylation of 50 μM NDEA was only about 27 % (24 % from untreated rat microsomes and 29% from morphine sulphate treated rat liver microsomes). So, administration of a single dose of morphine sulphate (20 mg/kg b.w. subcutaneously) to male rats seems to reduce the capacity of the liver of these rats to metabolize NDEA (and perhaps NDMA), and this might explain, at least in part, the increase in the metabolism of NDEA seen in the oesophagus of rats (and perhaps the

Table 3.6: The effect of different concentrations of morphine sulphate on the deethylation of NDEA (50 μM) by liver microsomes.

Inhibitor concentration	CH_3CHO produced Peak Area Units	Rate (nmol CH_3CHO/min/mg protein)
none	757, 414, 566, 497, 526, 494	**0.650** (± 0.124)
0.13 mM morphine sulphate	444, 499, 515, 427, 442, 546	**0.578** (± 0.094)
0.66 mM morphine sulphate	352, 435, 459, 412, 452, 443	**0.515** (± 0.095)
1.32 mM morphine sulphate	821, 464, 467, 475, 489	**0.661** (± 0.135)

Rates are given as mean (± SD) of 3 experiments carried out in duplicates with liver microsomes prepared from 3 different rats.

The microsomal suspension (0.4 mg microsomal protein/ml) was incubated with NDEA (50 μM) for 10 minutes and the amount of acetaldehyde produced measured, as the 2,4-denitrophenylhydrazone derivative by HPLC. Details of the procedure are given in Materials and Methods.

Table 3.7: Deethylation of NDEA (10 μM or 50 μM) by liver microsomes prepared from untreated or morphine sulphate (20 mg/kg b.w. subcutaneously 75 minutes before killing the rats) treated rats.

Microsomes prepared from	[NDEA]	CH₃CHO produced Peak Area Units	Rate of metabolism (nmol CH₃CHO/min/mg protein)
Untreated rats	10 μM	137, 146, 170, 156, 322, 273, 47, 250, 264	**0.262** (± 0.166)
Morphine treated rats	10 μM	52, 170, 96, 247, 238, 362, 142, 155, 172	**0.243** (± 0.129) P = 0.741
Untreated rats	50 μM	305, 470, 436, 630, 586, 585, 416, 394, 468	**0.645** (± 0.157)
Morphine treated rats	50 μM	306, 273, 260, 420, 490, 545, 307, 324, 350	**0.491** (± 0.144) P = 0.046*

* is significant using unpaired two-tailed student *t* test.

Rates are given as mean (± SD) of 3 experiments done in triplicates with liver microsomes prepared from different animals.

Assay conditions are described in Materials and Methods.

increase in the metabolism of NDMA seen in the kidney of rats), although other factors

also probably contribute to the alteration in the pharmacokinetics of the nitrosamines, as

will be discussed further below.

DISCUSSION

Because morphine sulphate is easier to use than opium and because most of the properties of opium are due to the pharmacological effects caused by morphine, the main alkaloid in opium representing about 20% of the dry weight, morphine was used in most of the experiments. The nitrosamine was given initially 45 minutes after the morphine administration in order to observe any possible short time effect produced by the alkaloid on the distribution and metabolism of nitrosamines. N-demethylation of morphine by rat liver *in vivo* has been shown to start as early as 15 minutes after administration of 10 mg morphine sulphate/kg b.w. subcutaneously and to continue over 60 minutes. (Fishman, 1978).

Initially, the equivalent of the therapeutic analgesic dose for men (10 mg/ 70 kg b.w.) of morphine sulphate was injected into male rats (ie 140 µg/kg b.w.) and 30 µg/kg [^{14}C]-NDMA given orally 45 minutes later. NDMA given orally at this dose to rats suffers extensive first pass clearance and the methylation in organs other than liver is barely detectable (Diaz Gomes, 1977; Swann, 1984b). Any decrease in first pass clearance of the NDMA would produce a significant increase in methylation of DNA of extrahepatic organs such as lung and kidney. This small dose of morphine produced no detectable change in the methylation of DNA of liver, lungs or kidneys of rats which received morphine sulphate 45 minutes before the NDMA, when compared to rats which were injected with water (control). By contrast, when a similar experiment, using a larger dose of morphine sulphate (30 mg/kg b.w.) was carried out, the rats which received the morphine sulphate before the administration of NDMA had a 30% reduction in the methylation of the N7 position of guanine at their liver DNA, and a 3.3-fold and 2.3-fold increase in the levels of this adduct in the DNA of kidney and lung, respectively when compared to rats which were injected water before NDMA administration. Subsequently, the effect of different doses of morphine sulphate was assessed. There was a dose related decrease in the levels of N7-methylguanine in liver

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DNA and a dose related increase in the levels of this adduct in DNA of kidney with increasing doses of the alkaloid. Remarkably, smaller doses of the alkaloid produced relatively higher increases in the levels of N7-methylguanine in kidney DNA. Thus, the increase in the levels of N7-methylguanine in the DNA of kidney in rats which were given 6 mg/kg, 18 mg/kg and 30 mg/kg sulphate before NDMA was 2.1-fold, 3.6-fold and 4.2-fold respectively, when compared to rats administered water before NDMA. This latter figure is slightly higher than in the previous experiment (3.3-fold). The difference probably reflects inter-experimental variations. This increase in the N7-methylguanine levels in kidney DNA produced by administration of 30 mg/kg morphine sulphate can be compared to the 4.6-fold correspondent increase produced by coadministration of 240 mg ethanol/kg b.w. with 35 μg NDMA/kg b.w. (Swann, 1982). The change produced by ethanol is the result of inhibition of first-pass clearance of NDMA by this alcohol (Swann, 1984b), and the dramatic change morphine produces in the alkylation of extrahepatic organs by NDMA probably has the same mechanism.

Since NDMA is not metabolized by the rat oesophagus (van Hofe, 1987), it was decided to study the influence of morphine sulphate on the distribution and metabolism of NDEA, a liver and oesophageal carcinogen in the rat (Druckrey, 1967; Lijinsky, 1992). The first experiment was done in order to see the effect of different dosage schedules of morphine sulphate, and of different time intervals between the administration of the alkaloid and the nitrosamine on the alkylation of DNA of liver, kidney and oesophagus by NDEA. A single administration of morphine sulphate (20 mg/kg b.w.) 45 minutes before an oral dose of NDEA (3 mg/kg b.w.) produced a 2.8-fold increase in the levels of N7-ethylguanine in oesophageal DNA when compared to control rats. This administration of morphine sulphate also produced a sharp decrease in the levels of N7-ethylguanine in the DNA of liver (43%) and kidney (53%). However, when the morphine was given to rats for 8 days (20 mg/kg b.w./day subcutaneously - 1 dose/day) and the NDEA given 45 minutes after the last dose of the alkaloid, there was a much smaller although still significant increase in the levels of N7-ethylguanine in the

DNA of the oesophagus (44%) which was accompanied by a smaller but detectable decrease in the levels of N7-ethylguanine in the DNA of liver (26%) and kidney (37%). However, when the NDEA was given 24 hours after the eighth dose of morphine sulphate, there was no effect on the alkylation in any of the organs when compared to control rats.

From this experiment, it could be concluded that presence of the morphine or some metabolic effect of the presence of morphine or its metabolites is essential for the effect caused by this alkaloid on the pharmacokinetics of NDEA. There also seemed to be an adaptation to successive administrations of morphine which decreases the effect of the alkaloid on the distribution and metabolism of NDEA.

The effect of different doses of morphine sulphate or an opium extract given 45 minutes before the administration of NDEA was measured in another experiment. It had seemed that (table 3.5) small doses of the alkaloid produce a relatively higher effect on the pharmacokinetics of NDEA than larger doses. 5 mg/kg b.w. morphine sulphate produced a 1.9-fold increase in the levels of N7-ethylguanine in oesophageal DNA, but 30 mg/kg b.w. produced only a 2.2-fold increase. Although this figure is lower than the corresponding figure in the last mentioned experiment (2.8-fold), it is in the same order of magnitude and the difference can be explained by inter-experimental variation. The relationship between morphine dose and the reduction in the levels of N7-ethylguanine in the DNA of liver and kidney was not as well defined as the reduction of N7-methylguanine in DNA of liver produced by NDMA. The increase in levels of N7-ethylguanine in the oesophageal DNA produced by NDEA when morphine sulphate was also given was also less dramatic than the increase in the levels of N7-methylguanine in the DNA of kidney after NDMA and morphine administration. A factor which may contribute to this difference is the difference in dose of each nitrosamine. NDMA given orally at 30 µg/kg b.w. is subjected to strong first pass clearance by the liver which keeps exposure of other organs to a minimum (Diaz

Gomes, 1977; Swann, 1984b). Even a small decrease in the capacity of the liver to metabolize NDMA produces dramatic changes in the alkylation of DNA of the extrahepatic organs. By contrast, NDEA at 3 mg/kg b.w. is not subjected to strong first pass clearance (Swann, 1984b; Graves, 1993) and is distributed throughout the animal body (Heath, 1962). Thus, a similar change in hepatic metabolism of NDEA would produce less dramatic change in the alkylation of DNA by this dose of NDEA than by the smaller dose of NDMA.

The opium extract also produced an increase in the levels of N7-ethylguanine in the DNA of oesophagus (1.4-fold), however this increase was not directly proportional to its morphine content (equivalent to 9.18 mg morphine sulphate/kg b.w.), and was smaller than the increase in the levels of N7-ethylguanine in the oesophageal DNA produced by a 5 mg/kg morphine sulphate dose (1.9-fold). It might be that some of the other components of opium or their metabolites are able to inhibit the metabolism of NDEA by the oesophagus, or that the effect on the pharmacokinetics of this nitrosamine produced by morphine is counterbalanced by some other component present in opium. Nevertheless, opium did produce a significant increase in the levels of N7-ethylguanine in the DNA of oesophagus when compared to control rats and this effect may be attributed to its main alkaloid, morphine.

To see whether the effect of morphine sulphate on the pharmacokinetics of nitrosamines was due to the inhibition of their metabolism in the liver, the effect of this alkaloid on the deethylation of NDEA by liver microsomes was assessed. Morphine sulphate did not produce any significant decrease in the rates of acetaldehyde formed from the deethylation of NDEA by liver microsomes, even when a vast excess concentration of the alkaloid (1.32 mM) relative to its *in vivo* concentration was present, thus showing that although morphine can be N-demethylated *in vivo* and *in vitro* (Fishman, 1978), the P450s responsible for this reaction are not the same as those responsible for the deethylation of NDEA, and that the change produced by morphine sulphate on the

distribution and metabolism of NDEA or NDMA *in vivo* is not due to inhibition of the metabolism of these nitrosamines by morphine in the liver. The possibility that either a metabolite of morphine produced *in vivo* could inhibit the metabolism of NDEA in the liver, or that an *in vivo* effect produced by morphine and/or a metabolite would produce a decrease in the metabolism of NDEA in the liver was assessed by pretreating rats with a 20 mg morphine sulphate/kg b.w. given subcutaneously, killing the animals 75 minutes later, and preparing liver microsomes from them. The rates of deethylation of NDEA (10 μM or 50 μM) by liver microsomes prepared from control or rats treated in this way were then compared. There was no significant difference between control and treated microsomes in the deethylation of 10 μM NDEA, but the deethylation of 50 μM NDEA by liver microsomes from morphine treated rats was significantly decreased (24% decrease - P for unpaired two-tailed t test = 0.05) when compared to the deethylation of 50 μM NDEA by control liver microsomes. The limits of sensitivity of the method used does not allow the difference in the rates of deethylation of 10 μM NDEA by either type of liver microsomes to be confidently measured, and the non-significant result from this concentration of NDEA may be due to the relatively high standard deviation (about 58% of the rate).

From the experiment *in vivo*, it was clearly seen that morphine alters the metabolism or distribution of both NDMA and NDEA, producing increased alkylation of the DNA of extrahepatic organs. Small doses of the alkaloid produce a relatively greater increase. The experiment with NDEA shows that the presence of the alkaloid in the body or of a pharmacological effect caused by it seems essential for this effect. There is also an apparent adaptation to morphine as successive administrations of morphine have a decreasing effect on NDEA pharmacokinetics. Opium produced a similar effect to morphine sulphate, though its effect could not be related directly to the morphine content of the opium. *In vitro* results showed the morphine is not an inhibitor of P450 2E1 and other P450(s) previously believed to be responsible for the deethylation of NDEA, but none the less liver microsomes prepared from rats given a single dose of

morphine sulphate (20 mg/kg b.w.) had a reduced capacity to deethylate NDEA.

It is not yet clear how morphine produces its effect on nitrosamine metabolism. Chronic or subacute administration of morphine to mature male rats has been shown to reduce P450 levels in the liver (Sladek, 1974; Amzel, 1980; Daniel, 1986; Blanck, 1990a) and to reduce the rate of some reactions catalyzed by liver microsomes (Cochin, 1958; Sladek, 1974; Amzel, 1980; Finnen, 1984; Daniel, 1986; Blanck, 1990a; Blanck, 1990b). The reactions affected seemed to be mainly catalyzed by male specific P450s or by P450s predominantly expressed in the liver, for instance N-ethylmorphine demethylation (P450 2C11). The effect produced by morphine treatment occurred only on mature male rats and not in immature male or mature or immature female rats (Sladek, 1974; Finnen, 1984; Blanck, 1990a; Blanck, 1990b). This effect of morphine seems to be mediated through an action of the alkaloid on the pituitary gland since hypophysectomized male rats were not affected by morphine treatment (Finnen, 1984; Blanck, 1990a). So, one way in which morphine could decrease the metabolism of NDEA in the liver could be by affecting the level of P450s controlled by hormones. Other mechanisms may involve the decrease in the availability of oxygen to the liver and the availability of cofactors such as NADP to the hepatic monooxygenase system.

Chronic administration of morphine has also been shown to reduce the protein and also the lipids of the liver microsomal monooxygenase system, with a decrease in the rate of metabolism of p-chloro-N-methylaniline by liver microsomes (Amzel, 1980). Amzel *et al* concluded that this effect is not caused by a direct action of the opiate on the liver cell, since liver cells incubated for 2 hours with the alkaloid were not affected (Amzel, 1980). Although some reactions were affected by this chronic administration of morphine to rats, aniline hydroxylation by liver microsomes was not affected (Amzel, 1980). By contrast another study showed that a single dose of morphine sulphate to mice (30 mg/kg b.w.) reduced the capacity of liver microsomes to hydroxylate aniline (Nabeshima, 1983), although there was no reduction in any of the components of the

hepatic monooxygenase system. P450 2E1 is responsible to a great extent for both aniline hydroxylation and for NDEA deethylation in liver microsomes of rats and mice (Yang, 1991b) and the reported effect of a single dose, but not of chronic administration, of morphine on the hydroxylation of aniline in these two rodent species mirror the effect of the alkaloid on the metabolism of NDEA and NDMA which is described in this chapter, although at the moment a definite connection between them has not been established.

Nevertheless, morphine and opium can dramatically change the distribution of NDMA and NDEA, with a reduction of alkylation of liver DNA and a subsequent increase in the alkylation of DNA of extrahepatic organs by NDMA and NDEA. This effect mimics the one produced by ethanol on the distribution of these two nitrosamines (Swann, 1984b), although the mechanisms involved seem to be different. Ethanol also alters the organotropism of a number of nitrosamines, as discussed in Chapter 1 and 2. Ethanol is the main factor associated with oesophageal cancer in the West (Craddock, 1993), and its effect on nitrosamine carcinogenesis and pharmacokinetics has been suggested to be the mechanism by which it produces oesophageal tumours (Swann, 1982; Swann, 1984b; Swann, 1987). So, if this is correct, one might expect that opium to act in the same way, although further experiments on the influence of morphine and opium on nitrosamine carcinogenesis, and the elucidation of the mechanism by which morphine and opium alter the distribution of nitrosamines are required.

CHAPTER 4

THE OESOPHAGEAL MONOOXYGENASE SYSTEM: COMPARISON TO THE LIVER AND RELEVANCE TO NITROSAMINE CARCINOGENESIS

INTRODUCTION

Nitrosamines present a striking organotropism in their carcinogenic action (Druckrey, 1967). Since they are precarcinogens, they must be metabolically activated in order to exert their carcinogenic action (Magee, 1989). Because the ultimate carcinogenic metabolite is unstable, it cannot travel through the body, and thus the capacity of each organ to activate nitrosamines is the most important factor in dictating its susceptibility to these carcinogens. The activation of nitrosamines has been exhaustively shown to be the result of the hydroxylation of the α-carbon of one of the alkyl chains of the nitrosamine, a reaction which is metabolized by cytochrome P450 (Yang, 1991b). So, the expression in a particular tissue of cytochrome P450s capable of metabolizing a nitrosamine will determine the organotropism of that particular nitrosamine. A number of nitrosamines (particularly asymmetric dialkylnitrosamines) have been shown to have a striking organotropism for the rat oesophagus (Druckrey, 1967), but since very little is known about the oesophageal monooxygenase system, the exact differences between oesophagus and other tissues in the kinds of P450s and in their activity which is responsible for this organotropism can only be speculated. In this chapter the oesophageal microsomal monooxygenase system is studied in depth and compared to the liver. Some components of the monooxygenase system in both organs are quantified through spectrophotometric and Western blot studies. The expression of specific P450 isoforms as well as the reactions catalyzed by liver and oesophageal microsomes and the effect of inducers upon them are examined. Finally, the relevance of the results of this investigation to the organotropism of certain nitrosamines for the rat oesophagus is discussed.

MATERIALS AND METHODS

PREPARATION OF HEPATIC MICROSOMES

Rat liver microsomes were prepared by the method of Labuc and Archer (Labuc, 1982) with some modifications, essentially as described in Chapter 2 and 3. For spectrophotometric studies, the microsomal pellet, prepared as described in Chapter 2 was sedimented in homogenizing buffer (tris 50 mM / 1.15% KCl (w/v) pH 7.4) and reprecipitated by another centrifugation (60 minutes; 105000 x g) in order to reduce contamination by haemoglobin.

PREPARATION OF OESOPHAGEAL MICROSOMES

Rat oesophageal microsomes were prepared based on the method of Labuc and Archer (Labuc, 1982) with some modifications. Male Sprague Dawley rats (200 g) were starved overnight and killed the next morning by CO_2 asphyxiation. The oesophagus was removed and immediate immersed in ice-cold homogenizing buffer (50 mM tris / 1.15 % KCl (w/v) pH 7.4). Oesophageal mucosas were obtained exactly as described previously (Chapter 2) and the mucosas were frozen in liquid nitrogen. All subsequent steps were carried out at 4 °C. The mucosas were thawed, weighed, opened longitudinally and chopped. They were homogenized in a 15 ml glass/glass Duall (Kontes, New Jersey) motor-driven homogenizer in 5 ml homogenizing buffer/g mucosa with 30 passes at speed 6 (3 x 10 passes with a 20 second interval between each group of passes to prevent heating). The homogenate was transferred to 19.4 ml centrifuge tubes (polycarbonate bottle with Noryl cap assembly, Beckman), the barrel and pestle were washed with 2 ml homogenizing buffer/g mucosa and this washing pooled with the original homogenate. The homogenate was then centrifuged at 9000 x g for 20 minutes in a L7 Beckman Ultracentrifuge using either a 70.1 Ti or a 75 Ti

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Beckman rotor. The supernatant was saved and the pellet was resuspended in 3 ml homogenizing buffer/g mucosa by the use of a 10 ml Teflon/glass motor-driven homogenizer with 5 passes at speed 2. the resuspended pellet was then centrifuged at 9000 x g for 20 minutes. The supernatants were pooled and centrifuged for 105000 x g for 60 minutes. The final microsomal pellet was transferred to a 6 ml Teflon/glass homogenizer using a small spatula and Pasteur pipette and resuspended in 1 ml 50 mM tris / 20% glycerol pH 7.4 /g mucosa. The final microsomal resuspension was divided into aliquots and stored at -70 °C.

For the spectroscopic measurement of cytochrome P450 and cytochrome b5, the 105000 x g pellet was resuspended as above, using 2 ml homogenizing buffer/g mucosa and centrifuged at 105000 x g for 60 minutes. The final microsomal pellet was then resuspended in 50 mM tris / 20% glycerol as described above. The protein concentration in the final microsomal suspension was 3.88 ± 0.38 mg/g mucosa.

MEASUREMENT OF NADPH-CYTOCHROME P450 REDUCTASE ACTIVITY

NADPH P450 reductase activity in the homogenate and in the various subcellular fractions of liver and oesophagus was measured using the artificial electron acceptor cytochrome c as described by Snell et al (Snell, 1987). The assay was carried out in 10 mm quartz cuvettes at 37 °C by monitoring the increase in absorbance at 550 nm in a Cary 3 spectrophotometer of the test cuvette relative to the blank cuvette which contained all the components present in the test cuvette, except NADPH. The assay mixture contained 0.1 M KH_2PO_4 pH 7.6, 50 μM cytochrome C (Sigma), 1 μmol PMSF (phenylmethylsulfonylfluoride, Sigma), 1mM KCN, the respective subcellular suspension prepared freshly (45 μl of oesophageal homogenate; 30 μl of oesophageal microsomes; 140 μl of oesophageal microsomal supernatant; 5 μl of liver homogenate; 1 μl of liver microsomes; or 50 μl of liver microsomal supernatant) and 0.4 mM

NADPH (in the test cuvette only) in a total volume of 2.5 ml. After 3 minutes preincubation at 37 °C, the reaction was started by adding 0.1 ml NADPH (from a 10 mM solution) to the test cuvette, followed by mixing and then the reaction was followed by the increase in absorbance at 550 nm. $E = 21$ mM^{-1}cm^{-1} for the reduced cytochrome C (Williams, 1962).

MEASUREMENT OF NADH CYTOCHROME B5 REDUCTASE ACTIVITY

The measurement of the NADH dependent cytochrome b5 reductase activity was done by measuring its NADH-ferricyanide reductase activity exactly as described by Mihara et al (Mihara, 1972). The reduction was carried out at 25 °C in 10 mm quartz cuvettes. The microsomal suspension (5 µl for oesophageal microsomes and 1 µl for liver microsomes) was diluted to 2 ml in 0.1 M KH_2PO_4 pH 7.5, 1mM potassium ferricyanide. The reaction was started by the addition of NADH, 10 µl from a 10 mM stock solution in 0.1 M KH_2PO_4 PH 7.5, prepared freshly (final concentration was 100 µM) and the reduction of ferricyanide was followed by recording the decrease in absorbance at 420 nm taking $E = 1.02$ mM^{-1}cm^{-1} for ferricyanide (Schellenberg,1958).

CYTOCHROME P450 MEASUREMENT IN LIVER MICROSOMES

Cytochrome P450 was measured by the method of Guengerich (Guengerich, 1989a) based on the original method of Omura and Sato (Omura, 1964). Liver microsomes, prepared as before for spectrophotometric studies, were diluted in 0.1 M KH_2PO_4 pH 7.5 / 1mM EDTA / 20% glycerol / 0.4% (w/v) Triton N101 / 0.5 % (w/v) sodium cholate to a protein concentration of 0.5 mg/ml. 1 ml was then placed in each of two cuvettes. The contents of both cuvettes were reduced by the addition of a few crystals of sodium dithionite and a baseline recorded between 500-400 nm. The sample cuvette

was then bubbled with carbon monoxide for 30 seconds (at 1 bubble/second) and the spectrum was re-scanned between 500-400 nm. The specific content of cytochrome P450 was determined by taking $E = 91$ $mM^{-1}cm^{-1}$ for the difference absorbance between 450 nm and 490 nm of the CO-reduced difference spectral peak.

CYTOCHROME P450 DETERMINATION IN OESOPHAGEAL MICROSOMES

The P450 content of oesophageal microsomes was determined by the method of Jackobson and Cinti (Jackobson, 1973), for samples contaminated with cytochrome oxidase, based on the original method of Omura and Sato (Omura, 1964). Oesophageal microsomes, prepared for spectroscopic studies as described before, were diluted in 0.1 M KH_2PO_4 pH 7.5 / 1mM EDTA / 20% glycerol / 0.4% (w/v) Triton N101 / 0.5% (w/v) sodium cholate to a protein concentration of around 1 mg/ml. 1 ml was then placed in each of two cuvettes. The sample and reference cuvette were bubbled with carbon monoxide for 1 minute (at 1 bubble/second) followed by the addition of 20 µl 0.5 mM sodium succinate. After 5 minutes a baseline was recorded between 500-400 nm. A few crystals of sodium dithionite were then added to the test cuvette only, the content gently mixed and the spectrum was re-scanned between 500-400 nm. The specific content of cytochrome P450 was determined by taking $E = 91$ $mM^{-1}cm^{-1}$ for the difference in absorbance between 450 nm and 490 nm of the sodium dithionite-reduced difference spectral peak.

CYTOCHROME B5 DETERMINATION IN LIVER MICROSOMES

The cytochrome b5 content in liver microsomes was determined by the method of Omura and Sato (Omura, 1964). Liver microsomes were diluted in 0.1 M KH_2PO_4 pH 7.5 / 1mM EDTA / 20% glycerol / 0.4% (w/v) Triton N101 / 0.5% (w/v) sodium

cholate as for the determination of cytochrome P450 from liver microsomes, and 1 ml was placed in each of two cuvettes. The baseline was recorded between 500-400 nm and 25 μl of a freshly prepared 2% NADH (w/v in water) stock solution was added to the test cuvette, gently mixed and the spectrum was re-scanned between 500-400 nm. The content of cytochrome b5 was determined by taking $E = 185$ mM^{-1}cm^{-1} for the absorbance change at 426 nm minus that at 409 nm.

DETERMINATION OF CYTOCHROME B5 IN OESOPHAGEAL MICROSOMES

The concentration of cytochrome b5 in oesophageal microsomes was determined by the method of Matsubara *et al* (Matsubara, 1974). Oesophageal microsomes were diluted as for P450 determination and helium was bubbled through the solution for 5 minutes. 1 ml was then placed in each of two cuvettes. The baseline was recorded between 500-400 nm followed by the addition of 25 μl of freshly prepared 2% NADH (w/v) to the sample cuvette only. After gently mixing, the spectrum was re-scanned between 500-400 nm and the cytochrome b5 content was determined exactly as with liver microsomes.

DETERMINATION OF PROTEIN CONCENTRATION

This was done by the method of Lowry (Lowry, 1951), as described in Chapter 2.

SDS/PAGE - ELECTROPHORESIS OF PROTEINS

It was done based on the original method of Laemmli (Laemmli, 1970) as modified by Phillips *et al* (Phillips, 1981).

Stock solutions :

30% acrylamide : 30% (w/v) acrylamide, 0.8% (w/v) methylene-bisacrylamide (Protogel, National Diagnostics).

pH 8.8 buffer : 1.5 M Tris - HCl pH 8.8 / 0.4% (w/v) SDS.

pH 6.8 buffer : 0.5 M Tris - HCl pH 6.8 / 0.4% (w/v) SDS.

An 8% resolving gel (6 cm high x 8 cm wide x 0.75 mm thick) was prepared by mixing thoroughly and quickly 30% acrylamide (8 ml), pH 8.8 buffer (7.5 ml), water (15 ml), 23 µl N,N,N',N'-tetramethylethylenediamine (TEMED, Sigma) and 1.5 ml 1.5% (w/v) ammonium persulphate. The gel was poured and immediately overlayed with 0.1% (w/v) SDS in water and left to polymerize for at least 90 minutes. The final gel composition was 8% acrylamide, 0.375 M Tris - HCl (pH 8.8), 0.1% SDS. A 10% acrylamide gel, which was used in Western Blot of cytochrome b5 reductase, was prepared by changing the volume of 30% acrylamide to 7.15 ml, water to 6.5 ml, and pH 8.8 buffer to 5 ml. A 13% acrylamide resolving gel, which was used in Western Blot analysis of cytochrome b5, was prepared by changing the volume of 30% acrylamide to 13 ml, and water to 8.5 ml, with the volume of buffer pH 8.8 being the same as for the 8% acrylamide gel. After the resolving gel had polymerized, the overlay solution was removed and the space washed with pH 6.8 buffer. A 3% acrylamide stacking gel was then prepared by thoroughly and quickly mixing 2 ml of stock acrylamide, 5 ml buffer pH 6.8, 12 ml water, 20 µl TEMED and 1 ml freshly prepared 1.5% (w/v) ammonium persulphate. The stacking gel was then poured over the resolving gel and left to polymerize for at least 1 hour.

The microsomal samples for electrophoresis were prepared by adding an equal volume of the microsomal suspension and the loading buffer (2% (w/v) SDS / 20 nM EDTA / 20 mM sodium phosphate buffer (pH 7.0) / 2% 2-mercaptoethanol / 30% (v/v) glycerol / 0.02 % (w/v) bromo phenol blue). The content was mixed, heated to 100 °C for 3 minutes and 2-3 µl of 2 -mercaptoethanol added. The samples were then quickly

centrifuged at 1000 x g for 1 minute and usually 15 μl of the supernatant loaded onto the gel. Samples were electrophoresed at constant current at 8 mA/slab gel (approximately 10 V/cm) until the dye entered the resolving gel. The current was then increased to 16 mA/slab gel and electrophoresis proceeded until the dye reached 0.5 cm from the bottom of the gel. The electrophoresis buffer was 0.025 M tris base / 0.192 M glycine / 0.1% (w/v) SDS (pH 8.4). The gels were stained with silver by the use of a silver staining kit (Silver Stain Plus, Bio Rad) and then fixed in 5% (v/v) acetic acid and photographed on an illuminated light box.

WESTERN-BLOT

As an Alternative to staining, the resolving gel was blotted to a nitrocellulose membrane (Amersham) and the membrane was subsequently blocked, incubated with antibodies and developed by the use of Enhanced Chemiluminescence (ECL) Western Blot kit (Amersham). Briefly: the resolving gel was placed on a nitrocellulose membrane. Two sheets of Whatman 3 MM chromatography filter paper cut to the approximate size of the gel were placed on either side to sandwich the gel/nitrocellulose. This was then placed between Scotch plastic sponge pads in a cassette and placed in a Bio Rad blotting tank containing 3 litres of "transfer buffer" (192 mM glycine / 25 mM tris pH 8.4 / 20% (v/v) methanol). The protein was transferred from gel to membrane by electrophoresis (overnight at 100 mA (approximately 30 V) and at 150 mA for the last hour). The membrane was removed and placed with the blotted side inwards in a Falcon 50 ml sterile tube. Any protein binding sites remaining on the membrane were blocked by a 5% (w/v) solution of the blocking reagent (milk powder, Amersham) in 20 mM tris pH 7.5 / 500 mM NaCl (total volume 10 ml) for 1 hour in a roller mixer at room temperature.

The primary antibodies (rabbit, anti-rat) against P450 2E1 and 1A1 were provided in

the Western Blot kit. The primary antibodies against P450 2B1/B2, cytochrome b5, cytochrome P450 reductase and cytochrome b5 reductase were a generous gift of Dr. E. Shephard (Phillips, 1983). After trial experiments to optimize the procedure in order to achieve minimum background, a 1/1000 dilution of these antibodies in 0.1% (w/v) blocking reagent in 20 mM tris pH 7.5 / 500 mM NaCl was used and they were incubated for 30 minutes with the membrane. The rest proceeded as stated in the kit manual, except that the membrane incubation with the second antibody (donkey anti-rabbit immunoglobulin) was carried out for 30 minutes as opposed to 1 hour as stated in the kit manual.

7-ETHOXYCOUMARIN DEETHYLATION BY LIVER AND OESOPHAGEAL MICROSOMES

The 7-ethoxycoumarin deethylase activity of liver microsomes was measured as described in Chapter 2, except that the amount of microsomal protein and the incubation time varied. The rate of formation of umbelliferone by liver microsomes was linear for up to 0.8 mg of protein and at least 8 minutes.

The 7-ethoxycoumarin deethylase activity of oesophageal microsomes was measured as with liver microsomes, except 0.5 mg of microsomal protein/tube was used. The 7-ethoxycoumarin concentration was 5 mM and the incubation time was 10 minutes. The NADPH-generating system used in the incubation with liver microsomes was replaced by 0.5 μmol NADPH and 0.5 μmol NADH for oesophageal microsomes (Greenlee, 1978). Under these conditions the rate of formation of unbelliferone was linear for up to 1 mg of microsomal protein and 15 minutes.

COUMARIN 7-HYDROXYLATION BY LIVER AND OESOPHAGEAL MICROSOMES

Coumarin 7-hydroxylation by liver microsomes was carried out as with the 7-deethylation of ethoxycoumarin, except that the substrate (coumarin) concentration was 10 mM, with 1 mg of liver microsomes and the incubation time was 30 minutes. Coumarin 7-hydroxylation by oesophageal microsomes was measured as with liver microsomes except 0.5 mg oesophageal microsomal protein was used. The NADPH-generating system in the incubation with liver microsomes was replaced by 0.5 μmol NADPH and 0.5 μmol NADH for the measurements with oesophageal microsomes. The rate of formation of umbelliferone was linear with respect to both time and protein content under the conditions used.

PENTOXYRESORUFIN AND ETHOXYRESORUFIN DEALKYLATION BY MICROSOMES

The dealkylation of pentoxyresorufin and ethoxyresorufin by hepatic and oesophageal microsomes was measured exactly as described in Chapter 2, except that up to 0.4 mg of oesophageal microsomal protein was used.

NDMA DEMETHYLATION BY LIVER MICROSOMES

The demethylation of N-nitrosodimethylamine by liver microsomes prepared from untreated and from ethanol treated rats was carried out as described in Chapter 2, except that the incubation mixture contained 1 mg of liver microsomal protein from untreated or 0.5 mg from ethanol treated rats, 0.2 mM NDMA and the reaction was carried out for 20 minutes. Under these conditions, the rate of production of formaldehyde from

the demethylation of NDMA by liver microsomes was linear with respect to both protein content and time.

TREATMENT OF RATS

Male Sprague Dawley rats (200 g) from the University College animal facility were kept in 12 hours light and dark cycle. They were starved overnight before being killed by CO_2 asphyxiation. After the rats were killed, livers and oesophageal mucosa were removed to ice-cold 50 mM tris pH 7.4 / 20% glycerol and frozen at -70 °C.

Phenobarbital Treatment

30 rats were fed 0.1% (w/v) phenobarbital (sodium salt, Sigma) in tap water for 4 days and given an intraperitoneal injection on the fifth day with a 4% (w/v) solution of sodium phenobarbital in 0.9% NaCl at a dose of 40 mg/kg b.w. Animals were killed 15 hours after the injection. This treatment causes maximum induction of P450 2B1/B2 in liver - ≥ 50-fold for P450 2B1 and ≥ 20-fold for P450 2B2 (Dr E. Shephard - personal communication).

Ethanol Treatment

Rats were treated as described by Yang *et al* (Yang, 1991a) in order to achieve a maximum induction of P450 2E1 in liver by ethanol. 30 rats were fed 15% (v/v) ethanol in their drinking water for 3 days and killed on day 4.

3-Methylcholanthrene Treatment

Rats were treated as described by Rodriguez *et al* (Rodrigues, 1991) in order to achieve maximum induction of P450 1A1 and 1A2 in the rat liver. 30 rats were given an intraperitoneal injection of 25 mg 3-methylcholanthrene/kg b.w. in 0.3 ml corn oil for 4 days and killed 24 hours after the last injection.

Control

Rats to be used as controls did not received any treatment.

RESULTS

The metabolism of nitrosamines in the oesophagus has been shown to be P450-dependent (NADPH-dependent and inhibited by CO), localized exclusively in the mucosa and in the microsomal fraction (Labuc, 1982). The oesophageal P450 content has been measured and shown to be approximately 15% of that of the liver (0.096 nmol P450/mg microsomal protein against 0.66 nmol/mg protein for oesophagus and liver respectively) (Labuc, 1982). However, no detailed study about the subcellular fractionation of the oesophagus and comparison with liver using an enzyme marker has been done. Such studies are important when dealing with extra-hepatic tissues to ensure that the microsomal fraction actually contains the endoplasmic reticulum and so that we know what proportion of the original endoplasmic reticulum has been recovered. Also, no measurements of the other components of the oesophageal monooxygenase system or its capacity to catalyze specific metabolites has been undertaken.

Several difficulties appear when studying the characteristics of oesophageal microsomes. The rat oesophagus is a very fibrous tissue, making it difficult to homogenize efficiently. Furthermore, only about 70 mg of oesophageal mucosa is obtained from each rat, and it possess a low P450 content when compared to the liver. Nevertheless, microsomes were prepared from the oesophageal mucosa by homogenization and differential centrifugation basically as described by Labuc and Archer (Labuc, 1982), but with some modifications as described in Materials and Methods. The precipitate obtained by centrifuging the homogenate at 9000 x g for 20 minutes was resuspended and recentrifuged at 9000 x g, and this supernatant pooled with the initial one. It has been reported that this procedure increases the recovery of the endoplasmic reticulum in the microsomal fraction of extrahepatic organs (Matsubara, 1974). The total protein recovered in the microsomal protein obtained in the study described in this thesis (3.88 ± 0.38 mg microsomal protein / g oesophageal mucosa) was a bit lower than reported by Labuc and Archer (4.49 ± 0 .17 mg/g mucosa)

(Labuc, 1982).

Since NADPH cytochrome P450 reductase has been considered to be the best possible marker for endoplasmic reticulum membrane (Depierre, 1975), it was measured spectrophotometrically as described in Materials and Methods. The oesophageal mucosa homogenate had a reductase activity (627.82 ± 111.27 μmol cytochrome C reduced/min/g tissue) and total protein content (63.31± 14.95 mg/g tissue) which were 6.8% and 71% of the respective values for liver homogenates (9183 ± 2280 μmol cytochrome C reduced/min/g tissue and 89.23 ± 11.02 mg protein /g tissue), thus showing the difference between the two organs in their reductase activity. Following differential centrifugation, the reductase activity of the oesophageal microsomal fraction was 45.88 ± 2.87 μmol cytochrome C reduced/min/mg protein and a total of 3.88 ± 0.38 mg of microsomal protein. The liver microsomal reductase activity was 558.65 ± 154.98 μmol cytochrome C reduced/min/mg protein, and a total of 9.44 ± 2.26 mg of microsomal protein was obtained / g tissue. Thus, whereas the oesophageal microsomal fraction has 30% of the reductase activity and 6% of total protein content of its homogenate, the liver had 59% and 11% respectively. This shows that the lower percentage of the oesophageal reductase activity recovered in its microsomal fraction is caused by the lower percentage of total protein recovered in the microsomal fraction when both figures are compared to the respective values obtained with liver. The enrichment factor (% recovery of reductase / % recovery total protein) for the reductase activity in the oesophageal microsomal fraction was 4.98, which is very similar to the one obtained in the liver which was 5.3, thus validating the method for preparation of microsomes from oesophagus.

In order to measure the total cytochrome P450 content in the oesophageal and liver microsomal fraction, microsomes were prepared as before, except a washing step of the microsomal precipitate was introduced in order to reduce the haemoglobin contamination, which may interfere with spectrophotometric studies. Microsomes

(between 0.5 and 1.0 mg/ml) were then solubilized according to Guengerich (Guengerich, 1989a) by addition of 0.1 M KH_2PO_4 pH 7.5 / 1 mM EDTA / 20% glycerol / 0.4% (w/v) Triton N101 / 0.5% (w/v) sodium cholate and the CO-ferricytochrome P450 difference spectrum was measured. The advantage of solubilizing the microsomes is that it makes the solution clearer, which allows a more accurate measurement of the P450 content when working with the small amounts of this protein found in the oesophagus. Figure 4.1 and 4.2 shows the CO-difference spectrum of sodium dithionite reduced liver and oesophageal microsomes respectively. The spectrum obtained with liver microsomes shows a maximum absorbance at 450 nm as reported originally by Omura and Sato (Omura, 1964), and no signs of haemoglobin contamination or P450 denaturation can be seen from the absence of any peak around 420 nm. The spectrum obtained with oesophageal microsomes on the other hand shows a maximum absorbance at 454 nm and another peak at around 417 nm. The shift of the maximum absorbance wavelength from 450 nm to 454 nm, with a trough at around 436 nm and a shoulder at 432 nm is due to cytochrome oxidase contamination of the microsomal fraction (Jackobson, 1973; Orrenius, 1973). The vigorous homogenization required to effectively break the mucosa cells probably disrupted mitochondrial membrane with consequent contamination with cytochrome oxidase (a mitochondrial enzyme) in the microsomal fraction. As there is only a low amount of P450 in the oesophagus, any small contamination with chromophores such as cytochrome oxidase and haemoglobin in the microsomal fraction would have a marked effect on the measurement of P450 in this tissue. To ensure that the cytochrome oxidase does not interfere with the measurement of P450, the method of Jackobson and Cinti (Jackobson, 1973) was used, which is based on the original method of Omura and Sato (Omura, 1964), but involves preincubating the microsomes with sodium succinate in the presence of carbon monoxide. Sodium succinate selectively reduces cytochrome oxidase anaerobically, and upon the addition of sodium dithionite to the sample cuvette, reduction of cytochrome P450 was achieved without interference from cytochrome oxidase as can be seen in figure 4.3. The oesophageal P450 spectrum shows a peak at

Figure 4.1: CO - difference spectrum of sodium dithionite - reduced
liver microsomes

Wavelength (nm)

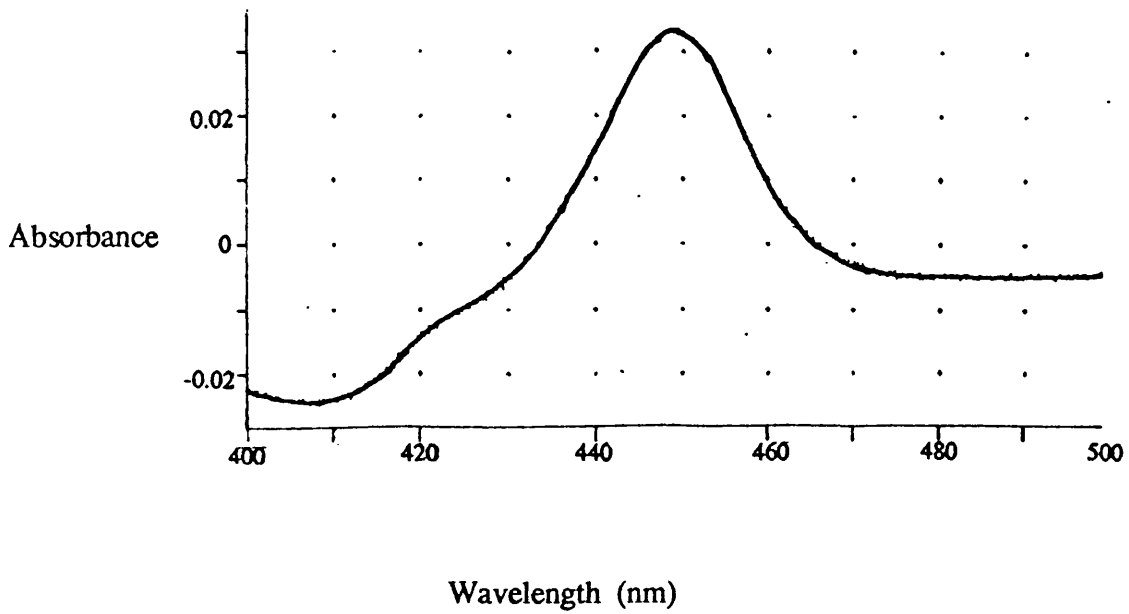Figure 4.2: CO - difference spectrum of sodium dithionite - reduced
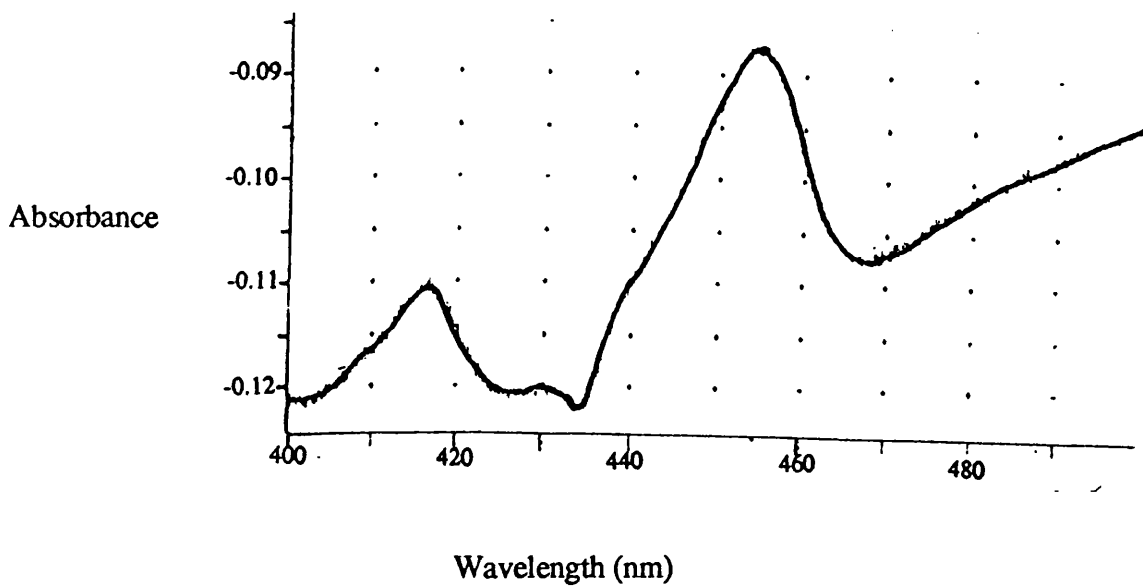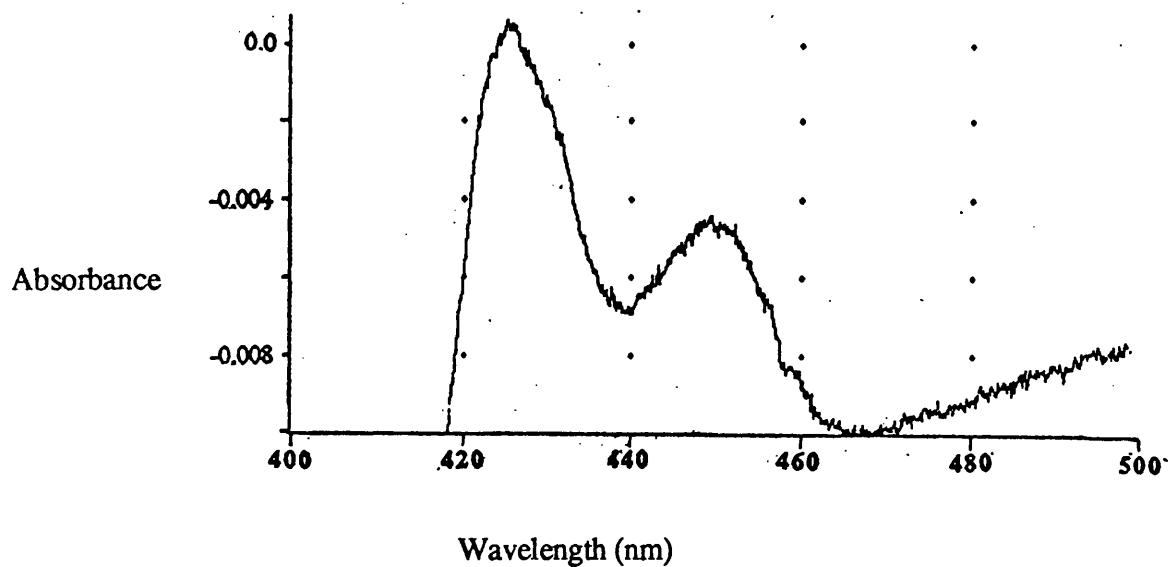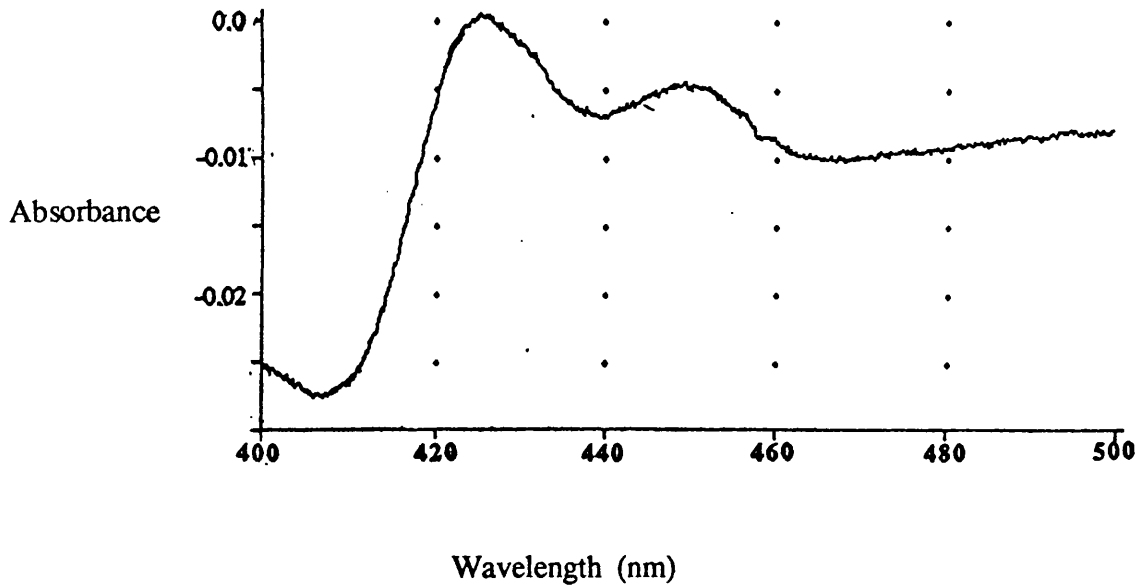oesophageal microsomes

Wavelength (nm)

Figure 4.3: sodium dithionite - difference spectrum of sodium succinate and CO - reduced oesophageal microsomes

The upper graph shows the whole spectrum. The lower graph has been magnified and shows only the spectrum over -0.01 absorbance units.

Wavelength (nm)

Wavelength (nm)

449 nm which is symmetrical above half-peak height and which was reproducible qualitatively and quantitatively using different batches of oesophageal microsomal preparation. The peak at around 423 nm may be due to cytochrome b5 which could have been reduced under the conditions utilized, or to haemoglobin contamination. If it were from the denaturation of P450, the measurement of P450 in the different batches of oesophageal microsomes would have shown a greater variation than was actually seen. The amount of total P450 measured in oesophageal mucosa microsomes was 0.068 ± 0.003 nmol/mg protein (from 4 determinations using different oesophageal microsomal batches) which is between the amounts reported by Labuc and Archer (Labuc, 1982) of 0.096 nmol/mg protein and that by Farinati et al (Farinati, 1984) of 0.035 nmol/mg protein. The amount of total P450 present in oesophageal microsomes was 6.9% of the amount of P450 present in liver microsomes which was 0.99 ± 0.043 nmol/mg protein (from 3 determinations, each done in duplicate and using a different liver microsomal preparation).

The next component of the monooxygenase system to be measured in the microsomes of liver and oesophageal mucosa was cytochrome b5. It was measured from the NADH-difference spectra of liver and oesophageal microsomes. The cytochrome b5 content of liver microsomes was 0.55 ± 0.062 nmol/mg protein (from 3 determinations, each done in duplicate and using a different liver microsomal preparation) which is about half the cytochrome P450 content, this is consistent with reports in the literature (Estabrook, 1978). The measurement of cytochrome b5 in the oesophageal microsomal fraction on the other hand was considerably more difficult due to the low amount of this enzyme present, and the haemoglobin contamination of the microsomal fraction. The method of Matsubara et al (Matsubara, 1974) was used, which consists of bubbling the microsomes with helium for 5 minutes and, after the baseline has been recorded, adding freshly prepared NADH (25 μl of a 2 mg/100 μl solution prepared freshly) to the sample cuvette. The amount of cytochrome b5 present in oesophageal microsomes was 0.04 ± 0.014 nmol/mg protein (mean of 3 determinations, using 3 different

oesophageal microsomal preparations) which was 7.3% of the value for liver microsomes.

The last component of the monooxygenase system to be measured was the NADH-cytochrome b5 reductase activity, using ferricyanide as the artificial electron acceptor as described by Mihara *et al* (Mihara, 1972). The measurement of the rate of reduction of ferricyanide by NADH-cytochrome b5 reductase was taken in the first minute of the reaction, although the reaction was linear for at least 5 minutes under the conditions used. The NADH-cytochrome b5 reductase activity was 7.13 ± 0.6 mmol ferricyanide reduced/min/mg protein (mean of 3 determinations, each done in duplicate and with different preparations of liver microsomes), for liver microsomes and 3.19 ± 0.11 mmol ferricyanide reduced/min/mg protein (mean of 3 determinations, each done in duplicate and with different oesophageal microsomal preparation) for oesophageal microsomes.

To confirm the amounts of cytochrome b5, cytochrome b5 reductase and cytochrome P450 reductase in oesophageal and liver microsomes, these enzymes were determined by immunoblotting. After separation of the protein by sodium dodecyl sulphate electrophoresis, the proteins were transferred to a nitrocellulose membrane and incubated with rabbit antibodies against the respective rat liver enzymes and subsequently with a biotinylated antibody against rabbit immunoglobulins. These second antibodies are then conjugated with streptavidin-horseradish peroxidase, which will catalyze the oxidation of luminol with consequent emission of light, which is detected on an x-ray film. This method of detection was used because of its greater sensitivity over the more conventional horseradish or streptavidin peroxidase methods. Figure 4.4 shows the Western blot of oesophageal and liver microsomal proteins using rabbit antibodies against the rat liver P450-reductase with the subsequent densitometric analysis. 4 different oesophageal microsomal preparations from untreated rats and 2 of phenobarbital treated rats, and 3 different liver microsomal preparations from untreated

Figure 4.4: P450 reductase in oesophageal and liver microsomes
detected by Western blotting with densitometric analysis
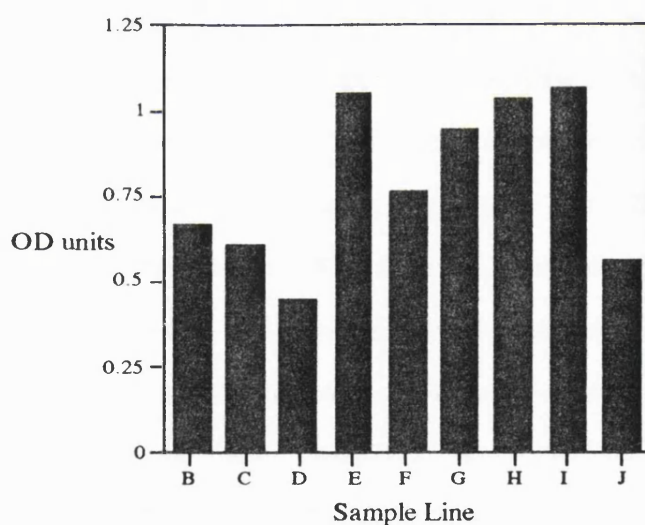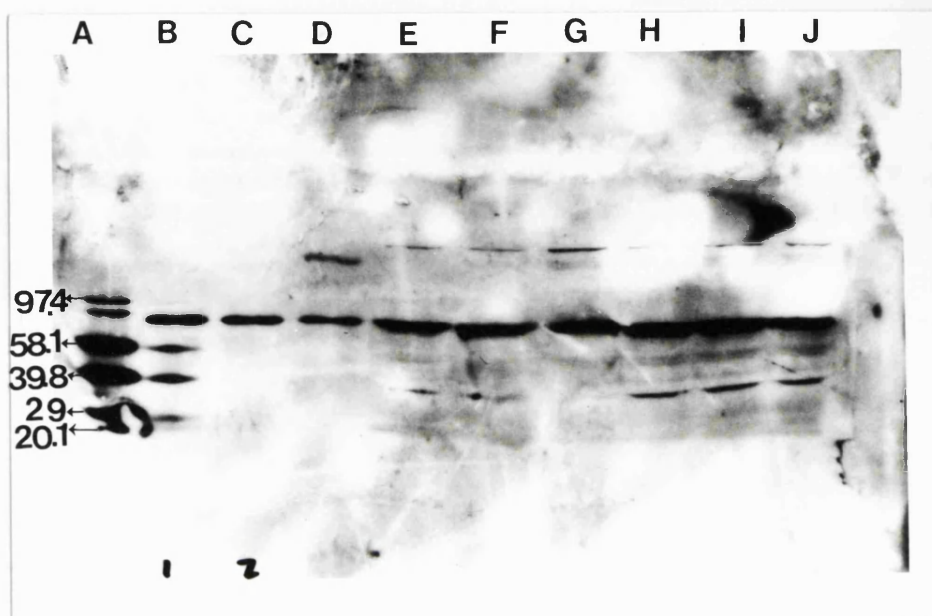
A- molecular weight markers. The main bands are: phosphorylase b (97.4 KD);
catalase (58.1 KD); alcohol dehydrogenase (39.8 KD), carbonic anhydrase (29 KD);
trypsin inhibitor (20.1 KD)
B-Liver microsomes sample 1 (1.51 μg)
C-Liver microsomes sample 2 (0.98 μg)
D-Liver microsomes sample 3 (0.497 μg)
E-Oesophageal microsomes from untreated sample 1 rats (18.14 μg)
F-Oesophageal microsomes from phenobarbital treated rats (18.14 μg)
G-Oesophageal microsomes from phenobarbital treated rats (18 μg)
H-Oesophageal microsomes from untreated sample 1 rats (18.67 μg)
I-Oesophageal microsomes from untreated rats sample 2 (22.43 μg)
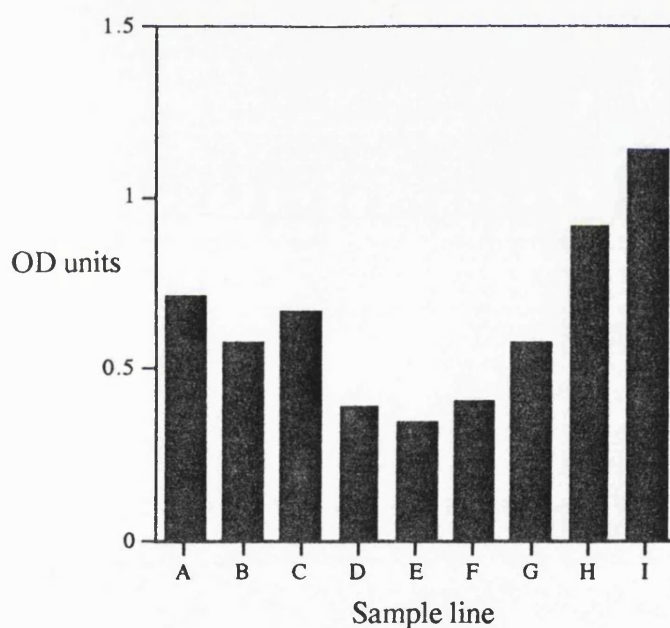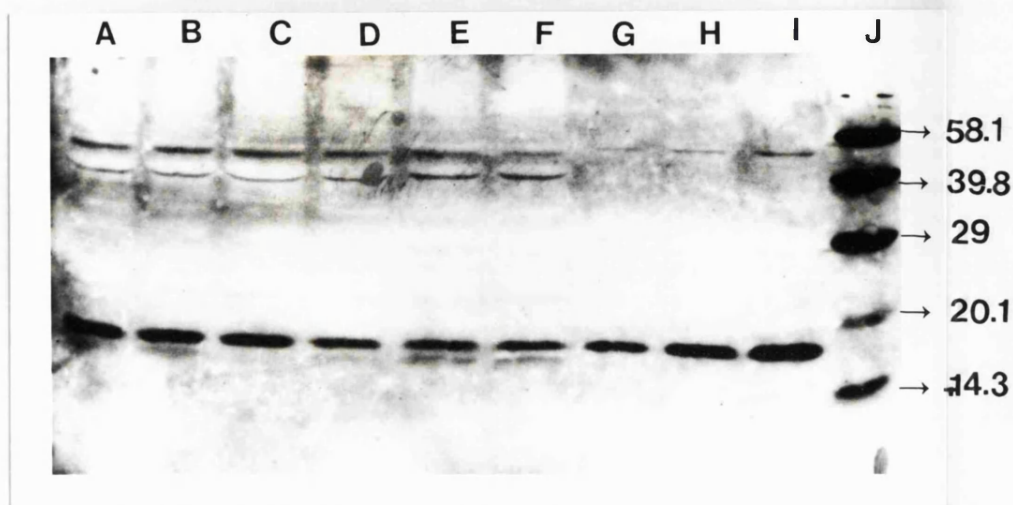J-Oesophageal microsomes from untreated rats sample 2 (20.57 μg)

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rats were used. Samples applied to all lanes showed an immunoreactive band at 76 KD, which is the reported molecular weight of the P450 reductase. The mean relative absorbance value for the immunoreactive band for the liver samples applied, after subtracting the background around the spot, was 0.654 ± 0.233 units / µg liver microsomal protein, whereas for the oesophageal samples applied (with the exception of those derived from phenobarbital treated rats) was 0.047 ± 0.012 units / µg oesophageal microsomal protein (ie. 7.1% the levels in liver microsomes). This result agrees well with the difference of the NADPH-P450 reductase activity measured between oesophageal and liver microsomes as showed above.

Figure 4.5 shows the Western Blot detection of cytochrome b5 in oesophageal and liver microsomal proteins from untreated rats. There was an immunoreactive band detected at around 16 KD (the reported molecular weight of cytochrome b5) in all lanes in which either liver or oesophageal microsomal samples had been applied. The mean value for the immunoreactive band in the liver samples, after subtracting from the background around the spot was 0.95 ± 0.292 units / µg protein, whereas for the oesophageal samples it was 0.027 ± 0.007 units / µg protein. Thus the cytochrome b5 present in oesophageal microsomes is 2.8% of that in liver microsomes as seen with Western Blot, which is less than that determined spectrophotometrically (7.3%). At the very low levels of cytochrome b5 found in oesophageal microsomes the value obtained by immunoblotting is less subjected to interference than that measured spectrophotometrically. Nevertheless, both results are of the same order of magnitude.

Figure 4.6 shows the Western Blot detection of cytochrome b5 reductase of oesophageal and liver microsomes from untreated rats. There was an immunoreactive band in all liver and oesophageal microsomal samples at around 33 KD, which is the reported molecular weight for cytochrome b5 reductase. Quantification of these bands by densitometry showed that the amount of this enzyme in oesophageal microsomes is 17.3% of that in liver microsomes (0.0168 ± 0.0049 units / µg protein against 0.0972

Figure 4.5: cytochrome b5 in oesophageal and liver microsomes
detected by Western blotting with densitometric analysis

A-Oesophageal microsomes prepared from untreated rats sample 1 (20.57 μg protein)
B-Oesophageal microsomes prepared from untreated rats sample 1 (20.57 μg)
C-Oesophageal microsomes prepared from untreated rats sample 2 (18.67 μg)
D-Oesophageal microsomes prepared from untreated rats sample 2 (18 μg)
E-Oesophageal microsomes prepared from untreated rats sample 3 (18.14 μg)
F-Oesophageal microsomes prepared from untreated rats sample 3 (18.14 μg)
G-Liver microsomes prepared from untreated rats sample 1 (0.497 μg)
H-Liver microsomes prepared from untreated rats sample 2 (0.979 μg)
I-Liver microsomes prepared from untreated rats sample 3 (1.506 μg)
J-Molecular weight markers. Main bands are: catalase (58.1 KD); alcohol
dehydrogenase (39.8 KD); carbonic anhydrase (29 KD); trypsin inhibitor (20.1 KD);
lysozyme (14.3 KD)

Figure 4.6: cytochrome b5 reductase in liver and oesophageal
microsomes detected by Western blotting with densitometric analysis
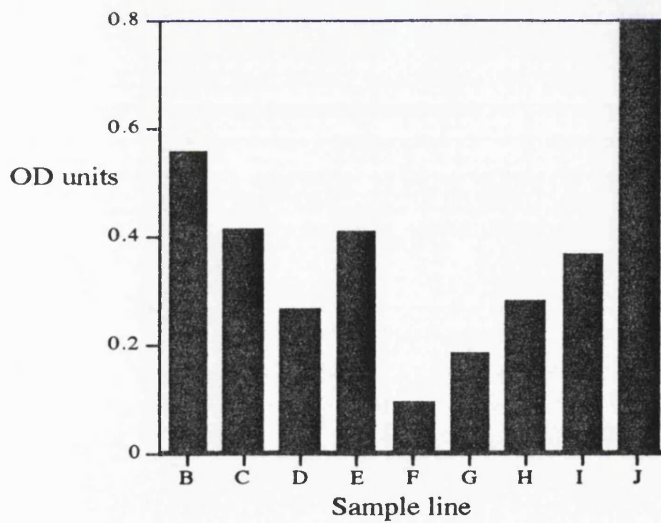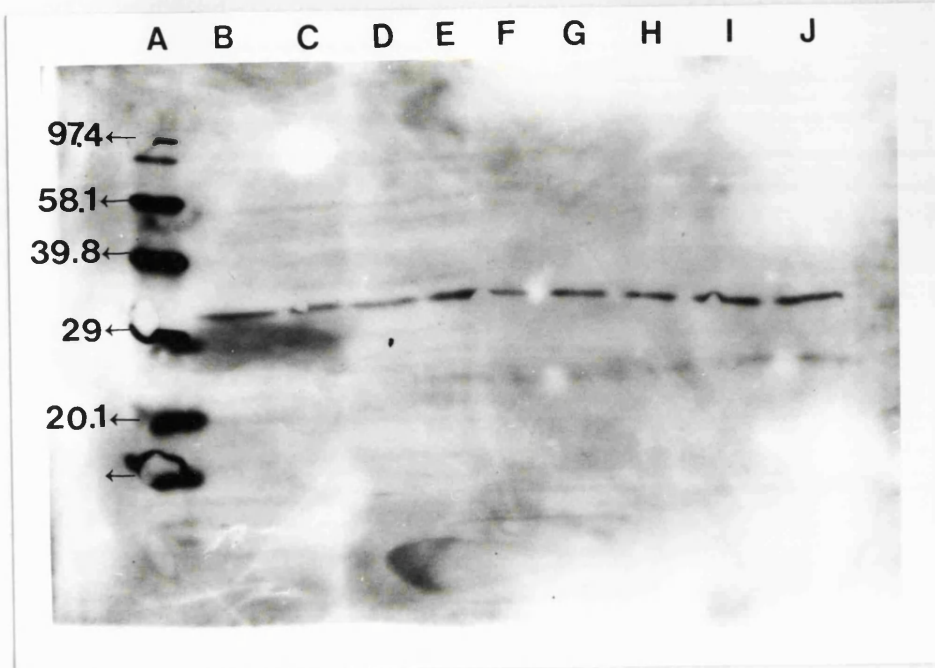
A-Molecular weight markers. Main bands are: phosphorylase b (97.4 KD); catalase
(58.1 KD); alcohol dehydrogenase (39.8 KD); carbonic anhydrase (29 KD); trypsin
inhibitor (20.1 KD); lysozyme (14.3 KD)
B-Oesophageal microsomes sample 1 (27.1 µg protein)
C-Oesophageal microsomes sample 1 (18.1 µg)
D-Oesophageal microsomes sample 2 (22.4 µg)
E-Oesophageal microsomes sample 2 (33.6 µg)
F-Liver microsomes sample 1 (1.64 µg)
G-Liver microsomes sample 1 (3.29 µg)
H-Liver microsomes sample 2 (1.88 µg)
I-Liver microsomes sample 2 (3.76 µg)
J-Liver microsomes sample 2 (5.64 µg)

± 0.0049 units / μg protein, respectively). A similar result was obtained from another gel (ie. that oesophageal microsomes have 18.9% the amount of b5 reductase present in liver microsomes, 0.0093 ± 0.0025 for oesophagus against 0.0493 ± 0.021.

In order to try to detect the presence of specific P450 isoforms in the oesophagus, and to study the effect in oesophagus of known inducers of these isoenzymes in liver microsomes, the following experiment was done: groups of 30 male rats (200 ± 20 g) were pretreated with either ethanol, phenobarbital or 3-methylcholanthrene and after being killed their livers and oesophagus were removed to ice cold tris/KCl buffer, the oesophageal epithelium removed and the tissues frozen in liquid nitrogen. Microsomes were prepared from the livers and oesophagus as described earlier. The regimen of administration of ethanol (Yang, 1991a), phenobarbital (Dr E. Shephard, personal communication) and 3-methylcholanthrene (Rodrigues, 1991) described in the Materials and Methods section, was chosen in order to provide the highest induction in the liver of the P450 isoenzymes induced by these chemicals. The amount of protein recovered in the oesophageal microsomal fraction of rats receiving the different pretreatments were 3.92 mg/g mucosa for rats pretreated with ethanol, 3.78 mg/g for rats pretreated with phenobarbital, 4.71 mg/g for rats pretreated with 3-methylcholanthrene and 4.32 mg/g for untreated rats. For liver microsomes the amount of protein was 10.67 ± 2.63 mg/g liver for ethanol pretreated rats, 15.77 ± 1.41 mg/g of liver for phenobarbital pretreated rats, 13 ± 1.58 mg/g liver for 3-methylcholanthrene pretreated rats and 13.19 ± 2.43 mg/g liver for untreated rats.

Figure 4.7 and 4.8 shows the silver stained SDS-PAGE of microsomes prepared from the liver and oesophagus, respectively, of untreated, ethanol treated, phenobarbital treated or 3-methylcholanthrene treated rats. No degradation of proteins which might have occurred during the preparation of liver or oesophageal microsomes could be seen. Liver and oesophageal samples showed a very different pattern of bands at around 50 KD, with only 3 bands being visible with oesophageal samples. There was a difference

Figure 4.7: SDS-PAGE of liver microsomes prepared from rats after treatments with 3-methylcholanthrene, phenobarbital, and ethanol
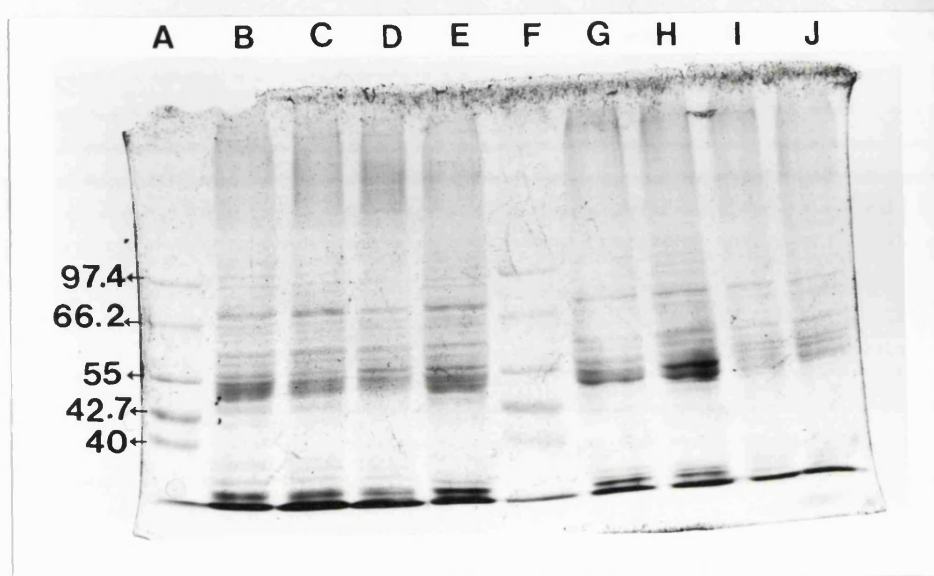
The polyacrylamide gel was stained with silver as described in Materials and Methods.

A-Molecular weight markers. The main bands are: phosphorylase b (97.4 KD); bovine serum albumin (66.2 KD); glutamate dehydrogenase (55 KD); ovalbumin (42.7 KD); Aldolase (40 KD)

B-Liver microsomes prepared from 3-methylcholanthrene treated rats sample 1 (2 μg protein)

C-Liver microsomes prepared from 3-methylcholanthrene treated rats sample 2 (2 μg)

D-Liver microsomes prepared from phenobarbital treated rats sample 1 (2 μg)

E-Liver microsomes prepared from phenobarbital treated rats sample 2 (2 μg)

F-Molecular weight markers

G-Liver microsomes prepared from ethanol treated rats sample 1 (2 μg)

H-Liver microsomes prepared from ethanol treated rats sample 2 (2 μg)

I-Liver microsomes prepared from untreated rats sample 1 (2 μg)

J-Liver microsomes prepared from untreated rats sample 2 (2 μg)

Figure 4.8: SDS-PAGE of oesophageal and liver microsomes prepared from rats after treatments with 3-methylcholanthrene, phenobarbital, and ethanol
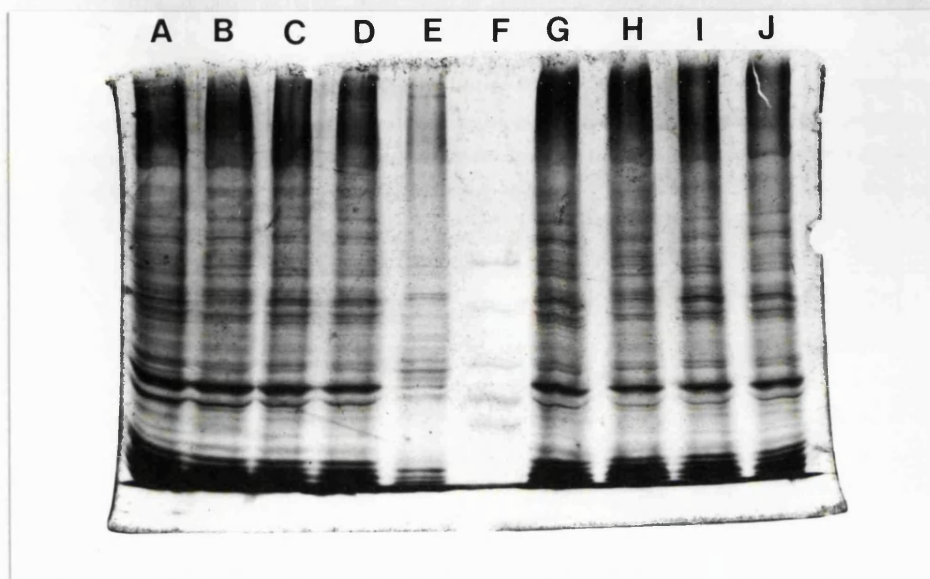
The polyacrylamide gel was stained with silver as described in Materials and Methods.

A-Oesophageal microsomes prepared from 3-methylcholanthrene treated rats (18 µg protein)

B-Oesophageal microsomes prepared from ethanol treated rats (18 µg)

C-Oesophageal microsomes prepared from phenobarbital treated rats (18 µg)

D-Oesophageal microsomes prepared from untreated rats (18 µg)

E-Liver microsomes prepared from untreated rats (2 µg)

F-Molecular weight markers. The main bands are: phosphorylase b (97.4 KD); bovine serum albumin (66.2 KD); glutamate dehydrogenase (55 KD); ovalbumin (42.7 KD); aldolase (40 KD)

G-Oesophageal microsomes prepared from 3-methylcholanthrene treated rats (18 µg)

H-Oesophageal microsomes prepared from ethanol treated rats (18 µg)

I-Oesophageal microsomes prepared from phenobarbital treated rats (18 µg)

J-Oesophageal microsomes prepared from untreated rats (18 µg)

in the pattern of bands around 50 KD in the gel of the liver microsomes prepared from rats subjected to different treatments, but the oesophageal samples prepared from the animals which received different treatments showed no difference in the profile of bands at around 50 KD.

In order to check that the ethanol adminstration had induced P450 2E1 in liver microsomes, and to look for the presence of this enzyme in the oesophagus, microsomal protein from the liver and oesophagus of untreated and ethanol treated animals were subjected to SDS-electrophoresis, blotted and immunostained using a rabbit antibody against cytochrome P450 2E1 as the primary antibody. Figure 4.9 shows that with all of the liver samples applied to the acrylamide gel, the antibody against P450 2E1 reacted with only a single protein band at around 51 KD, the reported molecular weight for P450 2E1. However, surprisingly, densitometry did not suggested any significant difference in the amount of P450 2E1 present in the liver microsomes of untreated and ethanol treated rats. No immunoreactive protein band could be detected with oesophageal microsomes prepared from either untreated or ethanol treated rats, which shows that this enzyme is absent from the oesophageal mucosa of rats.

Yang *et al* (Yang, 1991a) have reported that a similar ethanol pretreatment of rats produced a 5-fold increase in the demethylation of NDMA by liver microsomes, a reaction catalyzed exclusively by P450 2E1 at low substrate concentration. To confirm this, liver microsomes (1 mg protein) from untreated rats or from ethanol treated rats (0.5 mg protein) were incubated with 200 μM NDMA for 20 minutes and the formaldehyde produced quantified spectrophotometrically as described by Yang *et al* (Yang, 1991a). These conditions were used to assure linearity in the rate of the production of formaldehyde. The experiment was done in triplicate with 3 different preparations of liver microsomes from ethanol treated and untreated rats. The results are shown in Table 4.1. The rate of demethylation of NDMA by liver microsomes prepared

Figure 4.9: P450 2E1 in oesophageal and liver microsomes detected by Western Blotting with densitometric analysis

A- Moleculae weight markers. The main bands are: Phosphorylase b (97.4 KD), catalase (58.1 KD), alcohol dehydrogenase (39.8 KD), Carbonic anhydrase (29 KD)
B- Liver microsomes prepared from untreated rats sample 1 (0.66 μg protein)
C- Liver microsomes prepared from untreated rats sample 2 (0.66 μg)
D- Liver microsomes prepared from untreated rats sample 3 (0.66 μg)
E- Liver microsomes prepared from ethanol treated rats sample 1 (0.66 μg)
F- Liver microsomes prepared from ethanol treated rats sample 2 (0.66 μg)
G- Liver microsomes prepared from ethanol treated rats sample 3 (0.66 μg)
H- Oesophageal microsomes prepared from untreated rats (28.05 μg)
I- Oesophageal microsomes prepared from ethanol treated rats (28.05 μg)

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from ethanol treated rats was 4-fold higher than that of liver microsomes prepared from untreated rats. Thus the induction of P450 2E1 in the liver of rats given ethanol could be detected by measuring the rate of demethylation of NDMA, but not at the apoprotein level as seen by Western Blot analysis.

Table 4.1: rates of demethylation of NDMA by liver microsomes prepared from untreated or ethanol treated rats (given as nmols formaldehyde / minute / mg microsomal protein).

UNTREATED	ETHANOL TREATED
0.634	**2.51**
(± 0.053)	(± 0.307)

Results are expressed as mean (± SD) of 3 experiments with different microsomal preparations each done in triplicate.

To check that phenobarbital administration had produced the expected induction of P450 2B1/B2 in the liver, and to investigate whether these isoenzymes are present in oesophageal microsomes prepared from untreated or phenobarbital treated rats, liver and oesophageal microsomal samples prepared from untreated and phenobarbital treated rats were subjected to Western Blot analysis using a rabbit antibody against P450 2B1 which crossreacts with P450 2B2 due to the high (98%) homology in their aminoacid sequence (Waxman, 1992). Figure 4.10 shows that 2 µg of microsomes prepared from different livers of untreated rats similarly produced 2 immunoreactive protein bands. Their apparent molecular weights were around 51 KD and 52 KD, the reported molecular weights of P450 2B1 and 2B2, respectively. Microsomes (0.5 µg protein) prepared from livers of rats treated with phenobarbital produced a strong diffuse immunoreactive band, around the same molecular, thus showing the induction of P450 2B1 and 2B2 in the liver of rats treated with phenobarbital. By contrast, oesophageal microsomes (18 µg) prepared from untreated or phenobarbital treated rats

Figure 4.10: P450 2B1 and 2B2 in oesophageal and liver microsomes detected by Western blotting

A-Liver microsomes prepared from untreated rats sample 1 (2 µg protein)
B-Liver microsomes prepared from untreated rats sample 2 (2 µg)
C-Liver microsomes prepared from phenobarbital treated rats sample 1 (0.5 µg)
D-Liver microsomes prepared from phenobarbital treated rats sample 2 (0.5 µg)
E-molecular weight markers. The main bands are: catalase (58.1 KD); alcohol dehydrogenase (39.8 KD); carbonic anhydrase (29 KD)
F-Oesophageal microsomes prepared from untreated rats (18 µg)
G-Oesophageal microsomes prepared from untreated rats (27 µg)
H-Oesophageal microsomes prepared from phenobarbital treated rats (18 µg)
I-Oesophageal microsomes prepared from phenobarbital treated rats (27 µg)
J-Oesophageal microsomes prepared from phenobarbital treated rats (27 µg)

failed to produce any reactive protein band with the antibody against P450 2B1/B2. Since these proteins could be detected when only 0.3 μg of microsomes prepared from the liver of untreated rats were loaded on the gel, 60-fold less than the amount of oesophageal microsomes, it seems probable that both cytochrome P450 2B1 and 2B2 are absent from the oesophageal mucosa of either untreated or phenobarbital treated rats. Phenobarbital administration to rats cause the induction of not only some P450s, but also a battery of enzymes, including P450 reductase (reviewed by Waxman, 1992). However, the Western Blot analysis of oesophageal microsomal protein immunostained with antibodies against P450 reductase did not show any particular induction in the rats which received phenobarbital as shown in figure 4.4.

The induction of P450 1A1 in the liver of rats treated with 3-methylcholanthrene and the presence and induction of this isoenzyme in the oesophageal mucosal microsomes prepared from untreated and 3-methylcholanthrene treated rats was also studied by immunoblotting analysis. Figure 4.11 shows that microsomes (1.5 μg protein) from the liver of untreated rats failed to show any immunoreactive protein band with 1A1 antibody. By contrast, microsomes (0.5 μg protein) prepared from livers of 3-methylcholanthrene treated rats produced a strong and diffuse immunoreactive protein band, showing the induction of P450 1A1. Oesophageal microsomes prepared from both untreated, and 3-methylcholanthrene treated rats showed an immunoreactive protein band with anti-1A1 antibody, which comigrated with the band present in liver microsomes of 3-methylcholanthrene treated rats and which had a molecular weight (55.5 KD) around the reported molecular weight for rat P450 1A1. The immunolabelled band present in the oesophageal microsomes of 3-methylcholanthrene treated rats was visibly stronger than the band from untreated rats, and densitometric analysis showed a 11.8-fold induction (0.103 ± 0.033 units / μg protein for untreated oesophageal microsomes, against 1.215 ± 0.148 units / μg protein for 3-methylcholanthrene treated oesophageal microsomes). Subsequently, the amount of P450 1A1 present in oesophageal and liver microsomes prepared from 3-methylcholanthrene treated rats was

Figure 4.11: P450 1A1 in oesophageal and liver microsomes detected by Western blotting with densitometric analysis

A-Oesophageal microsomes prepared from untreated rats (20.6 µg protein)
B-Oesophageal microsomes prepared from untreated rats (20.6 µg)
C-Oesophageal microsomes prepared from 3-methylcholanthrene treated rats (20.6 µg)
D-Oesophageal microsomes prepared from 3-methylcholanthrene treated rats (20.6 µg)
E-Molecular weight markers. The main bands are: phosphorylase b (97.4 KD); catalase (58.1 KD); alcohol dehydrogenase (39.8 KD); carbonic anhydrase (29 KD); trypsin inhibitor (20.1 KD)
F-Liver microsomes prepared from untreated rats (3.76 µg)
G-Liver microsomes prepared from 3-methylcholanthrene treated rats sample 1 (0.5 µg)
H-Liver microsomes prepared from 3-methylcholanthrene treated rats sample 2 (0.5 µg)
I-Oesophageal microsomes prepared from untreated rats (20.6 µg)
J-Oesophageal microsomes prepared from 3-methylcholanthrene treated rats (20.6 µg)

analyzed by Western Blot and densitometric analysis. Figure 4.12 and 4.13 show that after treatment with 3-methylcholanthrene the amount of P450 1A1 in the oesophageal microsomes is 3.75% that in liver microsomes (0.0317 ± 0.0033 units / µg protein, against 8.296 ± 0.589 for liver; and for oesophagus in Western blot 2, 0.0408 ± 0.0046 units / µg microsomal protein, against 11.151 ± 2.016 for liver). Liver microsomes of rats which received a similar 3-methylcholanthrene treatment to the one described in this thesis have been reported to contain 1.4 nmol P450 1A1/mg protein, whereas 1A1 has been reported to be absent from liver microsomes of untreated rats (Soucek, 1992a; Funea, 1993; Kamataki, 1993). Using the published figures as a guide the amount of P450 1A1 in the oesophageal microsomes of rats treated with 3-methylcholanthrene can be roughly calculated to be 53 pmol/mg protein and in oesophageal microsomes of untreated rats to be 5 pmol/mg protein.

Since some compounds are metabolized by only one specific P450 or by a group of P450s, the presence, or absence, of the single, or the group of P450s in the oesophagus could be assessed by measuring whether the oesophagus can metabolize these substrates. As the amount of P450 present in the oesophageal mucosa is small, the amount of metabolism of the compounds to be studied would be expected to be low, making their detection and quantification difficult. For this reason it was considered essential to choose compounds which form fluorescent metabolites.

Rat liver microsomes hydroxylate coumarin at the 7-position at a very low, but detectable (3 pmol umbelliferone/min/mg protein) (Raunio, 1988). 3-methylcholanthrene did not change the rate, but pyrazole and phenobarbital increased the rate of this reaction by 2- and 10-fold, respectively. The 7-hydroxylation of coumarin by microsomes prepared from the liver of untreated rats was measured in two experiments, done in duplicate with different microsomal preparations, was 9.87 ± 1.65 pmol umbelliferone/min/mg protein and the rate of coumarin 7-hydroxylation by oesophageal microsomes prepared from untreated rats was 2.79 ± 1.06 pmol

Figure 4.12: P450 1A1 in oesophageal and liver microsomes detected by Western blotting with densitometric analysis

A-Molecular weight markers: The main bands are: phosphorylase b (97.4 KD); catalase (58.1 KD), alcohol dehydrogenase (39.8 KD); carbonic anhydrase (29 KD)

B-Liver microsomes prepared from 3-methylcholanthrene treated rats sample 1 (0.15 µg protein)

C-Liver microsomes prepared from 3-methylcholanthrene treated rats sample 2 (0.139 µg)

D-Oesophageal microsomes prepared from 3-methylcholanthrene treated rats (22.43 µg)

E-Oesophageal microsomes prepared from 3-methylcholanthrene treated rats (22.43 µg)

F-Oesophageal microsomes prepared from 3-methylcholanthrene treated rats (16.83 µg)

Figure 4.13: P450 1A1 in oesophageal and liver microsomes detected by Western blotting with densitometric analysis

A-Molecular weight markers. Main bands are: phosphorylase b (97.4 KD); catalase (58.1 KD); alcohol dehydrogenase (39.8 KD); carbonic anhydrase (29 KD)

B-Liver microsomes prepared from 3-methylcholanthrene treated rats sample 1 (0.15 µg protein)

C-Liver microsomes prepared from 3-methylcholanthrene treated rats sample 2 (0.139 µg)

D-Oesophageal microsomes prepared from 3-methylcholanthrene treated rats (22.43 µg)

E-Oesophageal microsomes prepared from 3-methylcholanthrene treated rats (16.83 µg)

F-Oesophageal microsomes prepared from 3-methylcholanthrene treated rats (33.64 µg)

umbelliferone/min/mg protein (table 4.2). Because the oesophagus contains very little P450 the rate of coumarin 7-hydroxylation / nmol total P450 is 4-fold higher than that of liver microsomes prepared from untreated rats. Table 4.2 also shows the rate of coumarin 7-hydroxylation by oesophageal microsomes prepared from rats treated with ethanol, phenobarbital or 3-methylcholanthrene. The standard deviation for these results was relatively high due to the low amounts of umbelliferone measured. Nevertheless, oesophageal microsomes prepared from rats which received 3-methylcholanthrene as treatment could metabolize coumarin at a significant higher rate (P=0.05 for a two-tailed unpaired t test) than did oesophageal microsomes prepared from untreated rats. This result is in contrast to Raunio $et\ al$ (Raunio, 1988) who had shown that 3-methylcholanthrene does not change the rate of coumarin 7-hydroxylation by rat liver microsomes.

Table 4.2: rates of 7-hydroxylation of coumarin by oesophageal microsomes prepared from rats treated with different inducers (given as pmol umbelliferone / minute / mg microsomal protein)

CONTROL	ETHANOL	PHENOBARBITAL	3-MC
2.79	2.36	3.58	4.99 *
(± 1.06)	(± 1)	(± 0.64)	(± 1.39)

* is $P \leq 0.1$ (probably significant)
Results are expressed as mean (± SD) of 3 determinations. Assay carried out with 0.5 mg protein, 10 mM coumarin for 30 minutes

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The deethylation of 7-ethoxycoumarin by liver and oesophageal microsomes prepared from untreated or treated rats has also been investigated. The P450 with the highest rate of metabolism for 7-ethoxycoumarin deethylation is P450 1A1, although other enzymes such as 1A2, 2B1, 2C11 and 2E1 can also carry out this reaction (Guengerich, 1982; Funea, 1993). The deethylation of 7-ethoxycoumarin by liver microsomes was induced 2.1, 3, and 5.1-fold upon administration of ethanol (induces mainly P450 2E1),

phenobarbital (induces mainly 2B1, 2B2) and 3-methylcholanthrene (induces mainly 1A1 and 1A2), respectively. By contrast, the only treatment which induced the deethylation of 7-ethoxycoumarin by oesophageal microsomes was 3-methylcholanthrene (2.9-fold) (table 4.3). Oesophageal microsomes metabolise 7-ethoxycoumarin faster than they metabolise coumarin.

Table 4.3: Rates of 7-ethoxycoumarin deethylation by liver and oesophageal microsomes prepared from rats treated with different inducers (given as pmol umbelliferone / minute / mg microsomal protein).

	CONTROL	ETHANOL	PHENOBARBITAL	3-MC
LIVER	430 (± 121)	897 (± 61)	1300 (± 332)	2190 (± 400)
OESOPHAGUS	13.1 (± 4.4)	10.4 (± 3.3)	11.3 (± 4.2)	37.4 (± 11.1)

Results are expressed as mean (± SD) for 2 experiments done in triplicate.
Assay for liver carried out with 0.4 mg protein, 0.3 mM 7-ethoxycoumarin and for 5 minutes. Assay for oesophagus carried out with 0.25 mg protein, 5 mM 7-ethoxycoumarin and for 15 minutes.

Since the metabolism of 7-ethoxycoumarin by oesophageal microsomes was induced by 3-methylcholanthrene, but not by ethanol or phenobarbital, and the administration of this compound to rats was previously shown to induce P450 1A1 in their oesophagus, it is probable that the metabolism of 7-ethoxycoumarin in the oesophagus is carried out by P450 1A1, although the involvement of some other P450(s) in the metabolism of this compound in oesophageal microsomes cannot be ruled out.

There are many problems involved in trying to study the metabolism of nitrosamines by microsomes of tissues like the oesophagus, which is obtained in low amounts from the

rat and possesses a low amount of P450. The assay is difficult to perform and the conditions used *in vitro* have to be so stretched that the interpretation of the results cannot always be correlated with the situation *in vivo*. Since the deethylation of 7-ethoxycoumarin was the highest metabolic rate detected with oesophageal microsomes, it was decided to study the effect of nitrosamines on the rate of 7-ethoxycoumarin deethylation by oesophageal and liver microsomes from untreated rats in order to try to identify the P450s responsible for their metabolism in both tissues. In a preliminary experiment it was shown that the presence of NDMA, NDEA and NMBzA at the concentrations to be used in the incubation assays did not effect the fluorescence of umbelliferone. Each nitrosamine (0.6 mM to liver and 10 mM for the oesophagus) and 7-ethoxycoumarin (0.3 mM for liver and 5 mM for oesophagus) were used in the incubations. Table 4.4 shows that NDMA, but not NDEA or NMBzA, significantly inhibited the deethylation of 7-ethoxycoumarin (P = 0.01 for an unpaired two-tailed *t* test). This is consistent with the fact that P450 2E1, which is the sole isoenzyme involved in the metabolism of NDMA at low concentrations (Yang, 1991b), is also capable of deethylating 7-ethoxycoumarin (Funea, 1993). NDEA can also be metabolized by P450 2E1, but this is not the only isoenzyme involved in the metabolism of this nitrosamine (Heath, 1962; Yoo, 1990) and NDEA did not produced a significant reduction in the deethylation of 7-ethoxycoumarin. NMBzA which is possibly metabolized by a number of P450s including P450 2B1 and 1A1 (Kawanashi, 1983; Kawanashi, 1985) also did not inhibit the deethylation of 7-ethoxycoumarin by liver microsomes probably because the P450s involved in the metabolism of 7-ethoxycoumarin are different to those involved in the metabolism of NMBzA. In contrast to the liver, NDMA did not inhibit the deethylation of 7-ethoxycoumarin by oesophageal microsomes prepared from untreated rats, which confirms the lack of expression of P450 2E1 in the rat oesophagus. NDEA which is metabolized by an isoenzyme different from P450 2E1 in the oesophagus also did not significantly inhibit the deethylation of 7-ethoxycoumarin by oesophageal microsomes, but NMBzA (10 mM) significantly inhibited (by 46%) the deethylation of 5 mM 7-ethoxycoumarin by

oesophageal microsomes (P = 0.0004 for an unpaired two-tailed t test). Since it had been concluded from the previous experiment that P450 1A1 is responsible, for most, if not all, of the deethylation of 7-ethoxycoumarin in oesophageal microsomes, this suggests that this isoenzyme is strongly involved in the metabolism of NMBzA. The lack of inhibition by NMBzA of the deethylation of 7-ethoxycoumarin by liver microsomes is probably because 1A1 is absent, or present at very low amount in the liver of untreated rats (Soucek, 1992a; Funea, 1993) and other isoenzymes which can also metabolize 7-ethoxycoumarin and are expressed at high levels, such as 2C11, cannot metabolize NMBzA.

Table 4.4: Influence of NDMA, NDEA and NMBzA on the rates of deethylation of 7-ethoxycoumarin by liver and oesophageal microsomes prepared from untreated rats (given as pmol umbelliferone / minute / mg microsomal protein)

	CONTROL	+ NDMA	+ NDEA	+ NMBzA
LIVER	400 (± 60)	287* (± 60)	341 (± 40)	361 (± 50)
OESOPHAGUS	10 (± 1.7)	9 (± 1.8)	7.9 (± 0.8)	5.4 *** (± 1.4)

* is P ≤ 0.1 (significant); *** is P ≤ 0.001 (extremely significant)
Results are expressed as mean (± SD) for 2 experiments done in triplicate.
Assay for liver was carried out with 0.5 mg protein, 0.3 mM 7-ethoxycoumarin and for 10 minutes; The concentration of the nitrosamines was 0.6 mM. Assay for oesophagus was carried out with 0.5 mg protein, 5 mM 7-ethoxyresorufin and for 15 minutes; the concentration of the nitrosamines was 10 mM.

Incubation of oesophageal microsomes (up to 0.4 mg) with either ethoxyresorufin or pentoxyresorufin did not produce measurable levels of resorufin. This is consistent with Traber et al (Traber, 1992) who were also unable to detect ethoxyresorufin metabolism for oesophageal microsomes from untreated rats, although they claim to have detected the production of 5 pmol umbelliferone/min/mg protein by oesophageal microsomes prepared from β-naphthoflavone treated rats.

DISCUSSION

Nitrosamines need to be metabolically activated in order to produce their tumorigenic action. This activation has been exhaustively shown to be the result of the α-hydroxylation of one of the alkyl chains of the nitrosamine which is catalyzed by cytochrome P450s (Yang, 1991b). The α-hydroxylated compound is unstable and decomposes with the generation of an alkylating species, which reacts with DNA at specific sites producing mutations of genes involved in control of cell growth, eventually leading to the development of tumours. Since the α-hydroxylated parent compound has a short life (in the case of NDMA a half life of about 7 seconds (Mochizuki, 1980), it can diffuse from the endoplasmic reticulum to the nucleus of the cell or to neighbouring cells, but not to other organs. So, the ability of the organ to activate nitrosamines is an essential factor in influencing its susceptibility to tumours produced by these chemicals. Since the capacity of the organ to activate nitrosamines is a reflection of the P450s expressed in that organ and which can metabolize nitrosamines, a study of the monooxygenase system and of P450 isoforms present in a particular organ is essential for an understanding, at least in part, of the organotropism of some nitrosamines for that organ. Whereas such studies have been carried out in the liver of rats (reviewed by Yang, 1991b), and in some extra-hepatic tissues such as the nasal epithelium (reviewed by Dahl, 1993), information regarding the oesophageal monooxygenase system is almost nonexistent. Immunoblots carried out with antibody against P450 2E1 failed to show the presence of this isoenzyme in oesophageal microsomes prepared from untreated or ethanol treated rats (a known inducer of P450 2E1 in the liver) (Dewaziers, 1989; Shimizu, 1990; Huang, 1992), although in one study (Shimizu, 1990) the authors identified through immunohistochemistry something which crossreacted with an antibody against P450 2E1 in the oesophageal epithelium of rats treated with ethanol.

Oesophageal microsomes have been prepared before in some laboratories (Labuc, 1982; Barch, 1984; Farinati, 1984; Dewaziers, 1989; Shimizu, 1990) although previously no enzyme marker was used during the preparation to quantify the recovery of endoplasmic reticulum in the microsomal fraction. Labuc and Archer (Labuc, 1982) showed that the metabolism of NMBzA to benzaldehyde and formaldehyde by the rat oesophagus was P450-dependent and localized in the mucosa. They measured NMBzA activity during subcellular fractionation studies of oesophageal mucosa and showed that the microsomal fraction prepared by differential centrifugation (the method normally applied to the preparation of liver microsomes) retained 40% of the capacity to metabolize NMBzA relative to the homogenate (Labuc, 1982). They also showed that the amount of P450 present in oesophageal microsomes was 0.431 nmol total P450/g tissue or 0.096 ± 0.012 nmol/mg microsomal protein (Labuc, 1982), a value which is higher than the one published by Farinati et al (Farinati, 1984) (0.036 nmol/mg microsomal protein).

Since the method of Labuc and Archer (Labuc, 1982) to prepare microsomes from the oesophagus was the most detailed study carried out with that tissue, it was decided to prepare oesophageal microsomes based on that method. NADPH P450 reductase (measured as NADPH cytochrome C reductase) activity was measured as a biochemical marker for the recovery of endoplasmic reticulum in the microsomal fraction,. Although the recovery of NADPH P450 reductase activity in the oesophageal microsomal fraction was 30.4% of that of the homogenate, the recovery of total protein in the oesophageal microsomal fraction was 6.1% of the total amount of protein present in the oesophageal homogenate, as opposed to 9.94% recovery of total protein of liver homogenate in the liver microsomal fraction. Thus, the enrichment factor for NADPH P450 reductase activity (and endoplasmic reticulum) in preparations from both organs is very similar - 4.98 for the oesophagus and 5.30 for the liver - and the difference between oesophagus and liver microsomes in the percentage of reductase activity and protein recovered from the respective homogenates is because higher amount of endoplasmic reticulum are

present in hepatocytes than in the oesophageal mucosa cells.

Table 4.5: Comparison between the oesophageal and liver
monooxygenase system

	Oesophagus	Liver	Value in oes / liver x 100
P450 (nmols/mg protein)	0.068 ± 0.003	0.99 ± 0.043	6.9
P450 reductase activity*	45.88 ± 2.87	558.65 ± 154.98	8.2 (7.1 by densitometry**)
cytochrome b5 (nmols/mg protein)	0.04 ± 0.014	0.55 ± 0.062	7.3 (2.8 by densitometry)
b5 reductase activity*	3.19 ± 0.11	7.13 ± 0.6	44.7 (18 by densitometry)
microsomal protein mg/g tissue	3.88 ± 0.38	9.44 ± 2.26	41

P450 was measured as described in Materials and Methods.

* activities are given as μmol cytochrome C reduced/minute mg protein for P450 reductase; and mmol ferricyanide reduced/min/mg protein for b5 reductase.

** Ratios were determined by densitometry of the Western Blots shown in figures 4.4, 4.5, and 4.6; and refer to the enzyme rather than its respective activity.

Total cytochrome P450 had to be measured in oesophageal microsomes after incubation of the microsomes with sodium succinate, which selectively reduces contaminating cytochrome oxidase which had been released from mitochondria during vigorous homogenization necessary in order to break the cells of the fibrous oesophageal mucosa. If these precautions were taken, cytochrome P450 could be reproducibly

measured in oesophageal microsomes. The amount of P450 measured in oesophageal microsomes was 0.068 ± 0.003 nmol P450/mg protein or 0.264 nmol P450/g tissue. This can be compared to the value previously published by Labuc and Archer of 0.096 nmol P450/mg protein (Labuc, 1982) or 0.431 nmol/g/tissue and by Farinati *et al* (Farinati, 1984) of 0.036 nmol/mg protein. By contrast, the amount of P450 measured in liver microsomes prepared from untreated animals was 0.99 ± 0.043 nmol P450/mg tissue or 9.84 nmol P450/g tissue. So, the levels of total P450 in oesophageal microsomes are 6.9 % of that in liver microsomes of untreated rats.

Measurement of the amounts of the components of the monooxygenase system present in oesophageal microsomes relative to liver microsomes was carried out because it has been suggested that the high amounts of NADPH-P450 reductase activity present in microsomes from the olfactory epithelium of rodents (about 2-fold higher than in the liver) may be responsible for the high rates of the metabolism of some substrates seen with olfactory microsomes compared to liver microsomes (Reed, 1986). The amount of NADPH P450 reductase, cytochrome b5 and NADH cytochrome b5 reductase in oesophageal and liver microsomes were measured both spectrophotometrically and through immunoblotting analysis (table 4.5). The amount of these enzymes or respective activities in oesophageal microsomes relative to liver microsomes as determined spectrophotometrically were 8.2%, 7.3% and 44.7%, whereas determined through immunoblotting was 7.1%, 2.8% and 18% for NADPH-P450 reductase, cytochrome b5 and NADH-cytochrome b5 reductase respectively. It is probable that the cytochrome b5 content in oesophageal microsomes relative to that in liver microsomes was more accurately measured through Western Blot analysis than by spectrophotometry because of interference by haemoglobin. However, there is no immediate explanation for the difference which was observed between liver and oesophageal microsomes when NADH-b5 reductase was measured enzymatically, and when it was determined through Western Blot analysis. Nevertheless, the amount of cytochrome b5 reductase present in oesophageal microsomes relative to liver

microsomes appears to be at least twice the respective value of the other enzymes of the monooxygenase system. It is unlikely however, that this contributes to the relative high rates of the metabolism of nitrosamines by the oesophagus when compared to the liver because, although the donation of the second electron to cytochrome P450 by the cytochrome b5 electron chain can increase the rate of some reactions catalyzed by some P450s, the high level of b5 reductase is not accompanied by a high level of b5, and the b5 / P450 ratio in oesophagus is not materially different from that in liver.

In order to study the presence of P450 isoenzymes in the oesophagus and the effect of chemicals on their expression, Western Blot analyses with antibodies against 2B1/B2, 1A1 and 2E1 were carried out with oesophageal and liver microsomes prepared from untreated rats or rats treated with ethanol, phenobarbital and 3-methylcholanthrene. Ethanol has been shown to induce P450 2E1 in the liver of rats by about 5-fold (Peng, 1982), whereas phenobarbital induces a number of isoenzymes in the rat liver, but mainly 2B1 by about 100-fold and 2B2 by about 20-fold (Waxman, 1992) and 3-methylcholanthrene induces 1A1 preferentially to 1A2 in the rat liver (Rodrigues, 1991).

Ethanol administration to rats was shown to induce the demethylation of NDMA in liver microsomes by 4-fold, which is consistent with earlier reports (Peng, 1982) showing that this reaction is catalyzed exclusively by P450 2E1 at low NDMA concentration and that P450 2E1 is induced 5-fold upon a similar ethanol treatment (Peng, 1982). However, P450 2E1 was not detected in the oesophageal microsomes prepared from untreated or ethanol treated rats, which is consistent with the fact that NDMA is not carcinogenic for the rat oesophagus (Druckrey, 1967), is not metabolized by oesophageal mucosa (Swann, 1984b) and does not alkylate oesophageal DNA (van Hofe, 1987).

Phenobarbital administration to rats produced a dramatic increase in the levels of P450

2B1/B2 in liver microsomes, but these enzymes were not present in microsomes prepared from oesophageal mucosa of either untreated or phenobarbital treated rats. It has been reported (reviewed by Waxman, 1992) that phenobarbital increases the NADPH-P450 reductase in liver microsomes of rats, but NADPH-P450 reductase was also not induced in oesophageal microsomes by phenobarbital. It is perhaps surprising that P450 2B1 and 2B2 are not expressed or induced in the oesophagus, since they have been shown to be expressed and/or induced in a number of extrahepatic organs and tissues, including olfactory epithelium, adrenal glands, testis, lung and small intestine (Waxman, 1992).

P450 1A1 is absent from the liver of untreated rats but is constitutively expressed in almost every extrahepatic organ studied, including brain, lung, nasal epithelium, kidney and throughout the alimentary tract (Soucek, 1992a; Traber, 1992; Funea, 1993), but is absent or expressed at only minute amounts($\leq$ 0.5 pmol/mg microsomal protein) in the liver of untreated rats (Boobis, 1990; Soucek, 1992a; Funea, 1993). 3-methylcholanthrene causes induction of P450 1A1 in liver (table 4.11). It has been reported that the induction of P450 1A1 occurs preferentially over P450 1A2 (Rodrigues, 1991), which is constitutively expressed in that organ (Rodrigues, 1991). Microsomes prepared from oesophagus of untreated rats were shown to express low levels of P450 1A1, and administration of 3-methylcholanthrene to rats caused a 12-fold induction in P450 1A1. Oesophageal microsomes from 3-methylcholanthrene treated rats had 3.75% the amount of P450 1A1 in liver microsomes from 3-methylcholanthrene treated rats. The absolute amount of P450 1A1 in liver microsomes from 3-methylcholanthrene treated rats has been reported to be about 1.4 nmol/mg microsomal protein (Funea, 1993), So the amount in oesophageal microsomes prepared from untreated or 3-methylcholanthrene treated rats can be estimated to be about 5 pmol/mg protein and 53 pmol/mg protein, respectively. Thus P450 1A1 constitutes about 7% of the total P450 present in oesophageal microsomes of untreated rats.

Traber *et al* (Traber, 1992) using PCR on a cDNA preparation showed that P450 1A1 mRNA was expressed throughout the rat alimentary tract, including the oesophagus and that upon administration of polycyclic aromatic hydrocarbon to rats the level of P450 1A1 mRNA increased in all the tissues examined. They showed that the increase in P450 1A1 mRNA levels in the jejunum occurred, at least in part, through an increase in CYP 1A1 gene transcription, exactly as occurs in liver, and probably through the same mechanism, the binding of the polycyclic aromatic hydrocarbon to the Ah receptor. So, the 12-fold induction of P450 1A1 seen in the oesophagus probably also occurs as the result of activation of transcription involving the Ah receptor. Traber *et al* (Traber, 1992) also detected 7-ethoxyresorufin deethylase activity with oesophageal microsomes, but only after β-naphthoflavone administration (about 5 pmol resorufin format/min/mg protein).

Oesophageal microsomes from untreated rats were able to deethylate 7-ethoxycoumarin and to hydroxylate coumarin at the 7-position. The rate of metabolism of 7-ethoxycoumarin was higher than that of coumarin (13.05 pmol umbelliferone/min/mg protein against 2.79 pmol umbelliferone/min/mg protein respectively), but the latter was 4-fold higher than in liver microsomes when calculated as pmol umbelliferone formed/ minute/nmol P450. The deethylation of 7-ethoxycoumarin can be carried out by a number of P450s, including 1A1, 1A2, 2A2, 2B1 and 2E1 (Guengerich, 1982; Funea, 1993). P450 1A1 is the most active in the metabolism of this compound, but as it can be considered to be absent from the liver of untreated rats (Soucek, 1992b; Funea, 1993), this compound is metabolized by other isoenzymes in the hepatic microsomes of untreated rats. Administration of ethanol (induces P450 2E1), phenobarbital (induces mainly P450 2B1/B2) and 3-methylcholanthrene (preferentially induces 1A1) to rats increased the deethylation of 7-ethoxycoumarin by liver microsomes by 2.1, 3 and 5.1-fold respectively, thus showing the contribution of the induced isoenzymes to the metabolism of this compound in liver. By contrast, administration of 3-methylcholanthrene, but not of ethanol or phenobarbital, induced the deethylation of

7-ethoxycoumarin by oesophageal microsomes by 2.9-fold. Since P450-1A1 has been shown to be constitutively expressed in the oesophageal mucosa of untreated rats and to be induced 12-fold upon administration of 3-methylcholanthrene to rats, it can be concluded that, most of the deethylation of 7-ethoxycoumarin in the oesophagus is catalyzed by P450 1A1. Coumarin, is very poorly metabolized by liver microsomes prepared from untreated rats, but it is well metabolized by microsomes prepared from mouse or human liver (Raunio, 1988). The reason for that is the presence in mice livers of P450 2A5 and in human liver of the orthologous P450 2A6 (Camus, 1993). Interestingly, these enzymes also metabolize NDEA at a relatively high rate (Crespi, 1990; Yamazaki, 1992; Camus, 1993). The enzyme responsible for the low rate of metabolism of coumarin in liver microsomes of untreated rats is not known, however Raunio *et al* (Raunio, 1988) have shown that this metabolism is induced 2- and 10-fold upon administration to rats of pyrazole and phenobarbital, respectively. 3-methylcholanthrene treatment of rats did not induce the metabolism of coumarin by liver microsomes indicating that P450 1A1 is not involved in the metabolism of this compound, but treatment of rats with 3-methylcholanthrene, but not with ethanol or phenobarbital, induced the 7-hydroxylation of coumarin by oesophageal microsomes by about 2-fold. Thus, it seems that a P450 other than 1A1, which is constitutively present in the oesophagus and may be induced upon 3-methylcholanthrene administration, is involved in the metabolism of coumarin in oesophageal microsomes.

Since it was established that P450 1A1 is responsible for most, if not all the deethylation of 7-ethoxycoumarin in oesophageal microsomes from untreated rats, but is absent from liver microsomes from untreated rats, the effect of different nitrosamines on the deethylation of 7-ethoxycoumarin by liver and oesophageal nitrosamines was studied. With liver microsomes, only NDMA inhibited the deethylation of 7-ethoxycoumarin, a consequence of the competitive inhibition of 2E1 by this nitrosamine, which is a known substrate for this P450. With oesophageal microsomes, only NMBzA effectively inhibited the deethylation of 7-ethoxycoumarin, suggesting

that P450 1A1 might be involved in the metabolism of NMBzA. Further evidence that P450 1A1 metabolizes NMBzA comes from the observation that a known inhibitor of P450 1A1, ellagic acid, a phenolic compound present in plants, can reduce the incidence of oesophageal tumours, caused by NMBzA in rats (Mandal, 1990). Furthermore, ellagic acid inhibits the metabolism of NMBzA (0.25 mM and 5 mM) by oesophageal but not by liver microsomes (Barch, 1989).

Following administration to rats, NMBzA produces only oesophageal tumours (Druckrey, 1967) and methylates DNA preferentially in the oesophagus (N7-methylguanine in the oesophagus was 3 times higher than in the liver (Wiestler, 1984). This methylation, rather than benzylation, of DNA is responsible for the carcinogenic effects caused by this nitrosamine (Hodgson, 1980). Labuc and Archer (Labuc, 1982) showed that oesophageal microsomes metabolized NMBzA at the α-methylene carbon (generating a methylating agent and benzaldehyde) at 100 times the rate of the metabolism at the methyl group (generating a benzylating agent and formaldehyde), whereas this difference for liver microsomes was only 10. Thus the preferential debenzylation in the oesophagus plays as crucial role in the oesophageal carcinogenicity as the overall rate of metabolism in the oesophagus. Two other nitrosamines which also produce oesophageal cancer, N-nitroso methylbutylamine and N-nitrosomethylamylamine, also produce more methylation of DNA in the oesophagus relative to liver (van Hofe, 1987), and this also probably underlines their preferential organotropism for the oesophagus (Druckrey, 1967; van Hofe, 1987; Lijinsky, 1992).

Several studies have shown that liver microsomes prepared from 3-methylcholanthrene treated rats have a higher capacity to debutylate N-nitrosomethylbutylamine (Kawanashi, 1983; Kawanashi, 1985; Yang, 1985b; Kawanishi, 1992), depentylate N-nitrosomethylamylamine (Ji, 1989; Mirvish, 1991a; Mirvish, 1991b; Huang, 1992) and debenzylate NMBzA (Kawanashi, 1983; Kawanashi, 1985; Lin, 1990; Rodrigues, 1991), but a lower capacity to demethylate these nitrosamines than do liver microsomes

prepared from untreated animals, and antibodies against P450 1A1 have established the role of P450 1A1 in the dealkylation of these nitrosamines (Mirvish, 1991b; Huang, 1993a). Furthermore, mutagenicity assays carried out with NMBzA and liver fractions prepared from rats which received different treatments, and the effect of antibodies against specific P450 isoforms have also suggested a role for P450 1A1 in the debenzylation of NMBzA (Lin, 1990). Experiments with reconstituted purified P450s have shown that P450 1A1 could debenzylate NMBzA and debutylate N-nitrosomethylbutylamine very efficiently and in preference to demethylating them, whereas P450 2B1 could remove the benzyl or butyl group and the methyl group from these nitrosamines with equal efficiency (Kawanishi, 1992). Thus the P450 1A1 present in the oesophagus of rats can remove the larger alkyl group from, but not demethylate, the asymmetric methylalkylnitrosamines which are carcinogenic for that organ, whereas the liver and other tissues containing other isoenzymes such as P450 2B1 and P450 2E1 have a proportionally higher rate of demethylation to dealkylation (and thus a detoxifying pathway). So one might suggest that the combination of the presence of P450 1A1 and the absence of other P450s, such as P450 2B1 and 2E1 in the rat oesophagus is important in determining the organotropism of asymmetric methylalkylnitrosamines for this organ. However other organs, such as the jejunum, have a higher content of P450 1A1 than the oesophagus (Traber, 1992) and are relatively resistant to the carcinogenic action of nitrosamines.Furthermore, two studies have shown results which are inconsistent with the view that P450 1A1 plays an important role in the metabolic activation of NMBzA.Lee *et al* (Lee, 1989) have reported that the metabolism of NMBzA to benzaldehyde by liver microsomes prepared from 3-methylcholanthrene treated rats was not different from that by microsomes prepared from untreated animals and Labuc and Archer (Labuc, 1982) have reported that administration of 3-methylcholanthrene to rats did not increase the metabolism of NMBzA to benzaldehyde by either oesophageal or liver microsomes. The reason for this discrepancy is not known, but it may be due to the different age of the rats used in the different experiments and/or the mode of administration of 3-methylcholanthrene.

Nevertheless, experiments *in vivo* comparing the proportion of methylation in different organs of untreated or 3-methylcholanthrene treated rats and the influence of this treatment in the organospecific carcinogenesis of NMBzA could determine the role of P450 1A1 in influencing the organospecificity of this nitrosamine for the rat oesophagus.

The data presented in this thesis supports the possibility that P450 1A1 metabolizes NMBzA, probably at the methylene carbon (debenzylation), since P450 1A1 can also dealkylate, but not demethylate, other nitrosamines which are carcinogenic for the rat oesophagus, whereas other P450s such as 2B1 and 2E1, can demethylate and dealkylate these nitrosamines with equal efficiency (reviewed by Yang, 1991b). The involvement of P450 1A1 in the activation of these other nitrosamines to their ultimate carcinogens in the rat oesophagus seems possible, since P450 1A1 has been shown to be constitutively expressed in the oesophageal mucosa, constituting about 7% of the total microsomal P450 content of that tissue. To date, this P450 has been the only identified P450 in the rat oesophageal mucosa. However, the possibility that other P450(s) exclusively expressed in the oesophagus can also remove the benzyl group, but not demethylate NMBzA and can remove the larger alkyl group from the methylalkylnitrosamines which are carcinogenic for this organ has been suggested in a number of studies measuring the methylation produced by these nitrosamines in different organs (van Hofe, 1987; Ji, 1991) and requires further investigation. The early work of Yang focussed attention on the possible role of P450s of the 2 family, but these results suggest that family 1 should also be investigated.

In respect to NDEA, there is evidence for a non-hepatic P450 form being responsible for the metabolism of this nitrosamine in the oesophagus (Heath, 1962; Swann, 1984b). In this regard, the metabolism of coumarin by oesophageal microsomes requires further investigation. P450 1A1 does not seem to be able to metabolise coumarin (Raunio, 1988) and in human and mice liver, the orthologous enzymes

responsible for the high rate of 7-hydroxylation of coumarin (2A6 and 2A5) can also efficiently metabolise NDEA (Camus, 1993). The rate of coumarin 7-hydroxylation by oesophageal microsomes was 4-fold higher than that of liver microsomes when the activity was compared with the respective amount of total P450s in the microsomes of both tissues. This suggests that either the same P450 is expressed in both organs, but only at very minute amounts in the liver, or that the difference in the amount of metabolism of coumarin in the liver and oesophagus is due to different P450s expressed in the 2 organs. The identification of the P450s present in the rat oesophagus should solve this problem, as well as providing the reason for the organotropism of nitrosamines for the rat oesophagus. Only then the situation in man could be examined and predictabilities made in respect to the role of nitrosamines in causing human oesophageal cancer.

CONCLUSION

It has been known for a long time that nitrosamines produce oesophageal cancer in experimental animals and, at the present time, they are the only carcinogens known to produce cancer in that organ. Their activity towards the oesophagus is quite remarkable and in the rat oesophageal cancer is the most common cancer induced by nitrosamines. The observation in experimental animals, and the realization that everyone is exposed to nitrosamines, largely through natural endogenous synthesis, has led to the suggestion that nitrosamines may be a significant factor in the cause of human oesophageal cancer. However the fact that nitrosamines cause oesophageal cancer in animals, even with the knowledge that all of us are exposed to them, is not enough to prove their association with human cancer and other evidence is needed before one could be convinced that they cause the oesophageal cancer seen in man.

The nitrosamines are carcinogenic to the oesophagus because they are able to reach the oesophagus through the blood stream and, once there, can be metabolically activated by some member of the cytochrome P450 family which is perhaps expressed exclusively in the oesophagus. Thus to extrapolate from the observations in animals to man one would need to know that the pharmacokinetics of the nitrosamines in man and rat are sufficiently similar that one could be sure that the nitrosamines will reach the human oesophagus and that the human oesophagus possesses a P450 which will activate them. Once established this parallel would show that nitrosamines have the *potential* to cause human oesophageal cancer, but it would not prove that they actually cause the cancer. To advance that proof one would need to show that people from groups with a high expectation of getting oesophageal cancer (for example people who drink, or the opium takers of Northern Iran) are either exposed to more nitrosamines than other people, or that they are exposed to something which increases the effectiveness of nitrosamines as oesophageal carcinogens. Finally one would eventually expect to show that the human tumours possess a spectrum of mutations in oncogenes and anti-oncogenes similar to

the spectrum observed in nitrosamine induced oesophageal tumours in animals.

This thesis has concentrated on two aspects that have been mentioned above: study of the rat oesophageal monooxygenase system which, by activating the nitrosamines, is responsible for their carcinogenic activity in the oesophagus; and, the effect on the distribution of nitrosamines of two factors epidemiologically associated with oesophageal cancer.

The rat oesophageal monooxygenase system was studied in depth for the first time. It was shown that the high activity of the oesophagus to activate some nitrosamines is not due to one of the components of the monooxygenase electron chain being present at a proportionally higher amount than the others. This is important, since it has been suggest (Reed, 1986) that the high activity of the nasal epithelium in metabolizing certain compounds is because of the high amount of P450 reductase present in that tissue. P450 1A1, but not 2E1 or 2B1 and 2B2, was shown to be constitutively expressed in the oesophagus and to be induced upon 3-methylcholanthrene treatment. To date, this has been the only P450 which has been identified in the oesophagus. It is shown that it accounts for about 7% of the total P450 present in that organ. The possibility that P450 1A1 has a role in the metabolism of NMBzA, but not of NDEA, was presented, and the combination of the presence of P450 1A1 in the oesophagus and the absence of other P450s, such as 2E1 and 2B1 which can demethylate NMBzA and methylalkylnitrosamines, from the oesophagus has been suggested as a contributing factor to the organotropism of these carcinogens for the oesophagus. The presence of P450 1A1 mRNA (McDonnell, 1992), and the absence of P450 2E1 (Dewaziers, 1989) in the human oesophagus had been previously shown. However this work shows that the investigation and identification of the other P450s present in the rat oesophagus is of vital importance in establishing the reasons for the organotropism of some nitrosamines for that organ, since a number of studies (van Hofe, 1987; Ji, 1991) suggest that this organotropism is due to the presence of enzymes exclusively expressed in that organ.

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Attempts to purify P450s from the rat oesophageal mucosa for peptide sequencing were unsuccessful due to the vast number of animals required (about 700, or 70 g tissue). However the high enzymic activity of P450s from the rat oesophagus towards nitrosamines, and the small total amount of P450 present in the organ suggest that there are probably only a small number of different P450s present in this organ and that the nitrosamine metabolizing P450 (s) represents a substantial proportion of the total. Peptide sequencing would have allowed the synthesis of oligonucleotide probes for cloning, PCR etc. A viable study would be to investigate if the oesophagus of larger animals, such as pig, can effectively metabolize asymmetric nitrosamines, and in case they can, purification attempts should be made with the oesophagus of this animal. If successful, antibodies obtained from such P450s could then be used to investigate for the presence of the orthologous enzyme in the rat and human oesophagus.

Further experiments *in vivo* in the rat are needed to establish the extent of the contribution of P450 1A1 to the organotropism of nitrosamines for the rat oesophagus. In particular the effect of 3-methylcholanthrene on the organ to organ methylation by NMBzA and also on its carcinogenesis needs to be measured for that would give a measure of the importance of the presence of P450 1A1 in the rat.

The investigation of the enzyme responsible for the coumarin hydroxylation in the rat oesophagus is also important, since this activity in oesophageal microsomes was 4-fold higher than in the liver microsomes when correlated with the total amount of P450 present in microsomes from both organs and the metabolism of coumarin and NDEA (a nitrosamine carcinogenic for the rat oesophagus) both involve P450 2A6 in human and the orthologous P450 2A5 in mouse (Camus, 1993).

The second thing investigated in this thesis was the possibility that two factors associated with a high incidence of oesophageal cancer, opium and isoamyl alcohol, could alter the metabolism and disposition of nitrosamines making them more available

for extrahepatic organs. This was done because ethanol has been shown to alter the carcinogenesis of nitrosamines through changes in their pharmacokinetics (Swann, 1987) and that these changes have been suggested to be the mechanism for the association between ethanol and oesophageal cancer (Swann, 1982).

Morphine and opium, the factors associated with the extremely high incidence of oesophageal cancer in some parts of Iran and South-Russia (IARC, 1977), were shown to produce similar changes in the distribution of NDMA and NDEA as those produced by ethanol (Swann, 1984). These changes were a decrease in first pass clearance of NDMA, and a shift of the metabolism of NDEA from the liver to the oesophagus. Future experiments should investigate their influence on carcinogenesis of nitrosamines. The results were in sharp contrast to those obtained with isoamyl alcohol, for isoamyl alcohol inhibited nitrosamine metabolism in all organs without increasing alkylation of oesophageal DNA. Nevertheless, isoamyl alcohol could be used to help to investigate the identity of the oesophageal NDEA-metabolizing P450, since it was an inhibitor of NDEA metabolism in the oesophagus.

The parallel between the results got with opium and morphine and those previously got with ethanol suggests that morphine and opium could act by altering the pharmacokinetics of nitrosamines, increasing their availability, and thus effectiveness, to the oesophagus. However the extremely high rates of the disease seen in Iran suggests that other factors are involved. Although a superficial survey for the presence of nitrosamines in diet was carried out (IARC, 1977), this was not properly done, and a more thorough investigation of potential external sources, using the Thermal Energy Analyzer, and of endogenous formation of nitrosamines, using the NPRO test, should be conducted, particularly since people living in some parts of China, which have similar rates of the disease to Iran, have been shown to be exposed to higher levels of nitrosamines from external sources (Yang, 1980) and to have a higher rate of endogenous nitrosation (Wu, 1993).

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