IDENTIFICATION OF A NOVEL CYTOCHROME P450 cDNA IN RAT OESOPHAGUS: RELEVANCE TO CARCINOGENIC *N*-NITROSAMINE METABOLISM

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To my mother…

ABSTRACT

Oesophageal cancer is an invariably fatal form of cancer with about 7000 deaths *per annum* in the UK alone. Evidence from the epidemiology and the mutation spectrum in the *p53* gene suggests that the more common form of this cancer, squamous cell carcinoma, is caused by exposure to carcinogens. The *N*-nitrosamines are candidate carcinogens for this cancer in man. *N*-nitrosamines are metabolically activated by cytochromes P450 (P450s) and their organotropism is largely dependent on the distribution in the body of the particular nitrosamine and the P450s capable of metabolising it. The rat oesophagus is particularly susceptible to methylation and tumour formation by asymmetric *N*-nitrosamines, many of which selectively induce oesophageal tumours. This suggests that the oesophagus may contain a P450 that is absent or rare in other organs. The nature of this nitrosamine-metabolising P450 was investigated.

A combination of Reverse Transcription-Polymerase Chain Reaction (RT-PCR), Rapid Amplification of Cohesive Ends-Polymerase Chain Reaction (RACE-PCR) and oesophageal cDNA library screening showed the expression in the rat oesophagus of a novel P450 of the 2B subfamily. The deduced amino acid sequence of this P450 shows 84%, 83% and 77% identity to those of CYP2B1, CYP2B2 and CYP2B12 respectively. The catalytic activity of this new P450 is not yet known but metabolism and methylation studies *in vivo* using rats treated with phenobarbital, a CYP2B1/2B2 inducer, confirmed that these members of the 2B subfamily can metabolise the oesophagus selective nitrosamine, *N*-nitrosomethyl-*n*-butylamine, and that a major part of this metabolism was activating hydroxylation of the α-carbon of the butyl group. This supports the view that the novel P450 identified in rat oesophagus may be responsible for the metabolic activation and carcinogenicity of nitrosamines in the oesophagus. Further *in vivo* metabolic studies of *N*-nitrosomethyl-*n*-butylamine using untreated rats showed that it is metabolised in rat liver by CYP2E1. However, in this case 75% of the metabolism is detoxifying. An increase in methylation of oesophageal DNA by the nitrosamine after ethanol administration showed that CYP2E1 is not involved in the oesophageal metabolism of this nitrosamine.

ACKNOWLEDGEMENTS

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ABBREVIATIONS

AGT	O^6-Alkylguanine-DNA alkyltransferase
BSA	Bovine Serum Albumin
DEAE	Diethylaminoethyl
DEPC	Diethyl Pyrocarbonate
DMF	Dimethylformamide
DTT	Dithiothreitol
EDTA	Ethylaminediaminotetraacetic Acid
HPLC	High Performance Liquid Chromatography
IPTG	Isopropyl β-D-Thiogalactopyranoside
KOAc	Potassium Acetate
LB	Luria Bertani
MBF	Methylbutylformamide
NADP(H)	Nicotinamide Adenine Dinucleotide Phosphate (reduced)
NDEA	*N*-Nitrosodiethylamine
NDMA	*N*-Nitrosodimethylamine
NMAA	*N*-Nitrosomethyl-*n*-amylamine (*N*-Nitrosomethyl-*n*-pentylamine)
NMBA	*N*-Nitrosomethyl-*n*-butylamine
NMBzA	*N*-Nitrosomethyl-*n*-benzylamine
NNK	*N*-Nitroso-4-(methyl)-1-(3-pyridyl)-1-butanone
NNN	*N*-Nitrosonornicotine
NZ	Enzymatic Casein Digest
P450	Cytochrome P450
PCR	Polymerase Chain Reaction
RACE-PCR	Rapid Amplification of Cohesive Ends-PCR
RT-PCR	Reverse Transcription followed by PCR
SSC	Saline Sodium Citrate
SSPE	Saline Sodium Phosphate-EDTA
(S)TE	(Saline) Tris-EDTA buffer
TAE	Tris Acetate-EDTA buffer
TEMED	N, N, N′, N′-Tetramethylethylenediamine
TWEEN 20	Polyoxyethylenesorbitan monolaurate
X-gal	5-Bromo-4-Chloro-3-Indolyl β-O-Galactopyranoside

CHAPTER 1   Introduction

1.1   Oesophageal Cancer

Oesophageal cancer is one of the ten most common cancers in man (Parkin *et al.*, 1993) and is a leading cause of cancer deaths in certain parts of the world (Pisani *et al.*, 1993). It is relatively rare in the UK with about 7000 new cases each year. Survival rates are uniformly poor, which is evident from the incidence and mortality rates determined by Parkin *et al.* (1993) and Pisani *et al.* (1993) respectively. In the UK 70 to 80% of its victims die within a year of diagnosis and the 5-year survival rate is very low at 7% (Cancer Research Campaign Annual Report, 1990). Oesophageal cancer occurs in two forms – adenocarcinoma and squamous cell carcinoma. Historically, squamous cell carcinoma has been the most common of these cancers and still represents about 90% of all oesophageal cancers. However, there has been a rapid increase in adenocarcinoma of the oesophagus in the West (Blot *et al.*, 1991; Devesa *et al.*, 1998; Powell *et al.*, 1987; Yang and Davis, 1988). In the USA, the incidence of adenocarcinoma of the oesophagus in white males increased by over 350% during the 20-year period from 1974 – 1976 to 1992 – 1994, surpassing the incidence of squamous cell carcinoma, which has seen a steady decline of 35% within the same period. Adenocarcinoma of the oesophagus develops from a precursor lesion known as Barrett's oesophagus (Spechler *et al.*, 1996). Barrett's oesophagus is caused by prolonged oesophageal-gastric reflux, which causes damage and ulceration to the oesophagus. The lost squamous epithelium is replaced by columnar epithelium, which is normally found in the stomach and small intestine. The presence of columnar epithelium results in the secretion of various types of mucus and increases the rate of cell replication and the level of the enzyme ornithine decarboxylase, which is associated with cell replication. Most adenocarcinomas occur in the lower third of the oesophagus (Yang and Davis, 1988) most likely due to their association with Barrett's oesophagus. Adenocarcinomas are normally associated with the mucous glands, thus limiting experimental study of this type of tumour to animals in which these glands are present, e.g. dog, pig and monkey. In squamous cell carcinoma there is a proliferation of the squamous epithelial cells resulting in thickening of the

epithelium and eventually invasive carcinomas. Squamous cell cancers could also occur as papillomas, which are non-invasive pedunculated tumours that cause discomfort and death by oesophageal obstruction. The cause of oesophageal squamous cell cancer is unknown but, as discussed at greater length below, the pattern of mutation of the *p53* gene in adenocarcinoma is entirely different from that seen in squamous cell carcinoma indicating that they have a different aetiology (Hainaut and Hollstein, 1999). This thesis is concerned only with squamous cell carcinoma. To enable a better understanding of the different types of cancer, a brief mention of the structure of the oesophagus is necessary.

1.1.1   Structure of the Oesophagus
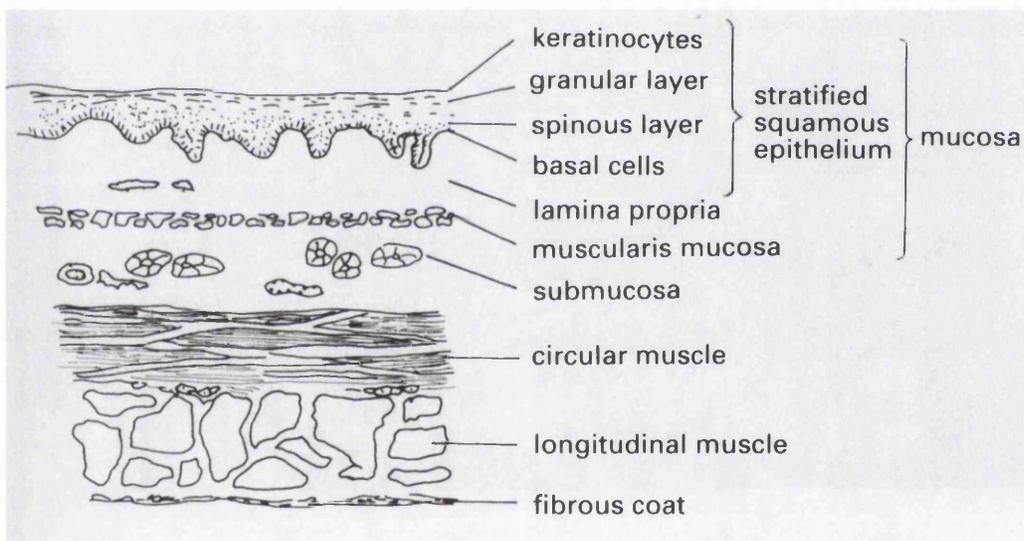
Figure 1-1   Diagram of transverse section of rat oesophagus. Taken from Craddock, 1993.

The oesophagus is a straight muscular tube running from the pharynx to the stomach and is about 25 cm long in man and 5 cm in the rat. The histological structure of the oesophagus of all mammals is very similar (Figure 1-1). The lumen of the oesophagus is lined with stratified squamous epithelium, which is continuous with that of the mouth and the skin. The squamous epithelium of the oesophagus is endodermal in origin, while that of the mouth and the epidermis of the skin are derived from the ectoderm. The squamous epithelium of the oesophagus is made up of different cell types – flat squamous cells, which may or may not be keratinised, at the luminal surface, followed by polygonal granular cells, large spinous cells and a

basal layer of cuboid replicating cells that rest on the basement membrane. Blood vessels do not extend into the epithelium, resulting in a decreasing supply of nutrients moving towards the lumen from the basement membrane, and hence a decrease in metabolic activity. A layer of loose connective tissue, the lamina propia, occurs below the stratified squamous epithelium. This contains scattered lymphocytes, and in humans, mucous glands. A layer of circular and longitudinal smooth muscle, the muscularis mucosa, is found external to the lamina propia, and separates it from the submucosa, which is composed of dense connective tissue. A layer of circular muscle, followed by a layer of longitudinal muscle occurs below the submucosa.

1.1.2   Epidemiology of Oesophageal Cancer

The widest variation in geographical distribution of any type of cancer is seen in oesophageal cancer (Day, 1975). There is a 300-fold variation in incidence across the world, with very large differences in occurrence from one country to another and from one region to another within any one country. The cause of this type of cancer is not known and a considerable number of external factors have been associated with it, none strong or consistent enough for any firm conclusions about their etiological role to be drawn. However, one factor that appears to be ubiquitous is socio-economic status, with this type of cancer being most prevalent among people from the lower socio-economic groups.

High incidence regions occur within the oesophageal 'cancer belt' of Central Asia, which stretches from Iran to China, parts of South Africa and Eastern Africa and the Normandy district of France (Figure 1-2).
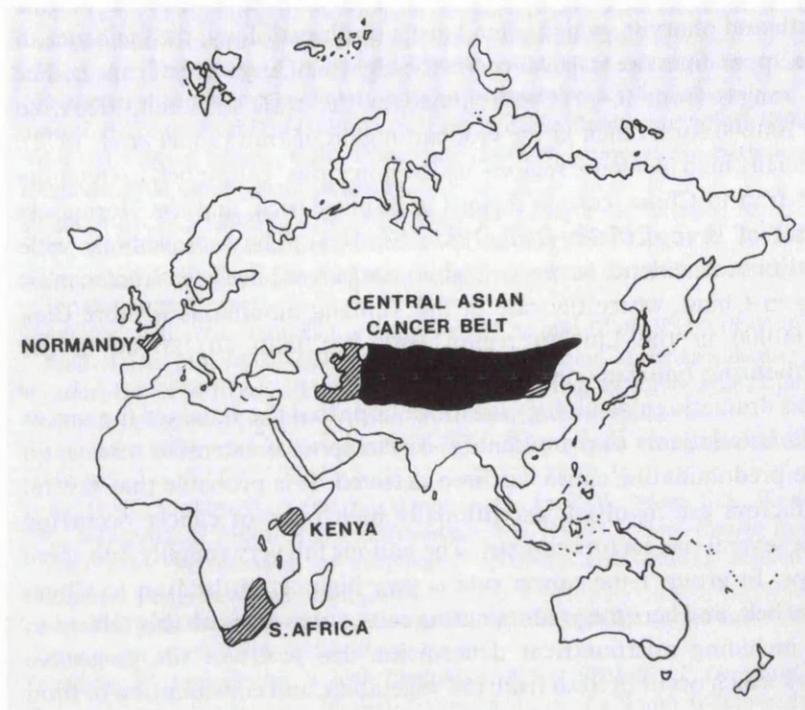
Figure 1-2 Geographical distribution of oesophageal cancer, showing the 'oesophageal cancer belt' of Central Asia. The black area represents very high incidence, the hatched area, high incidence and the white area, moderate to low incidence. Taken from Craddock, 1993.

In Western countries, alcohol and tobacco are the two most important etiological factors in oesophageal cancer (Tuyns, 1979; Yu *et al.*, 1988). The two act approximately multiplicatively (Tuyns, 1979; Wynder *et al.*, 1977) and account for up to 80% of oesophageal cancers in North and South America, Europe, South Africa and some Asian countries (reviewed by Russo and Franceschi, 1996). In Europe, particular reference is usually made to France, where the apple-based brandy, known as Calvados, when served hot, has been shown to carry a greater risk than other types of alcohol (Launoy *et al.*, 1997). Launoy *et al.* (1997) attributed a fall in oesophageal cancer incidence in the west of France over a 20-year period (Launoy *et al.*, 1994) to a decline in the custom of drinking hot Calvados. The heat may not be important however, because there is a similar high incidence of this cancer in the areas of Scotland where whisky is distilled (Kemp *et al.*, 1992).

In China and Iran, where the incidence of oesophageal cancer is several times higher than in Western countries, there is no association with alcohol or tobacco. Historical records show that cancer of the oesophagus, previously described as the "hard of swallowing disease," has existed in Linxian, a county in Henan province of China for

generations (Yang, 1980). In China, the presence of nitrosamines in food, a poor nutritional diet and the high consumption of pickled vegetables have been implicated as causes for oesophageal cancer. The consumption of food contaminated with fungus, which is able to convert nitrate to nitrite, a precursor of nitrosamine synthesis, was also implicated. In Iran, there is no direct association between exposure to nitrosamines and the high incidence of oesophageal cancer observed. In this part of the world, alcohol does not play a significant role and the incidence of oesophageal cancer has been associated with high opium consumption and poor nutrition (Cook-Mozaffari *et al.*, 1979; Hormozdiari *et al.*, 1975).

An interesting aspect of the epidemiology of oesophageal cancer is the steady decline that has been observed in parts of the world. Devesa *et al.* (1998) reported a 35% decrease in the incidence of squamous cell carcinoma in white males over a 20-year period ending in 1994. There was also a decline in the incidence in black males, although the levels were still higher than in white males.

1.1.3   Theories of causation of oesophageal cancer

The cause of squamous cell cancer of the oesophagus is not known, but several speculative theories have been proposed. Studies of the mutations in the *p53* gene in oesophageal cancer have been particularly informative. *p53* mutations are the most common cancer-related genetic change known (Hollstein *et al.*, 1991). The normal *p53* gene encodes a 53 kD nuclear phosphoprotein involved in the regulatory control of cell proliferation. A single base substitution in any of the five evolutionarily conserved regions of the gene results in proteins with altered growth regulatory properties. An interesting feature of *p53* base substitutions is that the kind of base substitution is related to the causative mutagen, i.e. whether endogenous or exogenous (reviewed by Hainaut and Hollstein, 1999). About 23% of all mutations in *p53* are G to A transitions in DNA sequences where the original G was in the sequence CpG. Essentially, all the C's in this sequence in *p53* are methylated to form 5-methylcytosine and the mutations are believed to originate from spontaneous deamination of these 5-methylcytosines to form G:T mismatches. By contrast, G:C to T:A transversion mutations are believed to result from carcinogen-induced damage to the DNA. The ratio of G to A transitions at CpG sites to G to T transversion

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mutations is about 5 in adenocarcinomas but only about 0.6 in squamous cell carcinomas (Hainaut and Hollstein, 1999). The great difference in this ratio seen in these two types of cancer shows that they must have a different aetiology, as one would have suspected from their very different epidemiology. The preponderance of G to T transversion mutations in squamous cell carcinoma of the oesophagus indicates that carcinogen-induced DNA damage plays a very significant role in this cancer. It is interesting that the only cancer with a greater preponderance of G to T transversions is lung cancer, where the role of carcinogens is well established (Hainaut and Hollstein, 1999).

Several factors have been associated with the aetiology of oesophageal cancer, but those that have been most extensively investigated are exposure to silica fibres, malnutrition and exposure to nitrosamines. No definite mechanism for the action of silica fibres has been identified, but it has been proposed that the silica fibres could stimulate cell replication by causing continuous irritation of the mucous membrane. O'Neill et al. (1980) were among the first to propose the possible relevance of silica fragments to oesophageal cancer. It was later observed that silica promoted the growth of cells in culture, possibly by providing anchorage (O'Neill et al., 1986). They also found that the inhabitants of the oesophageal cancer belt in northern Iran and China ate bread made from millet, one of the grasses that form natural silica needles on their seeds. The millet flour was found to contain up to 20% by weight of silica. Silica needles, probably from this source, were found associated with human oesophageal tumours in China (O'Neill et al., 1982), and in a subsequent experiment, millet derived silica was shown to promote tumour formation in mouse skin (Bhatt et al., 1984).

The influence of malnutrition on oesophageal cancer is more important in areas where there is a low socio-economic status, e.g. China, Iran and Africa. Low consumption of fresh fruit has been associated with oesophageal cancer in certain areas and epidemiological studies and animal experiments have shown that deficiencies in certain micronutrients, e.g. riboflavin and zinc, are associated with the development of oesophageal cancer (Yang, 1980). The fact that zinc is a trace element that occurs at the highest levels in more expensive foods like meat, fish and green vegetables could explain the correlation of zinc deficiency with low socio-economic status.

Laboratory experiments have shown that zinc deficiency increases the incidence of oesophageal cancer by apparently enhancing the effect of oesophageal carcinogens. Zinc deficiency in rats, induced by feeding soybean protein in which the zinc content has been reduced by EDTA-treatment, increases the oesophageal carcinogenicity of N-nitrosomethyl-n-benzylamine (Fong et al., 1997; Fong et al., 1978; Gabrial et al., 1982) and replenishment of zinc levels reduces this carcinogenic effect (Fong et al., 1998). There also appears to be some association between riboflavin deficiency and oesophageal cancer incidence, but the evidence for this is inconclusive. This is because trace micronutrients tend to occur in similar foods and a dietary deficiency of one of these micronutrients is often accompanied by deficiencies of several others (reviewed by Craddock, 1993). As mentioned earlier, because these micronutrients occur mainly in the more expensive foods like milk, meat and green leafy vegetables, it is people from the poorer rural communities and of lower socio-economic status who suffer from deficiencies. In support of this view, in the developing countries mentioned, it is in people from the poor rural areas that the incidence of oesophageal cancer is highest (Gammon et al., 1997). It has also been suggested that deficiencies in vitamins A and C could contribute to the development of oesophageal cancer but there is no experimental evidence for the contribution of a deficiency of either vitamin A and or vitamin C (reviewed by Craddock, 1993) to the development of oesophageal cancer. Being an antioxidant, vitamin C inhibits endogenous nitrosamine synthesis in the stomach (Ohshima and Bartsch, 1981; Wagner et al., 1985), which could therefore explain the protective effect of fresh fruit and vegetables for various types of cancer, including oesophageal cancer. Vitamin C inhibits endogenous nitrosation by reducing nitrite, which is the required nitrosating agent, to nitrogen oxide (Mirvish et al., 1972), a poor nitrosating agent.

Exposure to nitrosamines, with which this thesis is concerned, appears to be the most likely etiological factor in humans because nitrosamines are the only carcinogens that will induce tumours in laboratory animals. After the discovery in 1956 by Magee and Barnes (Magee and Barnes, 1956) that administration of N-nitrosodimethylamine to rats induced liver tumours there was a proliferation of studies of the carcinogenic properties of other nitrosamines notably by Druckrey and his colleagues (Druckrey et al., 1967). Over 300 nitrosamines have been tested for their carcinogenicity and it has been discovered that nitrosamines are active in all species. More than half of the

nitrosamines that have been tested in rats will induce oesophageal tumours independently of the route of administration (Druckrey *et al.*, 1967); reviewed by Lijinsky, 1992).

1.2 Nitrosamines and Cancer

Many nitrosamines have been found to be carcinogenic in all laboratory animal species so far examined, e.g. mice, rats, hamsters, guinea pigs, dogs and monkeys. The nitrosamines show striking organ specificity (Hodgson *et al.*, 1980; Kleihues *et al.*, 1981; Mehta *et al.*, 1984; Okada, 1984), and although almost all organs of the body are susceptible to the carcinogenic action of these nitrosamines, the most frequent target organs are the oesophagus, liver and kidney. Nitrosamines occur all around us – in industry (Spiegelhalder and Preussmann, 1983), in the environment, in food, in tobacco and tobacco smoke (Hecht and Hoffman, 1988; Preussman and Stewart, 1984), and can be synthesised within our bodies. Nitrosamines are *N*-nitroso compounds, of which there are two main groups – the *N*-nitrosamines and the *N*-nitrosamides. Whereas the *N*-nitrosamides are chemically reactive with a broad site action and have target organs dependent on the route of administration, the *N*-nitrosamines are chemically inert with striking organotropism and induce tumours in target organs independent of the route of administration (reviewed by Craddock, 1993). The nitrosamines are therefore absorbed into the general circulation and reach the target organs by this route, before being metabolically activated in these target organs. The ability for a particular organ to metabolically activate specific nitrosamines is dependent on the particular cytochrome P450 enzymes that the organ expresses.

The studies of the carcinogenicity of various nitrosamines by Druckrey *et al.* (1967) show that the susceptibility of various organs to a particular nitrosamine is dependent on the structure of the nitrosamine. They concluded from their findings that symmetrical dialkylnitrosamines induced liver tumours while asymmetrical dialkylnitrosamines induced oesophageal tumours. This has been shown by other researchers not to be entirely true. In the rat and hamster, the liver is susceptible to the carcinogenic action of a large number of nitrosamines. In some cases, however, nitrosamines that produce tumours in a particular organ in one species are not

carcinogenic for the same organ in another (Schulze et al., 1990). It has been shown for example that N-nitrosomorpholine will induce liver tumours in the rat, but not in the hamster (Lijinsky and Reuber, 1984), while N-nitrosomethyl-n-propylamine and N-nitrosomethyl-n-butylamine produce liver tumours in the hamster, but not in the rat (Lijinsky and Kovatch, 1988). But generally, of over 300 nitrosamines tested on various animals, no species has been found to be resistant to the action of these carcinogens. The simplest nitrosamine, N-nitrosodimethylamine induces primarily liver tumours when administered to rats in low doses in drinking water (Peto et al., 1984), but induces lung and kidney tumours when higher doses are given by gavage (Lijinsky et al., 1987) or intravesicular injection (reviewed by Lijinsky, 1992). The next higher homologue, N-nitrosomethylethylamine induces tumours of the liver and lung in rats, and when administered at high doses, tumours of the oesophagus are also induced (Lijinsky, 1992). The carcinogenicity of methylalkylnitrosamines for the liver decreases with increasing length of the alkyl chain, while carcinogenicity for the oesophagus increases until the C-9 compound (von Hofe et al., 1987). An interesting nitrosamine is N-nitrosodi-n-butylamine, which undergoes two stages of metabolism, hydroxylation of the α-carbon and of the ω-carbon. In the latter case 4-hydroxybutylnitroso-n-butylamine, a more potent carcinogen that induces exclusively urinary bladder tumours (Druckrey et al., 1967), is formed. When administered to rats at high doses, N-nitrosodi-n-butylamine induces mainly tumours of the liver, with a few of the oesophagus and urinary bladder. The organotropism shifts with decreasing dose size from the liver to the oesophagus and the bladder, with the bladder becoming the target organ (Druckrey et al., 1967).

A major factor that has been shown to affect the organospecificity and metabolism of nitrosamines is alcohol consumption. Alcohol, which plays an important role in human oesophageal cancer (Tuyns, 1979; Wynder et al., 1977), acts by altering the pharmacokinetics of the nitrosamine. Even though there is high correlation between alcohol consumption and oesophageal cancer, there is neither experimental nor epidemiological evidence to show that alcohol per se is carcinogenic. It however appears to increase the susceptibility of various organs to the carcinogenic action of nitrosamines and has a multiplicative effect on cancer incidence when combined with smoking (Wynder et al., 1977). In laboratory experiments administration of

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N-nitrosodimethylamine in 5% ethanol produced a six-fold increase in the methylation of kidney DNA as well as a slight decrease in liver DNA methylation (Swann, 1982). This change in organotropism was attributed to the inhibition of first pass clearance. *In vitro* experiments with rat liver microsomes or slices showed that alcohol was a competitive inhibitor of the metabolism of N-nitrosodimethylamine in liver (Peng *et al.*, 1982; Swann *et al.*, 1984). *In vivo* experiments with N-nitrosodimethylamine showed that co-administration of 1% ethanol produced a 4-fold increase in the incidence of lung tumours in mice and that there was an increase in lung tumour incidence with increasing ethanol concentration (Anderson *et al.*, 1992). Similar experiments carried out in rats showed that administration of N-nitrosodiethylamine in 10% ethanol increased the incidence of oesophageal tumours more than 4-fold and showed that ethanol alone was unable to induce oesophageal tumours (Aze *et al.*, 1993). In experiments using the oesophageal-specific carcinogen, N-nitrosomethyl-*n*-benzylamine, investigators reported that ethanol was only able to promote the occurrence of oesophageal tumours when administered after tumour initiation and that co-administration of the nitrosamine with ethanol inhibited tumour formation (Mufti *et al.*, 1989). Even though these last experiments with N-nitrosomethyl-*n*-benzylamine failed to show the importance of inhibition of hepatic metabolism of the nitrosamine to its carcinogenicity, they do show that ethanol is capable of enhancing oesophageal tumour incidence. This effect of ethanol has been attributed to the inhibition of first pass clearance of the nitrosamines from the portal blood, a process carried out by an ethanol-inducible cytochrome P450, CYP2E1, in the liver.

1.2.1 Sources of Nitrosamine Exposure

Nitrosamines are readily formed from secondary amines, and to a lesser extent tertiary amines, and nitrate once the pH is favourable (Figure 1-3). Humans are exposed to preformed nitrosamines present in the diet, in tobacco smoke (Hecht and Hoffman, 1988; Preussman and Stewart, 1984), and in the workplace. The major source of exposure, however, is probably from endogenous formation.

Exposure to preformed nitrosamines in the diet is probably not important in developed countries, but is considered to be an important factor in the Henan Province of China,

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where the incidence of oesophageal cancer rises over 100 per 100,000. In this area of China, N-nitrosodimethylamine and N-nitrosodiethylamine have been identified in food samples including wheat, corn, millet, dried sweet potato and pickled vegetables. Nitrosamines have very rarely been found in food samples from nearby low-incidence areas. Nitrosamines have been detected in cured meats to which nitrite is normally added to prevent the growth of *Clostridium spp.* The importance of food contaminated with fungi is based on the observation that fungi are able to methylate primary amines to secondary amines and then form nitrosamines from these secondary amines by nitrosation with sodium nitrite (Ji *et al.*, 1986). Nitrosamines are chemically stable compounds, and unless they are volatile, preformed nitrosamines in food remain there until they are metabolised by P450s in the body after the food has been eaten.

Tobacco smoke contains significant amounts of N-nitrosodimethylamine that is formed by nitrosation during smoking. However, the concentration of N-nitrosodimethylamine is higher at the smouldering end of the cigarette than in the mainstream smoke and could be more of a hazard to non-smokers who inhale the polluted air than to the smokers themselves (Preussman and Stewart, 1984). The tobacco-specific nitrosamines are formed from the alkaloids present in tobacco. The major alkaloid, nicotine, undergoes nitrosative dealkylation, and together with one of the minor alkaloids, nornicotine, a secondary amine, forms N-nitrosonornicotine, a potent nasal carcinogen (Singer and Taylor, 1976). A more potent tobacco-specific nitrosamine is 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), which induces tumours of the liver, lung and nasal mucosa (Hecht *et al.*, 1988; Hecht *et al.*, 1980). It is also formed from the nitrosative dealkylation, followed by ring opening of nicotine. Other minor alkaloids are anabasine and anatabine, also secondary amines, which form N-nitrosoanabasine and N-nitrosoanatabine respectively (Hecht and Hoffman, 1989). All these nitrosamines occur in cured tobacco and not in the growing plant suggesting that they are formed during the curing process by reaction of the alkaloids with nitrite produced by bacterial reduction of nitrate, or with nitrogen oxides in the flue gases.

The occurrence of volatile N-nitrosamines in industry has been investigated in depth by several researchers. Types of industries studied include the rubber and tyre and

metalworking industries (Spiegelhalder and Preussmann, 1983). Nitrosamines detected include *N*-nitrosodimethylamine, *N*-nitrosodiethylamine, *N*-nitrosodibutylamine and *N*-nitrosomorpholine among others. The highest environmental exposure to nitrosamines so far determined is in the rubber industry. Generally, nitrosamines are formed from secondary amines or their derivatives and a nitrosating agent. Tertiary amines and their derivatives have also been known to form nitrosamines, and this is seen in the rubber and tyre industry with certain chemicals that are used as vulcanisation accelerators, e.g. sulphanamides and dialkyl dithiocarbamates. Sources of nitrosating agents in this industry include nitrogen oxides from air, and nitrogen oxide-releasing compounds like *N*-nitrosodiphenylamine, which is used as a "retarder." It has been shown that the occurrence of specific nitrosamines is directly related to the use of the corresponding vulcanisation accelerators. A rubber curing process called "salt bath curing" practised in Western Europe is another likely source of nitrosamine formation. In this process, the rubber products are cured in a bath containing a mixture of molten nitrate and nitrite salts, thus directly exposing nitrosatable compounds in the rubber to the nitrite. Factory workers in the metalworking industry are exposed to cutting oils contaminated with *N*-nitrosodiethanolamine (Lijinsky *et al.*, 1980).

Experimental evidence has shown that nitrosamines are formed *in vivo* after the ingestion of harmless precursors (Ohshima and Bartsch, 1981) in a reaction influenced by the presence of activators and inhibitors of nitrosation. For example, it has been shown that the saliva of smokers contains large amounts of thiocyanate, a compound that catalyses nitrosation, and smokers have an increased ability to nitrosate administered proline (Hoffman and Brunneman, 1983). Also, administering vitamin C, a strong anti-oxidant, with the nitrosamine precursors causes inhibition of the nitrosation reaction (Hoffman and Brunneman, 1983). The stomach contains a wide variety of amines derived from food constituents and food additives or from drugs and medicines, and the acidic environment is favourable for nitrosation. The nitrosating agent is nitrite that is formed from the reduction by oral bacteria of nitrate present in saliva. Small amounts of nitrite are also obtained in the diet from cured meats and nitrite-preserved foods like bacon and fish. The carcinogenic effects of the nitrosamines formed would be determined by their structure and the amount produced.

1.2.2 Activation of Nitrosamines by Metabolism

The simplest, most common and most extensively studied dialkylnitrosamine is N-nitrosodimethylamine. Using ^{14}C-labelled N-nitrosodimethylamine it was shown that in animals CO_2 is the major measurable radioactive product of the *in vivo* metabolism of N-nitrosodimethylamine (Heath and Dutton, 1958). It was concluded from this finding that demethylation of N-nitrosodimethylamine occurs, followed by the oxidation of the removed methyl groups. It was also suggested that the metabolite, rather than N-nitrosodimethylamine itself, is responsible for the observed biological effects. Formaldehyde is a major product of *in vitro* metabolism. The metabolism of N-nitrosodimethylamine requires O_2, and is localised entirely in the microsomal and cytosolic fraction of liver preparations, with optimal metabolism requiring NADPH.

It has been accepted after extensive research in various laboratories that the metabolic activation of N-nitrosodimethylamine begins with hydroxylation of the carbon moieties, which is believed to be the critical, rate-limiting step. With N-nitrosodimethylamine, the hydroxylated N-nitrosodimethylamine formed is extremely unstable and is hydrolysed, yielding formaldehyde and N-nitrosomonomethylamine, another very unstable compound, which then decomposes spontaneously to form the methylating intermediate that reacts with macromolecules in the liver. The exact chemical nature of the methylating intermediate is not clearly understood, but several compounds have been suggested, including diazomethane, methylcarbonium ion and methyldiazonium hydroxide. Studies using deuterated N-nitrosodimethylamine have confirmed that the alkylation of nucleic acid occurs via a methylcarbonium ion (Wade *et al.*, 1987). The mechanism of the reaction is shown in Figure 1-3.

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$$
\begin{array}{c}
\underset{ONN}{\overset{CH_2R'}{\diagup}}_{CH_2R''} \longrightarrow \underset{ONN}{\overset{CHOHR'}{\diagup}}_{CH_2R''}
\end{array}
$$

$$
R'CHO \quad + \quad R''CH_2NNOH
$$

$$
R''CH_2{}^+ \quad + \quad N_2 \quad + \quad OH^-
$$
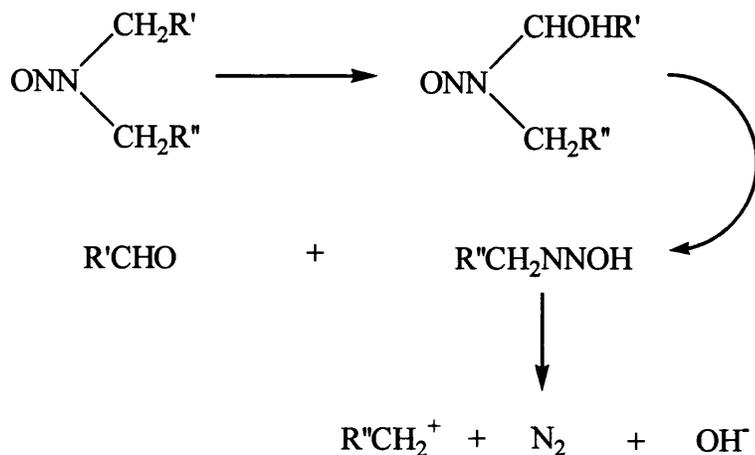
Figure 1-3   Mechanism of nitrosamine metabolism via the methylcarbonium ion intermediate.

The hypothesis that α-hydroxylation occurs is supported by experiments carried out using another nitrosamine, N-nitrosomethyl(acetoxymethyl)amine, which is more carcinogenic and more mutagenic than N-nitrosodimethylamine.   This α-acetoxynitrosamine is metabolised by esterases, which are present in all tissues, to α-hydroxylated N-nitrosodimethylamine, the unstable compound in the first step of N-nitrosodimethylamine metabolism.   Comparative measurements with N-nitroso-n-dibutylamine and N-ntiroso-n-di-$tert$-butylamine (Figure 1-4) have also shown that the metabolism and toxicity of nitrosamines are linked and that both carcinogenicity and toxicity require an unsubstituted α-carbon (the carbon adjacent to the nitroso group) that can be hydroxylated by the cell to form the unstable intermediate (Heath, 1962; Magee and Lee, 1963).

A.
$$
\underset{ONN}{\overset{CH_2CH_2CH_2CH_3}{\diagup}}_{CH_2CH_2CH_2CH_3}
$$

N-nitrosodibutylamine
toxic and carcinogenic

B.
$$
\underset{ONN}{\overset{C(CH_3)_3}{\diagup}}_{C(CH_3)_3}
$$

N-nitrosodi-$tert$-butylamine
non-toxic and not carcinogenic
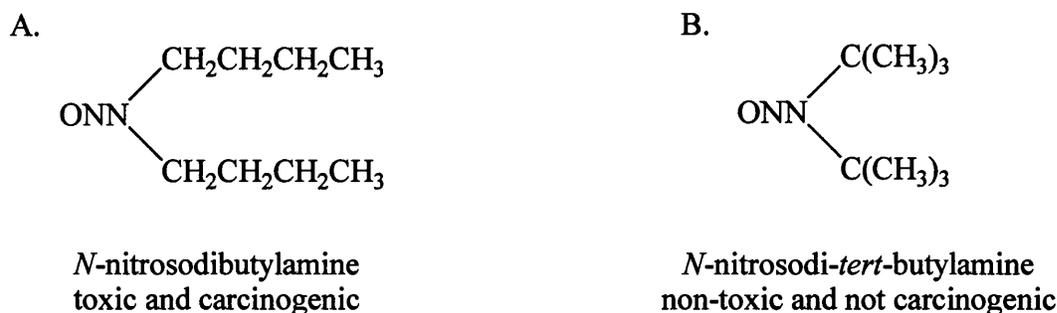
Figure 1-4   Structures of nitrosamines (A) with and (B) without unsubstituted α-carbon atoms available for methylation.

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1.2.3   The Carcinogenic Action of Nitrosamines

The methylation of organ macromolecules was demonstrated by Magee who administered ^{14}C-labelled N-nitrosodimethylamine to rats and found methylation of intact nucleic acids and proteins of the liver (Magee and Farber, 1962; Magee and Hultin, 1962). Later studies showed that administration of all carcinogenic N-nitroso compounds results in the alkylation of nucleic acids (Magee and Lee, 1964). The methylation of nucleic acids was predominantly at the 7 position of guanine and it was first believed that alkylation at this position was the cause of the carcinogenicity of N-nitrosodimethylamine until it was discovered that the degree of alkylation at this position showed no correlation with the incidence of tumours (Schoental, 1969; Swann and Magee, 1968). It has been shown that methylation of guanine at this position does not affect its pairing with cytosine. Also, the coding properties of DNA containing 7-alkylguanine are not lost, indicating that this modification is of little biological significance.

However, in 1969 Loveless showed that N-methyl-n-nitrosourea, which is carcinogenic, caused methylation of deoxyguanosine at the O^6 position while the weakly carcinogenic methyl methanesulfonate did not (Loveless, 1969). There is a positive correlation between the formation of O^6-methylguanine and the ability to induce mutations because O^6-alkylguanine in DNA does not base pair with cytosine. The alkylated base pairs with thymine instead and in the next round of DNA replication, adenine is incorporated at that point, resulting in a G→A mutation. Such a mutation in oncogenes and tumour suppressor genes, e.g. ras and $p53$ genes could result in their activation or suppression respectively. Point mutations in codons 12, 13 and 61 of H-, K-, and N-ras genes, resulting in the formation of a mutant ras p21 protein, are found in many tumour samples, e.g. G→A transition at codon 13 of the K-ras gene in patients with suspected vinyl chloride-induced liver cancer. With respect to nitrosamine-induced tumour formation, rat mammary tumours induced by N-methyl-N-nitrosourea, which is known to react with guanine to form O^6-methylguanine, contain a G→A transition mutation at codon 12. The occurrence of G→A transition mutations in codon 12 of H-ras in NMBzA-induced oesophageal tumours has also been reported (Lozano et al., 1994). To date, no ras mutations have been found in human oesophageal tumours (Victor et al., 1990), but several

investigators have detected *p53* mutations. The normal *p53* gene encodes a 53 kD nuclear phosphoprotein involved in cell proliferation. A single base substitution in any of the evolutionarily conserved codons of the gene results in proteins with altered growth regulatory properties. An interesting feature of p53 base substitutions is that the kind of base substitution is related to the mutagen, i.e. whether endogenous or exogenous. Also, the spectra of *p53* mutations differ according to the tumour type. There are five highly conserved domains in *p53*, coded for in exons 5 to 8. Investigators have observed that nearly all mutations in *p53* occur in this region. The major mutations observed are G→A transitions. A smaller percentage of G→T and G→C transversions as well as A→G transitions have also been detected. All the mentioned mutations have been observed in nitrosamine-induced rat oesophageal tumours, with G→A transitions being the most frequent. Most of these studies involve NMBzA-induced oesophageal tumours since this is the most potent oesophageal carcinogen.

Another site of alkylation in DNA is at the O^4 position of thymine. This modification is promutagenic because it causes T→C transitions. O^4-alkylthymine pairs with guanine instead of adenine resulting in the incorporation of cytosine in the next round of replication.

The strongest evidence supporting the view that O^6-alkylation of guanine is the most important reaction in nitrosamine-induced carcinogenesis has come from the discovery that living organisms, from bacteria to mammals, can remove O^6-alkylguanine residues from DNA (Goth and Rajewsky, 1974). The protein involved is O^6-alkylguanine-DNA alkyltransferase (AGT), which was initially isolated from *E. coli*. This enzyme removes alkyl groups from O^6-alkylguanine (reviewed by Lindahl, 1982; Pegg and Byers, 1992) and O^4-methylthymidine residues in DNA leaving the guanine and the thymine in place. The enzyme has since been identified in many different species including humans. This discovery emphasises the relevance of O^6-alkylation to biological systems. AGT catalyses the transfer of the alkyl group from the O^6 position of guanine to the sulfhydryl group of a cysteine residue within its own sequence, thus restoring the DNA to its unmodified form. The cysteine residue is not regenerated, and the enzyme is inactivated, resulting in a

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progressive decrease in the active AGT content of the cell. Thus tissues with a lower AGT content would be more susceptible to the carcinogen insult of methylating agents. The most effective inhibitor of AGT in mammalian tissues is O^6-benzylguanine.

In vitro studies have shown that bacterial preparations of AGT are able to remove alkyl groups from O^4-alkylthymine, but at much slower rates than from O^6-alkylguanine. Investigators have found that the bacterial enzyme readily repairs the substituted thymine, while an AGT preparation from rat liver does not (Dolan et al., 1984).

The modification, 7-alkyguanine, which as mentioned earlier, is the major product of DNA alkylation, is repaired by spontaneous depurination and the activity of 7-methylguanine glycosylase. There is no evidence of the involvement of AGT. The removal of O^6-alkylguanine residues is rapid while removal of 7-alkylguanine is slow. This may be an indication of their relative importance in mutagenesis and/or carcinogenesis. Due to the slow removal of 7-alkylguanine from DNA, measurement of 7-alkylguanine formed during methylation experiments gives a good index of the time integrated metabolism of the nitrosamines in a particular organ (Swann and Magee, 1968; Swann and Magee, 1971).

1.2.4   Enzymes Involved in the Activation of Nitrosamines

It has become widely accepted that nitrosamines are metabolised by a microsomal monooxygenase enzyme system due to the requirement of oxygen and NADPH for their metabolism. Furthermore, the metabolism of nitrosamines is markedly inhibited by carbon monoxide, a known inhibitor of the microsomal cytochrome P450 (P450) monooxygenase enzyme system. As far as the metabolism of nitrosamines by the oesophagus is concerned, Labuc and Archer (1982) showed that oesophageal microsomes were capable of the metabolism of N-nitrosomethyl-n-benzylamine and postulated the involvement of cytochrome(s) P450. Subsequent researchers studying the oesophageal metabolism of nitrosamines have also worked on the assumption that the metabolic activation is carried out by P450s (Murphy and Spina, 1994; Patten et al., 1998; Smith et al., 1998; von Weymarn et al., 1999) and the work in this thesis

makes a similar assumption. There have been speculations that the P450 monooxygenase system may not be solely responsible for the metabolism of nitrosamines, and that alternative mechanisms may exist. One suggestion for an alternative pathway is amine oxidase (Lake *et al.*, 1976). This was suggested due to the atypical binding spectra of nitrosamines with microsomes, which were different from the normal spectra for mixed function oxidases, and the fact that *N*-nitrosodimethylamine demethylation was inhibited by benzylamine, a substrate of monoamine oxidase. However, no definitive evidence for this pathway has yet been published.

1.3 The Cytochrome P450-Dependent Monooxygenase System

Cytochromes P450 (P450s) are a family of membrane bound haem-containing proteins present throughout most of the phylogenetic spectrum (bacteria and yeast, plants and animals – both invertebrates and vertebrates). The carbon monoxide complex of sodium dithionite-reduced P450 absorbs light maximally at 450 nm, hence the name P450 (Omura and Sato, 1964). P450s are found mainly in the smooth endoplasmic reticulum, with a very small proportion occurring in mitochondria. Over 480 P450s have been characterised so far at the level of their DNA sequences from 85 eukaryotic and 20 prokaryotic species. Currently 74 P450 gene families have been designated, of which 14 are present in all mammalian species so far examined (Nelson *et al.*, 1996). At the current state of knowledge, however, it is suggested that there are no more than 40 to 50 P450s in each species. Due to the complex nature of this family of hemoproteins, it is beyond the scope of this introduction to discuss them in detail. The more general aspects of mammalian P450s that have been associated with *N*-nitroso compounds will be discussed.

In mammals, P450 are found in many tissues, with the liver having the widest array of isozymes. They are the major constituent proteins of the microsomal mixed-function monooxygenases and are involved in the metabolism of a whole range of chemicals, both endogenous and exogenous. P450s play critical roles in the synthesis of some steroids in which case a deficiency causes potential health risks (Payne, 1990; Ryan, 1982). They are associated with the oxidative metabolism of steroids, fat-soluble vitamins, eicosanoids, fatty acids, and alkaloids, detoxification of xenobiotics,

including drugs, and with regard to this thesis, the activation of procarcinogens. P450s, which are part of the Phase I drug metabolising enzyme system, work together with the Phase II drug metabolising enzymes, e.g. glutathione-S-transferase, to rid cells of hydrophobic substances that would disrupt membrane function and could eventually lead to cell death if left to accumulate in cell membranes.

The major components of the cytochrome P450-dependent monooxygenase system are cytochrome P450, NADPH-dependent cytochrome P450 reductase and lipid, and in some cases cytochrome b_5 and cytochrome b_5 reductase.

1.3.1   P450 Nomenclature

To prevent incorrect assignments or duplication of gene or protein names, the designation of names to P450s requires the agreement of the P450 Nomenclature Committee of the International Union of Biochemistry, which prefers the term "heme-thiolate protein" instead of "cytochrome" to be used. This is because P450s are not "cytochromes" in the true meaning of its terminology and the term has only been maintained because of the name given to them when they were initially discovered (Nelson *et al.*, 1993).

The nomenclature of P450s relates to both genes and proteins. Each P450 gene generally produces a single protein, except in cases where alternative splicing may occur to produce a functional enzyme with a new catalytic activity or tissue-specific expression. In this case, there is differential processing of the initial P450 transcript resulting in the exchange of entire exons or portions of exons.

The nomenclature of cytochromes P450 and their genes is based on sequence similarity of the derived protein. In naming a P450 gene the italicised root symbol "*CYP*" ("*Cyp*" for the mouse), denoting *cy*tochrome *P*450 is used, followed by an Arabic number to designate the P450 family, a capital letter (small letter for the mouse) which indicates the subfamily when there are multiple subfamilies within a family, and an Arabic numeral to represent the individual gene, e.g. *CYP2B1*. With mouse genes, the final Arabic numeral is preceded by a hyphen, e.g. *Cyp2b-10*. "*P*" ("*p*" in mouse) after the gene number denotes a pseudogene. In naming the gene

product (protein) or mRNA, the same recommendations apply, but non-italicised in all species, e.g. CYP2B1. Proteins may also be designated "P450 2B1" or simply "2B1" (Nelson *et al.*, 1996).

The P450 superfamily is divided into families and subfamilies according to amino acid sequence similarities. The derived P450 protein sequence from one gene family usually has less than 40% amino acid identity to one from any other family. It has been found in P450 genes studied so far that those within a given subfamily are non-segregating and are located adjacent to one another on the same chromosome, thus forming a "gene cluster" for a particular family. Hence, within a single family, P450 protein sequences are usually more than 40% identical with some exceptions usually relating to insect or mitochondrial proteins. Mammalian sequences within the same subfamily are always more than 55% identical. All genes within a given family that have been examined so far contain the same number of exons and similar intron-exon boundaries.

Cytochromes P450 are either constitutively expressed in the tissues in which they are found or are induced by certain chemicals. Generally, the constitutively expressed P450s are involved in the metabolism of endogenous substances, while inducible P450s function in the metabolism of exogenous substances.

1.3.2 Mechanism of Catalysis by Cytochrome P450 Monooxygenases

Monooxygenases, as their name suggests, catalyse reactions in which only one of the two atoms of molecular oxygen is incorporated into the organic substrate, the other being reduced, usually to water. Since in their action two different substrates are oxidised simultaneously, monooxygenases are sometimes referred to as mixed function oxygenases. The general reaction catalysed by P450s may be represented by the stoichiometry:

$$RH + NADPH + H^+ + O_2 \rightarrow ROH + NADP^+ + H_2O$$
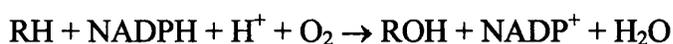
where RH represents a hydrophobic oxidisable substrate and ROH the hydroxylated product.

The cytochromes P450 catalyse, with some degree of overlapping specificity, a vast array of reactions that can be divided up into groups of reactions which include:

i.   Oxidative deamination of amphetamine (Shiiyama *et al.*, 1997; Yamada *et al.*, 1989),

ii. *N*-, O- and S-dealkylation of substances which include diazepam, codeine and 6-methylthiopurine respectively (Xu *et al.*, 1995; Yasumori *et al.*, 1993),

iii. Aliphatic and aromatic hydroxylation of compounds like pentobarbitone and lignocaine (Ellis *et al.*, 1992; Lu *et al.*, 1973; Oda *et al.*, 1989),

iv. Epoxidation of chemicals like benzene and benzo[α]pyrene,

v.   *N*- and *S*-oxidation of compounds like 3-methylpyridine, chlorpromazine and 2-acetylaminofluorine,

vi. Oxidative dehalogenation of haloethane (Olson *et al.*, 1990), and

vii. Alcohol oxidation of ethanol.

Microsomal cytochrome P450-dependent metabolism is not restricted to oxidative reactions. A wide variety of azo dyes are cleaved reductively to aromatic amine (Zbaida and Levine, 1990), and nitro compounds like chloramphenicol and nitrobenzene (Harada and Omura, 1980) are reduced to primary amines. Reductive dehalogenation also occurs in liver microsomes (Ahr *et al.*, 1982; Fujii *et al.*, 1981; Nastainczyk *et al.*, 1982).

1.3.3   P450s Involved in Xenobiotic Metabolism

The main P450s involved in xenobiotic metabolism belong in families 1 to 3. The CYP1 family has two subfamilies – 1A and 1B, which share a 68% amino acid sequence identity. Two separate genes have been identified in the 1A family, and as nomenclature dictates, have been named *CYP1A1* and *CYP1A2*. CYP1A1 has been purified from rats, mice and rabbits, but not from humans. The human form has however been expressed in several recombinant systems and has a 79% sequence identity with the rat enzyme. CYP1A1 is involved in the oxidation of polycyclic aromatic hydrocarbons and is induced by 3-methylcholanthrene, itself a polycyclic aromatic hydrocarbon (Sesardic *et al.*, 1990). It is active in the catalysis of 7-ethoxyresorufin *O*-deethylation, and this activity is used as a marker. It is expressed at very low levels in liver and other extrahepatic tissues. Only a small

percentage of human liver samples contain much CYP1A1, which is found predominantly in extrahepatic tissues. CYP1A2 has been purified from rats, mice, humans and rabbits, and the rat and human enzymes share a 75% sequence homology. The various CYP1A2 enzymes isolated catalyse the N-hydroxylation of many heterocyclic amines, and have 7-ethoxyresorufin O-deethylation, acetanilide 4-hydroxylation and phenacetin O-deethylation activities. CYP1A2 has only been identified in liver and is also induced by 3-methylcholanthrene in rat liver, but to a much smaller extent than CYP1A1 (Sesardic *et al.*, 1990). The CYP1B family has not been as extensively studied as the CYP1A enzymes and relatively little is known about it.

The CYP2 family of enzymes has six known subfamilies, designated A to G. CYP2A enzymes from mouse, rabbit, rat, hamster, monkey and humans have been investigated at great length. These enzymes metabolise a wide range of substrates and differ markedly in their catalytic specificity. For example, rat CYP2A enzymes have steroid 7α- and 15α-hydroxylation activities, which the human enzymes lack. The human enzymes are CYP2A6, the most extensively studied, CYP2A7, CYP2A9 and CYP2A13. All these enzymes are expressed at very low levels in human liver, with CYP2A6 being expressed in nasal mucosa as well (Su *et al.*, 1996). The rat enzymes are CYP2A1, CYP2A2 and CYP2A3. Rat CYP2A1 and human CYP2A9 catalyse steroid 7α-hydroxylation, while rat CYP2A2 catalyses steroid 15α-hydroxylation. Rat CYP2A3 and human CYP2A6 catalyse coumarin 7-hydroxylation in addition to having activity towards other xenobiotics. CYP2A3, which is induced by coumarin, was first identified in rat lung. It has since been shown that the rat nasal mucosa has a much higher content of CYP2A3 (Su *et al.*, 1996) and greater coumarin-7-hydroxylase activity than lung (Patten *et al.*, 1998). CYP2A4, CYP2A5 and CYP2A12 are present in mouse tissues, with CYP2A5 being expressed in liver, lung, kidney and nasal mucosa (Su *et al.*, 1996). CYP2A4 has been identified in mouse liver and nasal mucosa (Su *et al.*, 1996).

The CYP2B subfamily members are generally inducible by phenobarbital, an anti-epileptic drug. Induction of CYP2B1 and CYP2B2 in rats is at the transcriptional level (Waxman and Azaroff, 1992). These two enzymes differ in only 14 amino acid

residues and, to a large extent, catalyse the same reactions. The rate of catalysis by CYP2B2, however, is usually lower than by CYP2B1. The only CYP2B subfamily enzyme expressed in humans is CYP2B6. Other CYP2B enzymes have been isolated in several other mammals including CYP2B4 and CYP2B5 in rabbits, CYP2B11 in dogs, and CYP2B9 and CYP2B10 in mice. CYP2B enzymes are constitutively expressed at low levels in untreated animals, but high levels are observed in rat, mouse and rabbit liver after phenobarbital treatment. The human enzyme, CYP2B6, which is also phenobarbital-inducible (Gervot *et al.*, 1999) is poorly expressed in human liver and constitutes only about 1% of the human hepatic P450 content. CYP2B enzymes are generally associated with the detoxification of xenobiotics, but are also known to metabolically activate procarcinogens like cyclophosphamide. They also play roles in the activation of polyaromatic hydrocarbons and their derivatives that are primarily activated by other P450s. Human CYP2B6 is thought to be involved in the activation of the carcinogens 6-aminochrysene and 3-methoxy-4-aminoazobenzene to genotoxic intermediates (Mimura *et al.*, 1993).

The CYP2C subfamily has been extensively studied in rabbits and humans. The known human enzymes are CYP2C8, CYP2C9, CYP2C18 and CYP2C19. Homology between the various human forms of CYP2C is above 80%. CYP2C9 and CYP2C19 have tolbutamide methyl hydroxylation activity (Lasker *et al.*, 1998; Wester *et al.*, 2000). CYP2C9 has additional (*S*)-warfarin 7-hydroxylase activity. CYP2C8 hydroxylates retinoids and taxol, a drug used in treating breast cancer. There are 7 known rabbit CYP2C enzymes, which are 2C1, 2C2, 2C3, 2C4, 2C5, 2C14 and 2C16. These enzymes have 67% homology in their amino acid sequence. CYP2C3 is the predominant steroid 6β-hydroxylase in rabbit liver microsomes. CYP2C4 and CYP2C5 both have progesterone 21-hydroxylation activity, with the latter being more active.

CYP2D6 is the only known functional CYP2D subfamily member in humans and is the polymorphic debrisoquine 4-hydroxylase in humans (Gonzalez *et al.*, 1988; Kagimoto *et al.*, 1990). Other subfamily members in humans are only pseudogenes. Rats, however, have four CYP2D members referred to as 2D1, 2D2, 2D3 and 2D4, which have not been studied in great detail. Their activities are inhibited by quinine.

The human enzyme, on the other hand is inhibited by a stereoisomer of quinine, quinidine. CYP2D enzymes have also been identified in dogs (Sakamoto *et al.*, 1995) and non-human primates (Igarashi *et al.*, 1997).

Two members of the CYP2E subfamily have been identified, CYP2E1 and CYP2E2. CYP2E1 has been identified in several species including humans (Song *et al.*, 1986; Umenu *et al.*, 1988). CYP2E2 has only been identified in rabbit liver (Ding *et al.*, 1991). CYP2E1 is induced by chemicals like ethanol, acetone, pyrazole and physiological factors like diabetes (Dong *et al.*, 1988) and fasting (Tu and Yang, 1983). The increase in the hepatic level of CYP2E1 caused by the chemicals mentioned is due to protein stabilisation (Song *et al.*, 1989), while that caused by diabetes and fasting is due to RNA stabilisation (Dong *et al.*, 1988). CYP2E1 is also expressed in kidney, lung, nasal mucosa, testes, brain and ovaries. The human and rat isozymes share 78% amino acid identity (Song *et al.*, 1986).

The CYP3A subfamily is the only subfamily belonging to the 3 family of P450s. It is probably the most important drug metabolising family in humans with CYP3A4 making up over 40% of human liver P450 and metabolising a large number of different drugs. It was originally identified as nifedipine oxidase (Beaune *et al.*, 1986). Three other CYP3A subfamily members are expressed in human liver, CYP3A3, CYP3A5 and CYP3A7 (Aoyama *et al.*, 1989; Komori *et al.*, 1988; Watkins *et al.*, 1985). CYP3A1 and CYP3A2 have been identified in rat liver and are inducible by pregnenolone-16α-carbonitrile (Gonzalez *et al.*, 1985; Graves *et al.*, 1987). Other CYP3A enzymes have been identified in hamsters, dogs, mice, monkeys and rabbits.

1.3.3.1   Nitrosamine metabolism by P450s

The simplest and most extensively studied nitrosamine, *N*-nitrosodimethylamine, is metabolised by CYP2E1 (Yang *et al.*, 1985; Yang *et al.*, 1991) but at very high nitrosamine concentrations, CYP2B1/CYP2B2 are also capable of its metabolism (Yang *et al.*, 1985). It is, however, the metabolism of low concentrations of the nitrosamines that is of physiological importance since only small amounts of the nitrosamines are required to induce tumours in animals. Also, under normal

circumstances animals and humans will only be exposed to low concentrations of nitrosamines. CYP2E1 is also involved in the metabolism of N-nitrosodiethylamine (Yoo et al., 1990), but is not the sole P450 responsible for this metabolism as shown by inhibition studies with antibodies to CYP2E1. Whereas the anti-CYP2E1 inhibited N-nitrosodimethylamine metabolism by 73%, metabolism of N-nitrosodiethylamine was only inhibited by 31% (Yoo et al., 1990).

In the case of the longer chain methylalkylnitrosamines, researchers have shown that CYP2A6 is capable of the metabolism of N-nitrosodiethylamine (Bereziat et al., 1995; Camus et al., 1993). CYP2A6 is expressed in human nasal mucosa and it has been suggested that it may be responsible for the metabolism of nasal toxicants (Su et al., 1996). It has recently been reported that rat nasal microsomes metabolise N-nitrosomethyl-n-benzylamine, an oesophagus-specific carcinogen, to the same extent as the oesophagus (Patten et al., 1998). In that investigation, rat nasal microsomes were found to metabolise N'-nitrosonornicotine, which induces both nasal and oesophageal tumours. N-nitrosomethyl-n-benzylamine inhibited coumarin-7-hydroxylase activity and coumarin inhibited the metabolism of N-nitrosomethyl-n-benzylamine. Since coumarin-7-hydroxylase activity in rat nasal mucosa has been shown to be due to CYP2A3, it was suggested that CYP2A3 is responsible for the metabolism of N-nitrosomethyl-n-benzylamine in rat nasal mucosa. Another study using rat oesophageal and liver microsomes showed that coumarin inhibited the depentylation of N-nitrosomethyl-n-amylamine by 65% and it was suggested that CYP2A3 might be responsible for the activation of N-nitrosomethyl-n-amylamine in rat oesophageal microsomes (Chen et al., 1999). In a similar study to his experiments with N'-nitrosonornicotine and N-nitrosomethyl-n-benzylamine (Patten et al., 1998), Patten (Patten et al., 1997) came to the conclusion that CYP3A4 might be the enzyme responsible for 2'-hydroxylation of N'-nitrosonornicotine in human liver enzymes. No definite results have been shown to prove this suggestion. CYP2B1 has been shown to metabolise long chain dialkylnitrosamines including N-nitrosodibutylamine and N-nitrosodipropylamine (Shu and Hollenberg, 1996). Using two different concentrations of each nitrosamine, it has been shown that CYP2B1 has more than ten times as much activity towards the long chain asymmetric methyl-n-alkylnitrosamine, N-nitrosomethyl-n-butylamine, than N-nitrosodimethylamine (Yang et al., 1985).

CYP2B1 is also capable of metabolising methyl-*n*-alkylnitrosamines with the correct structural selectivity for producing a methylating agent as has been shown using *N*-nitrosmethyl-*n*-amylamine (Ji *et al.*, 1989; Lee *et al.*, 1989; Mirvish *et al.*, 1991). It is clear from work from Kleihues' laboratory (Ji *et al.*, 1991; von Hofe *et al.*, 1986; von Hofe *et al.*, 1987; von Hofe *et al.*, 1991) that the nitrosamine-activating enzyme of the oesophagus has to be chemically specific and that the highly oesophagus selective methy-*n*-lalkylnitrosamines have to be hydroxylated on the α-carbon of the alkyl chain. The alternative hydroxylation of the methyl group is a detoxifying reaction. Thus the reports by Lee *et al.* (1989), Ji *et al.* (1989) and Mirvish *et al.* (1991) that CYP2B1 and/or CYP2B2 preferentially hydroxylate *N*-nitrosomethyl-*n*-amylamine on the α-carbon of the amyl group is particularly significant.

1.3.4   Scope of the Thesis

This thesis is concerned with the metabolic activation of nitrosamines in the oesophagus of the rat because this activation is essential for their action as carcinogens, and exposure to nitrosamines appears to be the single most important determining factor for oesophageal carcinogenesis. However, nitrosamines are not central to this thesis because the basic question being investigated is whether there are drug metabolising enzymes peculiar to the oesophagus. All nitrosamines require metabolic activation and as the epidemiology and mutations in *p53* indicate that carcinogen-induced damage to DNA plays an important role in oesophageal cancer (Fong *et al.*, 1997; Hollstein *et al.*, 1991), knowledge of the enzymes in the oesophagus of man must be relevant to human cancer. These studies in the rat are a precursor to studies in man.

The experiments described in Chapter 2 show that the nitrosamine-metabolising P450 in rat oesophagus is different from the major nitrosamine-metabolising P450 in rat liver, CYP2E1. They also show that CYP2E1 essentially metabolises all the nitrosamine administered to rats and with methyl-*n*-alkylnitrosamines (except *N*-nitrosodimethylamine) most of this metabolism is hydroxylation of the methyl group. Rat oesophagus, however, has a greater ability than other organs to metabolically activate *N*-nitrosomethyl-*n*-butylamine, as is shown by the higher level of methylation in oesophagus than in any other organ (liver, kidney, lung and nasal

epithelium). This suggests that rat oesophagus expresses a P450 that is absent or very poorly expressed in these other organs. The experiments also show the different effects of ethanol on methylation of DNA of the oesophagus, liver, lung, kidney and nasal epithelium by *N*-nitrosomethyl-*n*-butylamine, proving that the oesophagus does not express CYP2E1. The Chapter also describes preliminary purification experiments carried with rat liver microsomes in an attempt to find a suitable protocol for the purification of the P450(s) expressed in rat oesophagus.

Chapter 3 describes molecular biology techniques used to identify the cDNAs of P450s expressed in rat oesophagus. Three different techniques are used – Reverse Transcription followed by Polymerase Chain Reaction (RT-PCR), Rapid Amplification of Cohesive Ends Polymerase Chain Reaction (RACE-PCR) and cDNA Library Screening. Combining these three techniques, it was possible to isolate the entire open reading frame of the cDNA of a novel P450 belonging to the 2B subfamily. This P450 has since been designated CYP2B21.

In Chapter 4 phenobarbital-treated rats are used to repeat some of the experiments described in Chapter 2 because phenobarbital treatment induces CYP2B1 and CYP2B2 in rat liver. Metabolic studies were carried out on these treated rats to determine whether CYP2B induction would increase the rate of metabolism of *N*-nitrosomethyl-*n*-butylamine, and whether the induced activity would have the necessary selectivity for the α-carbon of the butyl group. This was done by measuring the rate of metabolism of treated and untreated rats and comparing this to the levels of methylation of liver DNA.

CHAPTER 2   The Nitrosamine-Metabolising Enzyme in Rat Oesophagus

2.1   Introduction

This chapter describes experiments carried out to examine the P450(s) present in the rat oesophagus and compare the activity of the nitrosamine-metabolising P450 in the oesophagus to that in the rat liver. It also describes attempts at P450 purification from rat liver as a preliminary step to purification from oesophagus.

As mentioned in the previous chapter, the cytochromes P450 constitute a class of enzymes that are found in a wide range of living organisms, from micro-organisms to plants and humans. Four hundred and eighty one P450 genes and 22 pseudogenes from 74 gene families have been identified in 85 eukaryote and 20 prokaryote species. Of the 74 gene families, 14 are found in all mammals and comprise 26 mammalian subfamilies, of which 20 have been mapped to the human genome (Nelson *et al.*, 1996). Many of these P450s have been successfully isolated using purification techniques. The first reported success of P450 purification was partial purification of a P450 that had been induced in phenobarbital-treated rats (Lu and Coon, 1968). Since then, many different forms of P450 have been purified from animals and humans. The yields from the purification of P450s are always very low, usually in the range of 0.2 % for the electrophoretically pure (Yun *et al.*, 1991) to about 26 % for the partially purified protein (Van der Hoeven and Coon, 1974). P450s have been isolated from the livers of many mammals and from lung, brain, breast, intestine and extra-hepatic organs. No P450 has ever been isolated from the oesophagus of any animal. The ability to induce certain P450s so that there is a large quantity of protein available at the start of the purification is a very useful aid in the isolation of measurable amounts of cytochrome P450 after the final purification step. Unfortunately, no means of inducing the P450s of the oesophagus have been reported yet, so it was necessary to design a purification procedure that would result in minimal losses of P450, since the low amounts of constitutively expressed P450 would have to be purified from the oesophagus. The techniques that were used in this

thesis have been used routinely by other laboratories to isolate P450s that have been induced and are therefore available in large quantities (Guengerich *et al.*, 1982; Guengerich and Martin, 1980) or constitutively expressed P450s from other organs in which the P450s are in very limited quantities (Hellmond *et al.*, 1995). A third method which was reported to give a high yield of active P450 and be able to resolve different isozymes was also attempted (Isa *et al.*, 1992; Marriage and Harvey, 1986).

This chapter also contains a description of two related sets of experiments. The first were experiments carried out to measure the inhibition of nitrosamine metabolism by dimethylformamide (DMF), methyl-*n*-butylformamide (MBF) and diethylformamide (DEF). These experiments were undertaken initially in an attempt to find a competitive inhibitor that was selective for the nitrosamine-metabolising P450 of the oesophagus. There were several reasons for wanting a specific inhibitor of oesophageal P450 metabolism. In particular, substrates and inhibitors that are specific for one P450, or a small group of P450s, have been used frequently to show that particular P450s are expressed in various tissues. For example, it has been widely assumed that the ability to metabolise coumarin is diagnostic for the presence of P450s of the 2A subfamily. Second, it has been found that P450s that are difficult to isolate because of their lability can be isolated more successfully if substrates are included in all the solutions. For example, adding 4-methylpyrazole to samples and buffers during the purification of CYP2E2 stabilised the protein (Ding *et al.*, 1991). As it would not be safe to add nitrosamines to all the solutions used in the isolation of the P450, it was decided that a less toxic class of compounds would be used. The formamides constitute such a class. Dimethylformamide, which is one of the most common formamide derivatives, was first described as an inhibitor of hepatic *N*-nitrosodimethylamine metabolism by Heath (Heath, 1962). Heath chose this compound because it is isoelectronic with *N*-nitrosodimethylamine, but later work has shown that dimethylformamide and *N*-nitrosodimethylamine have a very similar molecular shape. In *N*-nitrosodimethylamine the two carbon atoms, the two nitrogen atoms and the oxygen atom are all in the same plane with restricted rotation about the N—N bond. Thus the actual structure is very similar to that shown:
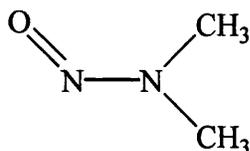
Dimethylformamide has a very similar structure, with similar restricted rotation about the C—N (carbonyl) bond:
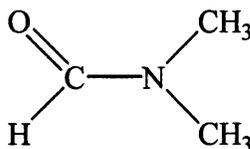
These electronic and structural similarities explain why DMF is an inhibitor of N-nitrosodimethylamine metabolism (Heath, 1962) and suggested that MBF would be a suitable inhibitor, and substrate, for the oesophageal nitrosamine metabolising P450 that metabolises N-nitrosomethyl-n-butylamine.
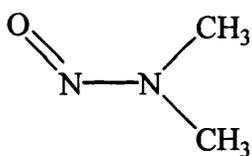
N-nitrosodimethylamine
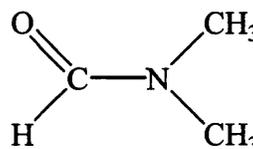
Dimethylformamide
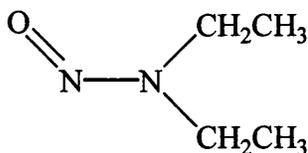
N-nitrosodiethylamine
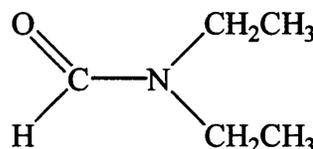
Diethylformamide
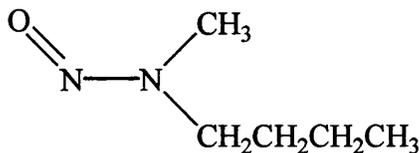
N-nitrosomethyl-n-butylamine
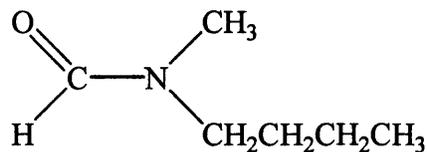
Methyl-n-butylformamide

Figure 2.1 Structures of some nitrosamines and their corresponding formamides

The second set of experiments was carried out to give a quantitative comparison of the activity of nitrosamine metabolism in the liver and the oesophagus. These experiments involve administering N-nitrosomethyl-n-butylamine, a nitrosamine that induces oesophageal cancer, to rats and then sacrificing them after several hours and removing the organs of interest. DNA is then extracted from these organs and analysed for the content of 7-methylguanine. Nitrosamines are metabolically activated by P450s in the specific organs that are susceptible to their carcinogenic action. Therefore, for a nitrosamine to induce oesophageal tumours, the oesophagus must contain the requisite P450(s) to metabolise the particular nitrosamine. This is because the carcinogenic intermediate that is formed has too short a half-life to be produced in one organ and transported in the general circulation of the body to the target organ. N-nitrosodimethylamine, the simplest nitrosamine, is not metabolised in the oesophagus, does not methylate oesophageal DNA and does not induce oesophageal tumours (von Hofe et $al.$, 1987). Previous experiments by von Hofe et $al.$ (1987) and Ji et $al.$ (1991) have shown that DNA in rat oesophagus is preferentially methylated by methylalkylnitrosamines with an alkyl chain length of 3 to 5 carbon atoms, the most effective being N-nitrosomethyl-n-butylamine (C4). These nitrosamines produce predominantly oesophageal tumours irrespective of the route of administration (Druckrey et $al.$, 1967). By contrast, methylation of rat liver DNA decreased with increasing length of the alkyl chain from N-nitrosodimethylamine (C1) to N-nitrosomethyl-n-dodecylamine (C12), with a rapid decrease within the first four members of the homologous series (von Hofe et $al.$, 1987). In particular, the level of DNA methylation after a single oral dose of N-nitrosomethyl-n-butylamine was greater in oesophagus than in liver. Ji et $al.$ (1991) showed that there was much greater DNA methylation in the oesophagus than in the liver after i.p. administration of N-nitrosomethyl-n-pentylamine and its positional isomers. In the oesophagus, 7- and O^6-methylguanine were formed by any of the isomers where the α-carbon of the pentyl group was unsubstituted (i.e. $-CH_2-$). In the liver, however, only those isomers with both unsubstituted α- and β-methylene groups produced 7- and O^6-methylguanine. These findings suggest that the P450 in the rat oesophagus responsible for the metabolism of these nitrosamines is present in a greater amount in the oesophagus than in the liver.

Because *N*-nitrosomethyl-*n*-butylamine treatment produces easily detectable levels of methylation in both oesophageal and liver DNA and is relatively simple to make labelled with [^{14}C], it was used as the nitrosamine in these studies. The methylation experiments were repeated, but this time nitrosamine was administered to the rats in the presence of ethanol. Previous experiments in this laboratory have shown that when NDMA and NDEA were administered to rats with ethanol, there was a decrease in the level of DNA methylation in the liver and an increase in that of extra-hepatic tissues (Swann *et al.*, 1984). This was attributed to the selective inhibition of the nitrosamine metabolising enzyme in the liver, which results in the inhibition of first pass clearance and an increase in the biological half-life of the nitrosamine. As a result there was an increased concentration of the nitrosamine in circulation, and hence an increased level of exposure of the extra-hepatic organs to these nitrosamines. These results with *N*-nitrosodimethylamine and *N*-nitrosodiethylamine depend on the fact that in the liver they are predominantly metabolised by CYP2E1. Ethanol is also a substrate for CYP2E1 and acts as a competitive inhibitor of the metabolism of these two nitrosamines. This experiment with ethanol had two objectives. If ethanol affected liver metabolism of *N*-nitrosomethyl-*n*-butylamine, then it could be concluded that metabolism of this nitrosamine in the liver is also largely by CYP2E1. Also, an increase in oesophageal DNA alkylation would serve as an important observation for the hypothesis on alcohol and human cancer.

2.2   Materials

Animals used were 200 to 250 g male Sprague Dawley rats that had been fed on a cubed diet TRM 9607 (Harlan Teklad, UK), and were purchased from the Biological Services Department, University College London.

Sodium cholate, butylated hydroxytoluene, cholic acid, NADPH, $NADP^+$, isocitrate, isocitrate dehydrogenase, cytochrome c, TEMED, DEAE Sephacel, Triton N-101, Polybuffer 96 and Polybuffer 74, Polyoxyethylene ether W-1 (Lubrol PX), p-chloroamphetamine, CHAPS and Tris were purchased from Sigma Chemical Co., UK. Emulgen 911 was purchased from Kao Atlas, Japan, Aquacide I was from Calbiochem, UK and Sepharose 4B from Pharmacia Biotech, UK. A strong cation exchange column, HC-X8, was purchased from Hamilton, Reno, USA. Ecoscint A was from National Diagnostics. Hydroxyquinoline was purchased from Fisons. Unlabelled nitrosamines, N-nitrosodimethylamine and N-nitrosodiethylamine, were purchased from Eastman Kodak, NY, USA. N-nitrosodi[^{14}C]methylamine and N-nitrosodi[1-^{14}C]ethylamine were synthesised by Prof. Swann from di[^{14}C]methylamine and di[1-^{14}C]ethylamine respectively, using the method of Dutton and Heath (1956). N-nitroso[^{14}C-methyl]methyl-n-butylamine was also synthesised by Prof. Swann from [^{14}C-methyl]methylbutylamine. All other chemicals were of analytical grade and were purchased from BDH, UK, unless otherwise stated.

2.3   Methods

2.3.1   Induction of Oesophageal Tumours by the Oesophagus-Specific Nitrosamine, N-Nitrosomethyl-n-benzylamine

A 1 mg/ml solution of N-nitrosomethyl-n-benzylamine in 25% dimethylsulfoxide (DMSO) was made up in normal saline and administered to 22 male rats (200 –250 g) by intra-peritoneal injection (2 mg/kg body weight) bi-weekly for 4 weeks. At the same time, 10 control rats were administered 25% DMSO in normal saline (2 ml/kg body weight). After 6 weeks the rats were killed by anaesthetising with an increasing concentration of CO_2, treated rats in groups of 4 or 5 per week and control rats in pairs. The oesophagus was removed and stripped of the surrounding muscle layer by gripping the oesophagus in the middle, across the length, with one pair of forceps and

pulling outwards from the centre with another pair and repeating the process at the other end to strip the other half. The stripped oesophagus was then slit open longitudinally and the luminal surface visually examined for any changes to the epithelium.

2.3.2 Comparison of the Methylation of DNA by *N*-Nitrosomethyl-*n*-butylamine in Liver, Oesophagus and Other Extra-hepatic Organs

Eight male Sprague Dawley rats (200 g) that had been starved overnight, but allowed water *ad libitum* were given *N*-nitroso[^{14}C-methyl]methyl-*n*-butylamine in deionised water (2 mg/kg body weight, specific activity 3.6 μCi/μmol) by gavage. After 4.5 hours the rats were killed as before. Kidneys, lungs, oesophagi and nasal epithelia were quickly removed, as well as 1 g of the median lobe of each liver and rinsed in ice cold saline sodium citrate (SSC). The nasal epithelium was obtained by stripping the skin off the head of the rat, splitting the head sagittally and scraping out the lining and bony plates of the nasal cavity, including the cartilage. The oesophagi were stripped of the surrounding muscle layer, as described previously. If necessary, the organs were stored at -20°C until required for DNA extraction.

2.3.3 Effect of Ethanol on the Methylation of DNA of Oesophagus, Liver and Other Extra-hepatic Organs by *N*-Nitrosomethyl-*n*-butylamine

Eight male Sprague Dawley rats (200 g) that had been starved overnight, but allowed water *ad libitum*, were given *N*-nitroso[^{14}C-methyl]methyl-*n*-butylamine in 5% ethanol (1.8 mg/kg body weight, specific activity 3.6 μCi/μmol) by gavage. As in the previous experiments, the rats were killed after 4.5 hours and the required organs removed, rinsed, prepared as before and stored at -20°C. DNA was extracted from the tissues by the methods described below.

2.3.3.1 Extraction of DNA from liver and kidney

DNA was extracted from liver and kidney by the method described by Kirby and Cook (1967). The frozen tissue was thawed on ice and homogenised with a Teflon pestle in a glass homogeniser in cold SSC (7 ml/g wet weight of tissue). The homogenate was transferred to 50-ml centrifuge tubes and centrifuged for 20 minutes (750g, 4°C). The pellet was washed with cold SSC (5 ml/g wet weight of tissue) by

vortex mixing and centrifuging as before. The pellet was transferred to a glass tissue homogeniser and quickly and thoroughly resuspended in 1 M NaCl (10 ml/g wet weight of tissue) with a hand-held glass pestle. The suspension was transferred to a 500 ml stoppered conical flask. 10% SDS (1 ml/g wet weight of tissue) was added and mixed quickly. Kirby phenol reagent (10 ml/g wet weight of tissue; 500 g phenol / 70 ml m-cresol / 0.5 g 8-hydroxyquinoline / 55 ml water) was added, the stopper was fitted and the mixture was shaken for 30 minutes in a mechanical shaker. The suspension was then centrifuged for 30 minutes (20,000g, 4°C). The aqueous upper layer was carefully aspirated and transferred to a 250 ml stoppered conical flask and an equal volume of ethanol was added to precipitate the DNA. The DNA was lifted out, quickly drained and dissolved in 20 ml of 5% (w/v) sodium acetate in water. Ribonuclease solution (1 mg in 10 ml sodium acetate) was added, the stopper was fitted and the mixture was incubated at 37°C for 15 minutes with occasional swirling. The DNA was reprecipitated with an equal volume of ethanol, lifted out, drained quickly and resuspended in deionised water (12 ml) in another conical flask. Equal volumes of 2.5 M Na_2HPO_4, pH 7.3 and methoxyethanol were added and mixed well. The suspension was then transferred to fresh centrifuge tubes, cooled on ice and centrifuged for 30 minutes (750g, 4°C) to remove polysaccharides. The upper layer was aspirated and the DNA precipitated with an equal volume of 1% CETAB (cetyltrimethyl-ammonium bromide). The DNA was washed twice with deionised water, drained quickly and allowed to stand for 15 minutes in 2% (w/v) sodium acetate/70% (v/v) ethanol to change the insoluble CETAB salt of DNA into the soluble sodium salt of DNA. The sodium acetate/ethanol mixture was poured off and the DNA was washed sequentially in ethanol, ethanol:diethyether (1:1) and twice in diethylether, and then placed in a vial and dried under vacuum.

2.3.3.2 Extraction of DNA from Oesophageal Epithelium, Nasal Epithelium and Lung

The oesophageal epithelium and nasal epithelium were transferred from the freezer into liquid nitrogen. They were then pounded into small pieces under liquid nitrogen using a mortar and pestle. The mortar was covered with a piece of shiny paper, with a hole cut out in the middle just big enough to fit and manoeuvre the pestle, to prevent bits of tissue flying out during pounding. The powdered tissue was mixed with 15 ml of 50 mM Tris/20 mM EDTA/20 mM NaCl/ 0.5% SDS, pH8, in a 50-ml conical tube. Protease K (2 mg) was added and the mixture was incubated at 37°C for 60 minutes

with occasional swirling and inversion. The material was transferred to a Wheaton homogeniser and homogenised by hand to break up any small pieces and then incubated for another 30 minutes. Phenol/*m*-cresol (15 ml) was added and the mixture was shaken for 10 minutes with a mechanical shaker and then centrifuged for 20 minutes (20,000*g*, 4°C). Most of the aqueous top layer was removed and 5 ml of 50 mM Tris/20 mM EDTA/ 20 mM NaCl/0.5 % SDS, pH 8, was added to the phenol layer. The mixture was shaken again for 10 minutes and centrifuged for 20 minutes as before. As much of the aqueous upper layer as possible was removed, making sure to avoid the phenol layer and any of the interfacial layer. Ethanol (20 ml) was added to the aqueous layer and the mixture was swirled gently to precipitate the DNA. The DNA was lifted out with a spatula, drained quickly, put into a vial and dried under vacuum.

2.3.4   Measurement of 7-Methylguanine

The extracted DNA from the various tissues was dissolved in deionised water (1 ml). HCl (1 M, 1 ml) was added and the mixture was boiled for 60 minutes. The hydrolysed DNA was injected onto a Hamilton HC-X8 cation exchange column and eluted at a rate of 1 ml/min with 0.4 M ammonium formate, pH 4.5 at 60°C. To maintain this temperature, the column was placed in a water bath at 60°C. 1-minute fractions were collected and the absorbance at 260 nm of each fraction measured. Ecoscint A (4 ml) was added to each fraction and the radioactivity determined by counting on a 14C program in a Packard 1600 scintillation counter.

2.3.5   CYP2E1 Metabolises *N*-Nitrosomethyl-*n*-butylamine in Rat Liver

The metabolism of ^{14}C-labelled nitrosamines in the living rat was followed by measuring the exhalation of $^{14}CO_2$ as described by Heath (Heath, 1962). A male rat (200 g) received a single i.p. dose of the appropriate nitrosamines: *N*-nitrosodi[^{14}C]methylamine   (0.216 mmol/kg);   *N*-nitroso[^{14}C-methyl]methyl-*n*-butylamine (0.216 mmol/kg); a   mixture of *N*-nitrosodi[^{14}C]methylamine and unlabelled *N*-nitrosomethyl-*n*-butylamine (0.216 mmol/kg each); or a mixture of *N*-nitroso[^{14}C-methyl]methyl-*n*-butylamine and unlabelled *N*-nitrosodimethylamine (0.216 mmol/kg each), and was immediately placed in a sealed glass metabolism

cage, Metabowl (Jencons, UK) from which air was drawn at a rate of 1 litre/min. Expired $^{14}CO_2$ was absorbed by two serially connected flasks each containing 350 ml of 2.5 M sodium hydroxide. At 30 minute intervals, 1 ml samples of the trapping alkali were removed from each flask, carrier sodium hydroxide (1 ml, 0.2 M) was added and the [^{14}C]carbonate was precipitated by adding excess barium choride solution (2 ml, 0.4 M). Distilled water (4 ml) was added to increase the volume. The precipitate was centrifuged (1,000g, 5 mins), washed with distilled water and then centrifuged again. The final precipitate was resuspended in distilled water (3 ml) and transferred into a scintillation vial. Ecoscint A scintillation fluid (15 ml) was added and the radioactivity determined by counting for 5 minutes on a ^{14}C programme in a Packard 1600 scintillation counter.

2.3.6 The effects of dimethylformamide, diethylformamide and methyl-*n*-butylformamide on the metabolism of nitrosamines by tissue slices

2.3.6.1 Oesophageal epithelium and N-nitrosodiethylamine

The metabolism of *N*-nitrosodiethylamine to carbon dioxide was measured in a Warburg apparatus using *N*-nitrosodi[1-^{14}C]ethylamine as described by Swann (Swann *et al.*, 1984). Male rats (200 g) were killed as before and their oesophagi removed to ice-cold Krebs-Ringer buffer. The following initial preparation of the oesophagi was done as quickly as possible to prevent possible cytochrome P450 degradation due to temperatures above 4°C. The muscle layer surrounding the mucosa of each oesophagus was removed as previously described. The mucosa was slit open along its length and cut into pieces about 1 cm long, i.e. each mucosa was cut into 4 or 5 pieces. A pleated paper wick made from a piece of Whatman No. 1 filter paper (1 cm × 2 cm) was placed in the centre well of each of twelve Warburg flasks. The pieces of mucosa were blotted dry on Whatman No. 1 filter paper and about 60 mg weighed and placed in the annulus of each of the Warburg flasks containing ice-cold Krebs-Ringer buffer (1.8 ml) containing 2 mg/ml glucose. The paper wicks were soaked with 2 M NaOH (200 µl) to trap any CO_2 produced. *N*-nitrosodi-[1-^{14}C]ethylamine in Krebs-Ringer buffer (100 µl) was put in the sidearm of each flask. Concentrations of unlabelled *N*-nitrosodiethylamine ranging from 6 to 25 µM, made up in Krebs-Ringer buffer, were also pipetted into the sidearm. To measure inhibition by dimethylformamide and methylbutylformamide, the inhibitors

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were added to flasks that had the same concentration of labelled and unlabelled nitrosamine as the controls to obtain final concentrations of 0.6 mM. The flasks were then attached to manometers and gassed with oxygen for 5 minutes while being shaken in a water bath at 37°C. The flasks were closed and equilibrated for another 5 minutes. The reactions were started by tipping the contents of the sidearm into the annulus of each flask. The respiration of the tissue was checked after 10 minutes by measuring the distance moved by the dye in the manometers attached to the flasks. After 40 minutes the reactions were stopped by pipetting perchloric acid (0.2 ml, 1.2 M) into the sidearm of each flask and tipping it into the annulus. The flasks were removed from the water bath and left, still sealed, at room temperature for 1 hour so that all the dissolved $^{14}CO_2$ would transfer to the NaOH. The paper wick in each flask was removed into an appropriately labelled 15 ml centrifuge tube and each well washed twice with 0.5 ml distilled water. The washings were transferred into the corresponding centrifuge tubes. Distilled water (4 ml) was added to each of the tubes and the paper wicks agitated and removed. As much water as possible was squeezed from the wicks by twisting between two pairs of forceps before discarding them. Sodium carbonate (1 ml, 0.2 M) was added to each tube, as a carrier for the radioactive carbonate, followed by barium chloride (2 ml, 0.4 M). The tubes were centrifuged (1,000g, 5 minutes) to sediment the barium carbonate precipitate. The precipitates were washed twice with distilled water (4 ml), centrifuging at 1,000g for 5 minutes between washes. The final precipitates were resuspended in distilled water (3 ml) and transferred into 25 ml scintillation vials. The tubes were washed with Ecoscint A scintillation liquid (5 ml) and the washings added to the scintillation vials. The vials were topped up with Ecoscint A (10 ml) and the samples were counted for 5 minutes on a ^{14}C programme in a Packard 1600 scintillation counter.

2.3.6.2 Liver slices and *N*-nitrosodiethylamine

This was carried out with the same procedure used for the oesophageal epithelia, but with 60 mg of 0.3 mm thick liver slices cut with a microtome. The concentrations of unlabelled *N*-nitrosodiethylamine that were used ranged from 17 to 70 μM and the reactions were incubated for 15 minutes instead of 40, due to the higher P450 content and hence higher rate of metabolism by the liver.

2.3.6.3 Liver slices and N-nitrosodimethylamine

This was carried out as described in the previous sections with N-nitrosodimethylamine concentrations ranging from 17 to 70 µM. Again, incubations were carried out for 15 minutes.

2.3.7 Preparation of Liver Microsomes

<u>Solutions</u>

Homogenising buffer: 0.1 M Tris/1 mM EDTA (pH 7.4) containing 1.15% KCl (w/v)

Wash buffer: 0.1 M Sodium pyrophosphate (pH 7.4)

Resuspension buffer: 0.1 M Tris/1 mM EDTA (pH 7.4) containing 20% glycerol (v/v)

Rats were killed by anaesthesia, using an increasing concentration of carbon dioxide as recommended by the Home Office. The livers were excised and immediately put into ice cold KCl (1.15% w/v) and rinsed of blood. All subsequent steps were carried out at 4°C. The livers were transferred into a beaker of homogenising buffer (4 ml/gram wet tissue), minced with scissors and homogenised with 4 passes of a motor-driven Teflon-glass homogeniser. The homogenate was centrifuged at 10,000g (20 minutes, 4°C). The supernatant obtained was then centrifuged at 105,000g (60 minutes, 4°C). This supernatant containing the cytosolic fraction was discarded. To reduce the amount of haemoglobin, the microsomal pellet remaining was washed by resuspending with 2 passes of a Teflon/glass homogeniser in a volume of wash buffer equal to the initial volume of homogenising buffer. The suspension was centrifuged at 105, 000g (60 minutes, 4°C) and the resulting microsomal pellet resuspended in resuspension buffer (1 ml/gram wet tissue) using a glass/glass hand homogeniser and stored at -70°C in 1.5-ml aliquots.

2.3.8 Preparation of Oesophageal Microsomes

<u>Solutions</u>

Homogenising buffer: 0.1 M Tris/1 mM EDTA (pH 7.4) containing 1.15% KCl (w/v)

Wash buffer: 0.1 M Sodium pyrophosphate (pH 7.4)

Resuspension buffer: 0.1 M Tris/1 mM EDTA (pH 7.4) containing 20% glycerol (v/v)

Rats were killed as previously described and their oesophagi were removed into ice cold KCl [1.15% (w/v)]. The following procedure was carried out in a cold room (4°C). The muscle layer surrounding the mucosa of each oesophagus was removed by gripping the oesophagus in the middle, across the length, with one pair of forceps and pulling outwards from the centre with another pair. The process was repeated at the other end to strip the other half. The mucosa was then weighed, slit open longitudinally and chopped into small pieces (approximately 1 – 2 mm length). The chopped up mucosa was homogenised in an ice-cooled tube in 4 times its weight of homogenising buffer using 6 passes of a motor-driven Ultra Turrax homogeniser. This rough homogenate was then further homogenised with 4 to 6 passes in an ice-cooled glass/glass Duall homogeniser (Kontes, New Jersey), with a 30-second interval between passes to prevent overheating. This final homogenate was centrifuged at 10,000g (20 minutes, 4°C). The supernatant was removed and centrifuged at 105,000g (60 minutes, 4°C). The microsomal pellet obtained was resuspended in a volume of wash buffer equal to that of the homogenising buffer, with 2 passes of a motor-driven Teflon/glass homogeniser and the resulting suspension centrifuged at 105,000g (60 minutes, 4°C). The final pellet obtained was resuspended in resuspension buffer (1 ml/gram mucosa), divided into single use aliquots (~ 1 ml) and stored at -70°C.

2.3.9   Estimation of Protein Content

<u>Solutions</u>

Alkaline copper reagent: 100 ml sodium carbonate [2% (w/v)] in 0.1 M NaOH, 1 ml potassium tartrate [2% (w/v)], 1 ml copper sulphate [1% (w/v)]

The protein concentration of the microsomes was determined according to the method of Lowry (Lowry *et al.*, 1951). Microsomes (5 µl) were measured into a test-tube and the volume made up to 100 µl with distilled water. Alkaline copper reagent (1 ml) was added. The contents of the tubes were vortex mixed and left to stand at room temperature for 15 minutes. Folin Ciocalteau reagent (0.1 ml, 1 N; Sigma) was added

to each tube, which was then shaken immediately and left to stand for a further 30 minutes at room temperature. The absorbance at 750 nm was read against a blank containing distilled water instead of microsomes. A protein standard curve was prepared with bovine serum albumin.

2.3.10 Determination of Cytochrome P450 Content of Microsomes

This was done according to the method of Omura and Sato (1964). Liver microsomes were diluted to a protein concentration of about 2 mg/ml. Oesophageal microsomes were used undiluted. Microsomes (100 μl) were measured into a test-tube and made up to 2 ml with 0.1 M potassium phosphate, pH 7.5 / 1 mM EDTA containing glycerol [20% (v/v)], sodium cholate [0.5% (w/v)] and Triton N-101 [0.4% (w/v)]. The tube was left on ice for about 5 minutes until the turbid solution became clear. A small amount of sodium dithionite (about 25 mg) was added to the tube and shaken. The mixture was equally divided between two quartz spectrophotometer cuvettes. A baseline of absorbance was measured by scanning the samples between 400 nm and 500 nm in a Cary 3 spectrophotometer (Varian). Carbon monoxide was bubbled through the sample cuvette at 1 bubble per second for about 2 minutes, and the CO-dithionite reduced spectrum obtained between 400 nm and 500 nm. The cytochrome P450 content of the microsomes was estimated using the difference between the absorbance readings at 450 nm and 490 nm assuming a molar extinction coefficient of 91,000.

2.3.11 NADPH-Cytochrome P450 Reductase Assay

Reductase activity was measured according to the method of Lake (1987), using the artificial electron acceptor cytochrome c. Cytochrome c (1 ml, 1.55 mg/ml) was measured into each of two spectrophotometer cuvettes. Microsomes (100 μl, 2 mg protein/ml) were added to each cuvette. KCN (200 μl, 15 mM) was added to each cuvette to prevent mitochondrial oxidation. The test sample was made up to 2.4 ml with KH_2PO_4 (0.1 M, pH 7.6) and the reference sample to 2.5 ml. Both cuvettes were inverted several times to mix their contents and placed in a Cary 3 spectrophotometer fitted with a temperature controller and left to equilibrate at 37°C for 3 minutes. NADPH (100 μl, 8 mg/ml) was added to the test sample and the absorbance at

550 nm monitored for 3 minutes. The NADPH-cytochrome P450 reductase content in nmol/min/ml was determined assuming a molar extinction coefficient 21,000.

2.3.12 SDS-PAGE of Protein

2.3.12.1 Preparation of SDS-PAGE Gel

<u>Solutions</u>

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

pH 6.8 buffer: 1.5 M Tris-HCl (pH 6.8) containing 0.4% (w/v) SDS

This was done according to the method of Phillips *et al.* (1981), based on the original method of Laemmli (1970). To make a 10% resolving gel, 30% (w/v) acrylamide/0.8% (w/v) methylene-bisacrylamide (7.15 ml, Protogel, National Diagnostics), pH 8.8 buffer (5 ml) and distilled water (6.5 ml) were mixed in a vacuum flask. This mixture was degassed by vacuum suction for 10 minutes and then TEMED (23 μl) and 15% (w/v) ammonium persulphate (1.5 ml) were added. This was mixed thoroughly but gently, and immediately poured between the glass plates of the electrophoresis apparatus (Minigel, Bio-Rad) assembled with 0.75-mm spacers. The gel was overlaid with 0.1% (w/v) SDS in water and left to polymerise for at least 2 hours. The overlay solution was poured off after the resolving gel had polymerised and the space was washed with pH 6.8 buffer. A 3% stacking gel was prepared by mixing 30% acrylamide (2 ml), pH 6.8 buffer (5 ml), TEMED (20 μl), 1.5% (w/v) ammonium persulphate (1 ml) and water (12 ml). The stacking gel was mixed thoroughly but quickly and carefully poured over the resolving gel. The required combs (0.75-mm thick) were inserted, ensuring that no bubbles were introduced. The gel was left to polymerise for about 1 hour.

2.3.12.2 Preparation and Electrophoresis of Samples

The microsomal sample was prepared for electrophoresis by adding an equal volume of loading buffer [2% (w/v) SDS / 20 mM EDTA / 20 mM sodium phosphate buffer, pH 7.0 / 2% β-mercaptoethanol / 30% (v/v) glycerol / 0.02% (w/v) bromophenol blue]. The mixture was heated to 100°C for 3 minutes and 2 μl of β-mercaptoethanol added to disrupt all disulphide linkages. The sample was then centrifuged briefly in a microcentrifuge at maximum speed to collect it at the bottom of the tube and sediment any debris. 15 μl of the supernatant was loaded onto the gel and electrophoresed in

0.025 M Tris/0.192 M glycine/0.1% (w/v) SDS, pH 8.4, at 10 V/cm until the dye entered the resolving gel. The current was then increased to 20 V/cm until the dye had migrated to within 0.5 cm of the end of the gel.

2.3.12.3 Staining of Gels

The gel was transferred to a container of 5 volumes of Coomassie blue staining solution (0.25 g of Coomassie Brilliant Blue R250 dissolved in 100 ml methanol:water:glacial acetic acid (45:45:10) and filtered through Whatman No. 1 filter paper) and left on a slowly rotating platform for at least 4 hours. The gel was destained with several changes of methanol:water:acetic acid (45:45:10) until the background was clear and the bands distinct. If no bands were detected or if they were very faint, the gel was completely destained, then re-stained with silver using Silver Stain Plus (Bio-Rad) according to the manufacturer's instructions and fixed in 5% acetic acid. The stained gel was photographed on an illuminated light box.

2.3.13 Western Blot

<u>Solutions</u>

Tris-buffered saline (TBS): 20 mM Tris (pH 7.5)/500 mM NaCl

TBS-Tween (TBS-T):   0.1% (v/v) Tween 20 in TBS

To detect the presence of particular P450s, the SDS-PAGE gel was blotted onto a nitrocellulose membrane (Hybond ECL, Amersham). Antigen detection was carried out using the Enhanced Chemiluminescence (ECL) Western Blot Kit (Amersham) according to the manufacturer's instructions. Briefly, the resolving gel was placed on a sheet of Hybond ECL cut to the same size as the gel. The gel/membrane was sandwiched between 2 sheets of Whatman 3MM chromatography paper of approximately the same size as the gel/membrane. This was then placed in a cassette between Scotch plastic sponge pads and inserted into a blotting tank (Bio-Rad) containing enough transfer buffer [192 mM glycine/25 mM Tris, pH 8.4/20% (v/v) methanol] to completely submerge the cassette. The protein was transferred from the gel to the membrane by electrophoresis (30 V) overnight. The membrane was removed and then placed, blotted surface inwards, in a sterile 50 ml conical tube. Remaining protein binding sites were blocked with a 5% (w/v) solution of blocking reagent (powdered milk, supplied with the kit) in TBS (10 ml) and rotating on a roller

mixer (1 hour, room temperature). The blocking reagent mixture was poured off and the membrane rinsed for 20 minutes with 3 changes of TBS-T (10 ml). The primary antibodies, diluted 1:1,000 in 0.1% (w/v) blocking reagent in TBS, were added to the tube and rotated as before for 30 minutes to 1 hour. The primary antibody solution was poured off and kept for future use and the membrane was washed with TBS-T as before. The secondary antibody was prepared as described by the manufacturer, poured into the tube and rotated for 30 minutes to 1 hour at room temperature. The membrane was washed again, incubated with streptavidin-horse radish peroxidase and washed, again as described by the manufacturer. The detection procedure was carried out according to the manufacturer's instructions.

2.3.14 Purification of Cytochrome P450 from Rat Liver

Attempts were made at purifying cytochrome P450 from rat liver, as this tissue is more readily available in large quantities. As the liver of a 200 g rat weighs about 10 g, while the oesophagus weighs only 0.07 g, one would need 150 rats to get a weight of oesophagus equal to the weight of a single liver. Furthermore, on a moles P450 per gram wet weight basis, since the oesophagus only contains about 5% of the P450 content of liver, a good yield would be required. Due to this, it was necessary to find a method for purifying the oesophageal P450 that would give the highest yield of active P450 that could be excised from an SDS-PAGE gel stained with Coomassie blue dye and used for N-terminal sequencing. Three methods used by different investigators were attempted.

2.3.14.1 N-Octylaminosepharose Column Chromatography

<u>Solutions</u>

Buffer A: 0.1 M potassium phosphate buffer (pH 7.25)/1.0 mM EDTA containing 20% (v/v) glycerol

Buffer B: 10 mM potassium phosphate buffer (pH 7.7)/0.1 mM EDTA containing 20% (v/v) glycerol, 0.1% (w/v) Lubrol PX and 0.2% (w/v) sodium cholate.

This was carried out according to the method of Guengerich and Martin (1980). It is a method that has been used to purify P450 from tissues where large quantities would be available, e.g. liver. This experiment was scaled down to cater for the much smaller quantities of P450 that would be encountered in oesophagus.

This procedure requires accurate quantities of the detergents used, so cholic acid used in the purification was recrystallised. Cholic acid (25 g) was dissolved in hot 50% ethanol (200 ml). Activated charcoal (4 spatula tips) and Celite (1 spatula tip) were added. The suspension was filtered twice in a Buchner funnel through Whatman No. 42 paper. The filtrate was left on ice for 30 minutes and then refiltered. The precipitate was dissolved in hot 50% ethanol (100 ml) and filtered as before. The filtrate was left on ice for 30 minutes and then refiltered. The precipitate was dried to constant weight *in vacuo*. Sodium hydroxide pellets were added to the recrystallised cholic acid and deionised water was added. The pH of the solution was adjusted to 7.5 by slowly adding 1 M acetic acid. The solution was then made up to 20% with deionised water and filtered through Whatman No. 1 paper.

The *N*-octylaminosepharose column was prepared in a fume cupboard. Sepharose 4B (400 ml) was stirred in a large beaker, with the pH and temperature being monitored. CNBr (100 g) dissolved in dioxane (300 ml), which was prepared immediately before use, was added drop-wise, with a separatory funnel over a ten-minute period. Drops of 5 M NaOH were added to maintain a pH of 11, and ice was added to keep the temperature between 20 and 25°C. After 20 minutes, crushed ice (about 600 g) was added to quench the reaction and the contents were poured into a large sintered glass funnel attached to a Buchner funnel and a water aspirator. The gel was washed *in vacuo* with 4 litres of cold water and added to a solution of 1,8-diaminooctane (114 g dissolved in 400 ml water). The pH of the solution was adjusted to 10 with 6 M HCl and the mixture stirred slowly overnight at 4°C. The gel was filtered *in vacuo*, washed with 10 litres of distilled water, with 2 litres of 0.20 M potassium phosphate buffer (pH 7.25) and another 10 litres of distilled water. About 25 ml of the gel was suspended in 400 ml water, deaerated *in vacuo* for 15 minutes and poured into a column (1.5 × 20 cm).

Washed liver microsomes were prepared as described earlier from about 35 g of liver from untreated male rats. 30 ml of the microsomal sample was made up to 100 ml with resuspension buffer and 20% (w/v) recrystallised sodium cholate added, with stirring, to a final concentration of 0.6%. After stirring for an additional 30 minutes at 4°C, the solution was centrifuged at 100,000 g (60 minutes, 4°C) to remove any

unsolubilised material. The supernatant fraction was applied at a rate of 0.4 ml/min to the prepared N-octylaminosepharose 4B column previously equilibrated with of Buffer A (40 ml) containing 0.6% (w/v) recrystallised sodium cholate. The column was washed with Buffer A (106 ml) containing 0.42% (w/v) recrystallised sodium cholate. Cytochrome P450 was eluted with Buffer A (240 ml) containing 0.33% (w/v) recrystallised sodium cholate and 0.06% (w/v) Lubrol PX (polyoxyethylene ether W-1, Sigma). Fractions 38 to 65, representing volumes of eluate between 118 ml and 208 ml, were collected as the peak fractions. These fractions were pooled to give a total volume of about 90 ml. The pooled fractions were concentrated overnight on a bed of Aquacide I, resulting in a final volume of about 45 ml. A small sample was taken for P450 and protein estimation. The remaining sample was dialysed for 2 hours against 2 changes of 20% (v/v) glycerol containing 0.1 mM EDTA (500 ml). The detergent-free sample was then dialysed against several changes of Buffer B (500 ml).

Further purification was carried out on a DEAE-Sephacel column (1.5 cm × 15 cm) that had been previously equilibrated with Buffer B (150 ml). The pH of the concentrated and dialysed sample from the N-octylaminosepharose 4B column was adjusted to 7.7 and the sample was applied at a rate of 0.6 ml/min to the DEAE-Sephacel column. The column was washed with Buffer B (80 ml) and the sample eluted with Buffer B (200 ml) containing 0.25 M NaCl. The peak fractions, 7 to 23, which came to about 80 ml, were pooled and concentrated to a final volume of about 15 ml on a bed of Aquacide I. A small sample was taken for the measurement of P450 and protein content.

2.3.14.2 *p*-Chloroamphetamine Column Purification

This was carried out according to an established method used by Hellmold *et al.* (1995) in the purification of P450 from extra-hepatic organs, especially brain and breast tissue. The *p*-chloroamphetamine column was prepared by Prof. Swann exactly as described by Hellmond *et al.* (1995) using 25 ml Affigel 10 (BioRad, UK). Preliminary experiments showed that sodium cholate interfered with elution of P450 from hyroxyapatite columns used to remove detergents, hence CHAPS was substituted for sodium cholate in the purification process.

Sodium cholate was substituted with twice the concentration of CHAPS because trials had shown the latter to be as effective a detergent for solubilising the microsomes. Liver microsomes were prepared as before and resuspended in 0.1 M Tris, pH 7.4, containing 1 mM EDTA and 20% glycerol. 3 ml of this sample was added to 30 mg CHAPS and 6 µl Emulgen 911 contained in a vial. The mixture was stirred for 2 hours and insoluble material removed by centrifuging at 100,000g for 60 minutes at 4°C. The supernatant was collected, measured and 3 volumes of dilution buffer [1× PBS containing 20% (v/v) glycerol and 1 mM EDTA] added to it. The extract was then applied at a rate of 0.5 ml/min to the column that had been previously equilibrated with 1× PBS containing 1 mM EDTA, 20% (v/v) glycerol, 0.2% (w/v) CHAPS and 0.04% (v/v) Emulgen 911. The column was washed through with 90 ml equilibration buffer and the sample eluted with solubilisation buffer [1× PBS containing 1 mM EDTA, 20% (v/v) glycerol, 1% (w/v) CHAPS and 0.2% (v/v) Emulgen 911]. 3-ml fractions were collected and the absorbance at 415 nm determined for each. The peak fractions were pooled and concentrated on a bed of Aquacide I and the P450 and protein content of this as well as the crude and solubilised microsomes measured.

2.3.14.3 Purification by Chromatofocussing

This was carried out according to the method of Marriage and Harvey (1986), using a Mono P column as described by Isa et al. (1992) in an attempt to improve resolution and speed. Liver microsomes were prepared as previously described and resuspended to a protein concentration of 3.5 mg/ml in resuspension buffer. Tergitol and CHAPS were added to final concentrations of 0.3% (w/v) and 0.5% (w/v) respectively. The mixture was left to solubilise by stirring for 1 hour at 4°C and then fractionated with 50% (w/v) PEG 8000 added in 500 µl aliquots. Fractions precipitating from 7% to 14% PEG were collected by centrifugation at 100,000g (60 minutes, 4°C), pooled, resuspended in 20 ml dialysis buffer [25 mM Tris/0.1 mM EDTA/20% (v/v) glycerol] and dialysed overnight at 4°C against 200 volumes of the same. Tergitol and CHAPS were added to final concentrations of 0.6% (w/v) and 1.0% (w/v) respectively. A Mono-P column (Bio-Rad) was equilibrated with 25 mM Tris-acetate buffer, pH 8.5, until the pH of the eluate was 8.5. The sample was applied to the column at a rate of 0.5 ml/min and eluted with Polybuffer 96: Polybuffer 74 (30:70) adjusted to pH 5.0 with acetic acid.

2.4   Results

2.4.1   Induction of Tumours

Tumours are easily induced in the rat oesophagus by administering small doses of the appropriate nitrosamine.   To demonstrate this, rats (200 g) were given bi-weekly doses of 200 mg per kg body weight of N-nitrosomethyl-n-benzylamine over a 4 week period, giving a total dose of 3.2 mg per 200 g rat.   Post-mortem examinations for the presence of tumours were conducted 6 to 10 weeks after the first injection.   Tumours were found in the oesophageal mucosa of 11 (50%) of the treated rats with thickening in the remainder of the mucosa (Figure 2-2).   There was thickening of the mucosa in 4 of the other oesophagi, but no distinct tumours were observed.   The oesophageal mucosa of another 4 rats appeared normal.   3 treated rats died, possibly from the effects of the toxicity of the nitrosamine.

Figure 2-2   Tumours obtained in the rat oesophagus after i. p. injection of the oesophageal-specific nitrosamine, N-nitrosomethyl-n-benzylamine
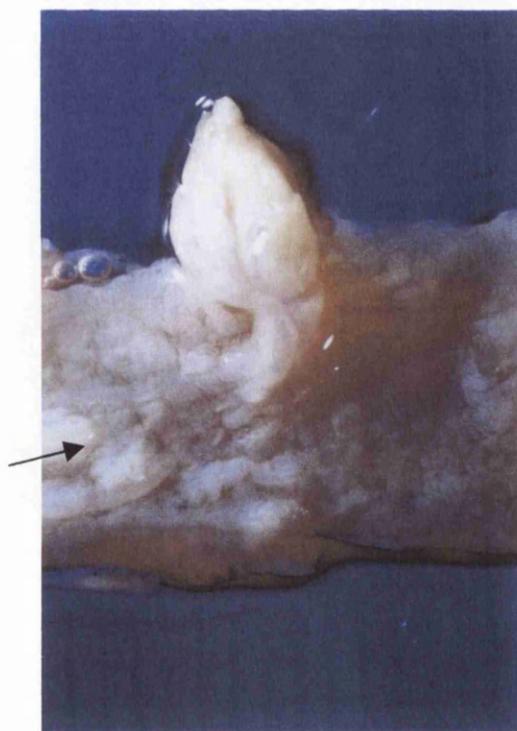
A.   A pedunculated tumour on the luminal surface of the oesophageal mucosa of one rat 7 weeks after the first injection. 22 rats were given 8 bi-weekly doses of N-nitrosomethyl-n-benzylamine and their oesophagi examined for visible changes. The arrow shows a region of thickening in the mucosa.

B.   A tumour on the luminal surface of the oesophageal epithelium 6 weeks after the first injection. 22 rats were given 8 bi-weekly doses of *N*-nitrosomethyl-*n*-benzylamine and their oesophagi examined for visible changes.

2.4.2   Tissue Distribution of Methylation of DNA by the Oesophageal Selective Carcinogen *N*-nitroso[^{14}C-methyl]methyl-*n*-butylamine

To gain a general overview of the relative ability of different rat tissues to metabolically activate oesophageal selective nitrosamines, 8 rats were administered a single dose of *N*-nitroso[^{14}C-methyl]methyl-*n*-butylamine (2 mg/kg body wt) by gavage, killed after 4.5 hours and the extent of methylation of DNA in various organs measured. As is shown later, rats can metabolise about 8 mg *N*-nitrosomethyl-*n*-butylamine/kg body wt/hour, so this small dose would have been rapidly metabolised. DNA methylation was determined by mild acid hydrolysis to liberate both methylated and non-methylated purines that were then separated by HPLC on a strong cation exchange resin. A typical elution profile from the HPLC column is shown in Figure 2-3.
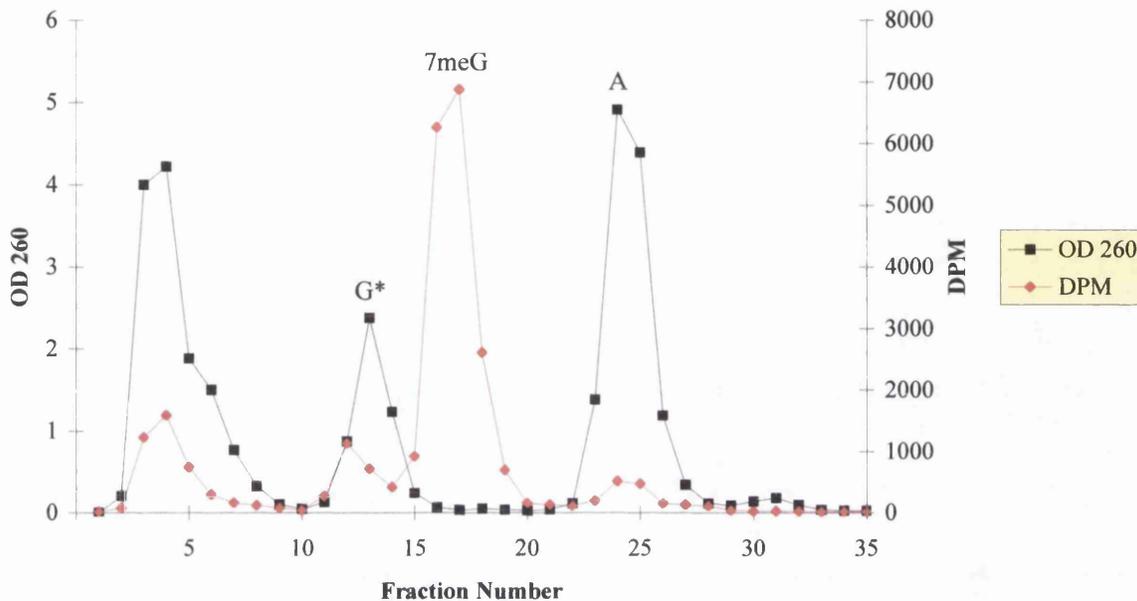
Figure 2-3   Chromatography of an acid hydrolysate of liver DNA from rats treated with N-nitroso[^{14}C-methyl]methyl-n-butylamine (2 mg/kg body wt) given in water.   8 rats were administered single doses of N-nitroso[^{14}C-methyl]methyl-n-butylamine in water and killed 4.5 hours later. DNA was extracted from the pooled livers, hydrolysed and the bases separated on a Hamilton Zorbax HC-X8 column eluted with sodium acetate.
*Abbreviations: A – adenine; G – guanine; 7meG – 7-methylguanine

Methylation was measured as 7-methylguanine (7meG), the major product of DNA methylation. The results are shown in Table 2-1. The highest level of methylation was observed in oesophagus, where the level was 4 times greater than in the liver. von Hofe *et al*. (1987) have also reported higher methylation in the oesophagus. Unfortunately, they do not give numbers, only histograms of their results, but it appears that in their experiments the methylation in the oesophagus was about 1.5 times higher than in the liver. DNA methylation in the organs examined decreased in the order: oesophagus > liver > nasal epithelium > lung > kidney (Table 2-1; Figure 2-4).   There was 4 times as much methylation per DNA-guanine in oesophagus (297.45 ± 5.98 μmol 7-meG/mole guanine) as in liver (74.36 ± 22.01 μmol 7meG/mole guanine) and 8 times as much as in nasal epithelium (38.66 ± 19.71 μmol

7meG/mole guanine). DNA methylation in the oesophagus was 21 and 114 times as much as in lung (13.89 μmol 7-meG/mole guanine) and kidney (2.61 ± 1.00 μmol 7meG/mole guanine) respectively.

Table 2-1 **Methylation of DNA in rat tissues by N-nitrosomethyl-n-butylamine administered in water.** Two sets of 8 rats were administered single doses of N-nitroso[^{14}C-methyl]methyl-n-butylamine in water and killed 4.5 hours later. The organs of interest were removed and pooled. DNA was extracted from each organ and hydrolysed and the bases separated on a Hamilton Zorbax HC-X8 column eluted with sodium acetate. The radioactivity in the 7-methylguanine fraction was measured and converted to μmol and methylation expressed as μmol of 7meG* per mole guanine.

Tissue	[^{14}C]-7meG* dpm	Alkylation of G (μmol 7meG/mole G	Mean (± s. e.) (μmol 7meG/mole G
Oesophagus	3618	261.4	297.45 ± 36.05
	1374	333.5	
Liver	17834	58.8	74.35 ± 15.55
	2495	89.9	
Nasal Epithelium	988	24.72	38.66 ± 13.94
	1233	52.6	
Lung	3398	11.36	13.89 ± 2.53
	681	16.42	
Kidney	519	1.9	2.61 ± 0.71
	133	3.32	

*Abbreviations: 7meG – 7-methylguanine; G – guanine; s.e. – standard error

65
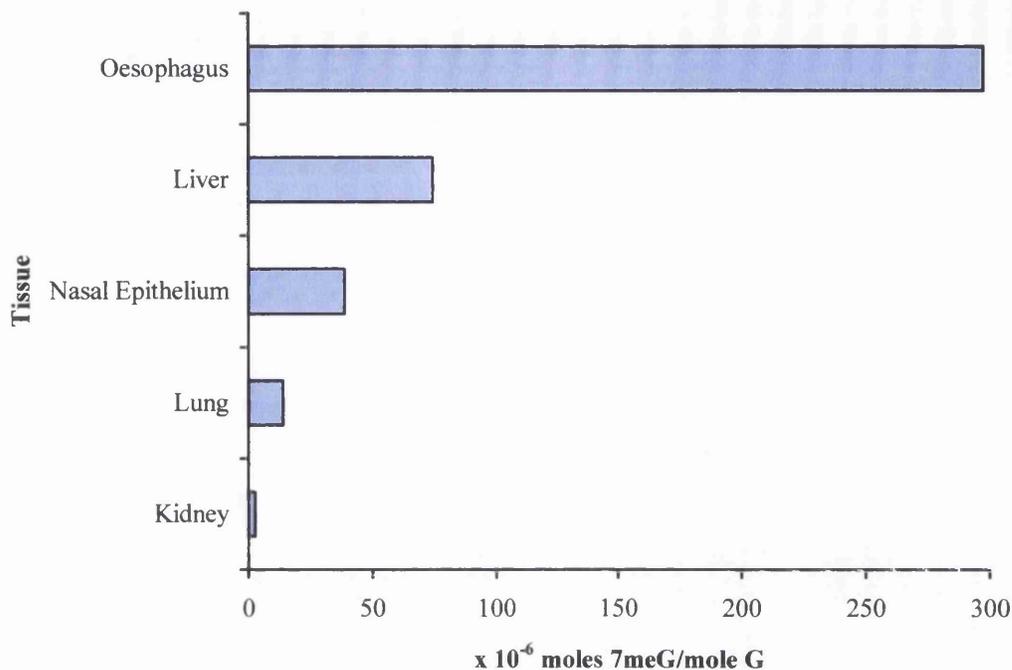
Figure 2-4   Methylation of DNA in various tissues after administration of a single dose of *N*-nitrosomethyl-*n*-butylamine.

2.4.3   Effect of Ethanol on the Tissue Distribution of Methylation of DNA by *N*-nitrosomethyl-*n*-butylamine

To investigate the effect of ethanol on DNA methylation in these organs, the experiments were repeated, this time administering the nitrosamine, *N*-nitroso[^{14}C-methyl]methyl-*n*-butylamine, in 5% ethanol. The 7-methylguanine content of oesophageal DNA (631 µmol 7meG/mole guanine) was 2.5 times greater than in the previous experiments without ethanol (Table 2-2; Figure 2-5). By contrast, the methylation of liver DNA was reduced by co-administration of ethanol to only 52 µmol 7meG/mole guanine, 30% less than the amount obtained when the experiments were carried out without ethanol. Thus in the alcohol-treated rats the level of 7-methylguanine in the oesophagus was 12 times that in liver. The methylation of the DNA in nasal epithelium in the rats given ethanol was 290 µmol 7meG/mole guanine, representing a 7.5-fold increase; methylation in lung was 33.93 µmol 7meG/mole guanine, a 2.5-fold increase. However, in the kidney, there was a decrease in methylation to 1.63 µmol 7meG/mole guanine, representing a 40% decrease, which is similar to that seen in the liver.

Table 2-2   Effect of co-administration of 5% ethanol on the methylation of DNA in selected tissues by *N*-nitrosomethyl-*n*-butylamine. 8 rats were administered single doses of *N*-nitroso[^{14}C-methyl]methyl-*n*-butylamine in 5% ethanol and killed 4.5 hours later. The organs of interest were removed and pooled. DNA was extracted from each organ and hydrolysed and the bases separated on a Hamilton Zorbax HC-X8 column eluted with sodium acetate. The radioactivity in the 7-methylguanine fraction was measured and converted to µmol and methylation expressed as µmol of 7meG* per mole guanine.

Tissue	[^{14}C]-7meG dpm	Alkylation of G (µmol 7meG/mole G)	Percentage of control (%)
Oesophagus	2579	631	212
Liver	3151	52	70
Nasal Epithelium	7448	290	750
Lung	2861	33.93	244
Kidney	519	1.63	62

*Abbreviations: 7meG – 7-methylguanine; G – guanine
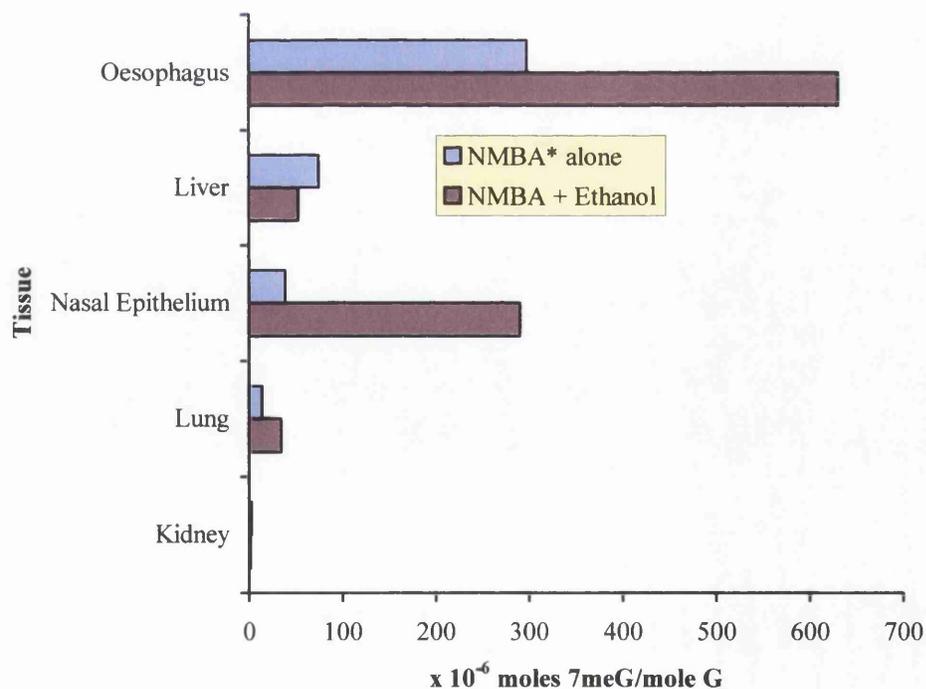
Figure 2-5   Effect of 5% ethanol on DNA methylation by *N*-nitrosomethyl-*n*-butylamine
    *Abbreviations: NMBA – *N*-nitrosomethyl-*n*-butylamine

2.4.4 Comparison of the Metabolism of *N*-nitrosomethyl-*n*-butylamine and *N*-nitrosodimethylamine in the Whole Rat

It was shown by Heath (1962) that the rate of metabolism of many nitrosamines in rats can be followed by measuring the rate of exhalation of $^{14}CO_2$ after a dose of the labelled nitrosamine has been given. In the following experiments rats were given either *N*-nitroso[^{14}C-methyl]methyl-*n*-butylamine or *N*-nitrosodi[^{14}C]methylamine and the exhalation of $^{14}CO_2$ measured. The doses were chosen to be sufficient to saturate the metabolising system so that the maximum rates could be determined. The results (Figure 2-6) show that the exhalation of $^{14}CO_2$ after *N*-nitroso[^{14}C-methyl]methyl-*n*-butylamine or *N*-nitrosodi[^{14}C]methylamine (both 216 μmol/kg body wt) administration followed a similar course. In both cases there was a short lag period, then the exhalation followed an essentially linear course between 30 min and 150 min after the dose. There was a plateau in the exhalation when 53% of the injected dose of *N*-nitroso[^{14}C-methyl]methyl-*n*-butylamine or 56% of the injected dose of *N*-nitrosodi[^{14}C]methylamine had been exhaled. Heath (1962) showed that the exhalation of $^{14}CO_2$ from a dose of *N*-nitrosodi[^{14}C]methylamine exactly paralleled the rate of disappearance of the nitrosamine from the blood and at the plateau for exhalation all the nitrosamine has been metabolised. Since *N*-nitroso[^{14}C-methyl]methyl-*n*-butylamine forms the same immediate radioactive metabolites as *N*-nitrosodi[^{14}C]methylamine, i.e. methanol and formaldehyde, although in different proportions, the same assumptions can be made for *N*-nitroso[^{14}C-methyl]methyl-*n*-butylamine as for *N*-nitrosodi[^{14}C]methylamine. So one can calculate that the rate of elimination of $^{14}CO_2$ between 30 minutes and 150 minutes after the administered dose (80.7 μmol $^{14}CO_2$/kg body wt/hour) represents metabolism of 80.7 μmol of *N*-nitroso[^{14}C-methyl]methyl-*n*-butylamine/kg body wt/hour, while exhalation of 159.3 μmol $^{14}CO_2$/kg body wt/hour from *N*-nitrosodi[^{14}C]methylamine represents metabolism of 79.65 μmol of *N*-nitrosodi[^{14}C]methylamine/kg body wt/hour. Co-administration of an equimolar amount of unlabelled *N*-nitrosodimethylamine with the *N*-nitroso[^{14}C-methyl]methyl-*n*-butylamine (216 μmol/kg body wt) strongly inhibited the rate of exhalation of $^{14}CO_2$ from the latter (Figure 2-7.A; compare with Figure 2-6.A). Similarly, unlabelled *N*-nitrosomethyl-*n*-butylamine inhibited the exhalation of $^{14}CO_2$ from an equimolar dose of *N*-nitrosodi[^{14}C]methylamine (216 μmol/kg body wt) as shown in Figure 2-7.B (compare with Figure 2-6.B). The

curves for the exhalation of $^{14}CO_2$ from the individual nitrosamines were not linear: the N-nitrosomethyl-n-butylamine was initially metabolised more rapidly than the N-nitrosodimethylamine. In the second set of experiments, the rate of exhalation of $^{14}CO_2$ was linear between 30 minutes and 250 – 300 minutes. The maximum proportions of the injected label eventually exhaled when the plateau was reached were essentially identical to the proportions obtained from the individual nitrosamines. If the same assumption is made, i.e. that the plateau of $^{14}CO_2$ exhalation is reached when all the nitrosamine has been metabolised, then the exhalation data shown in Figure 2-7 can be converted to moles of each nitrosamine metabolised (Figure 2-8).
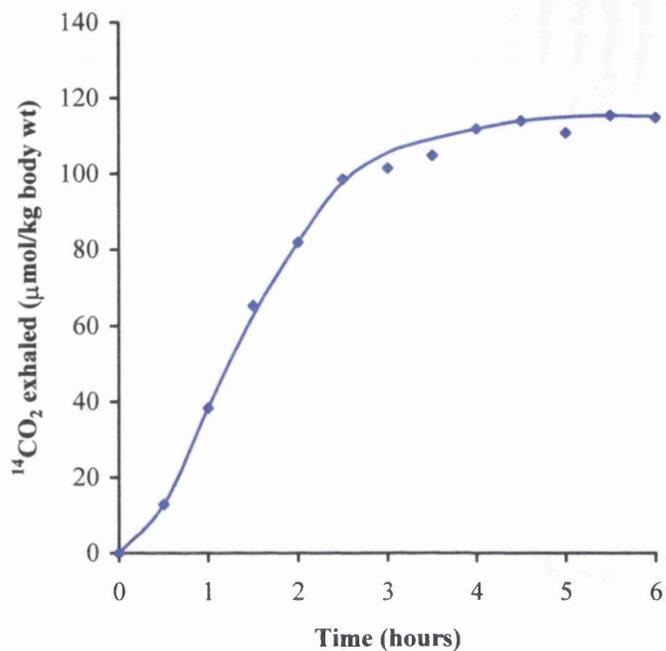
A. *N*-nitroso[^{14}C-methyl]methyl-*n*-butylamine
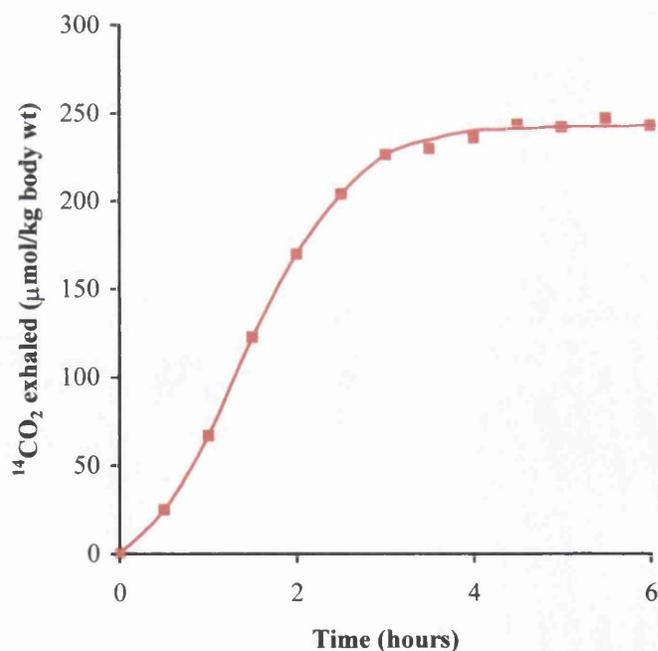
B. *N*-nitrosodi[^{14}C]methylamine

Figure 2-6 *In vivo* **metabolism in rats of individually administered methyl-*n*-alkylnitrosamines.** A rat was given a single intraperitoneal injection of 216 μmol/kg body wt of *N*-nitroso[^{14}C-methyl]methyl-*n*-butylamine (A) or *N*-nitrosodi[^{14}C]methylamine (B) and placed in a sealed chamber. $^{14}CO_2$ exhaled was trapped with NaOH and the radioactivity measured was converted to μmol of CO_2 exhaled.
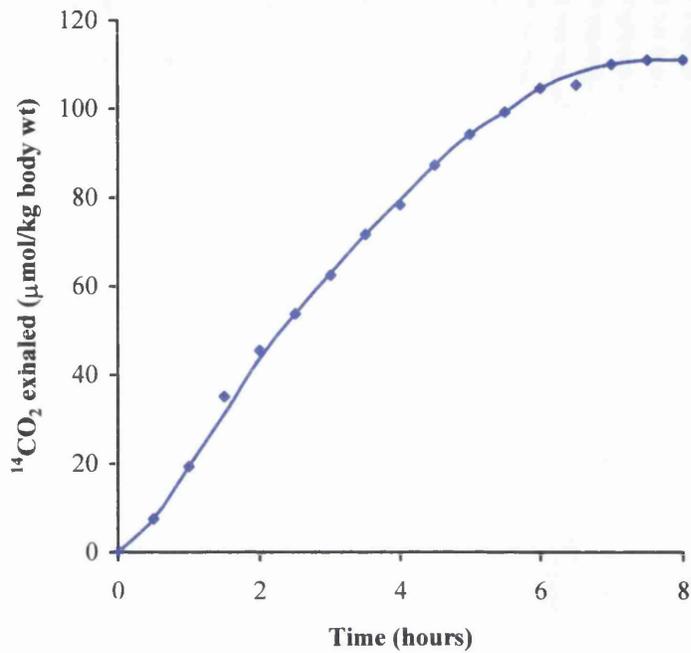
A.   N-nitroso[^{14}C-methyl]methyl-n-butylamine + unlabelled
N-nitrosodimethylamine
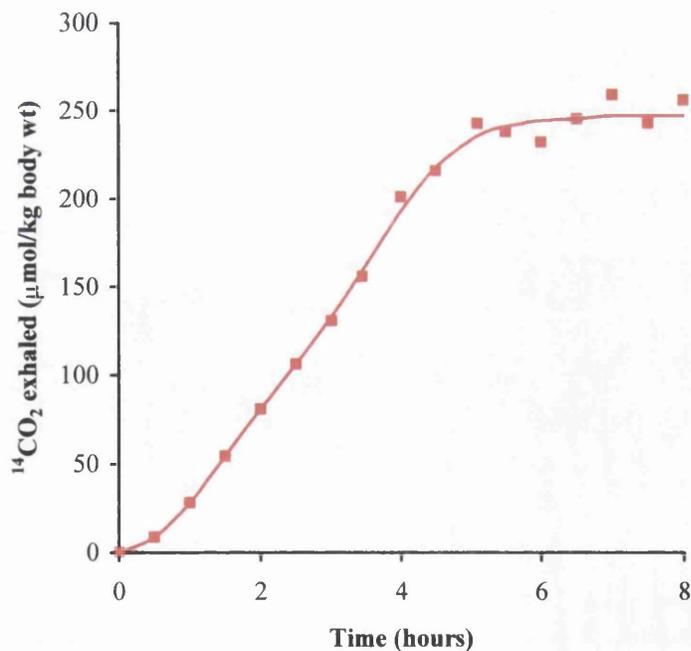
B.   N-nitrosodi[^{14}C]methylamine + unlabelled N-nitrosomethyl-n-butylamine

Figure 2-7   *In vivo* **metabolism of concomitantly administered methyl-*n*-alkylnitrosamines.** Two rats were given single i. p. injections of equimolar amounts (216 µmol/kg body wt) of both nitrosamines, one labelled and the other unlabelled, and placed in a sealed chamber. $^{14}CO_2$ exhaled was trapped with NaOH and the radioactivity measured was converted to µmol of CO_2 exhaled. (A) received labelled N-nitrosomethyl-n-butylamine and (B), labelled N-nitrosodimethylamine.
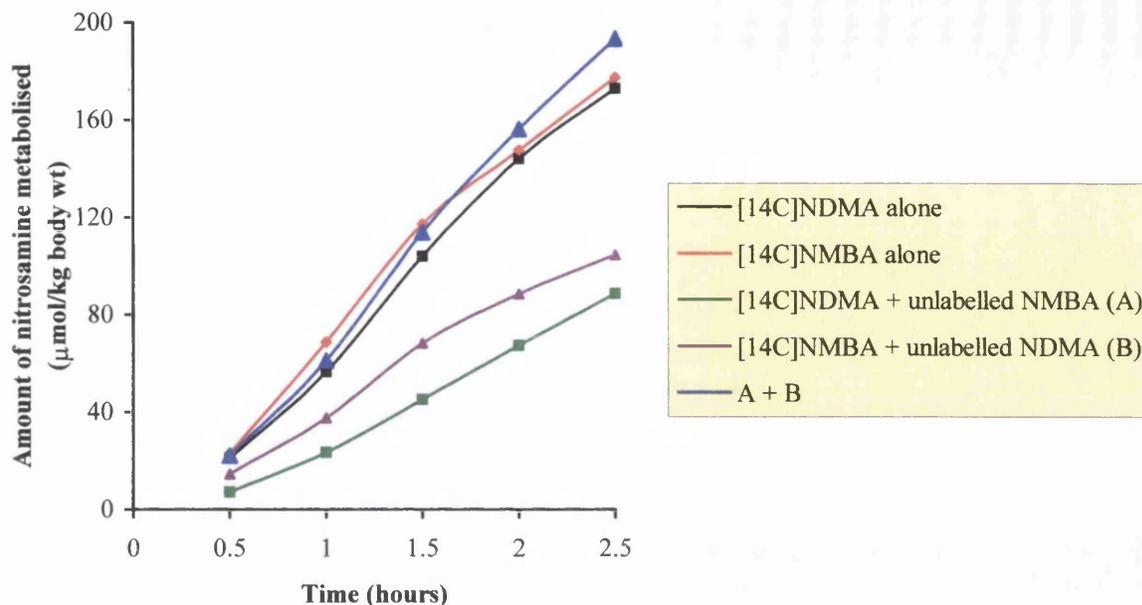
Figure 2-8 **Metabolism of *N*-nitrosodimethylamine, *N*-nitrosomethyl-*n*-butylamine and mixtures of both nitrosamines by whole rats.** This graph shows the results in Figures 2.6 and 2.7 expressed in terms of amount of nitrosamine metabolised and plotted on one axis.

The curve represented by 'A + B' in Figure 2-8 was obtained by adding the amount of nitrosamine formed from each [^{14}C]-labelled nitrosamine when the nitrosamines were administered together in equimolar amounts. This curve is essentially superimposed on the curves obtained from metabolism of the nitrosamines when they were administered individually.

An interesting feature of these experiments is the fact that after equilibration, the initial metabolism of the bulkier fat-soluble nitrosamine, *N*-nitrosomethyl-*n*-butylamine is not linear while the metabolism of *N*-nitrosodimethylamine, a water-soluble nitrosamine follows a linear trend.

2.4.5 Levels of Cytochrome P450 and NADPH-Cytochrome P450 in Liver and Oesophagus of Rats

Nitrosamines are metabolised by cytochromes P450. These P450s are largely microsomal proteins, so experiments were carried out to compare the levels of P450s and an associated protein, NADPH-cytochrome P450 reductase, in rat liver and oesophageal microsomes. The yield of microsomes from the oesophagus (2.6 ± 0.02

mg protein/g tissue) was 4 times less than from the liver (9.4 ± 1.1 mg protein/g tissue). Rat oesophageal microsomes contained 0.05 ± 0.02 nmol P450/mg microsomal protein, which was 10 – 20 times less than the P450 content of liver microsomes (0.85 ± 0.35 nmol P450/mg microsomal protein). However, the activity of NADPH-cytochrome c reductase (equivalent to NADPH-cytochrome P450) in rat oesophageal microsomes (0.048 ± 0.007 μmol/min/mg microsomal protein) was only 6 times less than in liver microsomes (0.28 ± 0.09 μmol/min/mg microsomal protein). Therefore the ratio of reductase to P450 in the oesophagus is 1:1, whereas in the liver it is only 0.36:1. There is therefore about 3 times as much reductase per nmol P450 in the oesophagus than in the liver.

2.4.6 Western Blot Analysis to Detect P450s of the 2A and 2E Subfamilies

Western blots of the oesophageal microsomal protein showed a reaction with an antibody to mouse CYP2A5 (Figure 2-9), but not rat CYP2E1 (Figure 2-10). The reacting protein had an apparent molecular size of 54 kD, which is similar to the molecular size of rat CYP2A3 (56.5 kD). These results suggest the presence of a P450 belonging to the 2A subfamily in rat oesophageal mucosa. They also show that CYP2E1 is not expressed in rat oesophageal mucosa or that if it is, the amounts are beyond the limits of detection by a Western blot.

The CYP2A5 antibody did not give a very clean reaction (Figure 2-9), but suggested the presence in oesophageal microsomes of a P450 belonging to the CYP2A subfamily. The intensities of the bands obtained for the oesophageal microsomes (10 μg protein) were visually the same as that obtained for 3 μg of liver microsomal protein suggesting that there is about 3 times as much CYP2A protein per milligram microsomal protein in liver as there is in oesophagus.
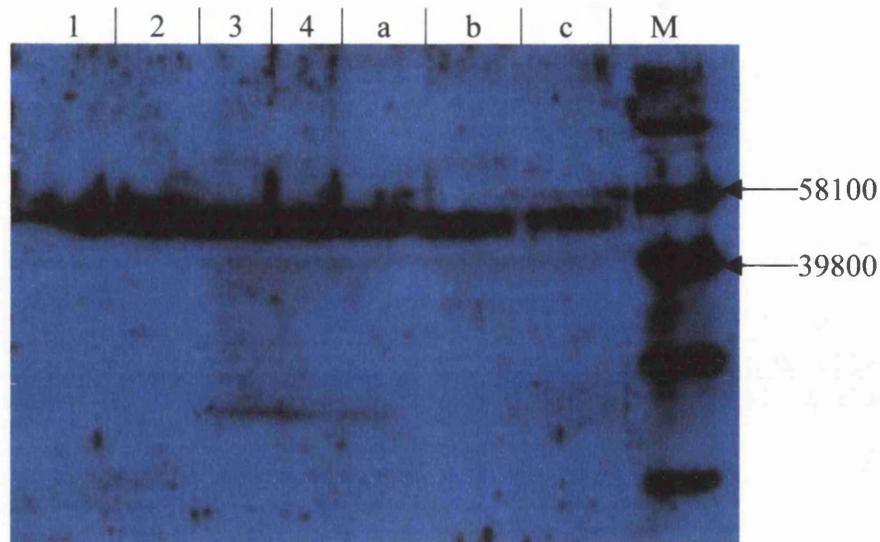
Figure 2-9   Western blot showing reaction of rat oesophageal and liver microsomal protein with mouse CYP2A5 antibody. The microsomal proteins were separated on a 10% SDS-PAGE gel and blotted onto a nitrocellulose membrane (Hybond ECL, Amersham). After hybridisation, the membrane was developed by ECL and exposed to radiographic film for 10 minutes.

1   -   oesophageal microsomes from preparation 1 (10 μg protein)
2   -   oesophageal microsomes from preparation 2 (10 μg protein)
3   -   oesophageal microsomes from preparation 3 (10 μg protein)
4   -   oesophageal microsomes from preparation 4 (10 μg protein)
a   -   liver microsomes (3 μg protein)
b   -   liver microsomes (2 μg protein)
c   -   liver microsomes (1 μg protein)
M   -   molecular weight markers
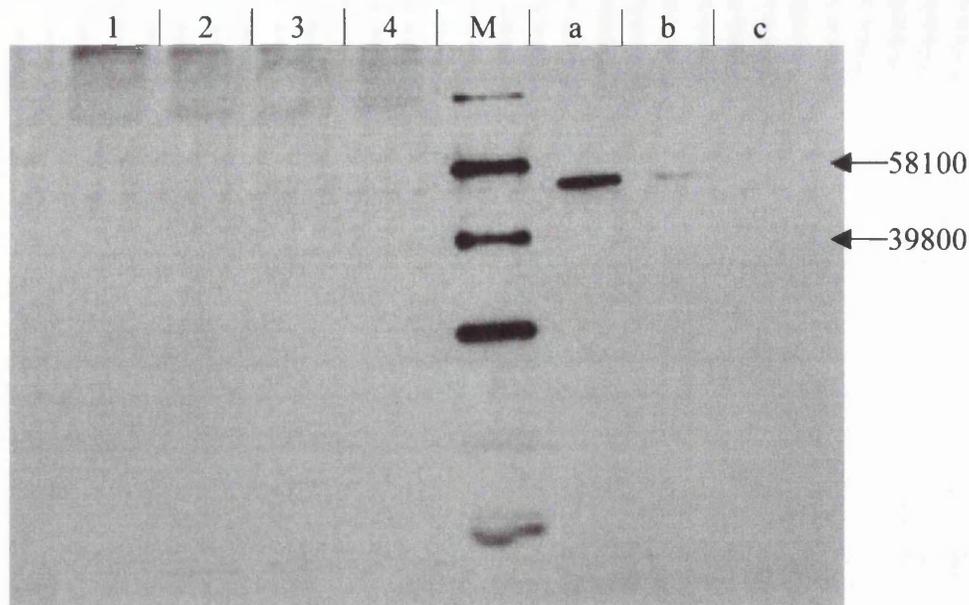
Figure 2-10 Western blot showing reaction of rat oesophageal and liver microsomal protein with rat CYP2E1 antibody. The microsomal proteins were separated on a 10% SDS-PAGE gel and blotted onto a nitrocellulose membrane (Hybond ECL, Amersham). After hybridisation, the membrane was developed by ECL and exposed to radiographic film for 10 minutes.

1 - oesophageal microsomes from preparation 1 (10 μg protein)
2 - oesophageal microsomes from preparation 2 (10 μg protein)
3 - oesophageal microsomes from preparation 3 (10 μg protein)
4 - oesophageal microsomes from preparation 4 (10 μg protein)
a - liver microsomes (3 μg protein)
b - liver microsomes (2 μg protein)
c - liver microsomes (1 μg protein)
M - molecular weight markers

2.4.7 Inhibition of the Metabolism of *N*-nitrosodimethylamine and *N*-nitrosodiethylamine by *N*-Dialkylformamides

In an attempt to find a selective inhibitor for the oesophageal nitrosamine-metabolising enzyme, experiments were carried out on the effect of dimethylformamide, diethylformamide and methyl-*n*-butylformamide on the metabolism of various nitrosamines by liver and oesophageal tissue. The effect of 0.2 mM of each of the *N*-dialkylformamides, dimethylformamide, diethylformamide and methyl-*n*-butylformamide, on the metabolism of *N*-nitrosodiethylamine to CO_2 by rat oesophageal mucosa was measured. Neither dimethylformamide nor

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diethylformamide had a significant effect (Table 2-3), but methyl-*n*-butylformamide was a strong, apparently competitive inhibitor of the oesophageal metabolism of *N*-nitrosodiethylamine (Table 2-3 and Figure 2-11).

Table 2-3   Effect of *N*-alkylformamides on *N*-nitrosomethyl-*n*-butylamine metabolism by oesophagus mucosa slices

Substrate Concentration (μM)	Rate of Reaction (nmol of substrate metabolised/min/mg tissue)					
	Control	+ 0.2mM DMF*	Control	+ 0.2 mM DEF*	Control	+ 0.2 mM MBF*
6.85	0.0083 ± 0.0011	0.0076 ± 0.0010	0.0061 ± 0.001	0.0051 ± 0.0004	0.0077 ± 0.0007	0.0028 ± 0.0002
12.00	0.0079 ± 0.0009	0.0106 ± 0.0013	0.0102 ± 0.0001	0.0066 ± 0.0006	0.0068 ± 0.0020	0.004 ± 0.0005
17.50	0.0138 ± 0.0006	0.0089 ± 0.0013	0.0120 ± 0.0012	0.0072 ± 0.0002	0.0118 ±0.0010	0.0071 ±0.0009
23.25	0.0117 ± 0.0015	0.0124 ± 0.0014	0.0123 ± 0.0010	0.0112 ± 0.0031	0.0144 ± 0.0010	0.0084 ± 0.0010

*Abbreviations: DEF – dimethylformamide, DEF – diethylformamide,
             MBF – methyl-*n*-butylformamide
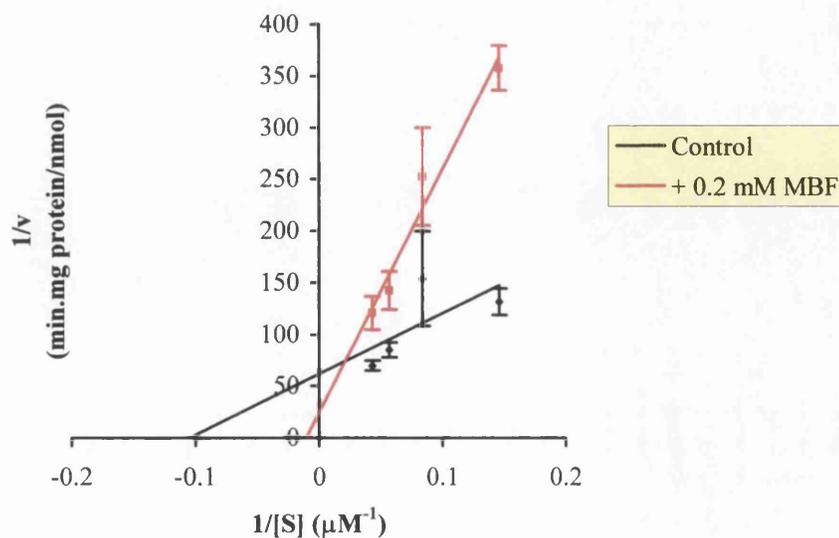
Figure 2-11   Lineweaver-Burke plot showing inhibition by methyl-*n*-butylformamide of *N*-nitrosodiethylamine metabolism by rat oesophageal mucosa

By contrast all three formamides were strong competitive inhibitors of the metabolism of *N*-nitrosodimethylamine by liver slices (Table 2-4 and Figure 2-12) with the greatest inhibition being from methyl-*n*-butylformamide.

Table 2-4   Effect   of   *N*-alkylformamides   on   *N*-nitrosodimethylamine metabolism of by liver slices

Substrate Concentration (µM)	Rate of Reaction (nmol of substrate metabolised/min/mg tissue)					
	Control	+ 0.2 mM DMF*	Control	+ 0.2 mM DEF*	Control	+ 0.2 mM MBF*
17.85	0.0641 ± 0.0042	0.0350 ± 0.0164	0.0764 ± 0.0134	0.0307 ± 0.0096	0.0625 ± 0.0187	0.0170 ± 0.0037
21.70	0.1125 ± 0.0166	0.0311 ± 0.0053	0.0987 ± 0.0308	0.0359 ± 0.0000	0.0728 ± 0.0118	0.0158 ± 0.0007
33.30	0.1203 ± 0.0231	0.0489 ± 0.0206	0.0759 ± 0.0000	0.0365 ± 0.0034	0.0810 ± 0.0032	0.0223 ± 0.0009
66.70	0.1155 ± 0.0299	0.0827 ± 0.0117	0.1132 ± 0.0238	0.0610 ± 0.0160	0.0871 ± 0.0307	0.0396 ± 0.0025

*Abbreviations: DMF – dimethylformamide, DEF – diethylformamide,
                   MBF – methyl-*n*-butylformamide

Figure 2-12   Liveweaver-Burke plots showing the effects of *N*-alkylformamides on the metabolism of *N*-nitrosodimethylamine by liver slices
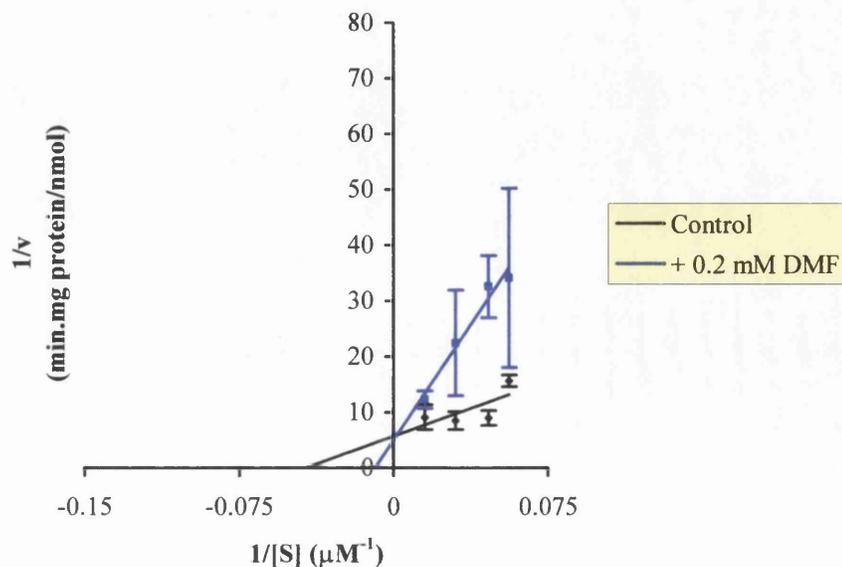
A.   Effect of dimethylformamide on *N*-nitrosodimethylamine metabolism by liver
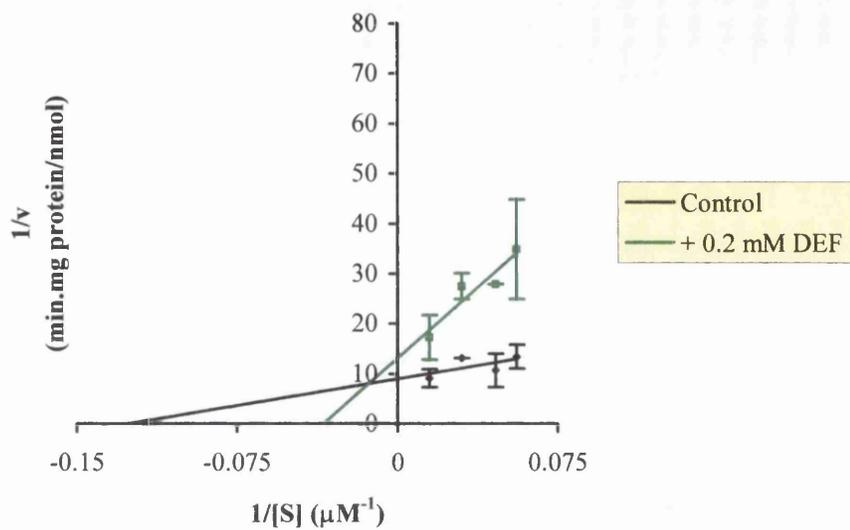
B.   Effect of diethylformamide on *N*-nitrosodimethylamine metabolism by liver
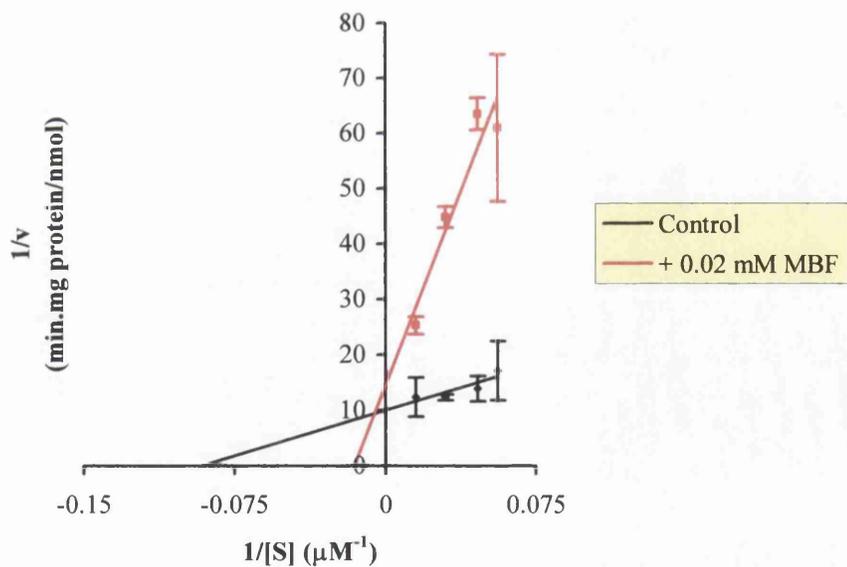
C.   Effect of methyl-*n*-butylformamide on *N*-nitrosodimethylamine metabolism by liver

The three formamides also inhibited the metabolism of *N*-nitrosodiethylamine by liver slices, but there was much less inhibition of metabolism of this nitrosamine than the metabolism of *N*-nitrosodimethylamine (Table 2-5 and Figure 2-13).

Table 2-5   Effect of *N*-alkylformamides on *N*-nitrosodiethylamine metabolism by liver slices

Substrate Concentration (μM)	Rate of Reaction (nmol of substrate metabolised/min/mg tissue)					
	Control	+ 0.2 mM DMF*	Control	+ 0.2 mM DEF*	Control	+ 0.2 mM MBF*
18.3	0.1191 ± 0.0153	0.0919 ± 0.0191	0.0790 ± 0.0116	0.0482 ± 0.0068	0.1531 ± 0.0175	0.0977 ± 0.0074
23.8	0.1627 ± 0.0190	0.1111 ± 0.0372	0.0953 ± 0.0015	0.0482 ± 0.0078	0.2042 ± 0.0059	0.1252 ± 0.0066
35.8	0.2319 ± 0.0394	0.1806 ± 0.0589	0.1232 ± 0.0274	0.0785 ± 0.0405	0.2744 ± 0.0681	0.1992 ± 0.0148
70.9	0.3834 ± 0.0564	0.2769 ± 0.0094	0.2082 ± 0.0480	0.1445 ± 0.0302	0.4079 ± 0.0101	0.4182 ± 0.1421

*Abbreviations: DMF – dimethylformamide, DEF – diethylformamide,
                   MBF – methyl-*n*-butylformamide

Figure 2-13   Lineweaver-Burke plots showing the effects of *N*-alkylformamides on the metabolism of *N*-nitrosodiethylamine by liver slices
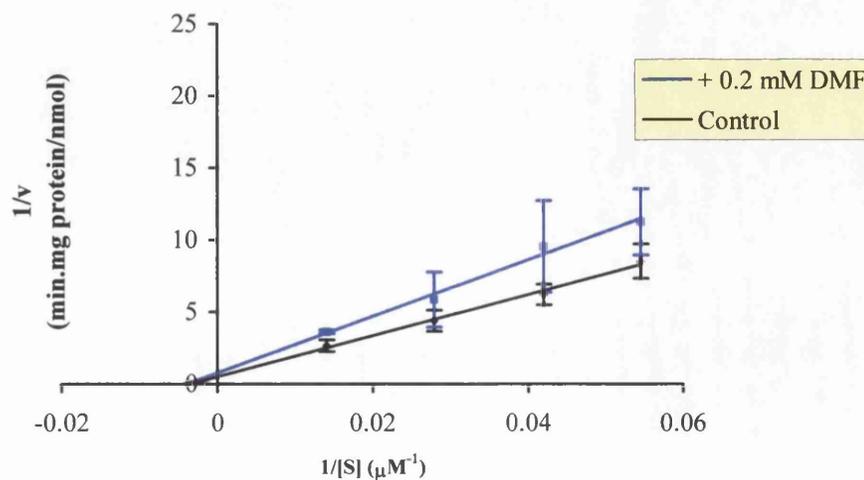
A.   Effect of dimethylformamide on *N*-nitrosodiethylamine metabolism by liver
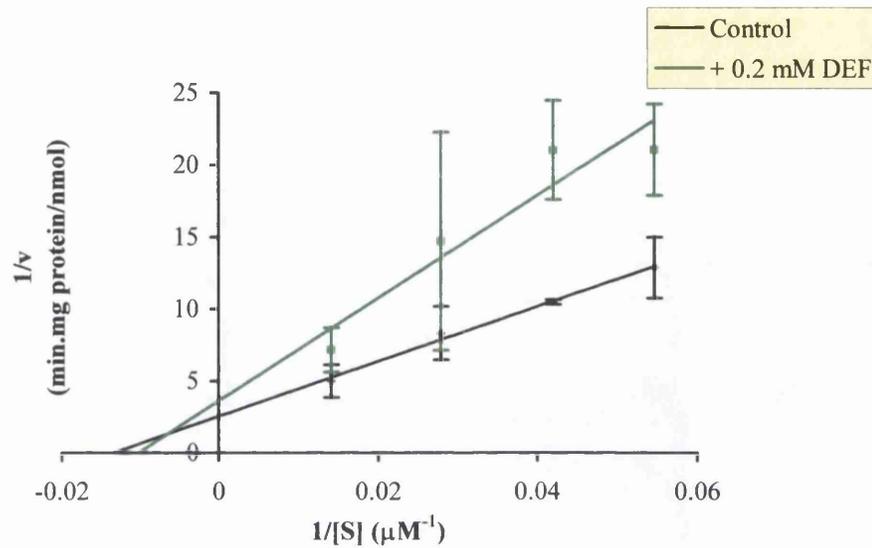
B.   Effect of diethylformamide on *N*-nitrosodiethylamine metabolism by liver
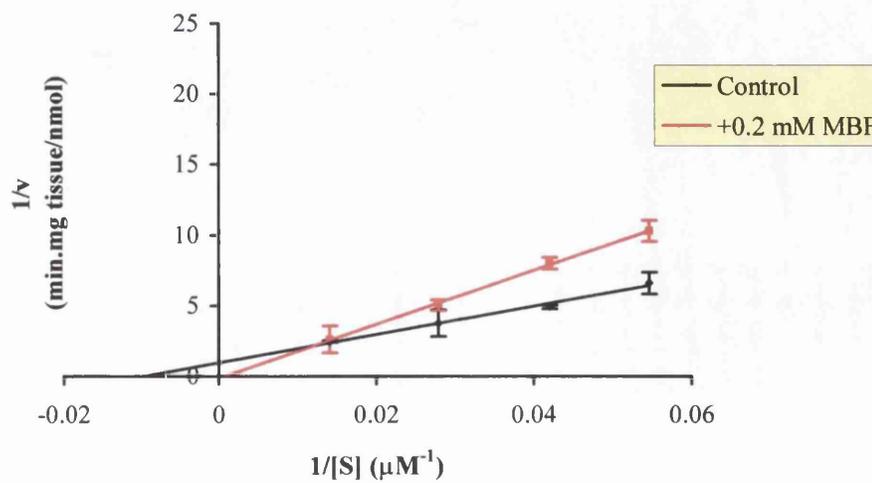
C.   Effect of methyl-*n*-butylformamide on *N*-nitrosodiethylamine metabolism by liver

2.4.8 Attempts at Purifying Cytochromes P450 from Liver Microsomes

Attempts were made at purifying constitutive P450 from rat liver to electrophoretic homogeneity in order to carry out N-terminal sequencing. It was hoped that if successful, the same procedure could be used for oesophageal P450. Three different purification procedures were carried out as described in the Methods and the results obtained are shown in the following pages. The purification procedures were carried out on liver microsomes because rat liver is more readily available in large quantities so it would be easier to perfect the technique with liver microsomes before applying it to the oesophageal microsomes. The first technique attempted was purification on an N-octylamine-coupled sepharose column followed by a DEAE sepharose column. The elution profiles obtained (Figure 2-14) were as demonstrated by previous researchers (Guengerich and Martin, 1980). Fractions 127 to 156 eluted from the N-octylaminosepharose column were pooled and the P450 content determined. The pooled fractions were then concentrated and dialysed and purified further on a DEAE sepharose column. Fractions 33 to 49 from this column were pooled and the P450 content measured. A 3-fold purification of the liver P450 was obtained, but the purified product was not electrophoretically homogeneous (Figure 2-15) and at this stage the yield was 31% (Table 2-6). Preliminary experiments using a hydroxyapatite column, which would have been the next step, had shown that only about 25% of the P450 loaded was eluted. Since this meant that only about 8% of the initial P450 could be recovered, this purification procedure was not pursued further.
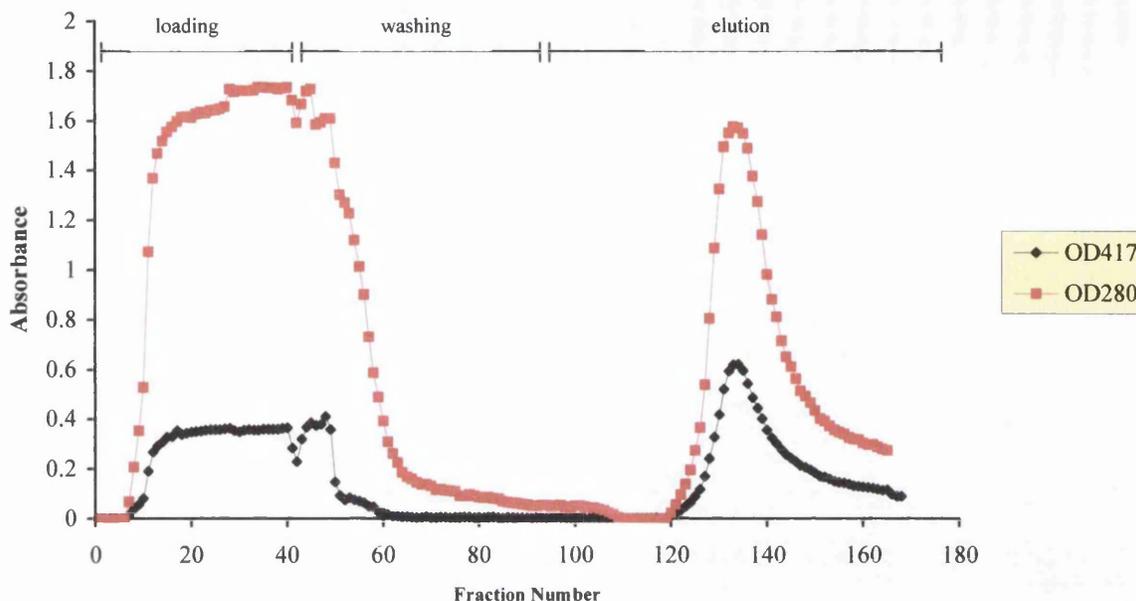
Figure 2-14. A   **Elution profile of *N*-octylaminosepharose column.**   Solubilised microsomes were applied to the column equilibrated with Buffer A (0.1 M potassium phosphate buffer (pH 7.25)/0.1 mM EDTA containing 20% (v/v) glycerol) at a rate of 0.4 ml/min.   The column was washed with 106 ml of Buffer A containing 0.42% (w/v) sodium cholate and P450 eluted with 240 ml of Buffer A containing 0.33% (w/v) sodium cholate and 0.06% (w/v) Lubrol PX.   3 ml fractions were collected.
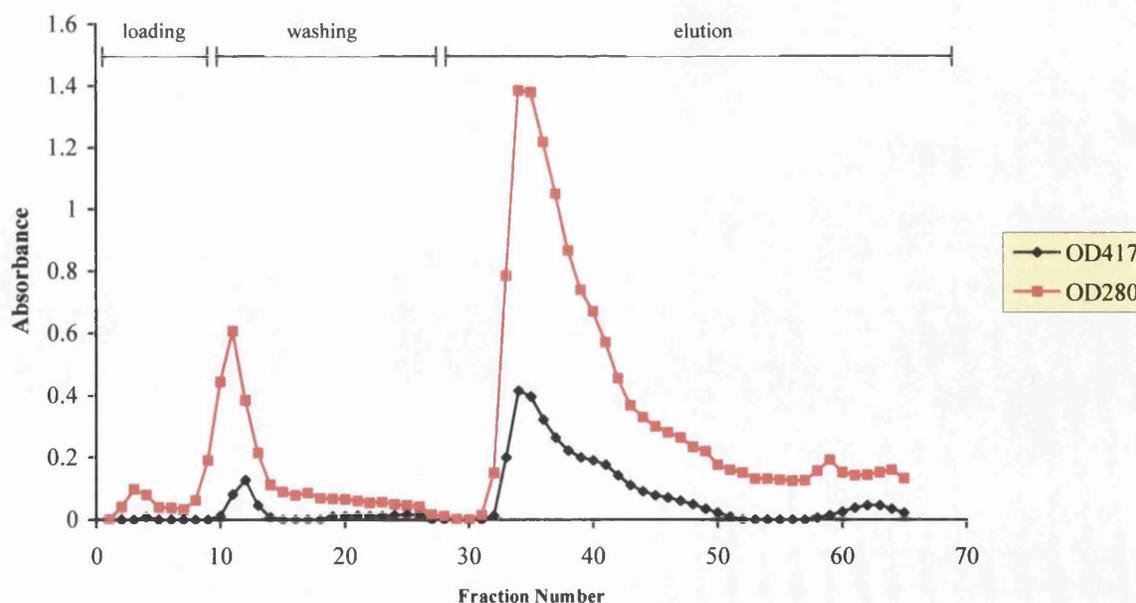
Figure 2-14. B   **Elution profile of DEAE-Sepharose column.**   The pooled P450 fractions from the *N*-octylaminosepharose column were loaded on to the column previously equilibrated with Buffer B (10 mM potassium phosphate buffer (pH 7.7) / 0.1 mM EDTA containing 20% (v/v) glycerol, 0.1% (w/v) Lubrol PX and 0.2% (w/v) sodium cholate.   The column was washed with 80 ml of Buffer B and P450 eluted with 200 ml of Buffer B containing 0.25 M NaCl.

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Table 2-6   **Results of purification on *N*-octylaminosepharose and DEAE sepharose columns**

Sample	P450 (nmol)	Protein (mg)	P450/protein (nmol/mg)	Fold Purification	Yield (%)
Crude microsomes	352	641	0.55	-	-
Solubilised microsomes	336	561	0.60	1.09	95
N-octylamino sepharose	191.7	108	1.78	3.24	54
DEAE sepharose	107.7	57.4	1.88	3.42	31

There was conversion of P450 to P420 during the purification step on the DEAE sepharose column. The *N*-octylaminosepharose column, however, appeared to reduce the proportion of P420. The crude and solubilised microsomes contained 66 and 63 nmol P420 respectively, 19% of the amount of P450 in each. After the purification step on the *N*-octylaminosepharose column there was 4.4 nmol P420, 2.3% of the amount of P450. 36 nmol P450 and 55.8 nmol P420 flowed through the column during loading. However after DEAE column purification the P420 content had risen to 10.6 nmol, 10% of the P450 content. When P420 was accounted for, the fold purification and yield after each separation step changed only marginally (Table 2-7).

Table 2-7   **Results of purification on *N*-octylaminosepharose and DEAE sepharose columns accounting for conversion of P450 to P420**

Sample	P450 + P420 (nmol)	Protein (mg)	P450 + P420/protein (nmol/mg)	Fold purification	Yield (%)
Crude microsomes	418	641	0.65	-	100
Solubilised microsomes	399	561	0.71	1.09	95
N-octylamino sepharose	196.1	108	1.82	2.8	47
DEAE sepharose	118.3	57.4	2.06	3.2	28
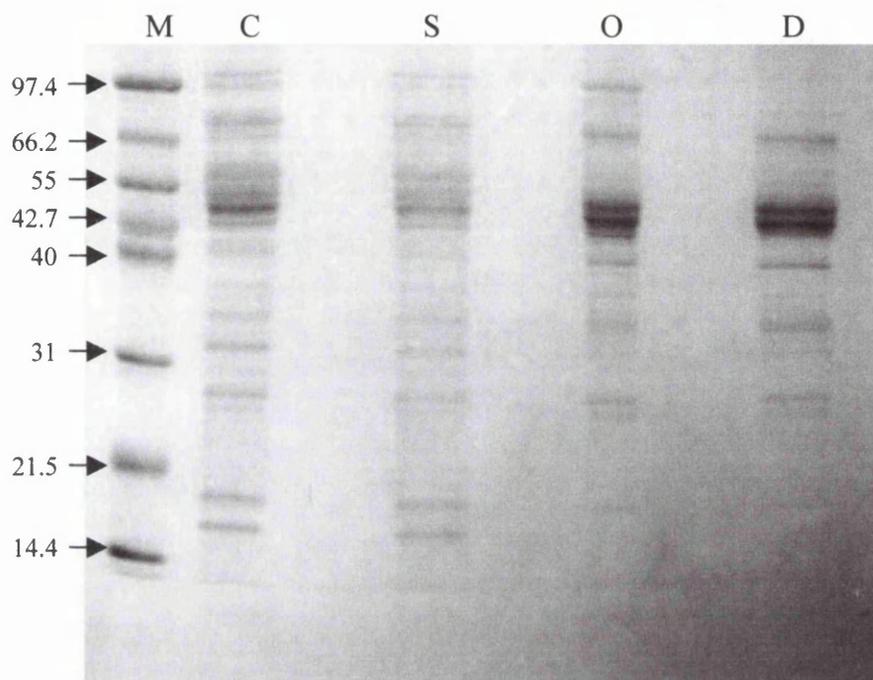
Figure 2-15   SDS-PAGE gel of rat liver microsomal protein at various stages in the purification process.

M - Molecular weight marker (Promega)
C - Crude microsomes (22 µg protein)
S - Solubilised microsomes (22 µg protein)
O - *N*-octylaminosepharose column eluate (22 µg protein)
D - DEAE sepharose column eluate (22 µg protein)

There was no significant change in the level of purification attained after applying the eluate from the *N*-octylaminosepharose column to the DEAE Sepharose column. Similar results were obtained for two attempts at purification using this technique.

Purification of the liver P450 on a *p*-chloroamphetamine-coupled sepharose column was attempted. The use of this technique has only been reported with tissues that are only available in small quantities e.g. rat breast and rat brain. The elution profile obtained is shown in Figure 2-16 and yield of P450 is represented in Table 2-8.

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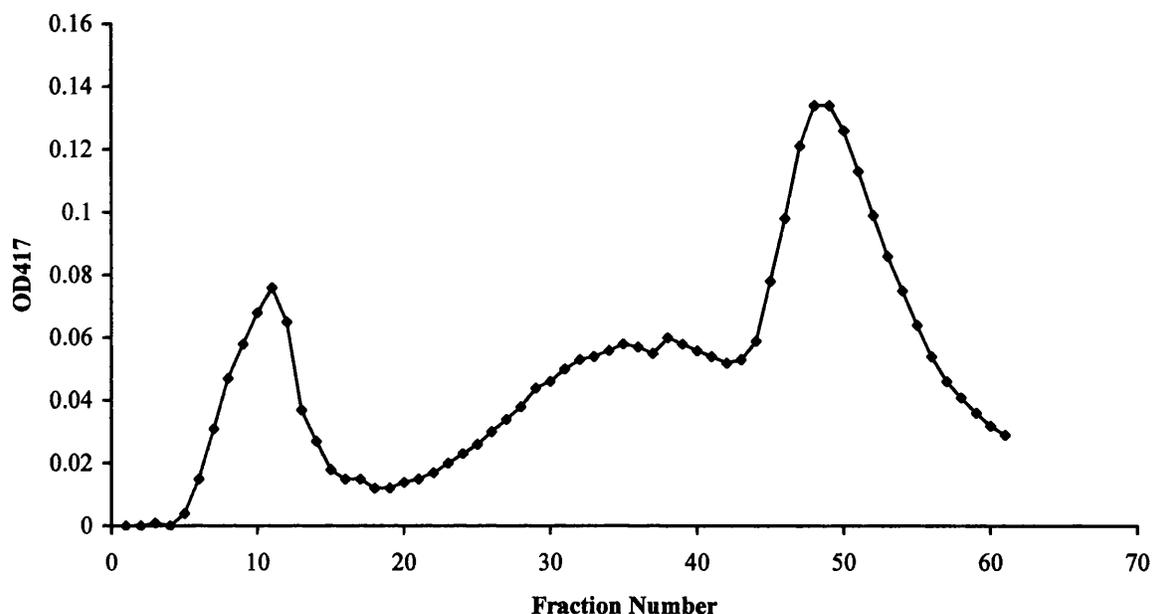
Figure 2-16   Elution profile of *p*-chloroamphetamine column. The solubilised P450 was loaded onto the column, equilibrated with PBS containing 20% (v/v) glycerol and 1 mM EDTA, at a rate of 0.5 ml/min and then eluted with 90 ml of PBS containing 1 mM EDTA, 20% (v/v) glycerol, 1% (w/v) CHAPS and 0.2% (v/v) Emulgen 911.

Table 2-8   Results of purification on *p*-chloroamphetamine column

Sample	P450 (nmol)	Protein (mg)	P450/protein (nmol/mg)	Fold purification	Yield (%)
Crude microsomes	28.6	32.4	0.88	-	100
Solubilised microsomes	22.7	11.9	1.91	2.2	79
Concentrated Eluate	8.3	1.7	4.88	5.5	29

These results show that better purification was achieved on the *p*-chloroamphetamine column than on the *N*-octylaminosepharose column at a relatively good yield. Also, the purified product was electropheretically homogeneous (Figure 2-17).
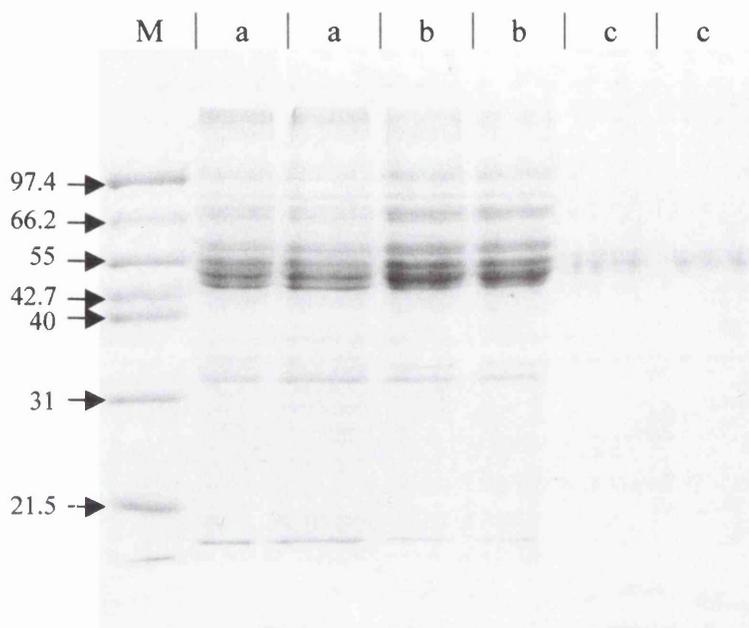
Figure 2-17 SDS-PAGE gel of rat liver microsomal protein at various stages of purification on a *p*-chloroamphetamine column.

a - crude microsomes (10 μg protein)
b - solubilised microsomes (10 μg protein)
c - purified protein (1 μg protein)
M - molecular weight markers (Promega)

As a final attempt at P450 purification, a chromatofocussing (Mono-P) column was used. The use of a Mono-P column had been reported to give electrophoretically pure P450 with very good yield (Isa *et al.*, 1992). The elution profile obtained was not as shown by other researchers (Isa *et al.*, 1992; Marriage and Harvey, 1986) and plans of purifying the oesophageal P450 were abandoned for a molecular biology approach.

2.5   Discussion

Results obtained from the induction of tumours show how easily tumours are induced in the rat oesophagus after small doses (Figure 2-2). Only eight injections totalling 3.2 mg (i.e. 21 μmoles) of the nitrosamine per 200 g rat were required to produce tumours. It is widely accepted that these tumours arise as a consequence of methylation of oesophageal DNA. To generate a methylating agent, nitrosamines are metabolically activated by hydroxylation by cytochromes P450. The oesophageal mucosa is particularly susceptible to tumour induction by asymmetric dialkylnitrosamines with one methyl group and another longer chain alkyl group. To generate a methylating agent from these asymmetric nitrosamines, the P450s must hydroxylate the longer chain alkyl group on the α-carbon, i.e. the carbon proximal to the nitroso group (Heath, 1962), producing the corresponding aldehyde and the methylating agent. As mentioned in the Introduction to this chapter, the methylating agent is very short-lived and cannot be transported to the target organs in the general circulation. It must therefore be generated in the affected organs by specific P450s. The methylating agent methylates different susceptible sites in DNA, including 7 and O^6 of guanine and O^4 of thymine. Methylation at O^6 of guanine, which is believed to be the main cause of the observed carcinogenicity, results in mispairing with thymine and hence incorporation of adenine in the next round of replication, i.e. a G:C→A:T transition. Methylation at 7 of guanine does not interfere with the normal pairing of bases and is hence not promutagenic, and is believed to play no direct role in carcinogenesis. The simplest way of assessing the extent of metabolic activation in any tissue is to measure the methylation of DNA produced by the nitrosamine. In these experiments as in those carried out by other researchers, the overall methylation was assessed by measuring the amount of 7-methylguanine because it is the most abundant and the most stable product of DNA methylation. Methylation at O^6 of guanine and O^4 of thymine was not measured because even though these adducts are believed to be directly responsible for the observed carcinogenicity, their methyl groups are rapidly removed by O^6-alkylguanine-DNA-alkyltransferase. Measurement of the quantities of these methylated bases cannot, therefore, be used as an index of the extent of metabolic activation in the tissue.

From the measurement of the amount of 7-methylguanine in the DNA of various tissues of the rat after a dose of N-nitrosomethyl-n-butylamine (Table 2-1 and Figure 2-4) it has been shown that there is greater activation in the oesophagus than in any other organ. The higher level of methylation of oesophageal DNA than of liver DNA is consistent with the results of von Hofe et al. (1987) although the preference of the oesophagus is much greater that reported by von Hofe et al. The trend obtained in this experiment is similar to that obtained in experiments by other investigators using N-nitrosomethyl-n-benzylamine (Hodgson et al., 1980) and N-nitrosomethyl-n-amylamine (Koenigsmann et al., 1988). In experiments carried out by Hodgson et al. (1980) there was 3, 5 and 100 times more 7-meG in oesophageal DNA than in liver, lung and kidney DNA respectively. Hodgson et al. (1980) also reported that although the concentration of the N-nitrosomethyl-n-benzylamine was highest in the liver, kidney and serum 10 minutes after the injections, the oesophagus had the highest level of ^{14}C-labelled metabolites at that time.

It is important to understand that the higher alkylation does not show that the rate of metabolism as a whole is greater in the oesophagus than in the other organs, but just that the rate of activation is greater. This distinction between metabolism and activation is necessary because hydroxylation of the methyl group of this nitrosamine, and possibly the hydroxylation of the 4-carbon of the butyl group (i.e. the carbon furthest from the nitroso group) are detoxifying reactions (Mirvish et al., 1988). As will be discussed below, the low alkylation in the liver is because the greater part of the metabolism is detoxifying. There have been several reports that suggested that the enzyme that activates nitrosamines in the oesophagus is different from that which catalyses metabolism in the liver. The most compelling of these is observed in experiments by Ji et al. (1991) that show that an isomer of N-nitrosomethyl-n-amylamine, N-nitrosomethyl-(2-methylbutyl)amine, is metabolically activated, i.e. DNA methylation can be detected, in the oesophagus, but not in the liver. This indicates that the oesophagus contains a P450 that is not expressed in the liver. However, this is the only report of such a clear difference between liver and oesophagus. With most methylalkylnitrosamines, as with N-nitrosomethyl-n-butylamine, there is methylation in liver as well as in oesophagus (von Hofe et al., 1987). The identity of the enzyme(s) that metabolise nitrosamines in the liver has

been disputed and various authors have shown that *in vitro* CYP2B1, CYP2B2 and CYP2E1 are capable of nitrosamine metabolism, and therefore might be involved.

The fact that this metabolism was being carried out by CYP2E1 in the liver and another P450 in the oesophagus was supported by the effect of co-administration of ethanol on the methylation (Table 2-2 and Figure 2-5) and by measurements of metabolism in the whole animal. Administration of *N*-nitrosomethyl-*n*-butylamine in 5% ethanol increased the methylation in oesophagus, nasal epithelium and lung, but decreased methylation in liver and kidney. This is consistent with the view that metabolism in the liver and kidney is predominantly carried out by CYP2E1, because ethanol is known to be a strong competitive inhibitor of this P450. The lack of inhibition in oesophagus, nasal epithelium and lung indicates that metabolism in these organs is carried out by a P450 that is not inhibited by ethanol. The actual increase is a reflection of the fact that inhibition of metabolism in the liver increases the lifetime of the nitrosamine in the animal, resulting in greater exposure to the organs that have the ability to activate it. The position in the lung is of particular interest because although the rat lung is known to metabolise *N*-nitrosodimethylamine, a nitrosamine that is metabolised by CYP2E1 (Lee *et al.*, 1989; Swann and Magee, 1968; Tu *et al.*, 1981; Tu and Yang, 1983; Yoo *et al.*, 1990), ethanol produced as big an increase in methylation of lung DNA as oesophageal DNA. This suggests that the lung expresses two P450s capable of nitrosamine metabolism – CYP2E1, which is inhibited by ethanol, and another, which is not inhibited by ethanol. The fact that the increase in DNA methylation in lung was similar to that in oesophagus suggests that this other P450 in lung carries out a major part of the activation of *N*-nitrosomethyl-*n*-butylamine.

The observation that metabolism of *N*-nitrosomethyl-*n*-butylamine in rat liver is carried out predominantly by CYP2E1 was confirmed by experiments on metabolism in whole rats (Figures 2-6 and 2-7). In these experiments rats were given a relatively large dose of *N*-nitrosodi[^{14}C]methylamine or *N*-nitroso[^{14}C-methyl]methyl-*n*-butylamine and the rate of metabolism measured by following the exhalation of $^{14}CO_2$, a technique used and justified in extensive studies by Heath (1962). These large doses saturate the metabolism system and the exhalation is linear with time over the main part of the course of the experiment. In the whole rat, the metabolism of the

nitrosamines is essentially due to metabolism in the liver due to its large size and high content of P450s. The apparent non-linearity observed during the metabolism of N-nitrosomethyl-n-butylamine may be due to the fact that being a lipophilic nitrosamine, N-nitrosomethyl-n-butylamine is partitioned between fat and blood. For this reason its initial concentration in the blood is less than that of N-nitrosodimethylamine, and the whole dose is not available to the metabolising enzyme. Later, as the nitrosamine in the blood is metabolised, more of it is released from the fat, and the lag imposed by this equilibration step results in the slight curve in the graph. All the radioactivity administered is not recovered as $^{14}CO_2$ due to the fact that some of it becomes incorporated, via folic acid and tetrahydrofolate, into macromolecules within the rats. The results expressed as µmoles of nitrosamine metabolised (Figure 2-8) show that both nitrosamines were metabolised essentially at the same rate, which strongly suggests that they were being metabolised by the same enzyme. This was confirmed when the two nitrosamines were administered together in equimolar amounts and the exhalation of $^{14}CO_2$ from each of them measured separately. This showed that both nitrosamines were metabolised at approximately half the rate they had been metabolised when they were given alone (Figures 2-7 and 2-8). However the rate of total nitrosamine metabolism was essentially identical to the rate of metabolism of each nitrosamine when given alone (Figure 2-8). This shows that the two nitrosamines are metabolised by the same enzyme, they bind to the same site on that enzyme, and the enzyme metabolises both nitrosamines with the same K_{cat}. Since it is known that N-nitrosodimethylamine is metabolised exclusively by CYP2E1 in the liver, then N-nitrosomethyl-n-butylamine must also be metabolised by CYP2E1.

Although N-nitrosodimethylamine and N-nitrosomethyl-n-butylamine are metabolised at the same rate in the liver, the level of methylation produced by the latter is only 25% that produced by N-nitrosodimethylamine (Table 2-1 and Swann et al., 1984). This indicates that 75% of the metabolism of N-nitrosomethyl-n-butylamine in the liver is detoxifying, probably due to hydroxylation of the methyl group. Similar non-activating metabolism probably also occurred in experiments reported by Ji et al. (1991). They demonstrated that N-nitrosomethyl-n-isoamylamine and N-nitrosomethyl-n-amylamine methylated liver DNA when administered to rats, but

another isomer, *N*-nitrosomethyl-(2-methylbutyl)amine, did not. This might be interpreted as showing that *N*-nitrosomethyl-(2-methylbutyl)amine was not metabolised in the liver. They showed that *N*-nitrosomethyl-*n*-isoamylamine produced the highest levels of methylation in all tissues examined, with the exception of liver. The amount of methylation in the tissues after administration of *N*-nitrosomethyl-*n*-amylamine was essentially identical to that obtained after administration of *N*-nitrosomethyl-(2-methylbutyl)amine. However, while there was a significant amount of methylation in the liver after *N*-nitrosomethyl-*n*-amylamine treatment, there was no methylation detected in liver after administering *N*-nitrosomethyl-(2-methylbutyl)amine. If *N*-nitrosomethyl-(2-methylbutyl)amine had not been metabolised in the liver, the level of methylation in the extrahepatic organs would have been much higher. Nitrosamines are neither excreted to a significant extent in urine nor exhaled (Heath and Dutton, 1958) and therefore the entire dose of nitrosamines is metabolised. If *N*-nitrosomethyl-(2-methylbutyl)amine had not been metabolised in the liver, its biological lifetime would have been greatly increased and the methylation of DNA in oesophagus and other extrahepatic organs would have been much greater than that produced by *N*-nitrosomethyl-(3-methylbutyl)amine. This shows that there must be metabolism of *N*-nitrosomethyl-(2-methylbutyl)amine in the liver, but that the metabolism must be entirely hydroxylation on the methyl group. The experiments shown in Figures 2-6 and 2-7 would suggest that this detoxifying metabolism is also carried out by CYP2E1. In the same manner, even though there was no methylation detected (in the same experiment by Ji *et al.*, 1991) in any of the organs after administering the other isomers, *N*-nitrosomethyl-(1-methylbutyl)amine, *N*-nitrosomethyl-(1,2-dimethylpropyl)amine or *N*-nitrosomethyl-(1-ethylpropyl)amine, the nitrosamines must have been metabolised on their methyl groups (detoxification) by CYP2E1 in the liver. The results of these experiments as well as those described in this thesis using whole rats show that methylalkylnitrosamines are metabolised by CYP2E1 in the liver.

The observation that *N*-nitrosodimethylamine and NMBA are metabolised at exactly the same rate by CYP2E1 despite the fact that they differ greatly in their structure and lipophilicity may be explained by recent studies which show that the K_{cat} of CYP2E1 is limited by a very slow step occurring after a very fast catalytic step (Bell-Parikh and Guengerich, 1999). This would allow the catalytic step for the two substrates to

differ without a significant effect on K_{cat}. The results support the view that the rate is limited by a conformational change in CYP2E1 after catalysis (Bell-Parikh and Guengerich, 1999) rather than being limited by product release as previously proposed (Bell and Guengerich, 1997). If the rate of the reaction were to be limited by product release, the individual rates of reaction for the two nitrosamines would not be identical since the intermediates formed from the two are very different and would influence product release. The proposal of a conformational change in CYP2E1 after substrate binding is however supported because that would not be influenced by a particular substrate and would agree with the identical rates of reaction observed. The hypothesis that the activity of CYP2E1 is limited by a conformational change (Bell-Parikh and Guengerich, 1999) suggests that there does not necessarily have to be as much P450 per gram tissue in the oesophagus as there is CYP2E1 in liver to obtain the activity observed in oesophagus. That is to say, if the activity of the oesophageal P450 responsible for the activation of nitrosamines is not limited by conformational changes and thus has a higher K_{cat}, then very little enzyme will be required to give the same levels of methylation caused by N-nitrosodimethylamine in the liver.

Reed *et al.* (1986) suggested that the high metabolising activity of nasal microsomes is due to a high NADPH-cytochrome P450 reductase to P450 ratio, and the high activity in the oesophagus might, in part, have a similar basis. Measurement of P450 in the oesophagus showed that there was only 0.05 nmol P450/mg microsomal protein, while the liver contained up to 20 times as much (~1 nmol/mg microsomal protein). The activity of NADPH-cytochrome c reductase (used as a measure of NADPH-cytochrome P450 reductase) in the liver (0.28 μmol/min/mg protein) was only 6 times greater than in the oesophagus (0.048 μmol/min/mg protein). This gives a ratio of reductase to P450 in the oesophagus of 1:1, as opposed to 0.36:1 in the liver and therefore 3 times as much reductase per nmol P450 in oesophagus than in liver. This could be important from the point of view of nitrosamine activation in the two organs. The higher reductase to P450 ratio in oesophagus could mean a more efficient electron transfer system than in the liver. This could explain the high activity seen in the oesophagus even though it contains so little P450 compared to the liver.

Because CYP2E1 metabolises these nitrosamines so well in the liver and also because there has been considerable speculation that CYP2A3, the rat orthologue of mouse CYP2A5 might metabolise nitrosamines in nasal epithelium, Western blot analysis of the oesophageal P450 was carried out using two available anti-P450 sera – anti-CYP2A5 and anti-CYP2E1. The results show that there was a reaction of anti-2A5 to an oesophageal microsomal protein of about 54 kD (Figure 2-9). This antibody reacted with a protein of the same size in rat liver microsomes. The intensity of the band obtained for 10 µg of oesophageal microsomal protein was about the same as that obtained for 3 µg of liver microsomal protein. This suggests that whatever P450 was reacting in the oesophageal microsomes was present at 30% of the level of that reacting in the liver microsomes. Without knowing what epitope this antibody recognises, it cannot be concluded that the oesophagus contains CYP2A3 or a CYP2A3-like P450, just that the oesophageal P450 reacts with anti-CYP2A5. Also since rat liver does not contain CYP2A3, the results obtained suggested that the antibody was not very suitable for the purpose. The results of Western blotting with anti-CYP2E1 showed clearly that there was no CYP2E1 in rat oesophageal microsomes (Figure 2-10). This observation was expected since the rat oesophagus does not metabolise *N*-nitrosodimethylamine (Swann *et al.*, 1984). In this respect there seems to be a significant difference between rats and humans since Smith *et al.* (1998) detected *N*-nitrosodimethylamine demethylase activity in human oesophageal microsomes. The oesophageal tissue was obtained from individuals in the USA who had died from accidents, or from surgically resected oesophagi from oesophageal cancer patients in China, in which case cancerous and non-cancerous sections of the oesophagi were separated. They determined the rate of metabolism of *N*-nitrosodimethylamine (i.e. formation of formaldehyde) as 10.7 – 14.7 pmol/min/mg protein. From these observations, Smith *et al.* (1998) concluded that human oesophagus contains CYP2E1. However, the level of activity was about 20 to 300 times lower than those reported by Yoo *et al.* (1988) for the metabolism of *N*-nitrosodimethylamine by human liver microsomes (0.18 – 2.99 nmol/min/mg protein), so the conclusion that human oesophagus contains CYP2E1 has to be drawn with caution since owing to their overlapping substrate specificities, the low activity could be due to many P450s. Other researchers have reported the detection of CYP1A, CYP2E1, CYP3A and CYP4A enzymes by Western blotting. These

observations have led to the researchers concluding that CYP2E1 could be involved in the metabolism of nitrosamines in the human oesophagus. The results reported in this thesis show that this cannot be the case in the rat because *N*-nitrosomethyl-*n*-butylamine, which induces oesophageal tumours, is metabolised by CYP2E1 in the liver at the same rate as *N*-nitrosodimethylamine, but is only poorly activated by CYP2E1 in the liver. Also, if CYP2E1 were responsible for the metabolism of nitrosamines in rat oesophagus, the activity would be inhibited by ethanol, which it is not. *N*-nitrosodimethylamine demethylase activity has been detected in the oesophagus of sub-human primates, and again this activity was not inhibited by ethanol (Anderson *et al.*, 1996), confirming that CYP2E1 is not the nitrosamine-metabolising enzyme in the oesophagus.

To identify the P450(s) present in the oesophagus, it was decided that attempts would be made to purify microsomal proteins to an extent that a single band of ~ 55 kD could be excised after SDS-PAGE for N-terminal sequencing. It would not be necessary to purify to electrophoretic homogeneity if a distinct band could be obtained. Having such a small amount of P450 in the oesophagus, it was necessary to set up a purification system that would give a very good yield of P450. The easiest way to achieve this would be to induce the P450 so that there would be a large starting amount before the purification procedure. Unfortunately, since so little is known about the oesophageal P450, it has no known inducers. Previous investigators had included 0.05 mM 4-methylpyrazole in all buffers during the purification of CYP2E2 from rabbit liver microsomes to stabilise the P450 during purification (Ding *et al.*, 1991). 4-methylpyrazole was chosen because being a substrate of CYP2E1, it would be bound by the P450, hence minimising degradation of the P450 and conversion to P420. In an attempt to perform a similar purification protocol on the oesophageal P450, it was decided to discover a substrate for the enzyme that metabolises nitrosamines in the oesophagus that could be used for a similar purpose. The *N*-alkylformamides were chosen since it would be dangerous to include *N*-nitrosamines, which are carcinogenic, in the buffers. Dimethylformamide, diethylformamide and methyl-*n*-butylformamide were investigated since they are isoelectronic to nitrosamines that are metabolised in liver and oesophagus and because it was known that dimethylformamide is a substrate for CYP2E1, the major enzyme involved in the activation of *N*-nitrosodimethylamine (Heath, 1962; Mraz *et*

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al., 1993). Methyl-*n*-butylformamide was found to be the most effective of the three at inhibiting *N*-nitrosodiethylamine metabolism by oesophageal mucosa (Table 2-3 and Figure 2-11). The poor inhibition by the other *N*-alkylformamides was expected since rat oesophagus does not activate *N*-nitrosodimethylamine and is a poor activator of *N*-nitrosodiethylamine. These experiments were carried out before it had been discovered that *N*-nitrosomethyl-*n*-butylamine is metabolised by CYP2E1, and it was therefore surprising to discover that methyl-*n*-butylformamide was a very efficient inhibitor of the metabolism of both *N*-nitrosodimethylamine and *N*-nitrosodiethylamine by liver slices. It was found to be more effective than both dimethylformamide and diethylformamide at inhibiting the metabolism of *N*-nitrosodimethylamine and was as effective as dimethylformamide at inhibiting the metabolism of *N*-nitrosodiethylamine (Tables 2-4 and 2-5 and Figures 2-12 and 2-13). This result is consistent with the observation that given a mixture of *N*-nitrosomethyl-*n*-butylamine and *N*-nitrosodimethylamine, the rat initially metabolises the *N*-nitrosomethyl-*n*-butylamine faster than the *N*-nitrosodimethylamine, indicating that the former has a greater affinity for CYP2E1. So even though methyl-*n*-butylformamide had been found to be a good inhibitor of the nitrosamine-metabolising P450 in oesophagus, its inhibitory effect was not specific to the oesophageal P450.

It was decided that different P450 purification techniques would be tried using rat liver as the source of microsomes in order to find a procedure that would give the best yield. The first method attempted was *N*-octylaminosepharose column chromatography, which has been used extensively in certain laboratories for purification of P450s from liver and lung especially (Guengerich *et al.*, 1982; Guengerich and Martin, 1980; Shimada *et al.*, 1992; Wang *et al.*, 1980; Yun *et al.*, 1991). The yields of P450 from this and other similar columns are generally very low. Guengerich and Martin (1980) obtained up to 6-fold purification of P450 from phenobarbital-treated rats with a yield of 18% for the most abundant P450. Wang *et al.* (1980) were able to purify P450 from human liver 72-fold, but their P450 yield was only 4.5% after the final purification step. The *N*-octylaminosepharose column used by them only purified the P450 3-fold, but the yield of P450 after this step was just 16%. Yun *et al.* (1991) obtained a 16-fold purification of CYP2A6 from human liver microsomes, but their yield of P450 was 18% after *N*-octylaminosepharose

column chromatography and only 0.2% after the final purification step. The results obtained in this thesis for the purification of constitutive P450 from rat liver microsomes (Tables 2-6 and 2-7) are similar to those shown above. The yield of P450 after *N*-octylaminosepharose chromatography was 47%, with a 3-fold purification. After the next purification step, DEAE sepharose chromatography, the yield had fallen to 28% without much difference in the level of purification (Figure 2-15). In the purification procedures reported above, the investigators started with large amounts of P450 (~3000 nmol) and so the P450 obtained after purification was still measurable even though the yields were so low. This technique is useful for purifying P450s from large tissues or those tissues that have an abundance of P450, but plainly of little use with the oesophagus.

Another apparently successful method of P450 purification is by running samples through a *p*-chloramphetamine-coupled sepharose column. This technique has been used extensively by researchers for the purification of P450 from tissues in which the P450 content is low, e.g. rat breast and brain, or in cases where the amount of tissue is small, e.g. prostate gland. Using this technique, it was possible to purify liver P450 to apparent electrophoretic homogeneity (Figure 2-17). But the knowledge that liver does not contain just a single P450 suggests that the preparation, although apparently homogeneous, was not actually homogeneous in its P450 content. This lack of homogeneity would cause big problems in N-terminal sequencing. Also yields were rather low and it was decided that this procedure would not be suitable for purifying P450(s) from rat oesophagus.

Marriage and Harvey (1986) introduced chromatofocussing as a method of resolving P450 isozymes due to its higher resolution power than other ion-exchange and affinity techniques. They reported a total recovery of P450 of 40% from microsomes prepared from both untreated and phenobarbital-treated rats, but noted that the fractions obtained were not homogeneous, containing different forms of P450. This was attributed to the way in which the fractions had been combined. A later paper by Isa *et al.* (1992) reported the use of HPLC using a chromatofocussing column, Mono-P (BioRad), to obtain a 17-fold purification of P450 from human liver microsomes. They also reported a 47% recovery of P450. When this technique was attempted with liver microsomes during this research project, the results obtained

were unsatisfactory and the elution profile obtained did not show any peaks of absorption at 417 nm, corresponding to the elution of haem-containing proteins.

By the time the final purification technique had been attempted, a lot of time had been spent on trying to establish a method for purifying oesophageal P450(s) without success and it was thought that another approach would have to be tried.

CHAPTER 3   Identification of Oesophageal Cytochrome(s) P450 Using Molecular Biology Techniques

3.1   Introduction

The results of experiments described in previous chapters suggest that the P450 in the oesophagus responsible for carcinogenic N-nitrosamine metabolism is not expressed to a significant extent in liver and that liver metabolism is essentially all carried out by CYP2E1. Initially, it was hoped that the required P450 could be purified from oesophageal mucosa. This is because many of the known mammalian P450s were initially identified by isolation and purification of the protein. These include the most important members of family 1, CYP1A1 (Botelho *et al.*, 1979), CYP1A2 (Botelho *et al.*, 1982); and family 2, CYP2B1 (Botelho *et al.*, 1979), 2C11 (Guengerich *et al.*, 1982), CYP2A10 and CYP2G1 from rabbit nasal mucosa (Ding and Coon, 1988), CYP2E1 (Ryan *et al.*, 1985) among many others. Purification methods are still being used by some of the major P450 groups to study P450s (Hellmond *et al.*, 1995). However, after several attempts at P450 purification using liver microsomes, it was realised from the yields obtained that it would not be possible to use the same methods for extracting P450 from the oesophagus, which only contains 5% of the P450 present in the liver. It was therefore decided that a molecular biology approach should be attempted, since this is more sensitive and small amounts of mRNA in the tissues would be more readily detected. Although, as discussed in the introduction, some authors have suggested that the oesophageal P450 responsible for nitrosamine metabolism is CYP2A3 (Patten *et al.*, 1998) or CYP2E1 (Smith *et al.*, 1998), it seemed likely that the P450 would turn out to be an entirely novel form.

Molecular biology techniques have been used increasingly to identify novel P450s without the prior purification of the protein. Many of these new P450s are polymorphisms of previously known genes, e.g. the CYP2D6 debrisoquine/sparteine-type polymorphism, which causes a clinically important genetic variation of drug metabolism observed in about 10% of the Caucasian population (Gonzales *et al.*, 1988). However, a number of entirely new, but important P450s have been

discovered by molecular biology techniques, especially library screening. These include CYP2A3 (Kimura *et al.*, 1989), CYP2A6 (Phillips *et al.*, 1985), CYP2A7 (Yamano *et al.*, 1990), CYP2B2 (Fujii-Kuriyama *et al.*, 1982), CYP2A11 (Peng *et al.*, 1993) and CYP3A9 (Wang *et al.*, 1996). Reverse transcription-polymerase chain reaction (RT-PCR) methods have also been used to identify novel P450s, but unfortunately in many cases only partial sequences have been obtained by this method, as in the case of CYP1B1 from mouse embryo fibroblasts (Shen *et al.*, 1993) and CYP3A9 from rat intestine (Gushchin *et al.*, 1999). The full sequence of the former was eventually obtained by screening a mouse embryo fibroblast library (Shen *et al.*, 1994).

In the search for the nitrosamine-metabolising P450 of the oesophagus, it seemed appropriate to concentrate on P450s belonging to the 2 family because previous investigators had shown that P450s of the 2A subfamily (Camus *et al.*, 1993; Crespi *et al.*, 1990; Patten *et al.*, 1998; Yamazaki *et al.*, 1992), the 2B subfamily (Schulze *et al.*, 1990; Shu and Hollenberg, 1996; Shu and Hollenberg, 1997) and 2E subfamily (Camus *et al.*, 1993; Crespi *et al.*, 1990; Yamazaki *et al.*, 1992; Yang *et al.*, 1985) are all capable of the metabolic activation of *N*-nitrosamines. At the time this work began, a P450 of the 2A subfamily seemed the most likely candidate for the oesophageal nitrosamine-metabolising enzyme because a Western blot had produced a positive reaction with a Cyp2A5 antibody (see Chapter 2; CYP2A5 is the mouse orthologue of rat CYP2A3 and human CYP2A6). Furthermore, CYP2A6 from human liver will 7-hydroxylate coumarin and metabolise NDEA (Camus *et al.*, 1993), so when it was shown that rat nasal microsomes would 7-hydroxylate coumarin, and by showing a degree of mutual competitive inhibition that the coumarin hydroxylase was also the enzyme that metabolised the oesophageal carcinogen, NMBzA (Patten *et al.*, 1998), it was suggested that a P450 of the 2A subfamily, most likely CYP2A3, was involved in the nasal metabolism of nitrosamines. Because NMBzA is pre-eminently an oesophageal carcinogen, the authors suggested that their results might be extrapolated to suggest that the P450 in the oesophagus was a member of the 2A subfamily (Patten *et al.*, 1998). However, other studies had suggested that other P450s might be involved in the metabolism of nitrosamines. The predominant nitrosamine-metabolising enzyme in rat and human liver is CYP2E1 (Anderson *et al.*, 1992; Bellec *et al.*, 1996; Yoo and Yang, 1985 and see Chapter 4) and there is also

evidence that members of the 2B subfamily can metabolise nitrosamines. Although it is clear that CYP2E1 itself is not active in the oesophagus and only one other member of the 2E subfamily is known – CYP2E2 in rabbits (Khani *et al.*, 1988a; Khani *et al.*, 1988b), it seemed possible that an unknown member of this subfamily could be expressed in the oesophagus. The particularly interesting aspect of nitrosamine metabolism by the CYP2B subfamily is that CYP2B1 has been shown to metabolise longer chain asymmetrical dialkylnitrosamines, which are particular substrates for the oesophageal enzyme and cause oesophageal cancer (reviewed by Lijinsky, 1992). Furthermore, the metabolism has the required structural selectivity for the α-carbon of the long alkyl chains (Mirvish *et al.*, 1991). *N*-nitrosamines are metabolically activated by hydroxylation at the α-carbons by cytochromes P450. It seems that for a methylalkylnitrosamine to be carcinogenic, the hydroxylation has to take place on the α-carbon of the alkyl chain. Hydroxylation of the methyl group is probably a detoxifying reaction. Longer chain methylalkylnitroamines are poor liver carcinogens in rats since CYP2E1, the major nitrosamine metabolising P450 in this organ, preferentially hydroxylates the methyl group. This requirement for a P450 that metabolises longer chain dialkylnitrosamines in the oesophagus with this structural selectivity meant that there was a possibility that the required enzyme belonged to the 2B subfamily.

The initial plan was to use Northern blotting as a first step to identify the P450 subfamilies expressed in the oesophagus, but preliminary experiments showed that this would not be possible, because none of the available probes tested produced any significant hybridisation to a Northern blot of oesophageal RNA. Northern blotting could only have been used as a first step towards the identification of the expressed P450 because the genes of each subfamily of cytochromes P450 are strongly conserved, so the detection by Northern blotting of an hybridising mRNA would not necessarily mean that the tissue in question expressed the particular P450 being used as a probe. The only conclusion that could be safely drawn is that a P450 belonging to the same subfamily is expressed in the tissue. Northern blotting was to be followed by a PCR-based approach, which seemed to be the quickest and most conclusive way to demonstrate the expression of a novel enzyme. In the first experiments, degenerate primers complementary to highly conserved regions of the cDNA sequences of P450s

belonging to the 2 family were designed in the hope of amplifying as many of these P450s as possible. These experiments were not successful. In a second set of experiments, degenerate primers containing inosine that had previously been designed by Shen *et al.* (1993) to reduce the complexity of the mixture of primers for use in the RT-PCR amplification of P450 cDNAs belonging to families 1, 2 and 3 were also tried, again without success. Finally, target primers were designed for each of the previously mentioned candidate subfamilies and used in RT-PCR. This led to the amplification of oesophageal cDNA fragments of the expected sizes from the CYP2B and CYP2E primers. The products obtained from amplification using the CYP2B primers were cloned and sequenced. When the sequence data showed that the cloned fragment was from a previously unknown gene, the experiments were continued to get a full-length cDNA clone. This also provided a probe for Northern blotting and for screening a rat oesophageal cDNA library.

3.2 Materials

3.2.1 Sources of Reagents and Kits

Male Sprague Dawley rats (200 – 250 g) were purchased from the Biological Services Department, University College London. Sarkosyl (N-lauroylsarcosine, sodium salt), Antifoam A emulsion, IPTG and X-Gal were purchased from Sigma, Poole, U.K. LB broth, NZ broth and agar in powder form were also purchased from Sigma. Phenol used in these experiments was saturated with 0.1 M citrate buffer, pH 4.3, and was purchased from Sigma. RNasin (ribonuclease inhibitor) and the pGEM-T Easy vector kit were from Promega, U.K. The PCR-Script Amp Cloning kit was purchased form Stratagene, as were the Zap-cDNA Synthesis Kit and the Gigapack III Gold Cloning Kit used in the library synthesis. 'Marathon' adapter primers (Clontech, CA, USA) for synthesising cDNA for RACE-PCR were kindly supplied by Dr. Alan Mackay, Department of Surgery, University College London. Restriction enzymes and modifying enzymes were purchased from GibcoBRL, and the DNA polymerases used for PCR, *Taq* polymerase (Ampli*Taq*), hot start *Taq* polymerase (Ampli*Taq* Gold) and a long distance polymerase (*rTth* polymerase XL) were from Applied Bioscience, Perkin Elmer, UK. The full-length cDNA for CYP2A6 of the marmoset, used in some of the experiments described, was a kind gift from the PhD students in Professor E. A. Shephard's laboratory. All other reagents were analytical grade chemicals routinely used in the laboratory.

3.3 Methods

3.3.1 Isolation of Total RNA

Total RNA was isolated from rat tissues by the guanidinium isothiocyanate-phenol-chloroform procedure (Chomczynski and Sacchi, 1987).

3.3.1.1 Isolation of Total RNA from Rat Oesophagus

To eliminate ribonuclease contamination through dust and skin contact, all glassware used was baked, plastics were autoclaved, solutions were prepared with water which had been previously treated with 0.1% diethylpyrocarbonate (DEPC) and autoclaved, and gloves were worn throughout the preparation. Immediately before use, the working solution of guanidinium isothiocyanate (GTC) was made by adding 360 µl of β-mercaptoethanol and 500 µl of Antifoam A emulsion to 50 ml of stock GTC

solution (4 M guanidinium isothiocyanate / 25 mM sodium citrate, pH 7.0 / 0.5% Sarkosyl).

Male Sprague Dawley rats (200 – 250 g) were killed by anaesthetising with an increasing concentration of carbon dioxide. The oesophagus was removed and stripped of the surrounding muscle layer, as described previously. The mucosa was immediately placed into liquid nitrogen and powdered under liquid nitrogen by pestle and mortar. Up to 500 mg of powdered tissue was added to 5 ml of an ice cold working solution of guanidinium isothiocyanate in a 15-ml Sarstedt tube. The tissue mixture was homogenised on ice with two 30 second bursts of a Polytron homogeniser at full speed. Five hundred microlitres of 2 M sodium acetate (pH 4) was added, followed by 5 ml of buffer-saturated phenol (pH 4) and 1 ml of chloroform. The contents of the tube were vortex mixed, chilled on ice for 20 minutes and then centrifuged at 3,000g (4°C, 20 minutes). The top layer was carefully separated into a fresh Sarstedt tube with a sterile Pasteur pipette. 5 ml of isopropanol was added and mixed and the RNA allowed to precipitate for at least 30 minutes at 4°C. The sample was centrifuged in a swinging bucket rotor at 3000g for 20 minutes to pellet the precipitated RNA. The supernatant was poured off and the tube inverted to drain off as much supernatant as possible. The RNA pellet was dissolved in 400 µl of DEPC-treated water by vortex mixing to obtain a final concentration between 1 mg/ml and 10 mg/ml, and transferred to a freshly autoclaved microcentrifuge tube.

3.3.1.2 Preparation of Total RNA from Liver, Kidney, Lung and Nasal Epithelium

Preparation of total RNA from rat liver, kidney and lung was done just as for rat oesophagus, with 0.5 g of tissue in each case. The nasal epithelium of one rat was used and was removed as described previously. These tissues did not require powdering prior to homogenisation. The RNA pellets were dissolved in DEPC-treated water, liver in 600 µl and kidney, lung and nasal epithelium in 400 µl.

3.3.2 Determination of RNA Concentration

The RNA concentration was calculated from the relation 1 OD unit = 40 µg RNA per millilitre. The 260 nm/280 nm ratio was calculated and used to determine the purity of the RNA sample, a pure sample having a 260 nm/280 nm ratio between 1.8 and 2.

3.3.3   Electrophoresis of RNA

3.3.3.1   Preparation of Denaturing Agarose Gel (1.2% Agarose, 2.2 M Formaldehyde)

Agarose (4.8 g) was measured into a 500 ml conical flask with 20 ml of 20× PB and 308 ml of distilled water and the agarose melted in a microwave oven at medium power. The agarose solution was allowed to cool to about 50°C (hand hot) and formaldehyde (72 ml, 37% w/v) was added, in a fume cupboard. The mixture was immediately poured into a 25-cm horizontal gel casting tray (Maxi Gel System, Hybaid, UK) in the fume cupboard until the depth of the liquid was 10 mm, the required combs were inserted and the gel was left to set. Only after the gel had set (after about 30 minutes) was the casting tray removed from the fume cupboard.

3.3.3.2   Agarose Gel Electrophoresis of Total RNA

Three volumes of denaturing mix [formaldehyde (24% w/v), formamide (8.8% w/v) in 13.3 mM phosphate buffer, pH 7.4 / 0.67 mM EDTA] was added to one volume of RNA (4 mg/ml). The RNA was denatured at 65°C for 30 minutes and then snapped cool on ice. 0.1 volumes of PB loading buffer [50% (v/v) glycerol, 0.4% (w/v) bromophenol blue in 10× PB / 1 mM EDTA] was added to the denatured RNA and 20 µl samples of the mixture electrophoresed on a 1.2% denaturing agarose gel overnight (~16 hours) at 20 V (0.8 V/cm) using 1× PB containing 0.1 µg/ml ethidium bromide as electrophoresis buffer. When the bromophenol blue had moved to within 3 cm of the edge of the gel, the gel was removed and photographed. The appearance of the ribosomal RNA bands and the absence of smearing were used as a measure of the integrity of the RNA. If the RNA was found to be undegraded the same gel was used for Northern blotting.

3.3.4   Northern Blotting of RNA

RNA was transferred from the gel to a nylon membrane (Hybond-N, Amersham) by capillary blotting overnight. The set-up was as shown in the diagram (Figure 3-1).

Weight (~ 1 kg)

Tissue paper

3MM paper

Hybond N

Gel (inverted)

3MM paper wick

Support
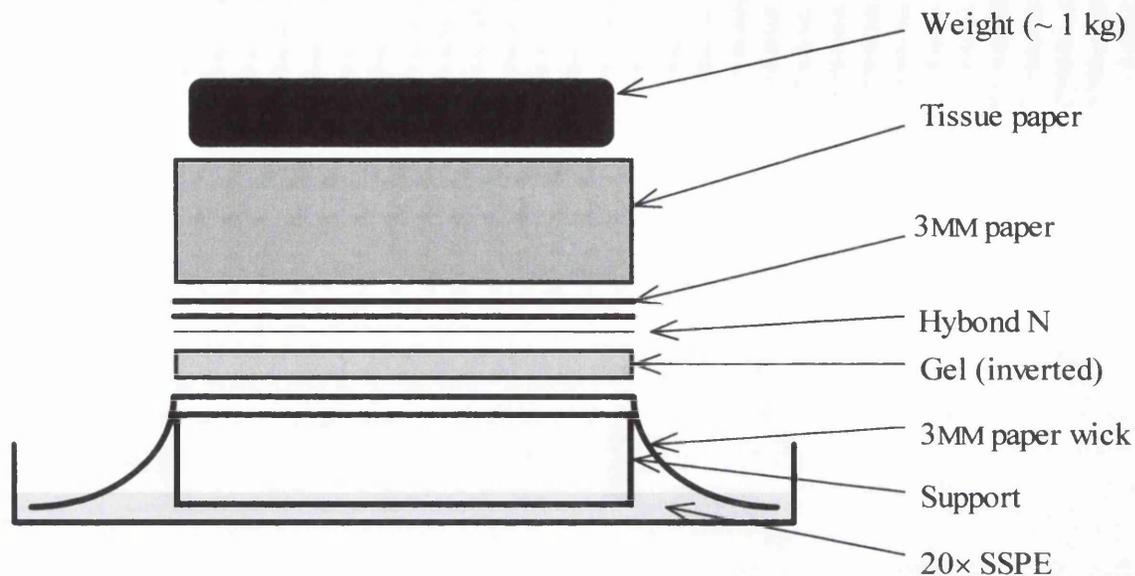
20× SSPE

Fig. 3-1   Capillary blotting set-up for Northern Blot

The set-up was dismantled and the membrane was rinsed in 20× SSPE (0.2 M phosphate buffer, pH 7.4 containing 2.98 M NaCl and 0.02 M EDTA), placed between 4 sheets of Whatman 3MM paper and baked in an oven at 80°C for 2 hours to bind RNA irreversibly. The gel was re-stained with ethidium bromide to verify complete RNA transfer. The blot was stored at 4°C until required for hybridisation.

3.3.5   Reverse Transcription of Total RNA

Total RNA was diluted to 2 mg/ml with diethyl pyrocarbonate (DEPC)-treated water. Total RNA (5 µg; 2.5 µl), oligo(dT)$_{12-18}$ (8 µl of 0.5 mg/ml) and RNasin (10 - 20 units, Promega) were measured into an autoclaved microcentrifuge tube and sterile distilled water added to a final volume of 12 µl. The mixture was heated at 70°C for 10 minutes to denature the RNA and the tube briefly centrifuged to collect the contents at the bottom and then placed on ice. Five times concentrated first strand buffer (4 µl, 250 mM Tris-HCl, pH 8.3 / 375 mM KCl / 15 mM MgCl$_2$), DTT (2 µl, 0.1 M) and dNTPs (1 µl, 10 mM) were added to the tube which was then heated at 42°C for 10 minutes. One microlitre (200 units) of Superscript II (Life Technologies) was added and mixed by pipetting up and down. The reaction was incubated at 42°C

for a further 50 minutes for first strand synthesis, heated at 70°C for 15 minutes to denature the enzyme and then snapped cool on ice.

3.3.6 Amplification by PCR of cDNA

3.3.6.1 Method I: Use of degenerate primers to amplify cDNA of any P450 of the 2 family

Degenerate primers were designed to amplify a 1200 base sequence in all cytochromes P450 of the 2 family. The antisense (reverse) primer was designed to incorporate the conserved cysteine in the haem binding site at about position 435 of the amino acid sequence of all cytochromes P450. The sense primers incorporated 2 conserved proline residues at about positions 34 and 35 of the amino acid sequences of P450s of the 2 family. The primers used were:

Sense   5'-MRNBWNCCNCCNGGNCC-3'

Antisense   5'-CNCCHRHRCANDNYCTYYT-3'

(where R = A or G; Y = C or T; M = A or C; W = A or T; H = A, T or C; B = G, T or C; D = G, A or T, N = A, C, G or T)

The three nucleotides complementary to the codon for the conserved cysteine in the haem-binding site are underlined. The double-stranded product formed in the reverse transcription reaction was used as the template for PCR. Each tube contained 1×reaction buffer, 0.2 mM dNTPs, 10 μM each of forward (sense) and reverse (antisense) primers, 2 μl of template and 0.5 μl (2.5 units) of Ampli*Taq* in a 50 μl reaction. A mineral oil overlay was added to prevent evaporation of the reaction during cycling.

The following sequence was used for thermal cycling:

- 94°C for 1 minute
-        30 cycles: 94°C for 30 seconds

               48°C for 1.5 minutes

               72°C for 2 minutes

-        72°C for 5 minutes

The tubes were transferred to a fridge (4°C) at the end of cycling.

Out of interest, a similar PCR reaction containing 1 µM each of sense and antisense primers specific for the sequence of CYP2A6 cDNA in the same region was carried out on the liver and oesophageal cDNA. CYP2A6 primers were used because a recombinant plasmid containing a marmoset CYP2A6 cDNA was available for use as a positive control. Hence a PCR reaction was also carried out on this plasmid. The sequences of these primers were:

Sense	5'-AAGCTGCCTCCGGGACC-3'
Antisense	5'-CTCCGAA<u>ACA</u>GTTCCGCTT-3'

These primers corresponded to bases 94 – 110 and 1306 – 1324 respectively, of the marmoset CYP2A6 sequence obtained from Professor Shephard's laboratory. The underlined nucleotides are complementary to the codon for the conserved cysteine in the haem-binding site. PCR reactions using primers designed to amplify a 500 bp region of human β-actin were also carried out as positive controls for the RT and PCR reactions. The reactions were kept at 4°C until ready for cloning.

3.3.6.2 Method II: Use of degenerate primers containing inosine

This method was based on a report by Shen *et al.* (1993) who used the primers below to amplify a fragment of a novel P450 cDNA from mouse embryo fibroblast cells. The downstream primer was designed by Shen *et al.* to anneal to the sequence around the conserved cysteine residue at about position 435 of the amino acid sequences of P450s. These primers were designed based upon known sequences of P450 cDNAs of families 1, 2 and 3, which are involved in xenobiotic metabolism, and were expected to amplify a 321 – 336 bp region. The primers used were:

Sense	5'-ARRITSCAIVARGARMTIGA-3', and
Antisense	5'-TCICCIAW<u>RCAI</u>HDICKYTTICC-3'

(R = A or G; Y = C or T; M = A or C; K = G or T; S = G or C; W = A or T; H = A, T or C; D = G, A or T; V = G, A or C; I = Inosine)

Specific primers were designed to amplify the corresponding region in CYP2A6, again to use for a positive control PCR reaction with the marmoset CYP2A6 cDNA. These primers were:

Sense	5'-AAGGTCCATGAGGAGATTGA-3', and
Antisense	5'-TCTCCGAA<u>ACA</u>GTTCCGCTTTCC-3'

Again, the underlined nucleotides encode for the conserved cysteine in the haem-binding site. The primers were used to amplify cDNA in oesophagus and liver, and the marmoset CYP2A6 DNA was used as a positive control. The primers corresponded to bases 976 – 995 and 1303 – 1325 respectively, of the marmoset CYP2A6 sequence. The constituents were used at the same concentrations as before.

Thermal cycling was carried out using the program:

- 94°C for 1 minute
- 30 cycles: 94°C for 30 seconds

  50°C for 1½ minutes

  72°C for 2 minutes
- 72°C for 5 minutes

The PCR products were kept at 4°C for cloning.

3.3.6.3   Method III: PCR, Cloning and Sequencing of Specific P450s in Liver and Oesophagus

Due to problems encountered when trying to use degenerate primers to amplify any P450 in the rat oesophagus, it was decided that less degenerate primers would be designed to amplify P450s belonging to specific subfamilies. The chosen ones were CYP2A, CYP2B and CYP2E, since these have been reported most frequently in the literature with respect to nitrosamine metabolism. The primers used were:

CYP2A	Antisense primer	5′-CCVAARCAGWTYCGCTTTCC-3′
	Sense primer	5′-GGCTTYYTGYTRCTCATGAA-3′
CYP2B	Antisense primer	5′-CCAAGACAAATVCGCTTYCC-3′
	Sense primer	5′-GGHTTCCTGCTCATGCTCAA-3′
CYP2E	Antisense primer	5′-CCARCACACACDCGYTTTCC-3′
	Sense primer	5′-GGGCTCCTGATYCTCATGAA-3′

(where R = A or G; Y = C or T; W = A or T; H = A, T or C; D = G, A or T; V = G, A or C). The sense primer occurs around nucleotide positions 945 to 965 of the coding sequences of the chosen P450s, and the antisense primer around positions 1310 to 1330.

The following thermal cycling program was carried out:

- 94°C for 1 minute
- 30 cycles: 94°C for 30 seconds

    60°C for 1½ minutes

    72°C for 2 minutes
- 72°C for 5 minutes

3.3.7   Cloning of PCR Products

3.3.7.1   Cloning into the pPCR-Script Amp SK(+) Cloning Vector

This was carried out with the PCR-Script Amp Cloning Kit (Stratagene) according to the manufacturer's instructions. Briefly, the PCR products were purified with the Strataprep PCR Purification Kit included in the cloning kit. Fifty microlitres of DNA-binding solution was added to each PCR reaction and the components mixed. The mixture was then transferred from beneath the mineral oil overlay with a pipette and transferred to a microspin cup that had been placed in a 2-ml receptacle tube. The cap of the receptacle tube was snapped onto the top of the microspin cup and the tube was centrifuged in a microcentrifuge at maximum speed for 30 seconds. The DNA binding solution in the receptacle tube was discarded and the microspin cup returned to the tube. Seven hundred and fifty microlitres of 1× PCR wash buffer (provided in the kit) was placed in the microspin cup and the tube was centrifuged again for 30 seconds. The wash buffer was discarded from the receptacle tube and the microspin cup replaced. The tube was centrifuged again for 30 seconds to ensure that all of the wash buffer, which contains ethanol, was removed from the microspin cup. The microspin cup was transferred to a fresh 1.5-ml microcentrifuge tube. Fifty microlitres of elution buffer (10 mM Tris-HCl, pH 8.5) was added onto the top of the fibre matrix at the bottom of the microspin cup and the tube incubated at room temperature for 5 minutes. The cap of the microcentrifuge tube was snapped onto the microspin cup and the tube was centrifuged in a microcentrifuge at maximum speed for 30 seconds to elute the DNA. The eluted DNA, which was in the microcentrifuge tube, was polished to generate a blunt-ended fragment to ligate into the pPCR-Script cloning vector. This was required because the PCR products had been generated with *Taq* DNA polymerase, which results in products with 3′ terminal adenosine residues at either end. The blunting (polishing) reaction comprised the purified PCR product

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(10 μl), dNTP mix [1 μl, 10 mM (2.5 mM of each dNTP)], 10× polishing buffer (1.3 μl from kit) and cloned *Pfu* DNA polymerase (0.5 units). The polishing reaction was mixed gently, a 20 μl mineral oil overlay added and the reaction incubated for 30 minutes at 72°C in a water bath. Four microlitres of the blunted reaction was used in a 10 μl ligation reaction which consisted also of 1 μl of 10 ng/μl pPCR-Script Amp SK(+) cloning vector, 1 μl of PCR-Script 10× reaction buffer, 0.5 μl of 10 mM rATP, 5 units of *Srf*I restriction enzyme and 4 units of T4 DNA ligase. The ligation reaction was mixed gently, incubated for 1 hour at room temperature and then heated for 10 minutes at 65°C to inactivate the ligase. The reaction was stored on ice until ready to use for transformation into competent cells.

3.3.7.2 Cloning into the pGEM-T Easy Vector

The pGEM-T Easy vector (Promega) has a 3′ terminal thymidine at either end and hence is convenient for cloning PCR products generated with *Taq* DNA polymerase or *Tth* DNA polymerase, which have 3′ terminal adenosine overhangs. The PCR products were ligated into the vector according to the manufacturer's instructions. Briefly, 3 μl of the PCR product was added directly to a 10 μl ligation reaction containing 5 μl of 2× Rapid Ligation Buffer (provided), 1 μl (50 ng) of the pGEM-T Easy vector and 1 μl (3 units) of T4 DNA ligase. The reaction was mixed by gently pipetting and incubated overnight at 4°C.

3.3.8 Transformation of Plasmids into Competent Bacteria

The cloned PCR products were transformed into XL1-Blue MRF′ Kan supercompetent cells (Stratagene). The cells were removed from a -80°C freezer, thawed on ice and gently mixed by hand. 40 μl samples of the cells were measured into chilled 15-ml Falcon 2059 polypropylene tubes and β-mercaptoethanol (0.7 μl, 1.44 M) added. The contents of each tube were swirled gently and incubated on ice for 10 minutes, swirling gently every 2 minutes. Two microlitres of the ligated PCR reaction was added to the cells and the reaction swirled gently. The reaction was incubated on ice for 30 minutes, heat pulsed in a water bath at exactly 42°C for 45 seconds and then incubated on ice for 2 minutes. LB broth (450 μl), preheated to 42°C was added and the tubes incubated at 37°C for 1 hour with shaking at 250 rpm. Using a sterile spreader, 100 μl to 200 μl of the transformation reaction was plated on

LB-ampicillin agar (50 µg ampicillin/ml) plates containing 50 µg X-gal/ml and 1 mM IPTG. The plates were incubated overnight (about 16 hours) at 37°C to allow for blue-white screening. The plates were transferred to 4°C until ready for streaking onto fresh plates to check the fidelity of blue-white selection. To do this, white colonies were picked from the original agar plates with autoclaved toothpicks and streaked onto fresh LB-ampicillin agar plates containing X-gal and IPTG. A fresh toothpick was used for each colony and different plates for each transformation reaction. About 2 or 3 blue colonies were also streaked to use as negative controls. The plates were incubated overnight at 37°C and then transferred to 4°C.

3.3.9   Colony lysis of Transformants

This was carried out to check for the presence of inserts in white colonies on the streaked plates and also to determine the sizes of the inserts, if present. About 2 mm of each of the white streaks was picked with an autoclaved toothpick and added to 50 µl of PBS/0.1 % (v/v) TWEEN in 1.5 ml screw cap microcentrifuge tubes. This was repeated for the blue colony controls. The tubes were placed in a boiling water bath for 10 minutes for cell lysis. The tubes were centrifuged in a microcentrifuge at maximum speed to separate the cells from the DNA. 2 µl of the supernatant containing the DNA was used in a 20 µl PCR reaction containing 1 µM each of T3 and T7 primers, 0.2 mM dNTP mix and 1 unit of AmpliTaq DNA polymerase. The following cycling parameters were used for DNA amplification:

- 94°C for 1 minute
- 30 cycles: 94°C for 30 seconds
  50°C for 1 minute
  72°C for 2 minutes
- 72°C for 5 minutes

Five microlitres of each PCR product was electrophoresed on a 1.2% agarose gel to check the sizes of the fragments obtained. Amplified fragments that were the same size as the blue colony negative controls (about100 bp) and represented plasmid without insert were not used in subsequent steps.

3.3.10 Small Scale Purification of Plasmid DNA

The plasmids containing the PCR fragments were purified from the host bacterial cells using QIAGEN miniprep spin columns according to the manufacturer's instructions. Briefly, bacteria containing the plasmid were grown overnight in 5 ml of LB medium containing 50 µg/ml ampicillin. The bacterial cells were pelleted by centrifuging at 3000 g for 20 minutes. The resulting pellet was resuspended in Buffer P1 (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 100ug/ml RNase A) making sure no cell clumps remained and transferred to a 1.5-ml microcentrifuge tube. The cells were lysed in Buffer P2 (200 mM NaOH, 1% SDS) to release DNA. The lysis reaction was mixed gently by inverting several times to avoid shearing of genomic DNA and left to proceed for about 3 minutes, ensuring that it did not go on for more than 5 minutes. The reaction was neutralised with Buffer N3 (3.0 M potassium acetate, pH 5.5), and the tube was inverted immediately several times to mix. The cloudy solution obtained was centrifuged in a microcentrifuge at maximum speed for 10 minutes. The supernatant was applied to a QIAprep spin column and centrifuged at maximum speed in a microcentrifuge for 30 - 45 seconds to bind the DNA. The column was then washed with 750 µl of buffer QE and the DNA was eluted with 10 mM Tris-HCl, pH 8.5. All the buffers mentioned were supplied with the QIAprep Spin kit.

3.3.11 Sequencing of PCR Products

The purified plasmids containing the PCR products were sequenced with the ABI PRISM dRhodamine Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Perkin Elmer, UK) according to the manufacturer's instructions. Briefly, Terminator Ready Reaction Mix (8 µl, from the kit), double stranded DNA (5 µl) and T3 or T7 primer (3.2 pmol) were mixed in a 20 µl reaction in order to sequence PCR products cloned into the pPCR-Script Amp SK(+) cloning vector. For PCR products cloned into the pGem-T Easy vector, T7 and SP6 primers were used. Cycle sequencing was performed using 25 cycles of 96°C for 30 seconds, 50°C for 15 seconds and 60°C for 4 minutes. The reactions were then held at 4°C until they were purified. To remove excess dye terminators, a 1.5-ml microcentrifuge tube was prepared containing 2.0 µl of 3 M sodium acetate, pH 5.2 and 50 µl of 95% ethanol for each sequencing reaction. The entire contents of the extension reaction was

pipetted into the tube and mixed thoroughly. The tube was vortex mixed and placed on ice for 10 minutes to precipitate the extension products. The tube was then centrifuged at maximum speed in a microcentrifuge for 30 minutes to collect the precipitate and then the supernatant was carefully aspirated and discarded. The pellet was rinsed with 70% ethanol and centrifuged for 10 minutes in a microcentrifuge at maximum speed. The supernatant was again aspirated and discarded. The pellet was dried in a vacuum centrifuge for 10 minutes and sent for sequencing on the ABI PRISM 377 DNA Sequencer (Applied Biosystems, Perkin Elmer, UK).

3.3.12 Blotting of RT-PCR Products

The gel was denatured in about 5 volumes of denaturing buffer (1.5 M NaCl/0.5 M NaOH) for 30 minutes and rinsed briefly in distilled water. It then washed twice in 5 volumes of neutralisation buffer (1.5 M NaCl/0.5 M Tris-HCl, pH 8.0), first for 30 minutes and then for 15 minutes. The gel was rinsed again in distilled water and the DNA blotted onto a nylon membrane as described for Northern blotting. The blot was placed between 2 sheets of 3MM chromatography paper and baked in an oven for 2 hours at 80°C. It was then stored, wrapped in Saranwrap, at room temperature until required for hybridisation.

3.3.13 Hybridisation of Blots

3.3.13.1 Preparation of Probes

Probes used were cloned DNA fragments amplified by RT-PCR from RNA prepared from the appropriate rat tissues – CYP2A3 from lung, CYP2B from oesophagus and CYP2E1 from liver. The CYP2A3 DNA fragment was amplified from lung using the primers shown below:

Sense	5'-CGGTCCTGTTTCACCATCCACCTGG-3'	
Antisense	5'-GTCAATGAGGGCACCGTTCGTCTTTC-3'	

The CYP2E1 DNA fragment used was one initially amplified from liver by the CYP2E-specific primer pair while the oesophageal CYP2B fragment was amplified using the CYP2B-specific primers. In addition a probe designated "CYP2Boes" was used. This was a full-length cDNA obtained by library screening (Section 3.3.15).

The RT-PCR fragments had been ligated into vectors (pPCR Script or pGem-T Easy), transformed into competent or supercompetent cells, grown up overnight and plasmids purified using Qiagen's maxi prep purification kit as described in previous sections. The plasmids were then digested with the appropriate enzymes (EcoR1 for pGem-T Easy; Xba1 and Not1 for pPCR Script) as described here. The restriction digestion reaction consisted of 30 µl of plasmid in a 100 µl reaction containing 1× React 3 buffer (50 mM Tris HCl, pH 8.0/10 mM MgCl/100 mM NaCl) supplied with the enzymes and 16 to 30 units of the required enzymes.

The digested probe was run on a 1.2% agarose gel to separate the plasmid from the insert. The insert was extracted from the gel using the QIAex II Gel Extraction Kit (Qiagen) according to the manufacturer's instructions. The procedure is described briefly. The band of interest was cut out of the gel with a scalpel blade and put into a clean microcentrifuge tube. Three hundred microlitres of solubilisation and binding buffer QX1 per 100 mg of the gel slice and 10 to 30 µl QIAex II suspension (silica gel particles), depending on the amount of DNA, was added to the gel slice in the tube. The sample was incubated at 50°C for about 10 minutes to melt the gel slices, shaking every 2 minutes to keep the matrix in suspension. The tube was centrifuged in a microcentrifuge at maximum speed for 30 seconds to collect the QIAex matrix with the bound DNA at the bottom. The supernatant was removed and discarded. Five hundred microlitres of QX1 buffer was added and the contents of the tube were vortex mixed to re-suspend and wash the matrix. The matrix was collected at the bottom of the tube by centrifuging again, and the supernatant was discarded. The matrix was washed twice with 500 µl of high salt buffer PE containing 80% ethanol. The final supernatant was removed completely and the matrix was left to air dry for 10 to 30 minutes, depending on the original volume used, until it turned white. The DNA was eluted by adding 20 µl of water, mixing by vortex and leaving to stand for 5 minutes at room temperature. The tube was centrifuged at maximum speed in a microcentrifuge to pellet the matrix and the supernatant containing the DNA was transferred to a fresh microcentrifuge tube with a pipette.

3.3.13.2 Labelling of Probes

This was done with the Megaprime™ DNA labelling system (Amersham, UK) with slight modifications to the manufacturer's instructions. Five microlitres (25 ng) of

each of the prepared probes was measured into 1.5-ml screw cap microcentrifuge tubes. Five microlitres of the random nanomer primer solution provided was added to each and the tubes placed in a boiling water bath for 5 minutes to denature the probes. The contents of the tubes were collected by briefly centrifuging. Ten microlitres of labelling buffer (dATP, dGTP and dTTP in Tris/HCl, pH 7.5, β-mercaptoethanol and $MgCl_2$), 2 μl of enzyme solution (1 unit/μl DNA polymerase I Klenow fragment) and water were added to make the volume up to 50 μl. [^{32}P]-dCTP (5 μl, 3,000 μCi/μl, Amersham) was added to each tube in a fume cupboard behind a Perspex screen and the contents of the tubes were mixed by pipetting up and down. The tubes were incubated at 37°C for 10 to 30 minutes. The reactions were stopped by adding 50 μl of 40 mM EDTA to each tube.

3.3.13.3 Removal of unincorporated nucleotides

Pharmacia's Nick Spin columns were used for this. Each column was prepared as described in the manufacturer's instructions by equilibrating with TE buffer. A labelled 1.5-ml screw cap microcentrifuge tube was placed in the collecting tube provided. The column was then placed in the collecting tube, ensuring that its tip was in the microcentrifuge tube, and the radioactively labelled probe was loaded onto the centre of the matrix behind a Perspex screen in a fume cupboard. The set-up was centrifuged at 500g for 4 minutes to elute the probe. The column was removed and discarded in a radioactive bin, and the sample tube was retrieved from the collecting tube with a pair of forceps and covered.

3.3.13.4 Hybridisation procedure

The Northern and Southern blot filters were removed from the fridge and pre-wetted by floating them on 2× SSPE (20 mM phosphate buffer, pH 7.4 containing 0.3 M NaCl and 2 mM EDTA) and then submerging them, making sure not to introduce air bubbles. Northern blots were pre-hybridised by shaking at 42°C for at least 1 hour in 50 ml hybridisation buffer (5× SSPE [50 mM phosphate buffer, pH 7.4 containing 0.75 M NaCl and 5 mM EDTA] containing 50% formamide, 5× Denhart's solution, 1% SDS and 0.5 mg/ml sonicated salmon sperm DNA) to saturate non-specific binding sites on the filters (both bare nylon and non-specific RNA). Southern blots were prehybridised in the same buffer, but at 65°C. The hybridisation solution was then changed and replaced with a smaller volume (~25 ml) containing the denatured probe. The Northern blots were left to hybridise overnight with shaking at 42°C, and

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the Southern blots at 65°C. After overnight hybridisation the radioactive hybridisation solution was poured down a sink suitable for radioactive waste, washing down with plenty of water. The filters were transferred into a 1 litre container and washed with buffers of successively lower salt concentrations. They were first washed twice for thirty minutes each time in 2× SSPE containing 0.1% SDS with shaking at 42°C for Northern blots and 65°C for Southern blots. They were then washed twice as before in 0.1× SSPE containing 0.1% SDS, again at 42°C for Northern blots and 65°C for Southern blots. The filters were then rinsed briefly in 2× SSPE, wrapped in SaranWrap, placed in a film cassette and exposed to radiographic film (Hyperfilm) for at least 16 hours at -70°C.

3.3.14 cDNA Amplification and RACE-PCR of Oesophageal "CYP2B"

3.3.14.1 Preparation of Polyadenylated RNA (mRNA)

Polyadenylated RNA (poly A^+ RNA or mRNA) was isolated from total RNA using Dynal Dynabeads according to a scaled up version of the manufacturer's instructions. Total RNA was diluted to a concentration of 1 mg/ml. Five hundred microlitres of 2× binding buffer (20 mM Tris-HCl, pH 7.5/1.0 M LiCl/2 mM EDTA) was added to 500 μl of the diluted RNA. One millilitre of Dynal Dynabeads was measured into a 1.5 ml microcentrifuge tube and prepared by binding to a magnet (Dynal MPC) for 30 to 45 seconds, removing the supernatant and washing once with 2× binding buffer. The Dynabeads were then resuspended in 500 μl binding buffer. The RNA mixture was heated at 65°C for 2 minutes and added to the prepared Dynabeads. The mRNA present in the mixture was bound to the beads by rotating the tube for 5 minutes at room temperature. The beads were bound to the magnet and the supernatant was removed and discarded. The beads were thoroughly washed twice with 1 ml washing buffer (10 mM Tris-HCl, pH 8.0/0.15 M LiCl/1 mM EDTA), binding to the magnet between washes before removing the supernatant. The final supernatant was removed, 50 μl DEPC-treated water was added and the tube was kept at 65°C for 2 minutes to elute the mRNA. The beads were bound to the magnet again and the supernatant containing the mRNA was removed and transferred to an autoclaved 0.5 ml microcentrifuge tube. The isolated mRNA was quantitated by taking

absorbance readings of a 1:500 dilution at 260 nm and 280 nm as was done for total RNA.

3.3.14.2 cDNA Amplification

Oesophageal cDNA amplification was carried out according to the Marathon cDNA Amplification protocol (Clontech, USA), with a few modifications. An overview of the method is shown in Figure 3-2. Poly A^+ RNA (1 μg, 4 μl) and oligo(dT)$_{12-18}$ (1 μl; 10 μM stock) were measured into a sterile autoclaved 0.5-ml microcentrifuge tube. The contents of the tube were mixed by pipetting up and down and then collected at the bottom of the tube by spinning briefly in a microcentrifuge. The tube was incubated at 70°C for 2 minutes, cooled on ice for 2 minutes and centrifuged in a microcentrifuge to collect the contents at the bottom. 5× first strand buffer (2 μl), dNTP mix (1 μl; 10 mM stock), DTT (1μl; 100 mM stock) and reverse transcriptase (1 μl; Superscript II, Life Technologies) were added to the contents of the tube and mixed by gentle pipetting. The tube was centrifuged briefly to collect the contents at the bottom and then incubated at 42°C in a water bath for 1 hour. The reaction was terminated by heating the tube at 65°C for 15 minutes. The reaction mixture was collected at the bottom of the tube by centrifuging briefly and then placed on ice.

Second strand enzyme cocktail (20× concentrated) was made by mixing of *E. coli* DNA polymerase I (8 μl, 40-80 units), *E. coli* DNA ligase (1.2 μl, 1.2 units) and *E. coli* ribonuclease H (0.8 μl, 0.8-3.2 units). All reagents used in this step were pre-chilled on ice. The whole first strand reaction (10 μl) was used for second strand synthesis. Sterile water (56.4 μl), 10× *E. coli* DNA ligase reaction buffer (8 μl), dNTP mix (1.6 μl; 10 mM) and 20× second strand enzyme cocktail (4 μl) were added to the first strand synthesis tube. The contents of the tube were mixed by gentle pipetting and the tube was centrifuged to collect the contents at the bottom. The tube was then incubated in a cooled water bath maintained at 16°C for 1.5 hours. The water bath was made by filling a polystyrene container to about an inch from the rim with water and adjusting the temperature of the water to 16°C with ice. The bath was then kept covered with its polystyrene lid to maintain this temperature. T4 DNA polymerase (2 μl, 10 units) was added to the tube and mixed with gentle pipetting. The tube was then incubated at 16°C for a further 45 minutes. 4 μl of 20×

EDTA/glycogen mix (0.2 M EDTA containing 2 mg/ml glycogen) was added to terminate second strand synthesis. One hundred microlitres of phenol:chloroform:isoamyl alcohol (25:24:1) was added and the reaction was thoroughly vortex mixed. The tube was centrifuged in a microcentrifuge at maximum speed to separate the phases. The top aqueous layer was carefully transferred into a clean 0.5-ml microcentrifuge tube. The interface and lower phase were discarded. 100 µl of chloroform was added to the top layer and the contents of the tube were again vortex mixed. The tube was centrifuged at maximum speed to separate the phases. The top aqueous layer was again removed into a 0.5-ml microcentrifuge tube. One-half volume (about 35 µl) of 4 M ammonium acetate was added to the contents of the tube. Two-and-a-half volumes (263 µl) of room temperature 95% ethanol was also added and the reaction was vortex mixed. The tube was centrifuged at maximum speed at room temperature for 20 minutes to collect the precipitate at the bottom. The supernatant was carefully removed by mild suction. The pellet was gently overlaid with 300 µl 80% ethanol and centrifuged at maximum speed for 10 minutes. The supernatant was aspirated by mild suction. The pellet was air dried for about 15 minutes to evaporate residual ethanol and dissolved in 10 µl of sterile water.

All this double-stranded cDNA (10 µl), cDNA adapter (4 µl; 10 µM) with the sequence shown below and sterile water (6 µl) were added to a tube of Ready-to-Go T4 DNA ligase (Pharmacia) and vortex mixed. The reaction was incubated overnight in a cooled water bath at 16°C and then heated at 70°C to inactivate the ligase. Samples of the adapter-ligated cDNA (1 µl each) were diluted with either 50 µl or 250 µl of Tricine-EDTA buffer (10 mM tricine-KOH, pH 8.5/0.1 mM EDTA) for RACE-PCR. The undiluted tube of adapter-ligated cDNA was stored at -20°C for future use.

The two tubes of diluted double-stranded cDNA were heated at 94°C for 2 minutes to denature the cDNA and then cooled on ice for 2 minutes. The tubes were briefly centrifuged to collect the contents at the bottom. These were then stored at -20°C until needed for RACE PCR.

The sequence of the adapter is shown:

"Marathon" Adapter

5'-CTAATACGACTCACTATAGGGCTCGAGCGGCCGCCCGGGCAGGT-3'

3'-H₂N-CCCGTCCA-PO₄-5'

3.3.14.3 RACE-PCR of Oesophageal "CYP2B"

RACE-PCR was carried out to amplify the 5' and 3' ends of the adapter-ligated cDNA. The diluted adapter-ligated cDNA from the previous section was used for these reactions. Each reaction contained adapter-ligated cDNA (5 μl), "Marathon"adapter primer 1 (AP1, 0.2 μM), gene specific primer (GSP, 0.2 μM), magnesium acetate (1 mM), a long-distance polymerase (2 units, r*Tth* polymerase XL) and HPLC-grade water to 50 μl. In order to amplify the 5' end (5' RACE-PCR), the antisense GSP (GSP1) was used and the sense GSP (GSP2) used to amplify the 3' end (3' RACE-PCR). The sequences of the primers used are shown below.

Sequences of RACE-PCR primers:

Adapter primer 1 (AP1):   5'-CCATCCTAATACGACTCACTATAGGGC-3'

Antisense GSP (GSP1):   5'-TCATTCTTCTTCAGTGCCCCATTGGC-3'

Sense GSP (GSP2):   5'-CTCAAGTACCCCCATGTTGCAGAG-3'

Touch down PCR was carried out using the following cycle sequence:

- 94°C for 1 minute
- 5 cycles: 94°C for 30 sec
  72°C for 4 min
- 5 cycles: 94°C for 30 sec
  70°C for 4 min
- 25 cycles   94°C for 20 sec
  68°C for 4 min
- 72°C for 10 minutes

3.3.14.4 "Nested" RACE-PCR of Oesophageal "CYP2B"

Multiple bands were obtained in the original RACE-PCR reaction and in the case of the 5' end the band that occurred at the expected position (1,200 bp) was quite faint, and several attempts to clone it into a vector failed. To overcome this problem

"nested" PCR was performed. This is a PCR amplification carried out using primers designed to amplify a region within the band of interest and is a method of enriching the required product. Each reaction contained 5 μl of the original RACE-PCR product, 0.2 μM "nested" adapter primer 2, 0.2 μM "nested" gene specific primer, 1 mM magnesium acetate, r*Tth* polymerase (units) and HPLC grade water to 50 μl. The "nested" antisense GSP (NGSP1) was used to re-amplify the 5′ end and the "nested" sense GSP (NGSP2) to re-amplify the 3′ end. The primers used were:

"Nested" adapter primer (AP2):

5′-ACTCACTATAGGGCTCGAGCGGC-3′

"Nested" Antisense GSP (NGSP1):

5′-GTGTGGCAGACCAATCGGGACAAG-3′

"Nested" Sense GSP (NGSP2):

5′-CATTGTTCCGAGGGTACCTGCTCCC-3′

Thermal cycling was performed using the same cycling sequence used for the original RACE-PCR. The products from these PCR reactions were cloned into the pPCR-Script cloning vector, transformed into *E. coli* XL1 Blue MRF ′ cells and colony lysis carried out as previously described. Clones containing inserts were then grown up and the plasmids purified and sequenced as previously described.

5′ – ▬▬▬▬▬▬▬▬▬AAAA–3′
Poly A⁺ RNA

First Strand Synthesis
Oligo(dT)$_{12-18}$ + Superscript II

5′ – ▬▬▬▬▬▬▬▬▬AAAA–3′
3′ – ▬▬▬▬▬▬▬▬▬TTTT–5′
RNA/DNA hybrid

Second Strand Synthesis
Second strand enzyme cocktail
+ T4 DNA polymerase

5′ – ▬▬▬▬▬▬▬▬▬AAAA–3′
3′ – ▬▬▬▬▬▬▬▬▬TTTT–5′
Double-stranded cDNA

Adapter Ligation
Adapters (▬▬▬)
+ T4 DNA ligase

Sequence amplified
by GSPs

5′ – ▬▬▬▬▬▬▬▬▬AAAA ▬▬ –3′
3′ – ▬▬▬▬▬▬▬▬▬TTTT ▬▬ –5′
Adapter-ligated double-stranded cDNA

5′-RACE-PCR
Antisense GSP
+ adapter primer
+ r*Tth* polymerase

3′-RACE-PCR
Sense GSP
+ adaper primer
+ r*Tth* polymerase

Adapter primer

5′ – ▬▬▬▬▬▬AAAA ▬▬ –3′   5′ – ▬▬▬▬▬▬▬▬▬ –3′
Antisense GSP   Sense GSP
3′ – ▬▬▬▬▬▬TTTT ▬▬ –5′   3′ – ▬▬▬▬▬▬▬▬▬ –5′

Adapter primer   Adapter primer

First PCR cycle uses only antisense GSP,
producing a strand with a binding site for the
adapter primer

First PCR cycle uses only sense GSP,
producing a strand with a binding site for the
adapter primer

5′ – ▬▬▬▬▬▬▬▬▬ –3′   5′ – ▬▬▬▬▬▬▬▬▬ –3′
3′ – ▬▬▬▬▬▬▬▬▬ –5′   3′ – ▬▬▬▬▬▬▬▬▬ –5′
5′-RACE product   3′-RACE product

Figure 3-2   Overview of cDNA synthesis and RACE-PCR

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3.3.15 Preparation of cDNA Library

3.3.15.1 cDNA Synthesis

This was done with Stratagene's cDNA Synthesis Kit according to the manufacturer's instructions with slight omissions. The procedure is described below and simplified by means of a diagram in Figure 3-3. Poly(A)$^+$ RNA was prepared as previously described and 5µg reverse transcribed in a reaction containing first strand buffer, first strand methyl nucleotide mixture (30 pmol each dATP, dGTP, dTTP plus 15 pmol 5-methyl dCTP), oligo(dT)$_{18}$ linker-primer (2.8 µg, see sequence below), ribonuclease inhibitor (RNase block; 40 units) and Moloney murine leukemia virus reverse transcriptase (MMLV-RT; 75 units) in a 50 µl reaction. To do this all the reagents, except the MMLV-RT, were measured into a 1.5 ml microcentrifuge tube. The primer was allowed to anneal to the template for 10 minutes at room temperature and then the MMLV-RT was added. The sample was mixed gently and the contents of the tube were collected at the bottom of the tube by centrifuging. This first-strand synthesis reaction was incubated at 37°C for one hour. The reaction was removed from 37°C and placed on ice.

Oligo(dT)$_{18}$ linker-primer

$$5'\text{-GAGAGAGAGAGAGAGAGAGAACTAGTCTCGAG(T)}_{18}\text{-3'}$$

"GAGA" sequence   *Xho* I

The "GAGA" sequence is required to protect the *Xho* I enzyme recognition site, which allows the finished cDNA to be inserted into the Uni-ZAP XR vector in a sense orientation (*Eco*R I-*Xho* I) with respect to the *lacZ* promoter.

The 5-methyl dCTP in the first strand methyl nucleotide mix ensures that the complete first strand will have a methyl group on each cytosine base, protecting the cDNA from the restriction enzymes used in the subsequent cloning steps.

3.3.15.2 Second Strand cDNA Synthesis

Second strand cDNA was synthesized with the whole first strand reaction in a 200-µl reaction containing 1× second strand buffer, second strand dNTP mixture (6 pmol each of dATP, dGTP and dTTP and 15.6 pmol dCTP), RNase H (3 units) and DNA polymerase I (99 units). The contents of the tube were quickly vortex mixed and the contents of the tube collected at the bottom by centrifuging. The reaction was

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incubated for 2.5 hours in a 16°C water bath, made as previously described. After 2.5 hours the reaction was removed and placed in ice. The RNase H was added to nick the RNA bound to the first-strand cDNA to produce fragments that would serve as primers for DNA polymerase I, which would "nick-translate" the RNA fragments into second-strand cDNA.

3.3.15.3 Blunting of cDNA Termini

The cDNA termini were blunted in a 125 μl reaction containing the whole second strand reaction, blunting dNTP mix (57.5 pmol dATP, dGTP, dTTP and dCTP) and cloned *Pfu* DNA polymerase (5 units). The mixture was vortex mixed briefly and the tube centrifuged briefly to collect the contents at the bottom. The blunting reaction was then incubated at 72°C for exactly 30 minutes. The DNA was separated from the enzymes used in the reaction by adding 200 μl of phenol:chloroform (1:1), vortex mixing and spinning for 2 minutes in microcentrifuge. The upper aqueous layer containing the cDNA was carefully removed and transferred to a new microcentrifuge tube. 110 μl of the aqueous layer was retrieved. An equal volume of chloroform (110 μl) was added and the contents of the tube were vortex mixed. The mixture was centrifuged in a microcentrifuge for 2 minutes at room temperature and the upper aqueous layer transferred to a new microcentrifuge tube. Sodium acetate (20 μl; 3 M) and absolute ethanol (400 μl) were added to the DNA and the contents of the tube were vortex mixed. The DNA was allowed to precipitate overnight at 20°C. The reaction was centrifuged in a microcentrifuge at maximum speed for 60 minutes at 4°C to collect the precipitated DNA. After carefully removing and discarding the supernatant, the pellet was washed by adding 500 μl of 70% (v/v) ethanol to the side of the tube away from the pellet and spinning in a microcentrifuge at maximum speed for 2 minutes at room temperature. The ethanol was aspirated and the pellet dried *in vacuo* for about 10 minutes.

3.3.15.4 Ligation of *Eco*R I Adapters

The DNA pellet from the previous section was resuspended in *Eco*R I adapters (9 μl, 3.6 μg; see below) and incubated at 4°C for at least 30 minutes to allow the cDNA to resuspend completely. One microlitre of the resuspended cDNA was taken for size analysis by gel electrophoresis. The whole blunted cDNA reaction (8 μl) was ligated with *Eco*R I adapters in a 11 μl reaction containing ligase buffer, rATP (10 nmol) and

T4 DNA ligase (4 units). The reaction was centrifuged in a microcentrifuge to collect it at the bottom of the tube and incubated at 4°C over the weekend. The ligase was heat inactivated by incubating the reaction at 70°C for 30 minutes.

*Eco*R I adapters

5′-OH-AATTCGGCACGAG-3′

3′-GCCGTGCTCp-5′

The adapters are composed of 9- and 13-mer oligonucleotides, complementary to each other and with an *Eco*R I cohesive end. The phosphorylated 9-mer oligonucleotide is able to ligate to other blunt termini (cDNA and other adapters), while the dephosphorylated 13-mer oligonucleotide is prevented from ligating to other cohesive ends.

3.3.15.5 Phosphorylation of *Eco*R I Ends

The heat inactivated ligation reaction (11 μl) was centrifuged in a microcentrifuge for 2 seconds and cooled at room temperature for 5 minutes. The adapter ends of the 13-mer oligonucleotides were phosphorylated by adding rATP (20 nmol), T4 polynuclotide kinase (10 units) and ligase buffer and sterile water to 21 μl and incubating at 37°C for 30 minutes. The kinase was then heat inactivated at 70°C for 30 minutes. The reaction was collected at the bottom of the tube by spinning in a microcentrifuge for 2 seconds and then equilibrated at room temperature for 5 minutes. This reaction was carried out to enable the ligation of the 13-mer oligonucleotide into dephosphorylated vector arms.

3.3.15.6 *Xho* I Digestion

This reaction was required to enable directional cloning of the cDNA in the library by releasing the *Eco*R I adapter and residual linker primer from the 3′ end of the cDNA. *Xho* I buffer (28 μl) and *Xho* I (3 μl, 120 units) were added to the phosphorylated cDNA (21 μl). The reaction was incubated at 37°C for 1.5 hours. STE buffer (48 μl) was added, followed by 100 μl of phenol:chloroform (1:1). The reaction was vortex mixed and centrifuged in a microcentrifuge at maximum speed for 2 minutes at room temperature. The upper aqueous layer was transferred to a fresh microcentrifuge tube. Chloroform (100 μl) was added to the aqueous layer and the reaction was vortex mixed, centrifuged and the top aqueous layer transferred to a fresh microcentrifuge tube as before. Ten times concentrated STE buffer (10 μl) and absolute ethanol

(250 μl) were added to the aqueous layer. The DNA was precipitated overnight at -20°C, then collected by centrifuging in a microcentrifuge at maximum speed for 60 minutes at 4°C. The supernatant was discarded and the pellet air-dried completely and resuspended in STE buffer (14 μl).

3.3.15.7 Size Fractionation of cDNA

A sterile 1-ml pipette was used as the column. The cotton plug in the pipette was partly teased out of the pipette with a needle leaving about 4 mm inside. The external portion of the plug was cut off with a pair of scissors. The remaining 4 mm of the cotton plug was pushed back into the top of the pipette. The top end of the 1-ml pipette was connected to the tip of a 10-ml syringe with a short length of plastic tubing, making sure there was no gap between the pipette and the syringe. The plunger of the syringe was rapidly and forcefully pushed into the syringe to thrust the cotton plug into the tip of the pipette. This was done several times to get the plug all the way down to the tip. The plunger was removed from the syringe, which then served as a buffer reservoir for the column, which was clamped in a vertical position for use. The column was loaded to within 0.25 inches of the top with Sepharose CL-2B gel filtration medium supplied using a glass Pasteur pipette, making sure no bubbles were introduced. It was then washed with 10 ml of 1× STE buffer. Three-and-a-half microlitres of loading dye (glycerol:saturated bromophenol blue:10× STE buffer, 50:40:10) was added to the cDNA sample and the sample was loaded onto the column. Collection of the eluent was started when the leading edge of the dye reached the 0.4-ml mark, and continued until the trailing edge reached the 0.3-ml mark. About 1.6 ml was collected in 4 fractions. To check the size range of the cDNA in the fractions, 1 μl of TAE gel loading buffer was added to 10 μl of each fraction and run on a 1% agarose gel. Fractions 1 to 3, which contained cDNA whose size was above 400 bases were used in the subsequent steps.

Each fraction was extracted with phenol-chloroform and chloroform as described in previous sections, the aqueous layers were pooled and the cDNA precipitated with 100% ethanol (1.2 ml) overnight at 20°C. The precipitated cDNA was collected by centrifuging in a microcentrifuge at maximum speed for 60 minutes at 4°C. The pellet was washed with 80% ethanol (200 μl), ensuring that it was not disturbed. The sample was centrifuged in a microcentrifuge at maximum speed for 2 minutes at room

temperature, and the ethanol removed with a pipette. The pellet was vacuum evaporated until dry and then resuspended in 3.5 μl of HPLC-grade water.

3.3.15.8 cDNA Ligation into Uni-ZAP Vector

Resuspended cDNA (1 μl, ~100 ng) was ligated into the Uni-ZAP vector (Lambda ZAP II vector digested with *Eco*R I-*Xho* I). The ligation reaction also contained rATP (5 nmol), Uni-ZAP XR vector (1 μg), 10× ligase buffer (0.5 μl; 500 mM Tris-HCl, pH 7.5 containing 70 mM MgCl$_2$ and 10 mM DTT) and sterile water to a final volume of 4.5 μl. After mixing all these reagents, T4 DNA ligase (0.5 μl, 2 units) was added. The reaction was incubated in a cooled water bath at 12°C overnight.

Linker-primer
5′-CTCGAGTTTTTTTTTT

3′-AAAAAAAAA ————— 5′
Messenger RNA template
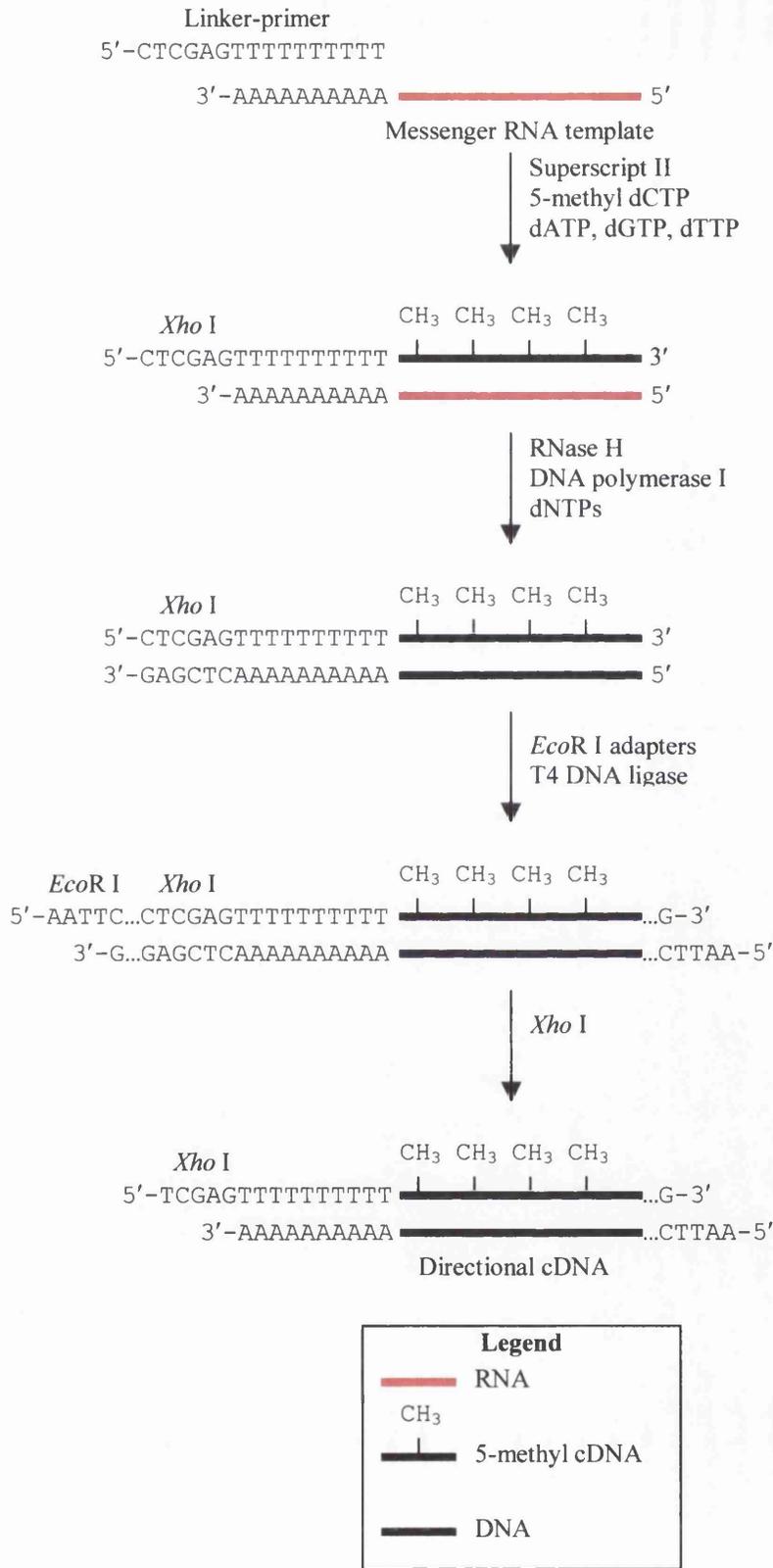
Step 1: Annealing of linker-primer that contains the recognition site for *Xho* I.

Superscript II
5-methyl dCTP
dATP, dGTP, dTTP

Step 2: First-strand synthesis. 5-methyl dCTP is used to protect the DNA from digestion by restriction enzymes used in subsequent steps.

Xho I   CH₃ CH₃ CH₃ CH₃

$Xho\ I$

CH3 CH3 CH3 CH3
5′-CTCGAGTTTTTTTTTTT |———|———|———|———— 3′
3′-AAAAAAAAA ————— 5′

RNase H
DNA polymerase I
dNTPs

Step 3: Second-strand synthesis. The mRNA is cut by RNase H and the fragments used as primers for DNA polymerase I.

Xho I   CH3 CH3 CH3 CH3
5′-CTCGAGTTTTTTTTTTT |———|———|———|———— 3′
3′-GAGCTCAAAAAAAAAA ————— 5′

*Eco*R I adapters
T4 DNA ligase

Step 4: Adapter ligation. The DNA fragments are ligated together and EcoR I adapter added to the ends of the completed cDNA.

*Eco*R I   *Xho* I   CH3 CH3 CH3 CH3
5′-AATTC...CTCGAGTTTTTTTTTTT |———|———|———|———...G-3′
3′-G...GAGCTCAAAAAAAAAA ————————...CTTAA-5′

Xho I

Step 5: *Xho* I restriction. The *Eco*R I adapter from the 3′ end is removed leaving cDNA suitable for directional cloning.

Xho I   CH3 CH3 CH3 CH3
5′-TCGAGTTTTTTTTTTT |———|———|———|———...G-3′
3′-AAAAAAAAA ————————...CTTAA-5′
Directional cDNA

Legend

———— RNA

CH3
|——— 5-methyl cDNA

———— DNA

Figure 3-3   Overview of cDNA Library Synthesis

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3.3.15.9 Packaging the Ligated cDNA

The ligated cDNA was packaged with Gigapack III Packaging Extract according to the manufacturer's instructions. Briefly, a microcentrifuge tube containing 25 μl of packaging extract was removed from the -80°C freezer and placed on dry ice. The extract was thawed by hand until it had just begun to thaw and 4 μl of the cDNA ligated into the Uni-Zap vector was added immediately, stirring with the pipette tip to mix well and making sure not to introduce air bubbles. The sample was centrifuged for about 3 seconds to collect it at the bottom of the tube and incubated at room temperature for exactly 2 hours. Five hundred microlitres of SM buffer (50 mM Tris-HCl, pH 7.5 / 0.1 M NaCl / 10 mM $MgSO_4 \cdot 7H_2O$ / 0.01% (w/v) gelatine) was added, followed by 20 μl of chloroform and the contents of the tube mixed gently. The tube was centrifuged briefly to sediment the debris and the supernatant transferred to a fresh tube. The supernatant containing the phage was stored at 4°C until ready for titration.

3.3.15.10 Phage Titration

A colony of XL1-Blue MRF′ cells was lifted from a tetracycline agar plate and grown overnight at 30°C in LB medium (50 ml) supplemented with 10 mM $MgSO_4$ and 0.2% (w/v) maltose in a 250 ml conical flask and swirled at 200 rpm on an orbital shaker. The bacteria were pelleted by centrifuging at 500g for 10 minutes. The cells were then gently resuspended in sterile 10 mM $MgSO_4$ (25 ml) and diluted to an OD_{600} of 0.5 with the same 10 mM $MgSO_4$.

To determine the phage titre of the packaged ligation product, two reactions were carried out. For the first, 1 μl of the packaged reaction was mixed with 200 μl of freshly prepared XL1-Blue MRF′ cells in sterile 10 mM $MgSO_4$ at an OD_{600} of 0.5. For the second, a 1:10 dilution of the packaged reaction was used. The phage and bacteria were incubated at 37°C for 15 minutes to allow the phage to attach to the cells. IPTG (15 μl; 0.5 M in water) and X-Gal (50 μl; 250 mg/ml in DMF) were added to 3 ml of autoclaved NZ top agar [NZ broth containing 0.7% (w/v) agarose], cooled to 48°C. This was added to the phage-bacteria mixture and poured immediately onto fresh NZ agar plates. The plates were left to set for 10 minutes, inverted and incubated overnight at 37°C. Recombinant (white) and background (blue) plaques

were counted. The remaining 2.5 μl of the ligation reaction was packaged as one reaction using one 25 μl tube of packaging extract according to the previously described procedure.

3.3.15.11 Amplification of the Uni-Zap XR Library

XL1-Blue MRF' (host) cells diluted to an OD_{600} of 0.5 in sterile 10 mM $MgSO_4$ were prepared as previously described. Twenty aliquots (3 μl of 1:10 dilution each) of the packaged library mixture, each containing about 25,000 plaque-forming units (pfu), were each mixed with 600 μl of the host cells in Falcon 2059 polypropylene tubes in order to amplify 0.5×10^6 plaques. The samples were incubated at 37°C for 15 minutes to allow the phage to attach to the cells. Autoclaved NZ top agar (10 ml), cooled to about 48°C, was added to each aliquot and immediately spread evenly onto freshly poured 150-mm NZ agar plates. The plates were incubated at 37°C for 8 hours, making sure that the plaques did not get larger than $1 - 2$ mm, and that they had not merged. The plates were overlaid with SM buffer (10 ml) and stored overnight at 4°C with gentle rocking to allow the phage to diffuse into the SM buffer. The bacteriophage suspension was poured off from each plate into sterile 50-ml conical Falcon tubes and each plate rinsed with an additional 2 ml of SM buffer. The rinse was added to the bacteriophage suspension in the Falcon tubes. Chloroform was added to a 5% final concentration, mixed well and incubated for 15 minutes at room temperature. The cell debris was collected by centrifugation at $500g$ for 10 minutes. The supernatant containing the amplified library was transferred to a sterile glass bottle, 600 μl of chloroform was added to obtain a final concentration of 0.3% and the library stored at 4°C. The amplified library was plated and titred as previously described using dilutions from 1:10 to 1:10,000.

3.3.15.12 DNA Screening

Fresh host cells were prepared in sterile 10 mM $MgSO_4$ as previously described. The amplified library was plated on twenty 2 day old NZ agar plates at 50,000 pfu/plate with 600 μl of host cells at an OD_{600} of 0.5 and 10 ml of NZ top agar as previously described. The plates were incubated at 37°C for 8 hours, then chilled at 4°C for 2 hours to prevent the NZ top agar from sticking to the nitrocellulose membrane when lifting the plaques.

Wearing gloves and using forceps, a nitrocellulose membrane (Hybond-C, Amersham, UK) was placed on each plate for 5 minutes. A hypodermic needle dipped in Indian ink was used to prick through the membrane and agar for orientation. The lifted DNA was denatured by submerging the membrane in denaturing solution (1.5 M NaCl / 0.5 M NaOH) for 5 minutes. The DNA was then neutralised by submerging the membrane in neutralisation solution (1.5 M NaCl / 0.5 M Tris-HCl, pH 8.0) for 5 minutes. The membrane was rinsed by submerging in 0.2 M Tris HCl, pH 7.5 and 2× SSPE buffer for no longer than 30 seconds and then blotted briefly on Whatman 3MM paper. The membrane was baked *in vacuo* for 2 hours at 80°C to crosslink the DNA. The agar plates were stored at 4°C to use after screening.

The DNA bound to the membrane was screened for the presence of the oesophageal "CYP2B" cDNA using the 350 bp fragment amplified from RNA by RT-PCR using the CYP2B specific primers. The screening was done by hybridisation as described for Northern and Southern hybridisation, but with a few modifications. The membranes were pre-wetted in 2 × SSPE as described previously and stacked in a suitable round Pyrex bowl with a lid containing 50 ml of hybridisation solution, making sure they were completely submerged. Since in a previous experiment it had been found that the 350 bp fragment being used as a probe hybridised relatively poorly to oesophageal RNA on a Northern blot, different hybridisation conditions were tried, and it was found that an aqueous hybridisation solution (5× SSPE / 5× Denhart's solution / 0.5% SDS) gave the best hybridisation conditions. Sonicated salmon sperm DNA was omitted in this hybridisation because trials carried out to optimise the hybridisation conditions showed that this was optimal. The membranes were pre-hybridised for 1 − 2 hours at 65°C and then the labelled probe was added and left to hybridise overnight. The membranes were transferred to a larger container containing about 500 ml 2× SSPE / 0.1% SDS and washed for 30 minutes at 65°C with shaking. The wash was repeated once with the same solution, and then twice with 0.1× SSPE / 0.1% SDS (500 ml). The membranes were then rinsed in 2× SSPE, wrapped in Saranwrap and exposed to radiographic film overnight at -80°C. A fluorescent marker (Stratagene) was also placed in the cassette to facilitate orientation of the film to the membranes. After developing the film, it was lined up with the membranes in the cassette, using the fluorescent marker as a guide, and spots were

marked on the film corresponding to the ink dots on the membranes. The stock plates were then placed on the film, the putative clones which lined up with the film spots were cut out with the wide end of a glass pasteur pipette and transferred into 1.5 ml microcentrifuge tubes containing SM buffer (1 ml) and chloroform (20 μl). The tubes were vortex mixed and several dilutions (1:100, 1:1,000, 1:10,000 and 1:100,000) plated with 600 μl of host cells on 100 mm 2 day old NZ agar plates. The plates were incubated overnight at 37°C. Plaque lifts were performed on pre-chilled plates as previously described. The lifts were prehybridised, hybridised and washed as before and exposed to radiographic film overnight at -80°C. Putative positive clones were cored from the secondary stock plates as previously described and transferred to sterile microcentrifuge tubes containing SM buffer (500 μl) and chloroform (20 μl). The contents of the tubes were vortex mixed to release the phage particles into the SM buffer and then incubated for 2 hours at room temperature if the clones were to be excised the same day or else stored at 4°C for later excision. The contents of this tube constituted the phage stock.

3.3.15.13 Single Clone Excision

Overnight cultures of XL1-Blue MRF′ and SOLR cells were grown in LB broth supplemented with 0.2% (w/v) maltose and 10 mM $MgSO_4$ at 30°C. The cells were collected by gentle centrifugation at 1000g for 10 minutes and resuspended at an OD_{600} of 1.0 in sterile 10 mM $MgSO_4$. Diluted XL1-Blue MRF′ cells (40 μl), phage stock (50 μl) and ExAssist helper phage (0.2 μl, ~2 × 10^5 pfu) were combined in a Falcon 2059 polypropylene tube and incubated at 37°C for 15 minutes. LB broth (600 μl) was added and the mixture incubated for 2.5 hours at 37°C with shaking. The reaction was then heated at 65°C for 20 minutes and centrifuged at 1000g for 15 minutes. The supernatant, containing the excised pBluescript phagemid, was decanted into a fresh sterile microcentrifuge tube. One hundred microlitres of this phage supernatant was added to diluted SOLR cells (200 μl) in a microcentrifuge tube and 10 μl of phage to another 200 μl of the same cells. The mixtures were incubated at 37°C for 15 minutes and 200 μl of each plated on separate LB-ampicillin agar plates. The plates were incubated overnight at 37°C. Single colonies were picked from the plates and grown up overnight in 50 ml LB broth for large-scale plasmid purification. A bacterial glycerol stock was prepared by adding 400 μl of the culture

to 500 μl glycerol in a 1.5-ml microcentrifuge tube. This was stored at -80°C for future use. A plasmid preparation was made from the rest of the culture. The purified plasmid was digested with *Eco*R I and *Xho* I restriction enzymes to determine the size of the insert.

3.3.15.14 Sequencing of Library Clones

The plasmid containing the insert was sequenced as previously described (Section 3.3.11) with T3 and T7 primers and also with a series of specific primers designed using the sequence obtained from the RACE-PCR products. This would produce overlapping sequences that would be used to confirm the final sequence. These primers were:

Sense 1	5′-GGTGTTTGAGCTTTTCCCTGGCTTCTTG-3′
Sense 2	5′-GACCAGCAGCACCACACTTCGCTATG-3′
Sense 3	5′-TCTTGTCCCGATTGGTCTGCCACAC-3′
Sense 4	5′-AGCGTGTTTGTCTTGGTGAAGGCATTG-3′
Sense 5	5′-TCACAGGCTTCTTGCTACTCTTGATCAGGG-3′
Sense 6	5′-TTTTCAGTGTCCAGCCCTGTGTCTCCTAAG-3′
Antisense 1	5′-CTTTCTCTGCAACATGGGGGTACTTGAGC-3′
Antisense 2	5′-CTGGTATGTTGGAGGTATTTTGGCAAAGCC-3′
Antisense 3	5′-TTCCCTTATGGTCTCGGTCCCATACAGC-3′
Antisense 4	5′-TGTGGAGAGATGATAAGTGAGGTCTTGGGC-3′

The sequencing data were then compared to sequences in the database using a BLAST search (Altschul *et al.*, 1990).

3.4   Results

3.4.1   RT-PCR

This chapter describes experiments carried out to identify a P450 in rat oesophagus that is not expressed in other organs, or is expressed at extremely low levels. A combination of molecular biology techniques, covering several experiments, was employed to achieve this. The first technique employed was reverse transcriptase-polymerase chain reaction (RT-PCR). Total RNA was used for this and several experiments were carried out using different sets of primer pairs. RNA was prepared from rat oesophageal mucosa and liver, quantitated and 10 - 40 µg examined on a 1.2% denaturing agarose gel to ensure that the ribosomal RNA bands were intact and that smearing, a result of RNA degradation, was at a minimum or absent.

In the first experiment, highly degenerate primers were designed based on the amino acid sequence similarity of two regions 1,200 nucleotides apart, present in all P450s belonging to the 2 family. The cDNA nucleotide sequences corresponding to these amino acid sequences of these P450 were examined and primers were designed. Also designed were specific primers to amplify the same region of CYP2A6. CYP2A6-specific primers were used because a plasmid containing cDNA for marmoset CYP2A6 was available and it was thought that this could be used as a positive control for the PCR amplifications. For the first RT-PCR, first-strand synthesis from oesophageal and liver RNA was carried out with an oligo(dT)$_{12-18}$ primer and the PCR performed using the degenerate primer pair (100 µM each) and the CYP2A6-specific primer pair (1 µM each). When the amplified products were electrophoresed on an agarose gel, no bands were observed for the reactions using either primer pair. The same was true for both oesophageal and liver cDNA (Figure 3-4). However, a PCR reaction carried out at the same time, using primers designed to amplify a 500 nucleotide region of human β-actin cDNA resulted in a product of the expected size. This showed that the reverse transcription had been successful and that the lack of products was most likely due to poor primers.
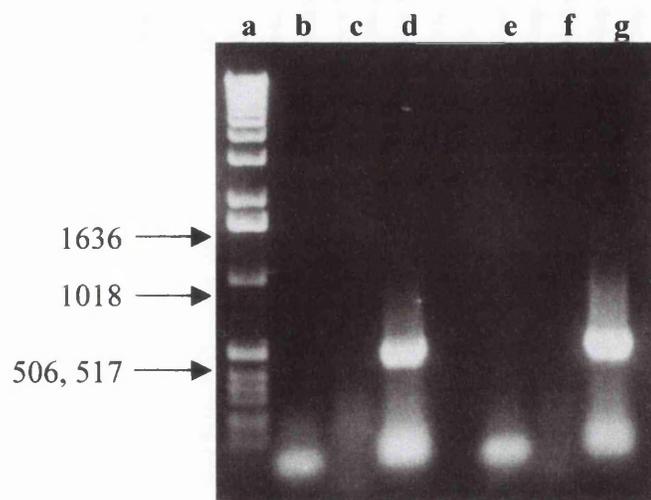
Figure 3-4 Gel electrophoresis of RT-PCR products obtained from oesophageal and liver RNA reverse transcribed with oligo(dT)$_{12-18}$. The RT-PCR products were run on a 1.2% agarose gel and visualised with ethidium bromide (0.1 µg/ml).
a: 1 kb DNA ladder
b: oesophageal cDNA amplified with degenerate primers
c: oesophageal cDNA amplified with CYP2A6-specific primers
d: oesophageal cDNA amplified with β-actin primers
e: liver cDNA amplified with degenerate primers
f: liver cDNA amplified with CYP2A6-specific primers
g: liver cDNA amplified with β-actin primers

Since amplification with these primers was unsuccessful using cDNA produced by reverse transcription with an oligo(dT)$_{12-18}$ primer, it was decided that a more specific cDNA should be produced. The CYP2A6-specific antisense primer was therefore used for first-strand synthesis. Even though the P450 expected was not CYP2A6, it seemed reasonable to use this primer because it spanned the region in the cDNA which codes for the conserved cysteine in the amino acid sequence, and bases in this region are usually quite conserved. These new cDNAs were amplified using the degenerate and CYP2A6-specific primer pairs as before. The results are shown in Figure 3-5. The degenerate primer pair still did not give any product with either oesophageal or liver cDNA, but the CYP2A6-specific primer pair gave several products of different sizes when used to amplify the oesophageal cDNA. The most abundant product (judging from the intensities of the bands) was 900 bp. There were other products of 500, 600 and 750 bp. There was also a band at 1,200 bp, representing a product of the expected size. Band intensity was not affected by

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doubling the concentration of first-strand cDNA used (Figure 3-5, lanes d and f). A single, very faint band of 750 bp, which was not observed in the previous experiment, was obtained on amplification of liver cDNA produced by oligo(dT)$_{12-18}$ reverse transcription using the CYP2A6-specific primer pair. A very faint band of 1,050 bp was obtained when the CYP2A6-specific primer pair was used to amplify this new liver cDNA. Again, there was no band of the expected 1,200 bp. The surprising result came from the positive control using the CYP2A6-specific primers to amplify the cloned marmoset CYP2A6 DNA. The expected 1,200 bp product was not obtained, but several products ranging in size from 600 to 6,100 bases were obtained. Two of these products were the same size as products obtained from the oesophageal PCR – 500 and 900 bp.
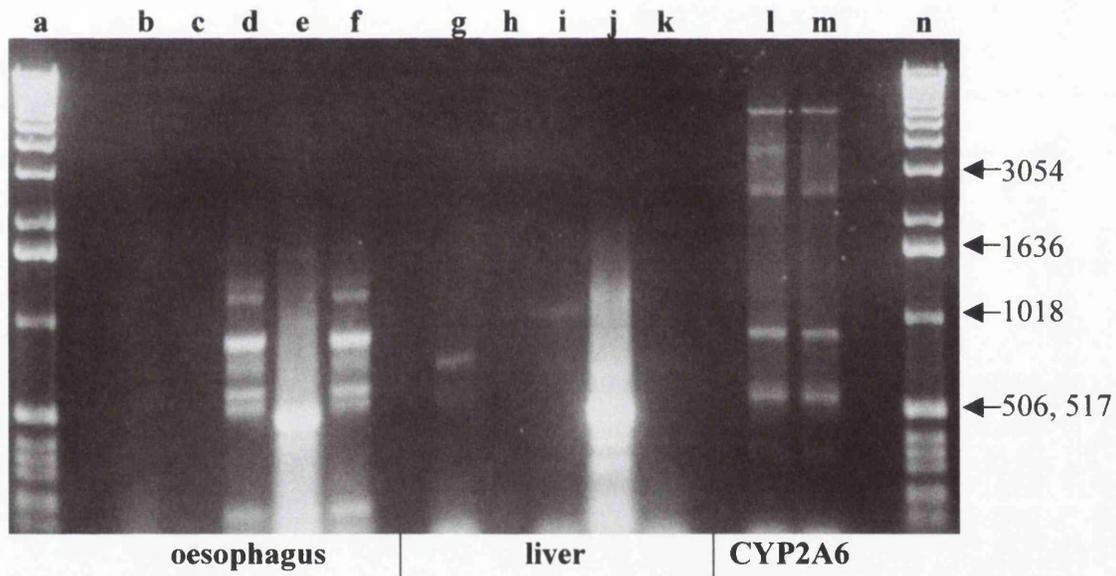
Figure 3-5   Gel electrophoresis of RT-PCR products showing the effect of using the antisense primers for first strand synthesis. Products were run on a 1.2% agarose gel and visualised with ethidium bromide (0.1 µg/ml).

a and n - 1 kb DNA ladder

b, c and e -   oesophageal cDNA (produced by reverse transcription with oligo(dT)$_{12-18}$) amplified with CYP2A6-specific, degenerate and β-actin primers respectively

d - 1 µl oesophageal cDNA (produced by reverse transcription with CYP2A6-specific antisense primer) and amplified with CYP2A6-specific primers

f -   same as d, but using 2 µl cDNA

g, h and j -   as b, c and e respectively, but using liver cDNA

i and k -   as d and f respectively, but using liver cDNA

l and m - plasmid containing marmoset CYP2A6 cDNA amplified with CYP2A6-specific and degenerate primers respectively.

The amplified products from the oesophagus were purified and cloned into the pPCR-Script cloning vector (Stratagene). Colony lysis PCR was performed on all white colonies obtained, and this showed that only the 900, 600 and 500 bp fragments could be cloned. None of the clones contained the 1,200 bp fragment. The fact that the CYP2A6-specific primers did not amplify anything in the marmoset cDNA clone suggested that the primers were not suited to the purpose and the cloned products were not pursued further. These primers were therefore abandoned and new PCR experiments using less degenerate primer pairs were planned.

A paper by Shen *et al.* (Shen *et al.*, 1993) showed that a degenerate primer pair, containing inosine, designed to amplify all P450s belonging to families 1, 2 and 3 had been used to identify a fragment of a novel P450 from mouse embryo fibroblast cells. Inosine had been introduced into these primers to reduce the complexity of primers in the mixture since inosine binds to all bases, and could therefore be inserted into the sequence at positions where any of the four regular bases occurred. CYP2A6-specific primers were designed based on the sequences of these primers to use as a positive control with the marmoset CYP2A6 cDNA clone. In this set of experiments first-strand synthesis of oesophageal and liver cDNA was carried out with oligo(dT)$_{12-18}$, the degenerate antisense primer, or the CYP2A6-specific antisense primer. PCR was performed on each first strand cDNA using both primer pairs - degenerate and CYP2A6-specific. The degenerate primers did not give any products with PCR, but the CYP2A6-specific primer pair appeared more successful, giving single products of 350 bp in most reactions, including the amplification of the marmoset CYP2A6 cDNA (Figure 3-6). A product was obtained irrespective of the first-strand cDNA used for the PCR. The sizes of the products (350 bp) correlated well with the expected size of 321- 336 bp. The products from amplification of the oesophageal cDNA were purified and cloned into the pPCR-Script cloning vector and sequenced. The sequences obtained from 6 clones did not correspond to P450s, but a structural protein, plectin.
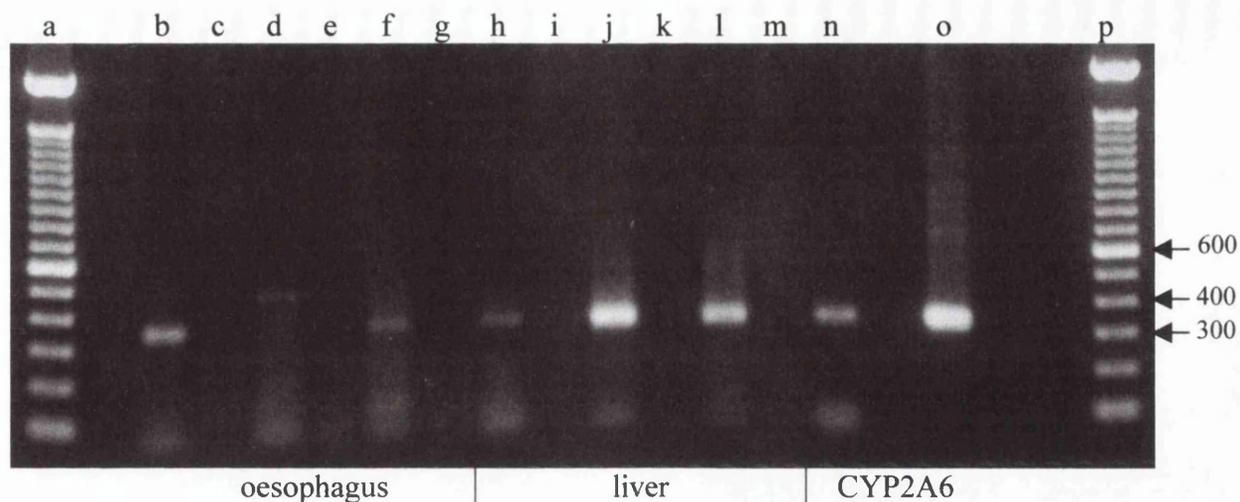
Figure 3-6   Gel of RT-PCR products using primers containing inosine.
Products were run on a 1.2% agarose gel and visualised with ethidium bromide.

b and h – RT primed from oligo(dT) and PCR with CYP2A6-specific primers

c and i – RT primed from oligo(dT) and PCR with degenerate primers

d and j – RT primed from antisense primer and PCR with CYP2A6-specific primers

e and k – RT primed from antisense primer and PCR with degenerate primers

f and l – RT primed from degenerate primer and PCR with CYP2A6-specific primers

g and m – RT primed from degenerate primer and PCR with degenerate primers

n and o – marmoset CYP2A6 DNA amplified with degenerate and CYP2A6-specific primers respectively

a and p – 100 bp markers (Life Technologies)

A third set of PCR experiments was carried out in which new primer pairs were designed to selectively amplify P450s belonging to separate subfamilies within the 2 family. The chosen subfamilies were 2A, 2B and 2E, since P450s belonging to these subfamilies have been shown to metabolise nitrosamines. The primers were based on the cDNA sequences of the P450s within each subfamily and were designed to amplify the same 350 bp region in each. It was decided to restrict the primers to subfamilies since there is a greater sequence homology between their members, therefore reducing the degeneracy of the primers and increasing their selectivity. PCR was performed on cDNA from oesophagus and liver reverse transcribed only with oligo(dT)$_{12-18}$. The results are shown in Figure 3-7. The CYP2A-specific primers amplified a 350 bp cDNA fragment from the liver cDNA, which was observed as a very faint band. No product was obtained from the oesophageal cDNA

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amplification using the same primers. The CYP2B-specific primers amplified a 350 bp cDNA fragment from both the oesophagus and liver cDNAs. The band amplified in liver, however, was more intense. CYP2E-specific primers did not amplify anything from oesophageal cDNA, but amplified a distinct 350 bp fragment from liver cDNA.
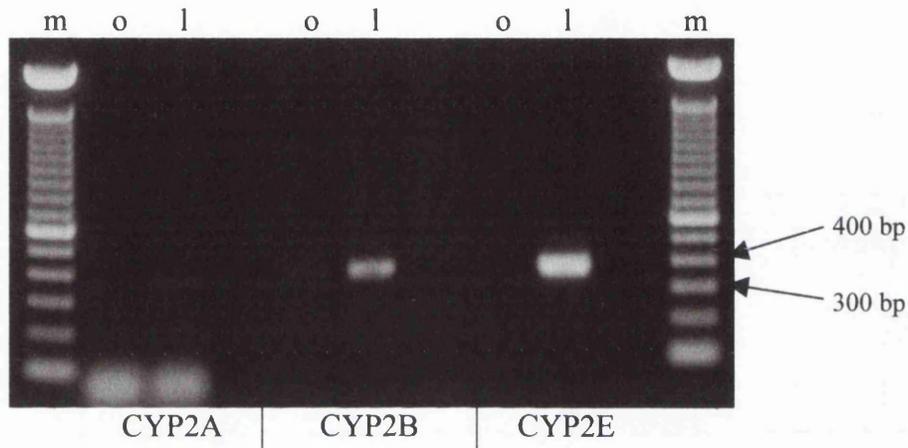
Figure 3-7   Gel of RT-PCR products using primers designed to amplify P450s belonging to specific subfamilies. Products were run on a 1.2% agarose gel and visualised with ethidium bromide.

o - oesophagus
l - liver
m - 100 bp DNA marker.

The CYP2B and CYP2E PCR products were cloned into the pPCR-Script cloning vector, the plasmids were grown up as before and the amplified cDNA fragments were sequenced on an automated sequencer. Only one liver CYP2B clone was sequenced and when a BLAST search was performed, this was found to be CYP2B2 (Figure 3-8 A). Several clones sequenced from the fragment amplified in the oesophagus by the CYP2B-specific primers were found to be exactly the same (Figure 3-8 B). The sequence had no exact matches in the database, but the closest match was to CYP2B12, a P450 found exclusively in rat sebaceous glands (Friedberg *et al.*, 1992). The product amplified from liver cDNA using CYP2E-specific primers was identified as a fragment of CYP2E1 (Figure 3-8 C), as expected. The oesophageal CYP2B product and the CYP2E1 fragment from the liver were grown for large-scale plasmid preparation and purified for use as probes.

A.

```
1       GTACCCCCAT GTCACAGAGA AAGTCCAAAA GGAGATTGAT CAGGTGATTG
51      GCTCTCACAG GCCACCATCC CTTGATGATC GTACCAAAAT GCCATACACT
101     GATGCAGTCA TCCACGAGAT TCAGAGATTT GCAGATCTTG CCCCAATTGG
151     TTTACCACAC AGAGTCACCA AAGACACCAT GTTCCGAGGG TACCTGCTCC
201     CCAAGAACAC TGAGGTGTAT CCCATCCTGA GTTCAGCTCT CCATGACCCA
251     CAGTACTTTG ACCATCCAGA CACCTTCAAT CCTGAGCACT TCCTGGATGC
301     CGATGGGACA CTGAAAAGA GTGAAGCTTT TATGCCCTTC TCCACA
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B.

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1       GTACCCCCAT GTTGCAGAGA AAGTTCAAAA GGAGATTGAT CAGGTGATTG
51      GCTCACACCG GGTCCCAACC CTTGATGACC GCATCAAAAT GCCATACACT
101     GATGCAGTCA TCCATGAGAT TCAGAGATTT TCAGATCTTG TCCCGATTGG
151     TCTGCCACAC AGAGTCACCA AAGACACATT GTTCCGAGGG TACCTGCTCC
201     CCAAGAACAT TGAGGTGTAC CCCATCCTGA GTTCAGCACT CCATGATCCA
251     CAGTACTTTG AACACCCGGA CACCTTCAAT CCTGAGCACT TCCTGGATGC
301     CAATGGGGCA CTGAAGAAGA ATGAAGCTTT TCTGCCCTTC TCCACA
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C.

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1       ATACCCAGAA ATTGAAGAGA AACTTCATGA AGAAATTGAC AGGGTTATTG
51      GGCCAAGCCG CGTCCCTGCT GTCAGAGACA GACTGGATAT GCCCTACATG
101     GATGCTGTGG TGCATGAGAT CCAGAGATTC ATCAATCTTG TCCCTTCCAA
151     CCTACCCCAT GAAGCAACCA GAGATACTGT GTTCCAAGGA TATGTCATCC
201     CCAAGGGTAC AGTTGTGATT CCAACTCTGG ACTCCCTCTT ATATGACAGC
251     CATGAGTTTC CAGATCCAGA GAAGTTTAAA CCTGAGCATT TCCTGAATGA
301     AAATGGGAAG TTCAAGTACA GTGACTATTT CAAGGCATTT TCTGCA
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Figure 3-8   Sequences amplified by RT-PCR using degenerate primers specific for the CYP2A, CYP2B or CYP2E subfamilies.
A -   CYP2B3 fragment amplified from rat liver
B -   novel CYP2B fragment amplified from rat oesophagus
C -   CYP2E1 fragment amplified from rat liver

When the product of oesophageal cDNA amplification with the CYP2B-specific primers was used to probe a Northern blot of oesophageal and liver RNA, it was found to hybridise to RNA of approximately 2,000 bases in both tissues. Hybridisation to liver RNA, however, was much stronger than to oesophageal RNA (Figure 3-9). This strong binding to a Northern blot of liver RNA suggests that the probe is not specific for the oesophageal P450 and is likely to bind to most P450s of the 2B subfamily.
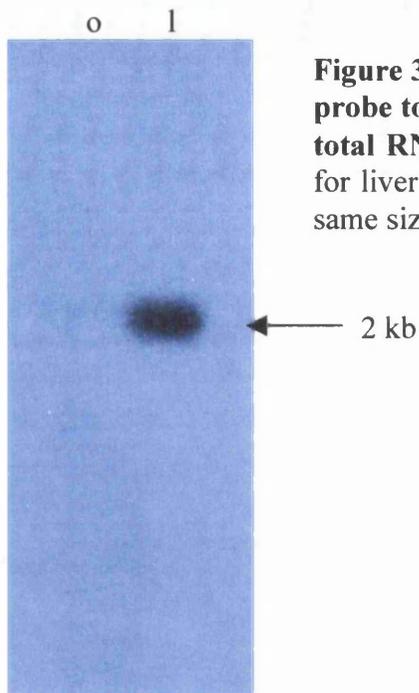
Figure 3-9 Hybridisation of the 350 bp "CYP2Boes" probe to a Northern blot of oesophagus (o) and liver (l) total RNA (40 μg each). Figure shows an intense band for liver RNA at 2000 bases, but a very faint band of the same size for oesophagus RNA.

3.4.2 RACE-PCR

New primers were designed within this fragment for use in RACE-PCR. It was necessary to use new primers to eliminate mispairing because the initial primers contained "wobble" bases and so the primer binding regions could not be confirmed. cDNA for RACE PCR was prepared using a Marathon cDNA synthesis protocol (Clontech), with some modifications as indicated in the Methods. The PCR was performed using an adapter primer and the newly designed antisense primer to amplify the 5′ end and the adapter primer and the sense primer to amplify the 3′ end. On agarose gel analysis the amplified 5′ end was found to be 1,150 bp (Figure 3-10a) and the 3′ end 1,000 bp (Figure 3-10b). The 3′ end was easily cloned into the multiple cloning site of the pPCR-Script cloning vector, but several attempts to clone the 5′ end into the same vector were unsuccessful. "Nested" RACE-PCR was therefore carried out for further, more specific amplification. A more intense band was obtained for the 5′ end, and this 1,000 base product was more easily cloned and the 3′ RACE-PCR product was even more distinct after "nested" PCR and this product was cloned (Figure 3-11).
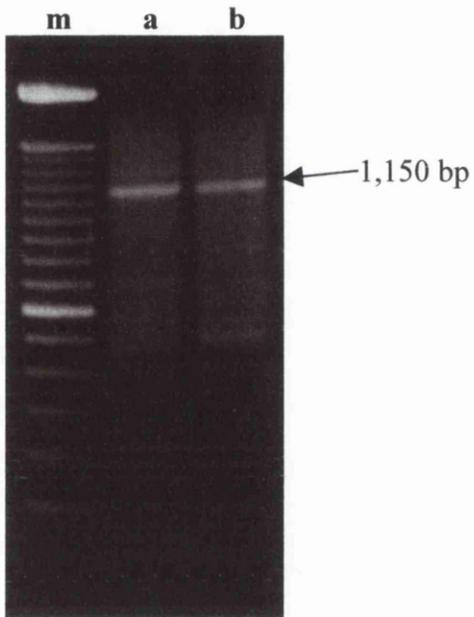
Figure 3-10a   Gel showing distinct 1,150 bp 5′ RACE-PCR products (a, b) from oesophageal cDNA in a smear of other amplified products.   Products were run on a 1.2% agarose gel and visualised with ethidium bromide.
m – 100 bp DNA ladder.
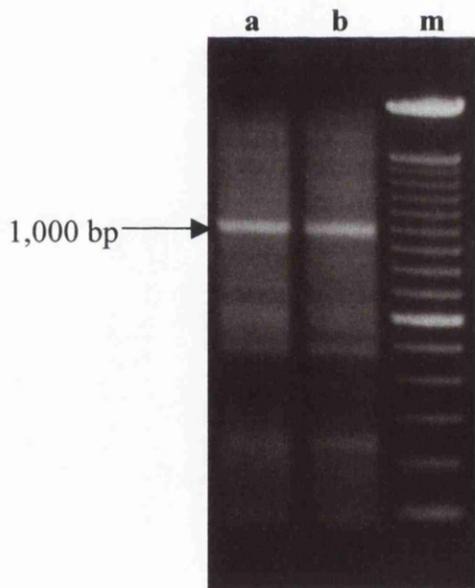
Figure 3-10b   Gel showing distinct 1,000 bp 3′ RACE-PCR products (a, b) from oesophageal cDNA in a smear of other amplified products.   Products were run on a 1.2% agarose gel and visualised with ethidium bromide.
m – 100 bp DNA ladder
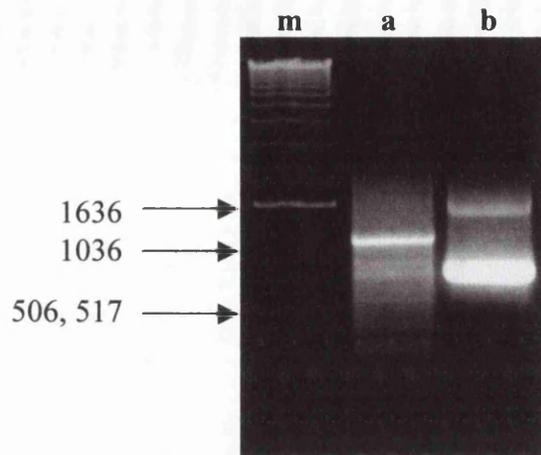
Figure 3-11   Gel showing the (a) 5′- and (b) 3′-nested RACE-PCR products from oesophageal cDNA. Products were run on a 1.2% agarose gel and visualised with ethidium bromide. The 5′ product is approximately 1000 bp and the 3′ product about 900 bp.

m – 1 kb DNA ladder

The plasmids were transformed into Stratagene's XL1-Blue MRF′ Kan Supercompetent cells. White colonies were picked and streaked on fresh LB agar plates containing IPTG and XGal. Colony lysis PCR was performed on a small sample of the streaks to check for the presence and sizes of inserts. The plasmids into which the fragment from the 3′ nested RACE-PCR had been cloned consistently contained inserts of 900 bp, while those into which the fragment from the 5′ nested RACE-PCR had been cloned contained inserts of varying sizes, ranging from 500 bp to 1,000 bp. The plasmids that contained the four longest 5′ RACE products and also four plasmids containing 3′ RACE products were sequenced. The 3′ RACE products were easily sequenced, but it was not possible to get the entire sequence of any of the 5′ RACE products. Attempts at sequencing the 5′ RACE products always resulted in bad sequence data towards both ends of the clones. It was therefore not possible at this stage to join the two ends of the cDNA together. Analysis of the sequence data showed that the clones of the 5′ RACE products were different lengths of the same fragment, while those of the 3′ RACE products were identical. BLAST searches conducted to compare the sequences obtained with those in the database showed high homology of the 5′ RACE products with CYP2B1, CYP2B2 and CYP2B3, while the 3′ RACE products showed high homology with CYP2B10 and CYP2B12.

3.4.3   cDNA Library Screening

Due to the difficulty in obtaining a full-length cDNA using RACE-PCR, it was decided that a new approach would be investigated. Since a probe that hybridised to RNA for cytochrome P450 in the oesophagus had been generated from the RT-PCR experiments,

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it was possible now to screen a cDNA library. An oesophageal cDNA library was constructed using Stratagene's Uni-Zap cDNA synthesis kit. The primary library contained 4×10^5 pfu/ml (4×10^5 pfu/µg of arms) and when 500,000 phage were amplified, a total volume of 200 ml was obtained containing 8×10^4 pfu/µl, giving a total of 1.6×10^{10} pfu. 0.5×10^6 clones were screened at a plaque density of 25,000 plaques per 150-mm plate. The oesophageal CYP2B probe obtained by RT-PCR was used and 9 positive clones were pulled out. On secondary screening, between 2 and 5 positive clones were obtained for each of the 9 clones. The clones were excised and transformed into XL1 blue cells and the plasmids were purified on a small scale. Three fragments were obtained for the first clone when digested with *Eco*R I and *Xho* I (Figure 3-12). Single enzyme digests showed that the cDNA had an internal *Eco*R I site, which resulted in cleaving 900 bases from one end of the clone. The sizes of the insert fragments were 900 and 1,100 bases, adding up to the expected total length of 2,000 bases. Further digests showed that clones 1 and 7 were possibly identical resulting in three fragments – linearised plasmid and two insert fragments of length 900 and 1,100 bases. Clones 3, 6 and 9 also resulted in three fragments on restriction digestion, the linearised plasmid, and two fragments from the insert of length 1,100 and 200 bases (Figure 3-13). The clones were then sequenced and the sequences aligned for comparison. It was observed that clones 1 and 7 were identical and that clones 3, 6 and 9 were shorter lengths of them. An internal EcoR1 site was observed 900 bases from the 5' end in clones 1 and 7, and 200 bases from the 5' end in clones 3, 6 and 9.

The sequences obtained were compared with sequences in the database using a BLAST search. The top 50 matches in this search were all to P450s of the 2B subfamily, the best matches being to CYP2B1/2.
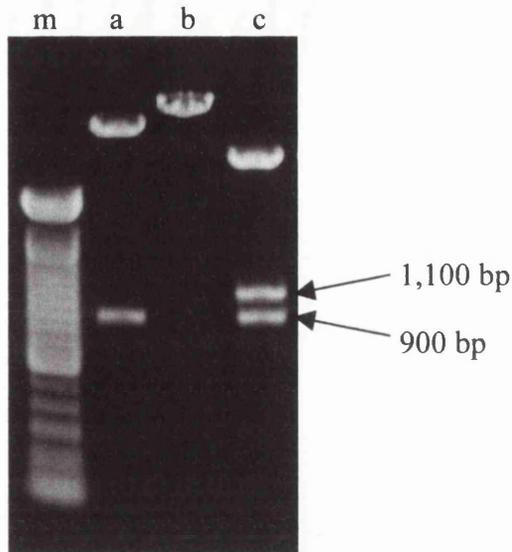
Figure 3-12   Gel electrophoresis of digested oesophageal cDNA library clone. The figure shows oesophageal cDNA library clone 1A digested with *Eco*R I alone (a), *Xho* I alone (b) and both *Eco*R I and *Xho* I (c). Clone contains an internal *Eco*R I site in the cDNA.

m – 100 bp DNA ladder.
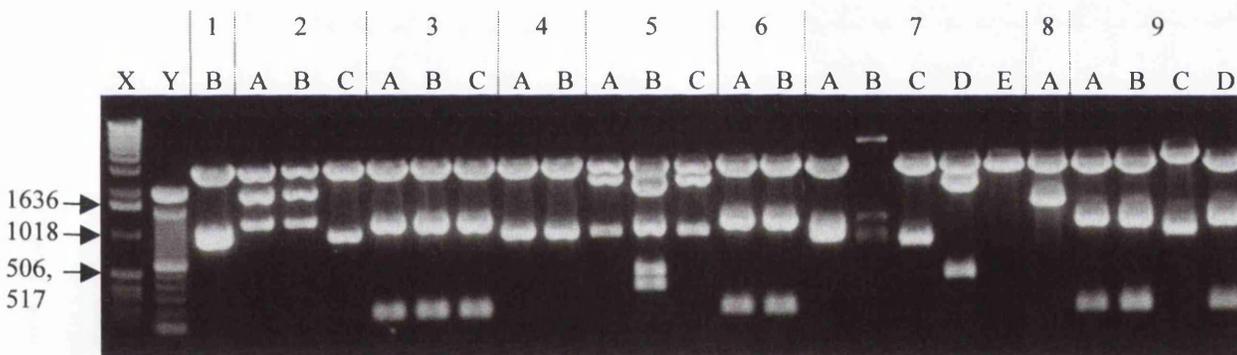
Figure 3-13   Gel electrophoresis of restriction digestion products of clones isolated from the oesophageal cDNA library using the product amplified from oesophageal cDNA by CYP2B-specific primers as a probe. The figure shows oesophageal cDNA library clones after digestion with both *Eco*R I and *Xho* I. Clones 1B and 7A are identical with insert fragments of 1,100 and 900 bases. All clones in 3, 6 and 9 (except 9C) are identical with insert fragments of 1,100 and 200 bases.

X – 1 kb DNA ladder, Y – 100 bp DNA ladder.

Comparison of the sequences of the library clones with those of the RACE-PCR products showed that all the sequence data represented one cDNA. The cloned cDNA sequence (Figure 3-14) and the deduced amino acid sequence (Figure 3-15) are shown. The 5′ nested RACE products, not being of the expected length (~ 1000 bp), could only be aligned with the central part of the library clones, while the 3′ nested RACE products could be completely aligned with the 3′ end of the library clones (Figure 3-14).

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                11          21          31          41
1      GGCACGAGTC  CAGGACC**ATG**  GATCCCAGTG  TCCTGCTCCT  TTTTGCTCTT
51     CTCACAGGCT  TCTTGCTACT  CTTGATCAGG  GGCCAGGGAA  ACGGATATGG
101    CCATCTTCCA  CCAGGACCCT  GTCCCCTGCC  CCTCTTGGGG  AACGTTTTGC
151    AAATGGACAG  AAGAGGTCTC  CTCAAGTCTT  TTATTCAGCT  TCGTGACAAA
201    TATGGAGATG  TGGTTACAGT  GCACTTGGGA  CCAAGGCCCA  TAGTCATGCT
251    GTATGGGACC  GAGACCATAA  GGGAAGCTCT  GGTGGACCAT  GCTGAGGCTT
301    TCTCTGGCCG  GGGAACAGTT  GCTGTGGTTC  AGCCAATTAT  ACAGGACTAT
351    GGTATGATCT  TTGCCAATGG  GGAACGTTGG  AAGATCCTTC  GGCGATTCTC
401    TCTAGCCACC  ATGAGAGACT  TTGGGATGGG  AAAGCGAAGT  GTGGAGGAGA
451    GGATAAAGGA  GGAGGCCCAA  TGTTTGGTGG  AGGAACTGAA  GAAATACAAG
501    GGAGCCCCAC  TGGACCCCAC  CTTCCACCTA  CAGTGCATTA  CAGCCAACAT
551    CATCTGCTCC  ATTGTCTTTG  GAGAACGTTT  TGACTACACA  GACCACCAGT
601    TTCTGCACCT  GCTCGACCTG  TTCTATGAGA  TCTTGTCACT  TGTTAGCTCA
651    TTCTCCAGCC  AGGTGTTTGA  GCTTTTCACT  GGCTTCCTGA  AGTACTTTCC
701    TGGCACCCAC  AGACACATCT  CCAAAAACAT  AGAGGAAATC  CTCAACTTCA
751    TTGGCCATTG  TGTGGAGAAG  CACAGGGCCA  CATTAGACCC  CAGCACTCCA
801    CGAGACTTCA  TAGATACCTA  CCTTCTCAGA  ATGGAGAAGG  AGAAATTAAA
851    CCACCACACA  GAATTCCATC  ACCAGAACCT  CATGATGTCT  GTGCTCTCTC
901    TCTTCTTTGC  TGGCACTGAG  ACCAGCAGCA  CCACACTTCG  CTATGGTTTC
951    CTGCTCATGC  TCAAGTACCC  CCATGTTGCA  GAGAAAGTTC  AAAAGGAGAT
1001   TGATCAGGTG  ATTGGCTCAC  ACCGGGTCCC  AACCCTTGAT  GACCGCATCA
1051   AAATGCCATA  CACTGATGCA  GTCATCCATG  AGATTCAGAG  ATTTTCAGAT
1101   CTTGTCCCGA  TTGGTCTGCC  ACACAGAGTC  ACCAAAGACA  CATTGTTCCG
1151   AGGGTACCTG  CTCCCCAAGA  ACATTGAGGT  GTACCCCATC  CTGAGTTCAG
1201   CACTCCATGA  TCCACAGTAC  TTTGAACACC  CGGACACCTT  CAATCCTGAG
1251   CACTTCCTGG  ATGCCAATGG  GGCACTGAAG  AAGAATGAAG  CTTTTCTGCC
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MDPSVLLLFALLTGFLLLLIRGQGNGYGHLPPGPCPLPLLGNVLQMDRRGLLKSFIQLR
DKYGDVVTVHLGPRPIVMLYGTETIREALVDHAEAFSGRGTVAVVQPIIQDYGMIFANG
ERWKILRRFSLATMRDFGMGKRSVEERIKEEAQCLVEELKKYKGAPLDPTFHLQCITAN
IICSIVFGERFDYTDHQFLHLLDLFYEILSLVSSFSSQVFELFTGFLKYFPGTHRHISK
NIEEILNFIGHCVEKHRATLDPSTPRDFIDTYLLRMEKEKLNHHTEFHHQNLMMSVLSL
FFAGTETSSTTLRYGFLLMLKYPHVAEKVQKEIDQVIGSHRVPTLDDRIKMPYTDAVIH
EIQRFSDLVPIGLPHRVTKDTLFRGYLLPKNIEVYPILSSALHDPQYFEHPDTFNPEHF
LDANGALKKNEAFLPFSTGKRVCLGEGIARNELFLFFTTILQNFSVSSPVSPKDIDLTP
KESGFAKIPPTYQICFLSRQLG
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Figure 3-15 Deduced amino acid sequence of the novel cytochrome P450 identified in rat oesophageal mucosa.

3.5   Discussion

The initial molecular biology experiments to identify nitrosamine-metabolising P450s in rat oesophagus involved RT-PCR using degenerate primers designed to amplify all P450s belonging to the 2 family. This was unsuccessful, probably due to the high content of "wobble" bases in the primer sequences. During primer design it was observed that although P450s belonging to the same family show > 40% amino acid homology, the codons used to denote any amino acid vary between different members of the same family. For example, even for the conserved cysteine residue in the haem binding site both codons for cysteine, TCT and TTT, are used. This made finding a stretch of identical nucleotides of any considerable length virtually impossible. The fact that the RT-PCR using primers designed to amplify human β-actin gave products of the expected sizes meant that the reverse transcription reaction had been successful. This suggests that the absence of products after PCR with the primers for P450 was due to the primers not annealing adequately to the cDNA. In a second experiment, the antisense primer designed for use in the amplification of CYP2A6 was used for first strand synthesis in the hope that any cDNA produced would belong to the 2 family or 2A subfamily. Even though CYP2A6 is not expressed in rat liver and was not expected to be expressed in oesophagus either, CYP2A6-specific primers were used because a positive CYP2A5 Western blot had suggested the presence of a P450 belonging to the 2A subfamily. It was hoped that if the annealing temperature of the PCR cycling program was lowered enough, the primers would anneal to any P450 belonging to the 2A subfamily. Also, a plasmid supposedly containing the cDNA of marmoset CYP2A6 was available to use as a positive control for PCR. These experiments using first strand cDNA synthesized with the CYP2A6-specific antisense primer appeared more successful, because unlike the previous experiment, some products were obtained, including one of the expected size of about 1,200 bp from oesophagus. However, unexpectedly the primers did not produce a fragment of the expected size of 1230 bp from the marmoset CYP2A6 clone. In retrospect it seems unlikely that this clone contained the required marmoset CYP2A6 cDNA because as well as this failure, the clone did not give the expected fragments on restriction digestion. Attempts were made to clone the 1,200 bp fragment produced from oesophagus, but only the smaller fragments could be cloned, probably because of the lower yield and larger size of the required 1,200 bp product. At this stage, still not knowing what P450s to expect in the

oesophagus, any PCR carried out would require primers with some degeneracy to be able to cover the spectrum of P450s belonging to the 2 family.

In a second set of RT-PCR experiments, degenerate primers containing inosine that had been used by Shen *et al.* (1993) were used. As mentioned in the Methods, inosine was included in these primers to reduce the complexity of primers in the mixture. This appeared to be even more successful since single fragments of the expected size (350 bp) were obtained in most cases. The products obtained were the same size when first-strand synthesis was carried out using oligo(dT)$_{12-18}$ primer, the CYP2A6-specific antisense primer or the degenerate antisense primer, and the PCR was carried out using the CYP2A6-specific primer pair. Two clones from the oesophageal PCR that were subsequently sequenced were found to be fragments of plectin, a structural protein. It is possible that if more clones had been sequenced, a P450 fragment would have been obtained.

Closer examination of the sequences of P450s belonging to the 2 family showed that the sequence homology within the subfamilies was very high, so it was decided that new degenerate primers would be targeted at specific subfamilies. Two arbitrary regions, 350 bp apart, with reasonable sequence homology within the specific subfamily, but different from other subfamilies, were chosen for P450s belonging to the major subfamilies studied in relation to nitrosamine metabolism: 2A, 2B and 2E. PCR experiments using the primers specific for CYP2B proved most successful and a 346 bp fragment corresponding to a novel P450 was isolated from oesophagus. This fragment showed greatest sequence homology to other P450s belonging to the 2B subfamily, especially CYP2B12, a rat sebaceous gland P450 (Friedberg *et al.*, 1992).

At this stage, the fragments that had been generated by RT-PCR were used to probe Northern blots with the hope of obtaining some idea about the P450(s) expressed in rat oesophagus. Northern blot analyses of RNA from liver and oesophagus showed that the 346 bp product amplified by the CYP2B-specific primers hybridised strongly to liver mRNA of 2 kb. There was very weak hybridisation to oesophageal mRNA of the same size. This could only just be detected on an autoradiograph after exposure over a weekend. Hybridisation of the blots of RNA from different organs to fragments of CYP2A3 and CYP2E1 showed that there were no detectable levels of hybridising

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mRNA in the oesophagus. An interesting feature of CYP2A3 hybridisation was the strong 2 kb band observed in nasal epithelium. There was little hybridisation to lung mRNA of 2 kb. Due to the similarity in the effect of ethanol on the methylation of DNA of oesophagus, nasal epithelium and lung, it was suspected that there might be similar P450s in the three organs. Also a recent paper had suggested that CYP2A3 could be the P450 involved in the metabolism of nitrosamines by the oesophagus (Patten *et al.*, 1998). The much higher level of CYP2A3 mRNA in nasal epithelium casts doubt on this assumption and this conclusion is supported in a recent paper (Gopalakrishnan *et al.*, 1999). Hybridisation of the Northern blots to a CYP2E1 fragment gave the expected results, with the highest level of hybridisation to liver mRNA of 2 kb (results not shown). The level of hybridisation decreased in the order kidney > nasal epithelium > lung. The hybridising mRNA in all cases was 2 kb.

Since only the CYP2B-specific primers had given any positive result in the oesophagus, new primers were designed for RACE-PCR based on the sequence of the fragment amplified, and the fragment itself used as a probe for screening an oesophageal cDNA library. The products obtained from RACE-PCR were cloned and sequenced. Attempts to join them to obtain a full-length sequence were unsuccessful because the entire 5' end had not been cloned. Three identical cDNA clones with the full coding sequence of a P450 were isolated from the library. The sequences of these library clones could be almost perfectly aligned to the sequences of the 5'- and 3'- RACE-PCR products, indicating that RACE-PCR and cDNA library screening had identified the same P450. Any errors in sequencing or PCR were checked by sequencing several independent clones of each product and sequencing overlapping regions. The fact that the same sequence was identified each time using these techniques suggests that this P450 is the predominant P450 in rat oesophagus. The low amount of P450 in the oesophagus coupled with the high activity of the oesophageal mucosa to certain nitrosamines suggests that this P450, being the most abundant, is the nitrosamine-metabolising P450 of the oesophagus. The cDNA obtained could be aligned with other rat P450s belonging to the 2B subfamily (Figure 3-16).

Figure 3-16   An alignment of the coding region of the cDNA of the P450 identified in rat oesophageal mucosa with the cDNA of other rat P450s belonging to the CYP2B subfamily. Only bases different from those in the novel P450 are shown for CYP2B1, CYP2B2, CYP2B3 and CYP2B12.

```
CYP2Boes     1 ATGGATCCCA GTGTCCTGCT CCTTTTTGCT CTTCTCACAG
CYP2B1       1 .....G.... ..A..T.... ...CC..... ..C...GTG.
CYP2B2       1 .....G.... ..A..T.... ...CC..... ..C..TGTG.
CYP2B3       1 ......A.T. .......T.. ...CC..... G.C...CTCA
CYP2B12      1 .....ATT.G .....T.... ...CC.CA.. ..CACTGTG.


CYP2Boes    41 GCTTCTTGCT ACTCTTGATC AGGG---GCC AGGGAAACGG
CYP2B1      41 ........T. ......AG.. ....---.A. .CCC...GTC
CYP2B2      41 ........T. ......AG.. ....---.A. .CCC...GTC
CYP2B3      41 .......... .T..C..G.. ..A.---... .T.C...A.T
CYP2B12     41 .......... .T....AG.. ..CCAAA.T. ..CC...GAC


CYP2Boes    78 ATATGGCCAT CTTCCACCAG GACCCTGTCC CCTGCCCCTC
CYP2B1      78 CCG....A.C T.C....... ....TC.... ...T......
CYP2B2      78 CCG....A.C T.C....... ....TC.... ...T......
CYP2B3      78 TC....T... .......... .....C.... .T.A......
CYP2B12     81 CC....G..C T.G....... ....TC.... .......T..


CYP2Boes   118 TTGGGGAACG TTTTGCAAAT GGACAGAAGA GGTCTCCTCA
CYP2B1     118 .........C .CC....GT. ........G.G ..C.......
CYP2B2     118 .........C .CC....GT. ........G.. ..C.......
CYP2B3     118 .....A...C .C.....G.. .......G.. ..CT.T.GT.
CYP2B12    121 .........C .C..A..G.. .A........ ..CT.T..G.


CYP2Boes   158 AGTCTTTTAT TCAGCTTCGT GACAAATATG GAGATGTGGT
CYP2B1     158 .T..C..C.. G........A ..A....... ........T.
CYP2B2     158 .T..C..C.. G........C ..A....... ........T.
CYP2B3     158 .......C.. ........AA ..A...C.C. ........T.
CYP2B12    161 .T.....C.. G.......AA ..A....... ........T.


CYP2Boes   198 TACAGTGCAC TTGGGACCAA GGCCCATAGT CATGCTGTAT
CYP2B1     198 C.....A... C......... ....TG.G.. ......A.G.
CYP2B2     198 C.....A... C......... ....TG.G.. ......A.G.
CYP2B3     198 C.....AT.. ..T.....T. ....TG.G.. ........G.
CYP2B12    201 C.....A... C......... .....G.G.. ...AT...G.


CYP2Boes   238 GGGACCGAGA CCATAAGGGA AGCTCTGGTG GACCATGCTG
CYP2B1     238 .....A..C. ......A... G......... .G...A....
CYP2B2     238 .....A..C. ......A... G......... .G...A....
CYP2B3     238 .....AC... .......... G......... ..........
CYP2B12    241 .....A..C. .......A.. G......... .....A....
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CYP2Boes   278 AGGCTTTCTC TGGCCGGGGA ACAGTTGCTG TGGTTCAGCC
CYP2B1     278 ...A...... ...T...... ...A.C.... ..A..G....
CYP2B2     278 ...A...... ...T...... ...A.C.... ..A..G....
CYP2B3     278 .......... .......... .T.A...... ..C.......
CYP2B12    281 .A........ ...T...... .......... ..C.C..T..

CYP2Boes   318 AATTATACAG GACTATGGTA TGATCTTTGC CAATGGGGAA
CYP2B1     318 ...CT.CA.. ..A......G .......... ..........
CYP2B2     318 ...CT.CA.. ..A......G .......... ..........
CYP2B3     318 ......G... ..A..C...G ..TC.....T T....AA...
CYP2B12    321 .G..G..... .GA......G .......... ..C.......

CYP2Boes   358 CGTTGGAAGA TCCTTCGGCG ATTCTCTCTA GCCACCATGA
CYP2B1     358 ..C.....G C......... ........G ..T.......
CYP2B2     358 ..C.....G C......... ........G ..T.......
CYP2B3     358 A.G....... .......... ...A.T.G.. ..........
CYP2B12    361 .......... C......... ........G .T........

CYP2Boes   398 GAGACTTTGG GATGGGAAAG CGAAGTGTGG AGGAGAGGAT
CYP2B1     398 .......... .......... A.G....... .A..AC....
CYP2B2     398 .......... .......... A.G....... .A..AC....
CYP2B3     398 .......... ...A...... .AG....... ....TCA...
CYP2B12    401 A...A..C.. A......... ..G.....A. .T........

CYP2Boes   438 AAAGGAGGAG GCCCAATGTT TGGTGGAGGA ACTGAAGAAA
CYP2B1     438 TC.......A .......... .......... ....CG....
CYP2B2     438 TC.......A .......... .......... ....CG....
CYP2B3     438 .......... ...A...... .A........ .........T
CYP2B12    441 .......... .....G.... .......... G.........

CYP2Boes   478 TACAAGGGAG CCCCACTGGA CCCCACCTTC CACCTACAGT
CYP2B1     478 .C.C...... .......... T......... .T.T.C....
CYP2B2     478 .C.C...... .......... T......... .T.T.C....
CYP2B3     478 C.TC...... T.T.C..... ...A..G... .T.T.C....
CYP2B12    481 .......... ....C...A. .......... .T.T.C..A.

CYP2Boes   518 GCATTACAGC CAACATCATC TGCTCCATTG TCTTTGGAGA
CYP2B1     518 ....C..... .......... .......... .G........
CYP2B2     518 ....C..... .......... .......... .G........
CYP2B3     518 ..G.C....G ......A... .......... ..........
CYP2B12    521 C...CG.... A....C.... .......... ..........

CYP2Boes   558 ACGTTTTGAC TACACAGACC ACCAGTTTCT GCACCTGCTC
CYP2B1     558 G..C...... .......... G......C.. ..G....T.G
CYP2B2     558 G..C...... .......... G......C.. ..G....T.G
CYP2B3     558 G..C...... ...G..... G......C.. ..G.T.....
CYP2B12    561 G..C...... ....A..... .......C.. ...T.....G
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CYP2Boes  598 GACCTGTTCT ATGAGATCTT GTCACTTGTT AGCTCATTCT
CYP2B1    598 ..G....... .CCG..C... T..C..CC.A ..T.......
CYP2B2    598 ..G....... .CCG..C... T..C..CC.A ..T.......
CYP2B3    598 ........G. ..AG..C... T..C..CA.A ..........
CYP2B12   601 ..T..AG... ..A...CA.C TGTC...A.G G....C..G.

CYP2Boes  638 CCAGCCAGGT GTTTGAGCTT TTCACTGGCT TCCTGAAGTA
CYP2B1    638 .......... ......T.C ...T....G. .......A..
CYP2B2    638 .......... ......T.C ...T....G. .......A..
CYP2B3    638 ........A. .......G.C .A.T...A.. ..........
CYP2B12   641 .......... .......... .A.T...... .......A..

CYP2Boes  578 CTTTCCTGGC ACCCACAGAC ACATCTCCAA AAACATAGAG
CYP2B1    578 .........T G......... .A........ ....C.CC..
CYP2B2    578 .........T G......... .A........ ....C.CC..
CYP2B3    578 .........T GT.......G .A....A... ....C.GA..
CYP2B12   681 .........T G.....A.. .A....T... ....C..C..

CYP2Boes  718 GAAATCCTCA ACTTCATTGG CCATTGTGTG GAGAAGCACA
CYP2B1    718 .........G .T.A...... ....AT.... ..........
CYP2B2    718 .........G .T.A...... ....AT.... ..........
CYP2B3    718 ...G.....G ...A.....A T...A..... .....C....
CYP2B12   721 .....G.... ...A...... ....AT.... ..........

CYP2Boes  758 GGGCCACATT AGACCCCAGC ACTCCACGAG ACTTCATAGA
CYP2B1    758 .......C.. ......A... G......... .......C..
CYP2B2    758 .......C.. .......... G......... .......C..
CYP2B3    758 .......T.. G......AT G....C.... ....T..C..
CYP2B12   761 ....A..C.. G.......T G......... .T.....C..

CYP2Boes  798 TACCTACCTT CTCAGAATGG AGAAGGAGAA ATTAAACCAC
CYP2B1    798 C..T...... ..GC.C.... .......... G.CG......
CYP2B2    798 C..T...... ..GC.C.... .......... G.CG......
CYP2B3    798 ...T.T.... ..GCAC.... .......... G..G......
CYP2B12   801 ...T...... ..GC.C.... .......... G.CC.....T

CYP2Boes  838 CACACAGAAT TCCATCACCA GAACCTCATG ATGTCTGTGC
CYP2B1    838 .......G. .......TG. .......... ..C..CC...
CYP2B2    838 .......G. .......TG. .......... ..C..CC...
CYP2B3    838 T....G..G. .......TTG ......A... ..A.......
CYP2B12   841 .......G. ..A....... ......TG.. ..C.......

CYP2Boes  878 TCTCTCTCTT CTTTGCTGGC ACTGAGACCA GCAGCACCAC
CYP2B1    878 .......... .......... .......... ..........
CYP2B2    878 .......... .......... ........G ..........
CYP2B3    878 ...T...T.. .C........ ......T.T. CA....A...
CYP2B12   881 .......... .......... ..C....... C.........
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CYP2Boes    918 ACTTCGCTAT GGTTTCCTGC TCATGCTCAA GTACCCCCAT
CYP2B1      918 ...C...... .......... .G........ ..........
CYP2B2      918 ...C...... .......... .......... ..........
CYP2B3      918 T..CT..... ..A....... .......... ......T...
CYP2B12     921 G..C..T.GC ACC......A .......... .........C

CYP2Boes    958 GTTGCAGAGA AAGTTCAAAA GGAGATTGAT CAGGTGATTG
CYP2B1      958 ..C....... ....C..... .......... ........C.
CYP2B2      958 ..CA...T.. ....C..... .......... ..........
CYP2B3      958 .......... ....C..... .......... ........C.
CYP2B12     961 .......... ....C..... .......... ..........

CYP2Boes    998 GCTCACACCG GGTCCCAACC CTTGATGACC GCATCAAAAT
CYP2B1      998 .......... .C.A...... .......... ...GT.....
CYP2B2      998 ....T...A. .CCA...T.. ........T. .T.C......
CYP2B3      998 .......G.. .......... .......... ..TC......
CYP2B12    1001 .......... .C.A...... .C.....T. .T.C......

CYP2Boes   1038 GCCATACACT GATGCAGTCA TCCATGAGAT TCAGAGATTT
CYP2B1     1038 .......... .......T. ....C..... ......G...
CYP2B2     1038 .......... .......... ....C..... ......G...
CYP2B3     1038 .......... ..G....... .......... ..........
CYP2B12    1041 ......T... .......... .......... ..........

CYP2Boes   1078 TCAGATCTTG TCCCGATTGG TCTGCCACAC AGAGTCACCA
CYP2B1     1078 .......... ....T..... AG.A...... ..........
CYP2B2     1078 G......... C...A..... .T.A...... ..........
CYP2B3     1078 ..T...G..T CT..A..G.. .T.....TG. ...A......
CYP2B12    1081 G......GA C......... ...A...... ..........

CYP2Boes   1118 AAGACACATT GTTCCGAGGG TACCTGCTCC CCAAGAACAT
CYP2B1     1118 .......CA. .......... .........T. .........C
CYP2B2     1118 .......CA. .......... .......... .........C
CYP2B3     1118 .......... .......... .......... .........C
CYP2B12    1121 .......CG. .......... .........T. .........C

CYP2Boes   1158 TGAGGTGTAC CCCATCCTGA GTTCAGCACT CCATGATCCA
CYP2B1     1158 ...A...... .......... .......T.. ......C...
CYP2B2     1158 ........T .......... .......T.. ......C...
CYP2B3     1158 .......... TT........ .......T.. ......C...
CYP2B12    1161 .......... .......... .......T.. ......C...

CYP2Boes   1198 CAGTACTTTG AACACCCGGA CACCTTCAAT CCTGAGCACT
CYP2B1     1198 .......... .C.....A.. ..G....... .....A....
CYP2B2     1198 .......... .C..T..A.. .......... ..........
CYP2B3     1198 .......... ....A..A.. .......... ..........
CYP2B12    1201 .G........ ....A..A.. ...T...... ..........
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CYP2Boes 1238 TCCTGGATGC CAATGGGGCA CTGAAGAAGA ATGAAGCTTT
CYP2B1   1238 .......... .......... .....A.... G.........
CYP2B2   1238 .......... .G.....A.. .....A.... G.........
CYP2B3   1238 .......... .......... .......AT G.........
CYP2B12  1241 .......... .......... .......... G.........

CYP2Boes 1278 TCTGCCCTTC TCCACAGGAA AGCGTGTTTG TCTTGGTGAA
CYP2B1   1278 CA........ .......... ....CA.... ......C...
CYP2B2   1278 .A........ .......... ....CA.... ......C...
CYP2B3   1278 CA........ ....T..... .....A.G.. ......A...
CYP2B12  1281 .......... ..T....... ....CA.... ......A...

CYP2Boes 1318 GGCATTGCCC GCAATGAGTT GTTCCTTTTC TTCACAACCA
CYP2B1   1318 .......... .A.....A.. ......C... .....C....
CYP2B2   1318 .......... .A.....A.. ......C... .....C....
CYP2B3   1318 .......... .A.G...A.. .......... .....T....
CYP2B12  1321 .......... .A.....A.. ....A..... .....CG...

CYP2Boes 1358 TCCTCCAGAA CTTCTCAGTG TCCAGCCCTG TGTCTCCTAA
CYP2B1   1358 .......... ......T... ..A....A.T ..G....C..
CYP2B2   1358 .......... ......T... ..A....A.T ..G....C..
CYP2B3   1358 .......... ..A....... .......... ..GA......
CYP2B12  1361 ....G..... ....A.TT.. G.A....... ..G.....G.

CYP2Boes 1398 GGACATTGAT CTCACTCCCA AGGAGAGTGG CTTTGCCAAA
CYP2B1   1398 .........C .....G.... .......... .A...GA...
CYP2B2   1398 .........C .....G.... T......... .A....A...
CYP2B3   1398 CAC....... A.G....... .......... A..A......
CYP2B12  1401 .........C .......... TTA.C.T... TG.A.G....

CYP2Boes 1438 ATACCTCCAA CATACCAGAT CTGCTTCTTA TCTCGCCAAT
CYP2B1   1438 .......... .G........ ........C. G....GTG.-
CYP2B2   1438 .......... .G........ ........C. G....GTG.-
CYP2B3   1438 G..G.C...G TG...A.... T.....TG.. G.C...TG.-
CYP2B12  1441 .....CT.TC .......... AAA....C.G ..C..TTG.-

CYP2Boes 1478 TGGGCTGA
CYP2B1   1478 --------
CYP2B2   1478 --------
CYP2B3   1478 --------
CYP2B12  1481 --------
```

The cDNA identified comprises 1894 nucleotides, consisting of a 17 bp 5′ untranslated region (UTR) and a 392 bp 3′ UTR which includes an 18 bp poly(A) tail. The short length of the 5′ UTR indicates that the clones isolated were not complete. The start codon, which occurs at positions 18 to 20, is in an optimal sequence context for the initiation of protein biosynthesis – there is a purine (guanine) three bases

upstream and a deoxyguanosine residue four bases downstream of the start of the open reading frame. The stop codon occurs at positions 1500 to 1502 and the poly(A) signal at 1852 to 1857. The codon for the conserved cysteine in the haem-binding site occurs at positions 1323 to 1325. This cDNA encodes for a protein of 494 amino acids (Figure 3-15), which is 3 amino acids longer than most of the other P450s of the 2B subfamily, with the exception of CYP2B11 and CYP2B15, which also have 494 amino acids. The cDNA and deduced amino acid sequences of this novel P450 show extensive sequence identity those of other rat P450s (Figures 3-16 and 3-17). Of the 1485 bases in the open reading frame of the novel cDNA sequence, 76 did not match corresponding bases in any of the other aligned sequences and there were 9 extra bases at the 3' end. This number is reasonable since 100 and 123 of the bases in CYP2B12 and CYP2B3 respectively did not match corresponding bases in the alignment. At the amino acid level, there is 84% homology with CYP2B1 and CYP2B2, 76% with CYP2B3 and 79% with CYP2B12. There are 39 amino acids that are not identical to amino acids in any of the other sequences. This correlates well with 55 and 71 in CYP2B12 and CYP2B3 respectively. 20% of the unique amino acids in the identified sequence are similar to the substituted amino acids of the other P450s, e.g. asparagine substituted for aspartic acid or *vice versa*, Val (V) substituted for Ile (I) and Phe (F) substituted for Thr (T).

Figure 3-17   An alignment of the deduced amino acid sequence of the P450 identified in rat oesophageal mucosa with the amino acid sequences of other rat P450s belonging to the CYP2B subfamily. Only amino acids different from those in the novel P450 are shown for CYP2B1, CYP2B2, CYP2B3 and CYP2B4

```
CYP2Boes    1 MDPSVLLLFA LLTGFLLLLI RG-QGNGYGH LPPGPCPLPL
CYP2B1      1 .E.TI...L. ..V......V ..-HPKSR.N F....R....
CYP2B2      1 .E..I...L. ..V......V ..-HPKSR.N F....R....
CYP2B3      1 ..T.....L. V.LS...F.V ..-HAKVH.. .....R....
CYP2B12     1 .EFG....LT .TV....F.V SQS.PKTH.. .....R...F

CYP2Boes   40 LGNVLQMDRR GLLKSFIQLR DKYGDVVTVH LGPRPIVMLY
CYP2B1     40 ...L..L..G ...N..M... E.....F... .....V...C
CYP2B2     40 ...L..L..G ...N..M... E.....F... .....V...C
CYP2B3     40 ...L.....G .FR......Q E.H...F..Y F....V...C
CYP2B12    41 ...L...N.. .F.N..M..Q E.....F... .....V.I.C
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```
CYP2Boes    80 GTETIREALV DHAEAFSGRG TVAVVQPIIQ DYGMIFANGE
CYP2B1      80 ..D..K.... GQ..D..... .I..IE..FK E..V......
CYP2B2      80 ..D..K.... GQ..D..... .I..IE..FK E..V......
CYP2B3      80 ..Q....... .......... II..L...M. E..VS.V.E.
CYP2B12     81 ..D....... .Q........ ....LH.VV. G..V...T..

CYP2Boes   120 RWKILRRFSL ATMRDFGMGK RSVEERIKEE AQCLVEELKK
CYP2B1     120 ...A...... .......... .......Q.. ........R.
CYP2B2     120 ...A...... .......... .......Q.. ........R.
CYP2B3     120 ......LFV .......I.. Q...DQ.... .K.......N
CYP2B12    121 ...T...... V..KE..... ...D...... ..........

CYP2Boes   160 YKGAPLDPTF HLQCITANII CSIVFGERFD YTDHQFLHLL
CYP2B1     160 SQ........ LF........ .......... ...R...R..
CYP2B2     160 SQ........ LF........ .......... ...R...R..
CYP2B3     160 HQ.VS..... LF..V.G... .......... .R.R...R..
CYP2B12    161 ......N... LF.S.A..T. .......... .K........

CYP2Boes   200 DLFYEILSLV SSFSSQVFEL FTGFLKYFPG THRHISKNIE
CYP2B1     200 E...RTF..L .........F .S........ A..Q....LQ
CYP2B2     200 E...RTF..L .........F .S........ A..Q....LQ
CYP2B3     200 ..L.RTF..I ......M..V YSD....... V..E.Y..LK
CYP2B12    201 ..V.KTSV.M G.L....... YS........ A.KQ.F..LQ

CYP2Boes   240 EILNFIGHCV EKHRATLDPS TPRDFIDTYL LRMEKEKLNH
CYP2B1     240 ...DY...I. .......... A......... .......S..
CYP2B2     240 ...DY...I. .......... A......... .......S..
CYP2B3     240 .V.DY.D.S. .N.......N A.......F. .H........
CYP2B12    241 .M..Y...I. .......... A......... .......S..

CYP2Boes   280 HTEFHHQNLM MSVLSLFFAG TETSSTTLRY GFLLMLKYPH
CYP2B1     280 ......E... I.L....... .......... ..........
CYP2B2     280 ......E... I.L....... ...G...... ..........
CYP2B3     280 Y.....W... I...F..L.. ..ST.N..C. ..........
CYP2B12    281 ....N....V I......... ...T.....C T..I......

CYP2Boes   320 VAEKVQKEID QVIGSHRVPT LDDRIKMPYT DAVIHEIQRF
CYP2B1     320 .......... .......L.. ....S..... ..........
CYP2B2     320 .T........ .......P.S ....T..... ..........
CYP2B3     320 .......... .....Q.... ....S..... E.........
CYP2B12    321 .......... .......L.. P...T..... ..........

CYP2Boes   360 SDLVPIGLPH RVTKDTLFRG YLLPKNIEVY PILSSALHDP
CYP2B1     360 .......V.. ......M... ......T... ..........
CYP2B2     360 A..A...... ......M... ......T... ..........
CYP2B3     360 ..VS.M...C .I........ ......T... F.........
CYP2B12    361 A..T...... ......V... ......T... ..........
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```
CYP2Boes   400 QYFEHPDTFN PEHFLDANGA LKKNEAFLPF STGKRVCLGE
CYP2B1     400 ...D...S.. .......... ...S...M.. .....I....
CYP2B2     400 ...D...... .......D.T ...S...M.. .....I....
CYP2B3     400 ....Q..... .......... ...C...M.. .I...M....
CYP2B12    401 R...Q..... .......... ...S...... .....I....


CYP2Boes   440 GIARNELFLF FTTILQNFSV SSPVSPKDID LTPKESGFAK
CYP2B1     440 .......... .......... ..HLA..... .......IG.
CYP2B2     440 .......... .......... ..HLA..... .......I..
CYP2B3     440 ....S..... .......Y.. ....D.NT.. M......L..
CYP2B12    441 ........I. ..A.....TL A...A.E... ...INI.VG.


CYP2Boes   480 IPPTYQICFL SRQLG
CYP2B1     480 .........S A.---
CYP2B2     480 .........S A.---
CYP2B3     480 VA.V.K...V A.---
CYP2B12    481 ..SP...N.. ..---
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The deduced amino acid sequence of the novel P450 contains all the regions that are known to be conserved in mammalian microsomal P450s. There are three highly conserved cysteine-containing regions that contain Cys 152, Cys 180 and Cys 436. The Cys 152 and Cys 436 regions are the most highly conserved of the three, with the latter being conserved in all prokaryotic and eukaryotic P450s so far discovered (Omura *et al.*, 1993). This cysteine residue, Cys 436, is the proximal thiolate ligand to the haem in P450s. Another highly conserved region found in this novel sequence occurs around Thr 302. This region occurs in the vicinity of the haem and makes up the oxygen-binding pocket of P450s. It is preceded by two conserved residues at positions 298 to 299, which are always Gly-Gly, Ala-Gly or occasionally, some other sequence of small amino acids. In this novel P450, Ala-Gly occurs at this position. Another feature of microsomal P450s that occurs in this novel sequence is the hydrophobic amino terminal portion, which occurs in nascent secretory proteins and cytochromes P450. In the secretory proteins, this signal sequence directs translocation to the cisternal space of the endoplasmic reticulum and is then cleaved to allow further translocation and hence secretion. In P450, however, this hydrophobic region is not cleaved and serves to anchor the protein to the membrane ensuring that the downstream portions of the protein are exposed on the cytoplasmic surface of the membrane (Ahn *et al.*, 1993; Nelson and Strobel, 1988; Szczesna-Skorupa *et al.*, 1988). Generally, these *N*-terminal segments contain a negatively charged residue preceding the hydrophobic core and several positively charged

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residues following it. The P450 identified conforms to this consensus to some extent, with an aspartic acid residue at position 2 and a histidine residue at position 30. The hydrophobic core occurs between these residues. The other CYP2B members found in the rat have more than one positively charged residue following the hydrophobic core, usually lysine at position 25 (26 for CYP2B12) and arginine or histidine at postion 27. Except for CYP2B6, all mammalian P450s belonging to the 2B subfamily have a lysine residue at position 25 (or 26 for CYP2B12 and CYP2B15). Like the newly identified P450, CYP2B6 has an asparagine residue at this position. It is interesting to note that while all the other CYP2B subfamily members, including the novel P450, have an arginine or a histidine residue at position 29 (30 for CYP2B12 and CYP2B15), CYP2B1, CYP2B2 and CYP2B10 have an asparagine residue at this position. CYP2B3 and CYP2B12 have another histidine residue at position 29 (30 for CYP2B12). A cAMP-dependent protein kinase site, which is found in most P450s of the 2B subfamily and has the sequence $Arg-Arg-X_1-Ser-X_2$, where X_1 and X_2 represent hydrophobic amino acids, occurs between positions 125 to 129. As in most P450s (Nelson and Strobel, 1988), X_1 and X_2 in this new P450 are Phe and Lys respectively.

The discovery of a novel P450 belonging to the 2B subfamily in rat oesophagus is rather promising because CYP2B1 has been shown to metabolise carcinogenic N-methyl-n-alkylnitrosamines with the correct structural specificity (Ji *et al.*, 1989; Mirvish *et al.*, 1991). However, the nitrosamine concentrations at which the metabolism occurs is too high for this metabolism to be of any physiological importance. It is possible that the unique amino acid residues in the novel P450 may be all that is necessary to alter the K_m to a level that is relevant to the concentrations of nitrosamines that animals (and humans) are naturally exposed to.

Previous work carried out in this laboratory showed that an antibody to CYP2B1 did not react with any protein in oesophageal microsomes prepared from untreated or phenobarbital treated rats when as much as 27 μg of microsomal protein was loaded on a gel (Pinto, 1994). This supports the suggestion that CYP2B1 is not the P450 responsible for the observed metabolism of nitrosamines in the oesophagus. To confirm that CYP2B1/2B2 does metabolise nitrosamines that are carcinogenic to the

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oesophagus and that this metabolism does lead to increased methylation of DNA, further experiments were carried out with phenobarbital treated rats.

CHAPTER 4   The Effect of Phenobarbital Treatment on the Metabolism of *N*-Nitrosamines in Rats

4.1   Introduction

The previous chapter described the identification and cloning from the rat oesophagus of the cDNA of a new member of the CYP2B subfamily. Because there is high nitrosamine metabolising activity in the oesophagus, but only a small total P450 content, one might assume that the P450 that metabolises the nitrosamines represents a large proportion of the total P450 of the oesophagus. The P450 that was identified was easy to amplify by RT-PCR and RACE-PCR and to isolate from a rat oesophageal cDNA library. This suggests that this P450 is relatively abundant in the oesophagus, and that in itself supports the view that it has some role in the metabolism of nitrosamines in that organ. This view is supported by reports that CYP2B1 is capable of the metabolism the assymetric methylalkyl- and methylaryl-nitrosamines that produce oesophageal cancer (Ji *et al.*, 1989; Lee *et al.*, 1989; Shu and Hollenberg, 1997; Yang *et al.*, 1985). However, these reports are of *in vitro* measurements with induced microsomes (Ji *et al.*, 1989), purified CYP2B1 (Lee *et al.*, 1989; Yang *et al.*, 1985) or those in which the involvement of CYP2B1 was inferred from inhibition studies using antibodies (Mirvish *et al.*, 1991). Because the conditions of these experiments are very artificial and commonly the substrate concentrations are very much greater than those found *in vivo*, the extrapolation of experiments of this kind to the whole animal is very difficult. For this reason measurements of the rate of metabolism of the oesophageal selective nitrosamine *N*-nitrosomethyl-*n*-butylamine were carried out in rats in which CYP2B1/CYP2B2 had been induced by phenobarbital treatment.

So far, three P450s of the 2B subfamily have been identified in rat liver. These are CYP2B1, CYP2B2 and CYP2B3. CYP2B1 and CYP2B2, which were the first to be completely sequenced, show 97% amino acid sequence identity. These two enzymes have broad and overlapping substrate specificities, but the activity of CYP2B1 in a reconstituted system may be 2- to 10-fold higher than CYP2B2 depending on the substrate (reviewed by Gonzalez, 1989). CYP2B1 is either absent or poorly

expressed in liver, but is induced up to 100-fold by phenobarbital (Waxman and Azaroff, 1992). The same P450 in lung and testis is constitutively expressed and not inducible by phenobarbital. CYP2B1 is also constitutively expressed in small intestine and inducible by various agents including phenobarbital. CYP2B2, on the other hand, is absent from these extra-hepatic tissues and cannot be induced in them. CYP2B2 expression in liver is increased 20-fold by phenobarbital treatment (Waxman and Azaroff, 1992). CYP2B3 shows 77% amino acid sequence identity to CYP2B1 and CYP2B2. It occurs constitutively in liver and is not induced by phenobarbital. Induction of *CYP2B1* and *CYP2B2* by phenobarbital occurs though transcriptional activation (Waxman and Azaroff, 1992). Other rat P450s belonging to the 2B subfamily are CYP2B12, expressed in rat sebaceous glands (Friedberg *et al.*, 1992) and CYP2B15 (Nakayama *et al.*, 1993).

The cDNA of two P450s belonging to the 2B subfamily, CYP2B6 and CYP2B7, have been isolated from human liver and sequenced. Their deduced amino acid sequences show 93% identity, but CYP2B7 contains a premature termination codon due to a C→T transition so does not encode a functional protein. There is a large degree of inter-individual variation in the amount of CYP2B6 protein and in the expression of its mRNA in human liver specimens. There is, however, no evidence that this P450 can be induced and no evidence of P450-inducing agents in patients whose liver specimens contain high levels of either the protein or the mRNA. Because there is a 70% amino acid sequence identity between CYP2B6 and rat CYP2B1, CYP2B6 is thought of as the human homologue of CYP2B1.

A recent paper has looked for the expression of various P450s in human oesophagus (Lechevrel *et al.*, 1999). The authors showed that there was no expression of CYP2B6. However, the antisense primer they used, corresponding to bases 1402 to 1421 of CYP2B6, was to a poorly conserved region of the CYP2B subfamily and would probably not have amplified other P450s belonging to this subfamily. This is unfortunate because Nakajima *et al.* (1996) claimed to have detected the expression of a member of the CYP2B subfamily in human oesophagus by Western blotting.

The inducing regimen used in the following experiments was recommended by Professor E. Shephard who has shown that it increases the total level of P450 in the liver about 3-fold, almost all of the increase being due to induction of CYP2B1/CYP2B2. The CYP2B1/CYP2B2 in the livers of rats induced in this way can represent up to 85% of the total P450.

There were two objectives of these experiments:

1.   To determine whether the induction of CYP2B1/2B2 increased the rate of metabolism of the oesophageal carcinogen, N-nitrosomethyl-n-butylamine, and

2.   If there were an increase, whether the CYP2B1/2B2 dependent metabolism would be more or less activating than the metabolism by CYP2E1.

To answer these questions the metabolism of N-nitrosomethyl-n-butylamine as well as the methylation of liver DNA by phenobarbital treated and normal rats were measured. The level of methylation due to N-nitrosomethyl-n-butylamine treatment was compared with the level produced by N-nitrosodimethylamine. The reason for doing this comparison was to determine what proportion of the metabolism of N-nitrosomethyl-n-butylamine was activating. With N-nitrosodimethylamine all metabolism, except denitrosation, is activating. But while hydroxylation of the α-carbon of the butyl group of N-nitrosomethyl-n-butylamine is activating, hydroxylation of the methyl group of this nitrosamine is detoxifying. Making this comparison of the amount of methylation of DNA of rat liver from equimolar doses of N-nitrosodimethylamine and N-nitrosomethyl-n-butylamine gives some idea of the proportion of N-nitrosomethyl-n-butylamine that is activated.

4.2   Materials

Animals used were 200 g to 250 g male Sprague Dawley rats that had been fed on a cubed diet TRM 9607 (Harlan Teklad, UK), and were purchased from the Biological Services Department of University College London.

A strong cation exchange column, HC-X8, was purchased from Hamilton, Reno, USA. Ecoscint A was from National Diagnostics. [^{14}C]-N-nitrosodimethylamine and N-nitroso-[^{14}C]methyl-n-butylamine were synthesised by Prof. Swann from [^{14}C]methyliodide using the method of Dutton and Heath (1956). All other chemicals were of analytical grade and were purchased from BDH, UK, unless otherwise stated.

4.3   Methods

4.3.1   Effect of Phenobarbital Treatment on Metabolism of Nitrosamines by Whole Rats

Two rats were given 0.1% phenobarbital in drinking water for 4 days and on the fifth day were given an i.p. injection of phenobarbital in saline (40 mg/kg body wt) using 4% phenobarbital in 0.9% sodium chloride. After 16 hours one rat was administered N-nitrosodi[^{14}C]methylamine (506 µmol/kg body wt) and the other was given N-nitroso[^{14}C]methyl-n-butylamine (517 µmol/kg body wt) by i.p. injection. Control experiments were set up using rats not treated with phenobarbital and giving them N-nitrosodi[^{14}C]methylamine (338 µmol/kg body wt) or N-nitroso[^{14}C]methyl-n-butylamine (345 µmol/kg body wt). The rats were placed in a metabolism cage and the $^{14}CO_2$ exhaled was measured as described in Chapter 2. The phenobarbital-treated rats were given a higher dose than the control rats because a preliminary experiment showed that they metabolised the nitrosamines much faster.

4.3.2   Effect of Phenobarbital Treatment on the Methylation of Liver DNA by N-Nitrosodimethylamine and N-Nitrosomethyl-n-butylamine

Eight rats were treated with phenobarbital as described in the previous experiment and then given N-nitrosodi[^{14}C]methylamine or N-nitroso-[^{14}C]methyl-n-butylamine (in both cases 474 µmol/kg body wt) 16 hours after the phenobarbital injection. The rats were killed 7 hours after nitrosamine treatment, DNA was prepared from the excised

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livers and the level of methylation determined by measuring 7-methylguanine as previously described (Chapter 2). Control rats that had received no phenobarbital treatment were also treated with the same dose of the nitrosamines and the level of methylation in their liver DNA also determined.

4.3.3 Determination of P450 and protein content of liver microsomes from phenobarbital treated rats

A 200 g rat received phenobarbital treatment as described above, was killed and liver microsomes were prepared as previously described (Chapter 2). The P450 and protein content of these microsomes, as well as microsomes prepared from the liver of an untreated rat, were determined as described in Chapter 2.

4.4   Results

Metabolism of the nitrosamines was monitored by measuring $^{14}CO_2$ exhaled from rats treated with N-nitrosodi[^{14}C]methylamine or N-nitroso[^{14}C]methyl-n-butylamine. The doses of the nitrosamines administered to the control rats, 338 μmol/kg body wt of N-nitrosodimethylamine and 345 μmol/kg body wt of N-nitrosomethyl-n-butylamine, saturated the metabolising enzyme as shown by the linear portions of the graphs (Figures 4-1 and 4-2). Figure 4-1 shows the metabolism of the nitrosamines represented by the radioactivity in the exhaled CO_2 expressed as a percentage of the input and shows that the percentage of the dose exhaled as CO_2 is reasonably independent of the size of the administered dose. Figure 4-2 shows the metabolism represented as μmoles of CO_2 exhaled. More CO_2 is exhaled from the phenobarbital treated rats because they were administered larger doses of the nitrosamines but more importantly it can be seen that the phenobarbital pre-treatment increased the rate of metabolism. In both the control and phenobarbital-treated rats there was a short lag, then metabolism followed a linear course, during which the rate of metabolism was independent of the concentration of the nitrosamine administered. Phenobarbital pre-treatment of rats before administering the nitrosamines increased the rate of metabolism of both N-nitrosodimethylamine and N-nitrosomethyl-n-butylamine during this linear period. The rate of metabolism of N-nitrosodimethylamine in the phenobarbital treated rat between 30 minutes and 2 hours after the dose was 1.45 times the rate in the control rat (Table 4-1). The rate of metabolism of N-nitrosomethyl-n-butylamine in the phenobarbital treated rat between 30 minutes and 1.5 hours after the dose was 2.3 times the rate in the control rat.

The P450 content of liver microsomes was increased 3-fold by phenobarbital treatment. This was accompanied by a 54% increase in the protein content of the microsomes (Table 4-1).

Phenobarbital pre-treatment decreased methylation by N-nitrosodimethylamine of liver DNA to 82% of the level in control rats, but increased methylation by N-nitrosomethyl-n-butylamine by 66% (Table 4-2).

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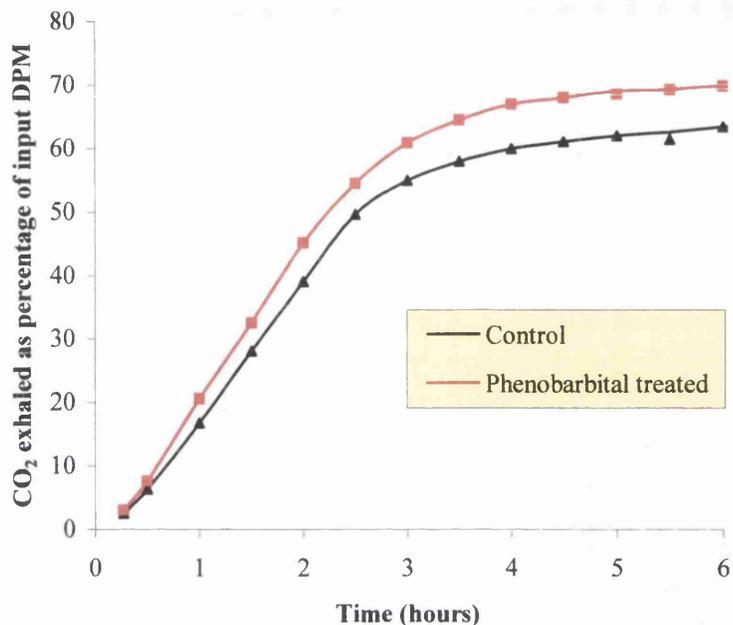
A.   Effect of phenobarbital pre-treatment on the metabolism of
N-nitrosodimethylamine (506 µmol/kg body wt) in a whole rat.
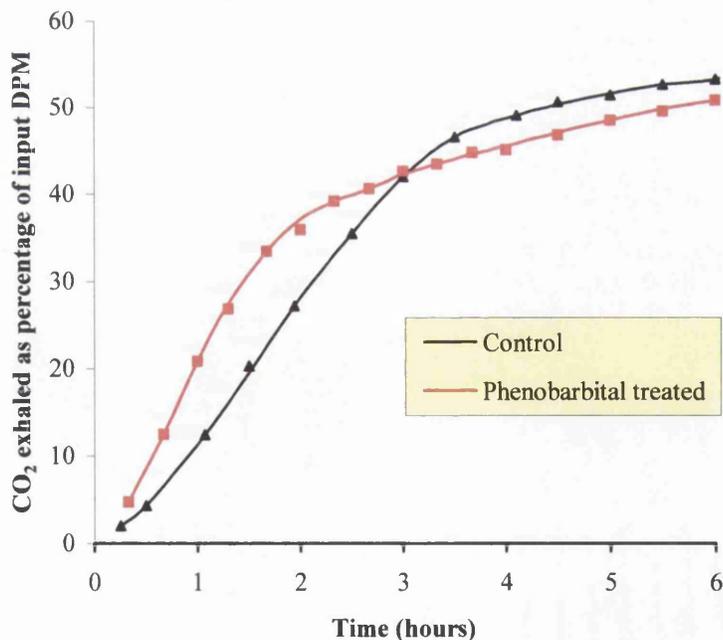
B.   Effect of phenobarbital pre-treatment on the metabolism of
N-nitrosomethyl-n-butylamine (517 µmol/kg body wt) in a whole rat.

**Figure 4.1   Effect of phenobarbital pre-treatment on nitrosamine metabolism
in the whole rat represented by counts exhaled as CO_2.** This is expressed as a
percentage of the input showing that the final proportion of $^{14}CO_2$ exhaled was
essentially the same in the phenobarbital-treated rats as in the control rats.

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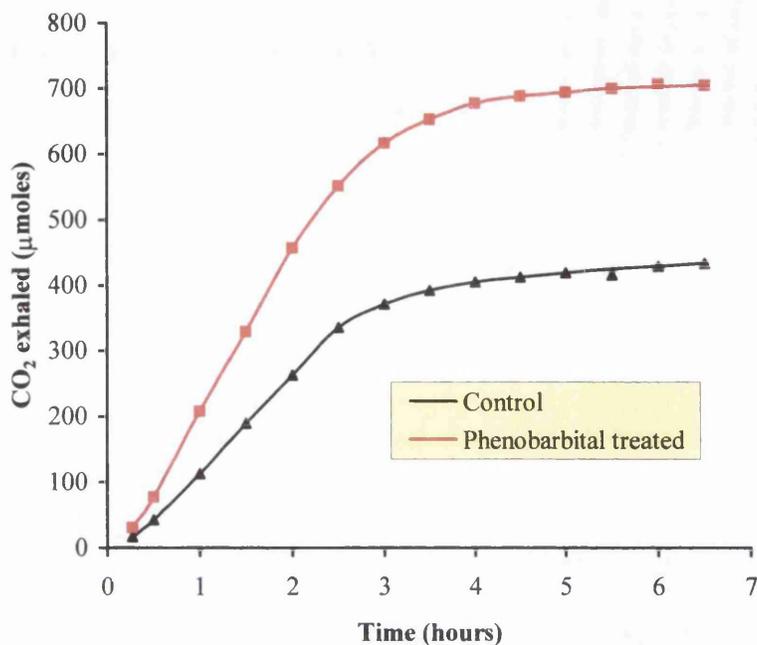
A.   Effect of phenobarbital pre-treatment on metabolism of
N-nitrosodimethylamine in the whole rat. Doses: control rat – 338 µmol/kg
body wt, phenobarbital-treated rat – 506 µmol/kg body wt.
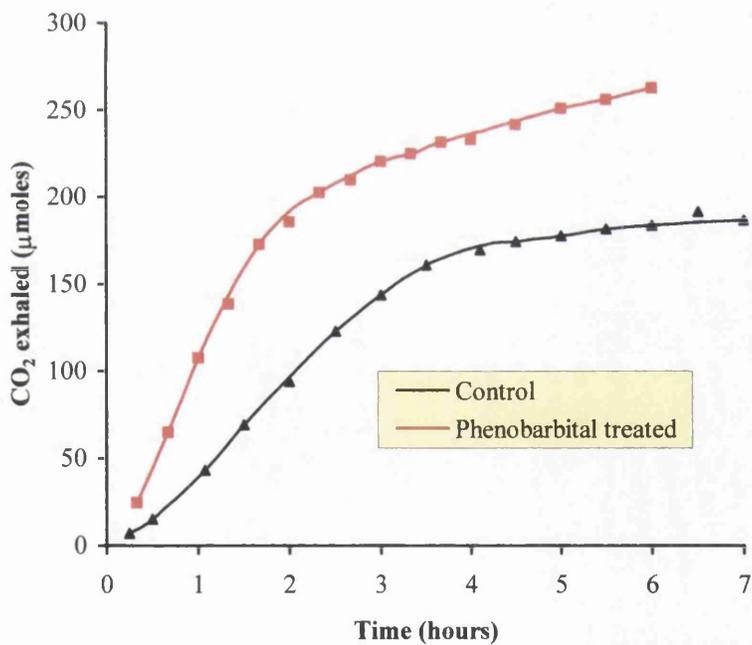
B.   Effect of phenobarbital pre-treatment on the metabolism of
N-nitrosomethyl-n-butylamine in the whole rat. Doses: control rat – 345
µmol/kg body wt, phenobarbital treated rat – 517 µmol/kg body wt.

**Figure 4-1   Effect of phenobarbital pre-treatment on nitrosamine metabolism in
the whole rat represented as µmoles of CO_2 exhaled.** The rate of metabolism of
N-nitrosodimethylamine and N-nitrosomethyl-n-butylamine during the linear period is
greater in the phenobarbital-treated rats than in the control rats.

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Table 4-1   Effect of phenobarbital pre-treatment on the P450 and protein content of liver microsomes and on the rate of metabolism of *N*-nitrosodimethylamine and *N*-nitrosomethyl-*n*-butylamine in the rat.

	Untreated	Phenobarbital-treated
Microsomal protein (mg/g tissue)	9.48	14.62
P450 content (nmol/mg protein)	7.08	23.31
Rate of NDMA Metabolism (μmol/kg/hour)	110	161
Rate of NMBA metabolism (μmol/kg/hour)	91	209

Table 4-2   Effect of phenobarbital pre-treatment on the methylation of liver DNA by nitrosamines. Each nitrosamine was administered at a dose of 474 μmol/kg body wt.

Nitrosamine administered	Phenobarbital pre-treatment	Alkylation of G (mmol 7meG*/mole guanine)	Percentage of control
NDMA*	No (Control)	5.24	-
	Yes	4.3	82
NMBA	No (Control)	1.5	-
	Yes	2.49	166

*NDMA - *N*-nitrosodimethylamine;   NMBA - *N*-nitrosomethyl-*n*-butylamine
7meG - 7-methylguanine

4.5   Discussion

The experiments reported in this chapter show that induction of CYP2B1/2B2 in the rat produces a 45% increase in the rate of metabolism of N-nitrosodimethylamine, but a 130% increase in the rate of metabolism of N-nitrosomethyl-n-butylamine. This indicates that the induced CYP2B1/2B2 has a strong preference for metabolism of the asymmetric N-nitrosomethyl-n-butylamine. However, it is important to note that these relatively small increases in metabolism of the nitrosamines used in this work were due to a 3-fold increase in total P450 and a likely 80-fold induction of CYP2B1/2B2. This would suggest that CYP2B1/2B2 itself is not the enzyme that gives the observed metabolism in the oesophagus, but probably a closely related enzyme. At a glance, the results obtained in the metabolism experiments with phenobarbital treated rats appear to be supported by the observations of other researchers (Lee *et al.*, 1989; Yang *et al.*, 1985). Yang *et al.* (1985) purified to apparent electrophoretic homogeneity different P450 isozymes from rats treated with various inducers and measured the demethylation of N-nitrosodimethylamine using a reconstituted enzyme system containing purified P450, NADPH:P450 reductase and dilauroylphosphatidylcholine. Using nitrosamine concentrations of 4 mM, they found that P450 isolated from phenobarbital treated rats, which we now know to be CYP2B1/2B2, was able to metabolise N-nitrosodimethylamine about 1.5 to 3 times faster than P450 from untreated rats. In the same experiment, P450 from phenobarbital treated rats metabolised N-nitrosomethyl-n-butylamine 7.5 times faster than P450 from ethanol treated rats (CYP2E1), but the metabolism of N-nitrosodimethylamine was carried out 38.5 times faster by CYP2E1 than by CYP2B1. Lee *et al.* (1989) also purified CYP2E1 and CYP2B1 from ethanol and phenobarbital treated rats respectively. They prepared a reconstituted enzyme system with the purified P450, NADPH:P450 reductase and dilauroylphosphatidylcholine and examined the formation of aldehydes from several methylalkylnitrosamines. They observed that debutylation of N-nitrosomethyl-n-butylamine occurred 2.2 times faster with CYP2B1 than with P450 from untreated rats. CYP2E1 had no effect on the rate of debutylation of N-nitrosomethyl-n-butylamine. In the experiments described in this chapter, metabolism of N-nitrosodimethylamine in phenobarbital treated rats was 1.45 times as fast as in the control rats, while metabolism of N-nitrosomethyl-n-butylamine in phenobarbital treated rats was 2.3 times as fast as in

the control rats. The results obtained by the other researchers (Lee *et al.*, 1989; Yang *et al.*, 1985) suggest that CYP2B1/2B2 is more efficient at metabolising *N*-nitrosodimethylamine than P450 purified from untreated rats. The results obtained in this thesis contradict this since a 3-fold increase in total P450 content did not produce as big an increase in the metabolism of *N*-nitrosodimethylamine in the whole rat, but only increased the metabolism by 45%.

The increased rate of metabolism of *N*-nitrosodimethylamine after phenobarbital pre-treatment is due to the larger amount of nitrosamine metabolising enzyme present. Since with *N*-nitrosodimethylamine, metabolism on either methyl group produces a methylating agent that is able to produce DNA adducts, increasing the overall rate of metabolism does not affect the amount of DNA methylation obtained, since the amount of methylating agent per mole of nitrosamine remains unchanged. This was confirmed with the DNA methylation experiments with untreated and phenobarbital treated rats (Table 4-2). There was a slight decrease in the level of 7-methylguanine in liver DNA. The small apparent decrease in the level of methylation in the phenobarbital treated rats may be a reflection of animal to animal variation, but a similar decrease has been reported in *in vitro* experiments by other researchers (Shu and Hollenberg, 1997). Thus, the difference is possibly worthy of more intensive study because the decrease could have been caused by an increase in denitrosation. In the case of *N*-nitrosomethyl-*n*-butylamine, phenobarbital pre-treatment again caused an increase in the rate of metabolism of the nitrosamine, but the increase was substantially greater than that seen with *N*-nitrosodimethylamine. Also, the phenobarbital pre-treatment produced a substantial increase in methylation of liver DNA by *N*-nitrosomethyl-*n*-butylamine (Table 4-2). The following calculations suggest that a major part of the 1.3-fold increase in overall metabolism of the nitrosamine involved activating hydroxylation of the butyl group rather than the inactivating hydroxylation of the methyl group.

If one assumes that the increase in the rate of metabolism of *N*-nitrosomethyl-*n*-butylamine is entirely due to CYP2B1/2B2 then 56% of the total dose would have been metabolised by CYP2B1/2B2 and 44% by CYP2E1. As the methylation produced by *N*-nitrosomethyl-*n*-butylamine in the control rat was 1.5 mmol 7meG/mole guanine, the amount of methylation in the phenobarbital treated rat would

be 0.44 × 1.5, i.e. 0.66 mmol 7meG/mole guanine. Therefore the 56% of the dose metabolised by CYP2B1/2B2 produced 1.83 mmol 7meG/mole guanine, i.e. the CYP2B1/2B2 produced 2.2 times as much activation per mole nitrosamine metabolised as did CYP2E1. If the induced CYP2B1/2B2 had metabolised the whole dose instead of just 56% of it, the expected methylation would have been 3.3 mmoles 7meG/mole guanine. However, even CYP2B1/2B2 did not produce from *N*-nitrosomethyl-*n*-butylamine as much methylating agent, on a mole for mole basis, as CYP2E1 produced from *N*-nitrosodimethylamine because CYP2E1 gave 5.24 mmol 7meG/mole guanine from *N*-nitrosodimethylamine.

These results support the suggestion that a CYP2B might be responsible for the activation of nitrosamines in the oesophagus. From the results of their experiments, Ji *et al.* (1989) postulated that although *N*-nitrosomethyl-*n*-amylamine is not normally carcinogenic for the liver, it could be carcinogenic for the livers of phenobarbital treated rats. The results reported in this thesis support this suggestion since phenobarbital pre-treatment increased the level of 7-methylguanine produced by *N*-nitrosomethyl-*n*-butylamine in rat liver.

CHAPTER 5   Conclusion

The importance of nitrosamines to oesophageal cancer has been investigated for many years, and with time there is increasing evidence for this.   After exhaustive examination of many possible carcinogens, nitrosamines remain the sole contender for the initiation of oesophageal carcinogenesis in humans.   They are the only carcinogens that will induce oesophageal tumours in laboratory animals and high levels of various nitrosamines have been detected in the urine of people in areas where the incidence of oesophageal cancer is high (reviewed by Craddock, 1993).

Nitrosamines are metabolically activated by cytochromes P450, resulting in the formation of an alkylating agent that reacts with DNA.   The activation of the nitrosamines must occur in the target organs in which the carcinogenic effect is observed because the alkylating agent is too short-lived to be transported to the target organs in the general circulation.   Methylation studies have shown that there must be at least one P450 that is expressed exclusively in the rat oesophagus (Ji *et al.*, 1991; von Hofe *et al.*, 1987) since certain nitrosamines produce a much higher level of methylation in this organ than in any other, including liver.

This thesis has been concerned with identifying the P450(s) in the rat oesophagus responsible for the metabolism of nitrosamines and showing that it is different from the P450(s) that metabolise the same nitrosamines in the liver.

It was shown by the methylation experiments that even though rat oesophagus contains only about 5% of the total amount of P450 present in rat liver, it is able to metabolically activate *N*-nitrosomethyl-*n*-butylamine to a much greater extent than liver (Chapter 2). It was also shown that whereas ethanol inhibited this metabolism in the liver, the metabolism in the oesophagus was enhanced. This was attributed to the inhibition of CYP2E1 in the liver, resulting in increased exposure to extra-hepatic organs. The metabolism in kidney, which contains the second highest amount of CYP2E1, was also inhibited by ethanol.

Experiments involving the metabolism of *N*-nitrosodimethylamine and *N*-nitrosomethyl-*n*-butylamine in the whole rat showed that these two nitrosamines are metabolised by the same P450, CYP2E1, in the liver. It is shown that, in part, the lower activation in the liver may be because the metabolism in the liver is by CYP2E1 and that this metabolism is predominantly deactivating. Since the rat oesophagus does not express CYP2E1, there must be a different P450 carrying out this metabolism in the oesophagus. Attempts at purification of the oesophageal P450 were unsuccessful because of the small size of the tissue and the little amount of P450 it contains. The yields of P450 from purification procedures are generally very low and it was realised that the oesophagi of several hundred rats would be required to achieve any meaningful purification. However, the low amount of P450 in the oesophagus suggested that there are probably only a few P450s expressed in this organ and its high activity towards certain nitrosamines also suggested that the nitrosamine-metabolising P450 would be the most abundant. The protein purification was planned to give a sufficiently pure sample for N-terminal sequencing for subsequent primer design and PCR, but since this appeared to be unlikely RT-PCR was carried out.

Using a combination of RT-PCR, RACE-PCR and cDNA library screening a novel P450 belonging to the CYP2B subfamily was identified in rat oesophagus. The deduced amino acid sequence of this P450 has 76 – 84% identity with other P450s of the CYP2B subfamily. Since CYP2B1 has been shown to metabolise nitrosamines that are capable of inducing oesophageal tumours, it is possible that this P450 is the nitrosamine-metabolising P450 in the rat oesophagus. To establish this, however, several more experiments need to be carried out.

Since the work described in this thesis was completed, a gridded rat genomic library from the Human Genome Mapping Project (HGMP) was screened with the cDNA of the oesophageal CYP2B. The library contained 60,000 clones and of these only 22 hybridised to the cDNA. The hybridisation to 3 of these clones was particularly strong and it is possible that these clones contain the gene for this P450. It would be interesting to obtain the 5′-flanking sequence of this gene, since it may be possible to find the sequences that confer oesophagus selective expression of this P450. It will

also be necessary to express this P450 in insect cells using a baculovirus vector to determine whether the expressed P450 will indeed metabolise nitrosamines that induce oesophageal cancer. If it does not, it will be necessary to investigate the possibility that other P450s are expressed in the rat oesophagus.

Due to the high similarity in the sequences of P450s belonging to any one subfamily, it was not possible to measure the specific expression in the oesophagus of the novel P450 by Northern blotting. However, this P450 has a unique C-terminal amino acid sequence and since the completion of the work described in this thesis an antibody has been raised by immunising chicken with a peptide from this region. Other researchers have successfully raised antibodies to C-terminal peptides of other P450s (Adams *et al.*, 1997; Edwards *et al.*, 1995). Attempts can be made at using this antibody for Western blotting, and since the sequence is unique to the oesophageal P450, there should be no cross-reaction with known P450s in other organs. Also once the full-length protein has been successfully expressed in a baculovirus system, animals can be immunised to raise antibodies.

It is not always possible to extrapolate results from experiments in rats to humans because of the interspecies differences. It would be possible to use samples obtained from humans after oesophagectomy to carry out similar experiments to those described, but a recent report has shown that there are large interindividual differences in the expression of P450s in human oesophageal samples (Lechevrel *et al.*, 1999). In part, this may reflect the fact that most of the samples come from old people. It is well known that the expression of P450 decreases markedly in old age (Sotaniemi *et al.*, 1997). However, one would expect the gene to be strongly conserved among humans and subhuman primates. It is therefore possible that sub-human primates could be used in similar studies since they are bred in more controlled environments, and as there are large experimental colonies in the USA it should be possible to get samples from young adults.

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