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CHARACTERIZATION OF SHEEP LIVER AND LUNG MICROSOMAL ETHYLMORPHINE *N*-DEMETHYLASE

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Abstract—1. Ethylmorphine *N*-demethylase activity of the sheep liver and lung microsomes was reconstituted in the presence of solubilized microsomal cytochrome P-450, NADPH–cytochrome *c* reductase and synthetic lipid, phosphatidylcholine dilauroyl.

2. The K_m of the lung microsomal ethylmorphine *N*-demethylase was calculated to be 4.84 mM ethylmorphine from its Lineweaver–Burk graph and lung enzyme was inhibited by its substrate, ethylmorphine, when its concn was 25 mM and above, reaching to 67% inhibition at 50 mM concn.

3. The Lineweaver–Burk and Eadie–Hofstee plots of the liver enzyme were found to be curvilinear. From these graphs, two different K_m values were calculated for the liver enzyme as 4.17 mM and 0.40 mM ethylmorphine.

4. Ethylmorphine *N*-demethylase activities of both liver and lung microsomes were inhibited by $NiCl_2$, $CdCl_2$ and $ZnSO_4$. Ethylalcohol inhibited *N*-demethylation of ethylmorphine in lung and liver microsomes.

5. Acetone (5%) slightly enhanced the *N*-demethylase activity of the liver enzyme, whereas 5% acetone completely inhibited the lung enzyme.

6. Phenylmethylsulfonyl fluoride at 0.10 mM and 0.25 mM concn had no effect on liver enzyme activity, while at these concns, it inhibited the activity of the lung enzyme by about 35%.

INTRODUCTION

Ethylmorphine *N*-demethylase is a membrane-bound enzyme belonging to the group of enzymes known as cytochrome P-450 dependent microsomal monooxygenases or mixed-function oxidases. In 1970 Lu *et al.* first described the reconstitution of liver microsomal ethylmorphine *N*-demethylase activity of rat by combining solubilized microsomal cytochrome P-450, NADPH–cytochrome *c* reductase, and lipid fractions. Mixed-function oxidases are responsible for the metabolism of a variety of endogenous compounds such as steroids, fatty acids, cholesterol, bile acids and exogenous substances such as drugs, insecticides, carcinogens, and many other compounds (Lu and Coon, 1968; Lu *et al.*, 1970; Ryan *et al.*, 1975; Arınç and Philpot, 1976; Gielen *et al.*, 1976). The rate at which these various compounds are metabolized by this enzyme system varies widely and depends on the species, strain, age, tissue, nutritional status, and pretreatment of the animal (Lu and Levin, 1974; Conney and Burns, 1972).

Takemori and Mannering (1958) showed that mice liver microsomes contained highly active ethylmorphine *N*-demethylase. Holtzman *et al.* (1968) reported that the rate of *N*-demethylation of ethylmorphine was significantly higher in rabbit liver smooth endoplasmic reticulum than in the rough. Alvares and Mannering (1970) and Holtzman and Rumack (1973) studied the kinetic properties of liver microsomal ethylmorphine *N*-demethylase in Holtzman and Sprague-Dawley rats, respectively.

Ethylmorphine *N*-demethylase activity of rat, mouse, guinea-pig and rabbit liver microsomes and the influence of sex on liver ethylmorphine *N*-demethylase activity of these animals were studied by Nerland and Mannering (1978). From these studies they concluded that, except for mature rats, the animals did not exhibit enzymatic activity difference related with sex. Nerland and Mannering (1978) also found that *N*-demethylation capacity of rat, mouse, guinea-pig and rabbit liver microsomes were varied over a four-fold range.

Recently, there has been a great deal of interest in the study of drug metabolism in extrahepatic tisues such as intestine, lung, and kidney (Arınç and Philpot, 1976; Wattenberg *et al.*, 1962; Oppelt *et al.*, 1970; Bend *et al.*, 1972; Chhabra *et al.*, 1974; Gram, 1980; Arınç and İşcan, 1983; İşcan *et al.*, 1984). The study of detoxication–toxication mechanisms in extrahepatic tissues is important since some of these tissues can be the portal entry for environmental pollutants, and it is important to know the fate of these chemicals in these tissues.

The hepatic and pulmonary mixed-function oxidase system has been extensively studied in smaller laboratory animals especially in rat and rabbit. However, very little information is available on the metabolism of foreign compounds by larger animals such as sheep, even though sheep liver has a very high nutritional value and is extensively consumed by man. Gourley *et al.* (1977) reported the basal values for hepatic microsomal aniline 4-hydroxylase, *p*-nitroreductase, aminopyrine *N*-demethylase and *p*-nitroanisole *O*-demethylase activities in untreated, phenobarbital treated, and vitamin E-deficient Merino sheep of Australia.

Recently, in our laboratory some kinetic properties of sheep liver and lung microsomal aniline 4-hydroxylase were studied and microsomal aniline 4-hydroxylase activity of these tissues was reconstituted in the presence of cytochrome P-450, NADPH–cytochrome *c* reductase and synthetic lipid, phosphatidylcholine dilauroyl (Arınç, 1980).

In the present study, some properties of liver and lung microsomal ethylmorphine *N*-demethylase of sheep are described, compared and microsomal ethyl-

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morphine *N*-demethylase activity of these organs was reconstituted in the presence of cytochrome P-450, NADPH–cytochrome *c* reductase, and lipid.

MATERIALS AND METHODS

Chemicals

NADPH, NADH, $NADP^+$, NAD^+, bovine serum albumin (BSA), horse heart cytochrome *c* (Type VI), phenylmethylsulfonyl fluoride (PMSF) DL-dithiothreitol (DTT), D-glucose-6-phosphate monosodium salt, 3α, 7α 12α-trihydroxycholanic acid, sodium salt (cholic acid, sodium salt), yeast D-glucose-6-phosphate dehydrogenase (Type XI), *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), phosphatidylcholine dialauroyl, sodium deoxycholate and EDTA were purchased from Sigma Chemical Co., St. Louis, MO, USA. *p*-Aminophenol and formaldehyde were obtained from Fluka A.G., Buchs S.G., Switzerland. Carbon monoxide and aniline (99% pure) were purchased from Fisher Scientific Company, Fairlawn, NJ, USA. Glycerol and sodium dithionite ($Na_2S_2O_4$) were the products of E. Merck, Darmstadt, Germany. Ethylmorphine (Dionine) was obtained from Toprak Mahsülleri Ofisi, İstanbul, Turkey. All other chemicals were of the analytical grade and were obtained from commercial sources at the highest grade of purity available.

Isolation of microsomes

The livers and lungs from well bled Akkaraman sheep about 8–12 months old were obtained from the slaughterhouse (Et ve Balık Kurumu/Ankara) immediately after killing and were placed in plastic bags packed in crushed ice (gall bladders were excised from the livers and discarded in the slaughter-house). All subsequent steps were carried out at 0–4 C. The livers and lungs were homogenized in 0.15 M KCl and microsomal fractions were isolated by differential centrifugation (Arınç and İşcan, 1983). The microsomes were washed by homogenization in 0.15 M KCl containing 1 mM EDTA, collected by centifugation, suspended in 25% glycerol. Enzymatic reaction rates and cytochrome P-450 concn in microsomes were determined with these freshly made preparations. Additional microsomal suspensions in small aliquots were gassed with nitrogen and were immediately immersed into a tank containing liquid nitrogen and were kept in liquid nitrogen up to two weeks.

Preparation of solubilized cytochrome P-450 and NADPH–cytochrome c reductase fractions

Lung cytochrome P-450 was solubilized by treatment of microsomes with sodium cholate as described by Arinç and Philpot (1976). The solubilized microsomal fraction was fractionated with ammonium sulfate. Those fractions precipitating between 25 and 43% and 43 and 50% saturation of the supernatant solution with ammonium sulfate contained cytochrome P-450 and reductase, respectively. These reductase fractions will be referred to as cholate-reductase.

Liver microsomes were solubilized by sodium cholate under the same conditions used for lung microsomes and fractionated by ammonium sulfate. The fractions of 25 and 35% and 35 and 50% ammonium sulfate contained high concns of cytochrome P-450. Since the latter fraction also contained high cytochrome *c* reductase activity, it was discarded. Cytochrome *c* reductase was solubilized by treatment of microsomes with deoxycholate as described (Philpot and Arinç 1976). Microsomes were then subjected to ammonium sulfate fractionation. The fraction containing high reductase activity was precipitated between 25 and 50% saturation of the solution with ammonium sulfate. These reductase fractions will be referred to as deoxycholate-reductase.

Analytical procedures

The protein content of microsomes were determined by the procedure of Lowry *et al.* (1951). Crystalline bovine serum albumin was used as a standard.

Aniline 4-hydroxylase activity was measured by the quantitation of *p*-aminophenol (pAP) as described by Imai *et al.* (1966). Optimized assay conditions were used for determination of sheep liver and lung microsomal aniline 4-hyroxylase activity. Details of the assay conditions were given by Arinç and İşcan (1983).

Ethylmorphine *N*-demethylase activity was determined by measuring the quantity of formaldehyde formed according to the method of Nash (1953) as modified by Cochin and Axelrod (1959). In general, NADPH generating system is used as a cofactor. Assay conditions for lung and liver enzymes were optimized as described in "Results". Typical optimized assay mixtures contained 15 mM ethylmorphine, 100 mM HEPES buffer pH 7.7 (at 25 C), NADPH generating system consisting of 0.25 mM $NADP^+$, 2.5 mM $MgCl_2$, 2.5 mM glucose 6-phosphate, 0.5 unit of glucose 6-phosphate dehydrogenase (EC 1.1.1.49. 1 unit of glucose 6-phosphate dehydrogenase reduces 1 μmol of $NADP^+$ per min at 25°C), 14.6 mM HEPES buffer pH 7.8 (at 25 C), and 1–2 mg of liver microsomal protein or 2–4 mg of lung microsomal protein in a final vol of 1.0 ml.

The tubes containing formaldehyde standards, appropriate blanks (tissue-blank, zero time blank, no cofactor-blank) and microsomal incubation mixtures containing no NADPH generating system were placed in a shaking water-bath at 37°C. The reaction was initiated with addition of NADPH generating system, and carried out for 15 min aerobically with moderate shaking. Enzymatic reaction was stopped by addition of 1.0 ml of 0.75 N perchloric acid. The denatured protein was collected by centrifugation at 7500 *g* for 20 min. A 1.0 ml aliquot of the supernatant solution containing formaldehyde was removed and was mixed with freshly prepared 0.75 ml double Nash reagent. The mixture was incubated at 50°C for 10 min in a water-bath and the intensity of yellow color was measured at 412 nm.

Cytochrome P-450 was determined by carbon monoxide-difference spectra of dithionite-reduced microsomes on a Cary-14 spectrophotometer using an extinction coefficient of 91 $mM^{-1}cm^{-1}$ for A_{450}–A_{490} as described by Omura and Sato (1964a, b).

NADPH–cytochrome *c* reductase activity was determined spectrophotometrically with and without 1 mM KCN in 0.1 M potassium phosphate buffer, pH 7.8 at 25°C using the procedure of Masters *et al.* (1967) on a GCA/Mc Pherson Model EU-707-11 double beam spectrophotometer, equipped with GCA/Mc Pherson Model EU-205-11 strip chart recorder. One unit of reductase was defined as the amount of enzyme catalysing reduction of 1 nmol cytochrome *c* under the described conditions.

RESULTS

Ethylmorphine N-demethylase activities of sheep liver and lung microsomes

Ethylmorphine *N*-demethylase activities of different liver microsomal preparations were found to be 3.10, 4.20, 5.10, 3.72, 3.75, 5.18, 1.29, 2.80, 2.10, 3.61, 1.42 nmol formaldehyde formed per min per mg of microsomal protein. The average specific activity was calculated to be 3.30 ± 1.32 (mean ± SD, $N = 11$) as opposed to 1.32 ± 0.43 (mean ± SD, $N = 11$) for lung microsomes. *N*-Demethylase activities of different lung microsomal preparations was determined as 0.83, 0.67, 1.53, 1.51, 2.00, 1.60, 1.70, 1.65, 0.80, 1.12, 1.08 nmol formaldehyde/min/mg protein. Although, in general, ethylmorphine *N*-demethylase activity of liver microsomes was higher than that of lung microsomes, in some microsomal preparations enzymatic activity of lung was comparable to that of liver. The sp. act. of

both liver and lung microsomes remained constant at least for 2 weeks when the microsomes were suspended in 25% glycerol, gassed with nitrogen and stored in liquid nitrogen. However, enzymatic activities of the both preparations were lost by 40% when nitrogen-gassed microsomes were stored at −20°C for 24 hr.

Based on the ethylmorphine *N*-demethylase activity per mg of microsomal protein, sheep liver enzyme activity was found to be similar to liver enzyme activity of guinea-pigs (Nerland and Mannering, 1978, İşcan *et al.* 1984) and of immature rats (Nerland and Mannering, 1978). On the other hand, sheep lung ethylmorphine *N*-demethylase activity was higher than that of guinea-pigs (İşcan *et al.*, 1984).

Effect of pH on liver and lung microsomal ethylmorphine N-*demethylase*

Figure 1 shows the effect of pH on the lung and liver microsomal ethylmorphine *N*-demethylase activity. The maximal activity of the lung enzyme was exhibited over a rather broad pH range over 7.4 to 8.2, while for the liver enzyme the optimum activity was observed between pH 7.6 and 7.8.

Effect of incubation period and microsomal protein amount on the rate of liver and lung ethylmorphine N-*demethylation*

The effect of varying incubation period on enzyme activities is shown in Fig. 2. The time course of ethylmorphine *N*-demethylation by liver and lung microsomes was linear for 20 min under the conditions used.

As seen in Fig. 3 the rate of ethylmorphine *N*-demethylation was linear with protein concns up to approx 3 mg protein/ml and 6 mg protein/ml incubation mixture for liver and lung microsomes, respectively.

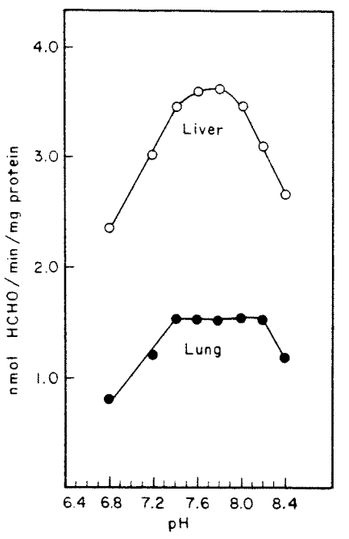

Fig. 1. Effect of pH on sheep liver and lung microsomal ethylmorphine *N*-demethylase activity. Incubations were carried out at optimum conditions. The point are means of 3–5 determinations.

Cofactor requirements of sheep liver and lung microsomal ethylmorphine N-*demethylase*

The NADPH generating system was required for maximal activity of ethylmorphine *N*-demethylase in liver and lung microsomes. It was found that NADPH alone could completely replace the NADPH generating system for liver *N*-demethylase activity. On the other hand, in lung microsomes NADPH alone supported only 65% of the activity seen with NADPH generating system (Table 1). However, as will be discussed later, in the presence of 2.5–10 mM $MgCl_2$ NADPH could completely replace lung microsomal ethylmorphine *N*-demethylase activity. As seen in Table 1, similar results were obtained when NADPH and NADH were used together. When other cofactors tabulated in Table 1 were used there was negligible enzyme activity in liver or lung microsomes. Formation of formaldehyde was also low when boiled microsomes were used and none was formed when cofactors were omitted.

Kinetics of ethylmorphine N-*demethylation in lung and liver microsomes*

Figure 4 illustrates substrate saturating curve for ethylmorphine *N*-demethylase of sheep lung microsomes. Lung enzyme was saturated by its substrate at approx 10 mM ethylmorphine concn. But the activity of the lung enzyme was inhibited by ethylmorphine when its concn was 25 mM and above. Ethylmorphine, at 40 and 50 mM concns, inhibited the lung enzyme activity by about 33 and 67%, respectively. The apparent K_m value was calculated as 4.84 mM ethylmorphine and V_{max} 2.22 nmol formaldehyde min mg protein from the Lineweaver–Burk plot shown in Fig. 5.

Formaldehyde formation during aerobic incubation of ethylmorphine with liver microsomes did not follow Michaelis–Menten kinetics. Both Lineweaver–Burk (Fig 6) and Eadie–Hofstee (Fig. 7) plots of liver ethylmorphine *N*-demethylase were curvilinear. The apparent K_m values were calculated as 4 and 0.39 mM ethylmorphine from Lineweaver–Burk plot and 4.35 and 0.40 mM ethylmorphine from Eadie–Hofstee plot.

In vitro *effects of divalent metal ions on liver and lung ethylmorphine* N-*demethylase activity*

The liver and lung microsomes used for effects of metal ions were additionally washed with 0.0013 M HEPES buffer, pH 7.7, containing 50 mM EDTA, to remove the endogenous metals which may be present in the microsomes and may affect the results.

Effect of magnesium ions

The effect of magnesium on ethylmorphine activity of both liver and lung microsomes was studied by adding various concns of magnesium chloride, ranging from 1.25 mM to 100 mM, to the assay mixture.

As seen in Fig. 8A when NADPH alone was used as a cofactor, magnesium ion had stimulatory effect on lung ethylmorphine *N*-demethylase activity at 1.25 to 10 mM concns. 2.5, 5, 10 mM magnesium ions were found to give maximal rate of *N*-demethylation. In the

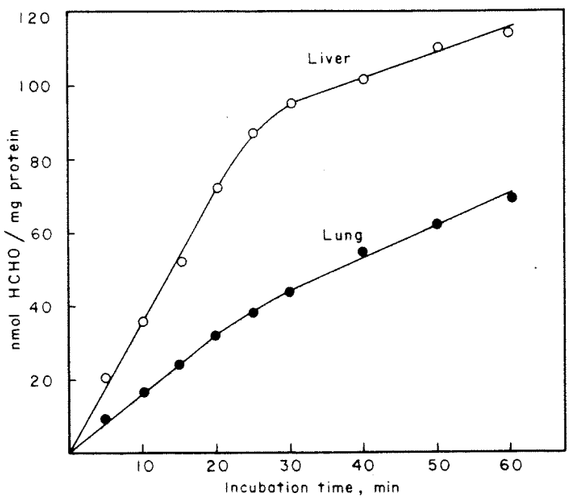

Fig. 2. Effect of incubation period on sheep liver and lung microsomal ethylmorphine *N*-demethylase activity under the optimum conditions. The points are means of duplicate determinations.

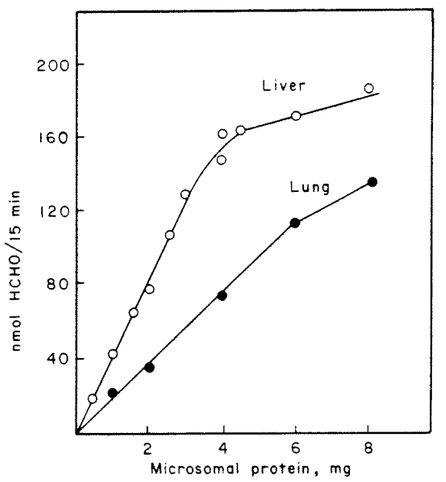

Fig. 3. Effect of microsomal protein amount on the rate of ethylmorphine *N*-demethylation of sheep liver and lung microsomes. The points are means of duplicate determinations.

Table 1. Cofactor requirements of sheep liver and lung microsomal ethylmorphine N-demethylase

Cofactor	Ethylmorphine *N*-demethylase Liver % Activity	Lung % Activity
NADPH generating system*	100.0	100.0
NADPH†	100.0	65.0
NADPH + $MgCl_2$‡	100.0	100.0
NADPH + NADH§	100.0	67.0
NADPH + NADH + $MgCl_2$‡	100.0	100.0
NADH†	11.3	4.5
NADP†	11.3	4.5
NAD†	11.3	1.5
Boiled microsomes‖	11.3	8.3
No substrate	11.3	7.0
No microsomes	11.3	8.3
No cofactor	0.0	0.0

*Incubation conditions were as described under "Materials and Methods".
†NADPH generating system was replaced by NADPH (0.5 mM in assay mixture or equivalent concns of other nucleotides in assay mixtures.
‡In the presence of 2.5 mM $MgCl_2$ for lung microsomes.
§NADPH 0.5 mM, NADH 0.5 mM or NADPH 0.25 mM, NADH 0.25 mM.
‖In the presence of NADPH generating system.

absence of magnesium ion, NADPH supported the *N*-demethylation of ethylmorphine about 65% when compared with the activity obtained by NADPH generating system. As illustrated in Fig. 8B, when NADPH generating system was used, magnesium ions at 1.25 mM to 10 mM concns did not affect activity of the lung enzyme. However, magnesium ion at 25 mM or above concns inhibited the lung enzyme in the presence of NADPH or NADPH generating system (Fig. 8B).

On the other hand, magnesium ion 1.25 to 100 mM concns had no effect on liver *N*-demethylase activity in the presence of NADPH generating system or NADPH.

Effects of nickel, cadmium and zinc ions

The effects of varying concns of added $NiCl_2$, $CdCl_2$ and $ZnSO_4$ on liver and lung microsomal ethylmorphine *N*-demethylase activity are given in Table 2. All

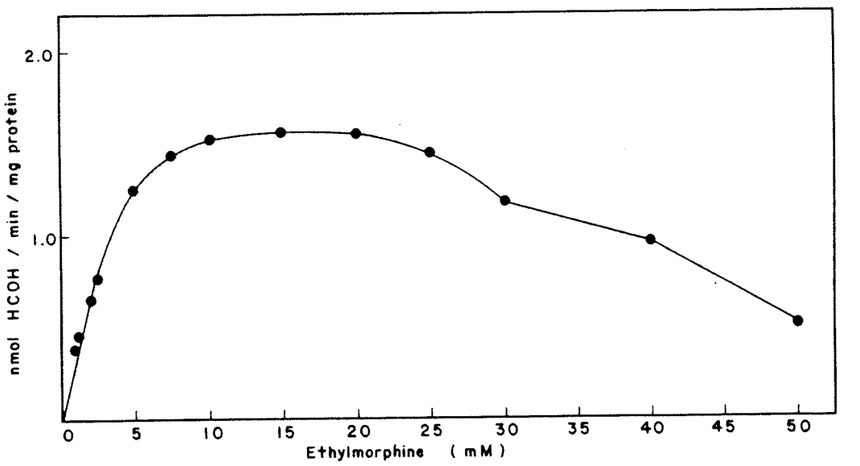

Fig. 4. Substrate saturation curve for sheep lung microsomal ethylmorphine under the optimum conditions. The points are the averages of two different sets of data and each point is the mean of duplicate determination.

metal ions strongly inhibited the activity of the enzymes in both tissues. Nickel ions at 0.5, 1.0 and 5 mM concns inhibited the liver ethylmorphine *N*-demethylase activity by 26, 34 and 50%, respectively, whereas the same metal ion concns caused 48, 84 and 100% inhibition, respectively for the lung enzyme.

As seen in Table 2, 1 mM $CdCl_2$ completely inhibited the ethylmorphine *N*-demethylase activity of both tissues. When 0.10 mM $ZnSO_4$ was added to the assay mixture only lung enzyme was inhibited by 40%, and 0.25 mM zinc sulfate was sufficient to inhibit ethylmorphine *N*-demethylase activity in lung, whereas 0.5 mM $ZnSO_4$ was required for complete inhibition of the liver enzyme.

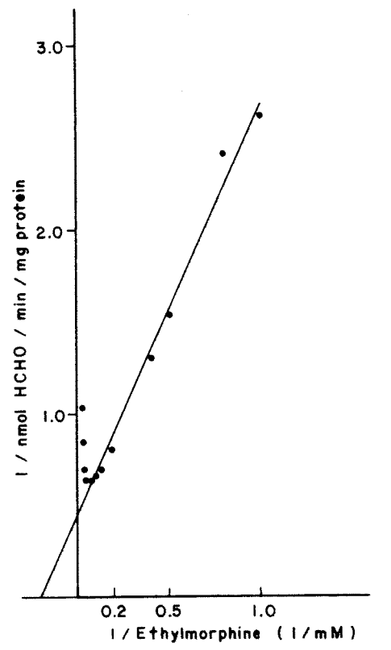

Fig. 5. Lineweaver–Burk plot of sheep lung microsomal ethylmorphine *N*-demethylase. The apparent K_m value was 4.84 mM ethylmorphine and V_{max} 2.22 nmol HCOH/min/mg protein. The points were fitted to a line by least-squares method.

In vitro *effects of ethylalcohol, acetone and PMSF on liver and lung ethylmorphine* N-*demethylase activity*

Ethylalcohol at 5 and 10% (v/v) concns inhibited ethylmorphine *N*-demethylase activity of both liver and lung enzymes (Table 3). Acetone at 5–15% (v/v) final concns had a slightly stimulatory effect on liver microsomal ethylmorphine *N*-demethylase activity while in the presence of 5% acetone, activity of the lung enzyme was inhibited completely (Table 3).

PMSF, proteolytic enzyme inhibitor, has been widely used during the solubilization and purification of NADPH cytochrome *c* reductase from microsomes (Dignam and Strobel, 1977; Mayer and Durrant, 1979; Kobayashi and Rikans, 1984). Thus the effects of PMSF on liver and lung ethylmorphine *N*-demethylase activity were examined. PMSF at 0.1 and 0.25 mM concns had no effect on liver enzyme activity while at these concns it inhibited lung enzyme activity by about 35% (Table 3).

Reconstitution of liver and lung microsomal ethylmorphine N-*demethylase activity*

The reaction rates of ethylmorphine *N*-demethylation in sheep lung and liver microsomal reconstituted systems are given in Table 4. Lung cholate-reductase supported ethylmorphine *N*-demethylase activity in the presence of lung or liver cytochrome P-450. However, liver deoxycholate-reductase was inactive in supporting ethylmorphine *N*-demethylase activity

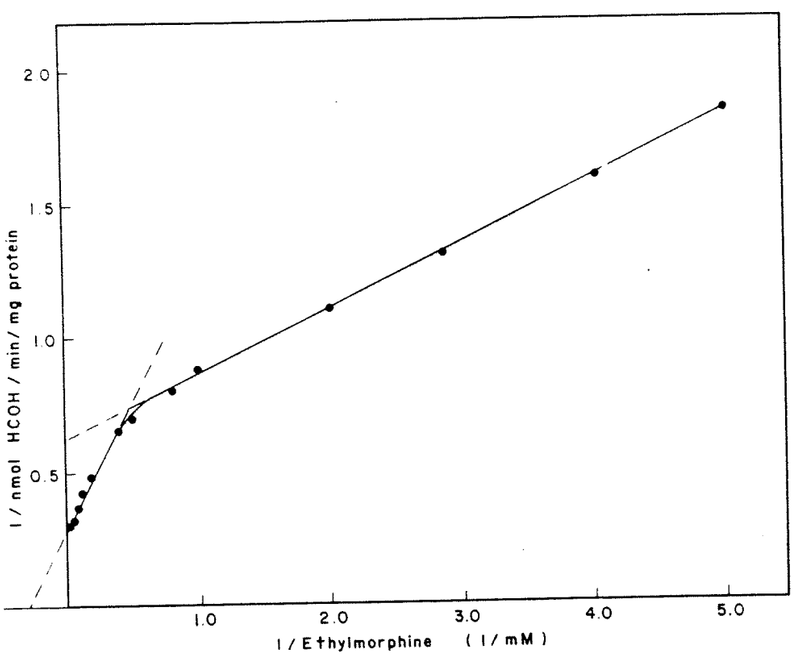

Fig. 6. Lineweaver–Burk plot of the sheep liver microsomal ethylmorphine *N*-demethylase. The apparent K_m values were 4.00 and 0.39 mM ethylmorphine and the apparent V_{max} values were 4.00 and 1.61 nmol HCOH/min/mg protein.

when combined with liver or lung cytochrome P-450, and this was not changed in the presence of synthetic lipid, phosphatidycholine dilauroyl. Similar results obtained during reconstitution of aniline 4-hydroxylase activities of sheep lung and liver microsomes were also given in Table 4. The results obtained in this study showed that sheep lung and liver microsomal ethylmorphine *N*-demethylase activities can be reconstituted in the presence of solubilized cytochrome P-450, NADPH–cytochrome *c* reductase and synthetic lipid, phosphatidylcholine dilauroyl.

DISCUSSION

Mixed-function oxidations of drugs and other compounds by microsomal preparations from different tissues or animals are, in general, similar. This metabolic process involves the participation of cytochrome P-450 and NADPH–cytochrome *c* reductase and requires oxygen and NADPH for activity. In our laboratory, lung and liver microsomal ethylmorphine *N*-demethylase (in this study) and aniline 4-hydroxylase (Arınç, 1980) activities of sheep were reconstituted by combining detergent solubilized cytochrome P-450, NADPH–cytochrome *c* reductase and synthetic lipid, phosphatidylcholine dilauroyl.

Some properties of microsomal ethylmorphine *N*-demethylase of sheep lung and liver were found to be similar. Their cofactor requirements, pH optima, time–activity relationships, and responses to nickel, cadmium, and zinc ions show this similarity. Similar results were also obtained for sheep lung and liver microsomal aniline 4-hydroxylase (Arınç and İşcan, 1983).

On the other hand, some differences were observed in lung and in liver microsomal ethylmorphine *N*-demethylase enzymes. In lung NADPH alone could only support 65% of the activity obtained by the generating system and in the presence of 2.5 to 10 mM $MgCl_2$ and saturating levels of NADPH, activity of the lung enzyme increased about 35% equalling the activity supported by NADPH generating system (Fig. 8A). Magnesium ion at 1.25 to 100 mM concentrations had no effect on liver enzyme activity in presence of NADPH generating system or NADPH whereas magnesium ion at 25 mM concentrations and above markedly inhibited the activity of the lung enzyme (Fig. 8A, B). 100 mM $MgCl_2$ inhibited the activity of the lung enzyme by 55 and 70% in the presence of NADPH generating system and NADPH, respectively (Fig. 8A and B). These results were in contrast to those obtained with sheep lung and liver aniline 4-hydroxylase enzymes (Arınç and İşcan, 1983).

It was observed that NADPH alone could completely replace the NADPH generating system for lung and liver aniline 4-hydroxylase activity and magnesium ion had a stimulatory effect on both liver and lung microsomal aniline 4-hydroxylase activities (Arınç and İşcan, 1983).

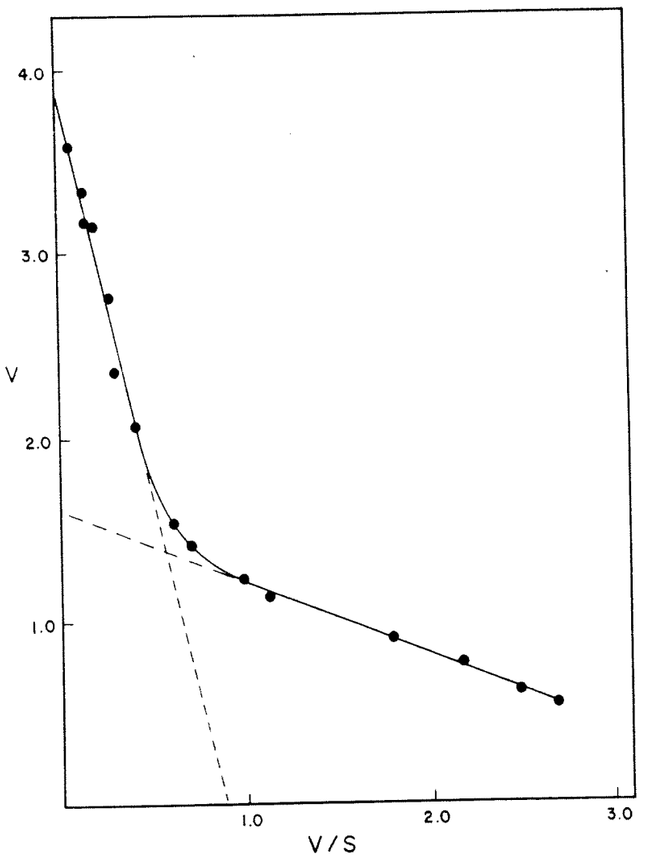

Fig. 7. Eadie–Hofstee plot of the sheep liver microsomal ethylmorphine *N*-demethylase. The apparent K_m values were 4.35 and 0.40 mM, and the apparent V_{max} values were 3.88 and 1.61 nmol HCOH/min mg protein.

Marked differences were noted in the kinetic properties of sheep lung and liver ethylmorphine *N*-demethylase enzymes. The Lineweaver–Burk (Fig. 6) and Eadie–Hofstee (Fig. 7) plots of the liver enzyme were found to be curvilinear, suggesting that the liver enzyme did not follow Michaelis–Menten kinetics. From these graphs, two different K_m values were calculated for the sheep liver enzyme as 4.18 and 0.40 mM ethylmorphine. The apparent V_{max} values were 3.94 and 1.61 nmol HCOH min/mg protein. These results may suggest the presence of two enzymes or two forms of the enzyme or one enzyme with two different active sites. This finding is in contrast to the reports of several investigators who studied kinetics of ethylmorphine *N*-demethylation in rat liver microsomes and found that enzyme followed simple Michaelis–Menten kinetics by giving straight line when double-reciprocal plot of ethylmorphine vs activity of the enzyme was plotted. (Alvares and Mannering, 1970; Hildebrandt and Estabrook, 1971; Holtzman and Rumack, 1973; Thompson and Holtzman, 1977.) The apparent K_m values calculated from Lineweaver–Burk plots were 0.26 mM (Alvares and Mannering, 1970), 0.19 mM (Hildebrandt and Estabrook, 1971), 0.25 mM (Holtzman and Rumack, 1973; Thompson and Holtzman, 1977) for male rat liver microsomal enzyme and 0.64 mM (Holtzman and Rumack, 1973) for female rat liver microsomal enzyme.

One reason for this controversy is likely to be due to the concentration of the substrate, ethylmorphine, used in kinetic studies of the enzyme. In the present study, ethylmorphine concentration ranging from 0.2 mM to 50 mM was used for kinetic studies of sheep liver and lung microsomal ethylmorphine *N*-demethylation. Lineweaver–Burk and Eadie–Hofstee plots (Figs 6, 7) show non-linearity at 2–2.5 mM ethylmorphine concentration. On the other hand, highest ethylmorphine concentration used by other investigators was 2 mM and K_m value, 0.40 mM calculated from

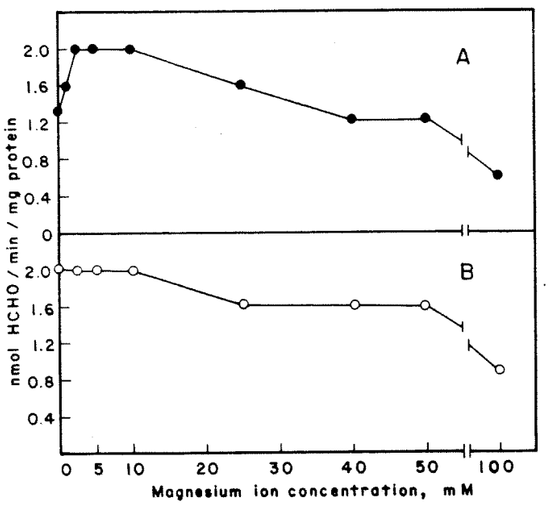

Fig. 8. Effect of magnesium ion concn on sheep lung microsomal ethylmorphine *N*-demethylase activity (A) NADPH was used as a cofactor; (B) NADPH generating system was used as a cofactor. The points are the averages of two different sets of data and each point is the mean of duplicate determinations.

Table 2. Effects of $NiCl_2$, $CdCl_2$ and $ZnSO_4$, added *in vitro*, on sheep liver and lung microsomal ethylmorphine *N*-demethylase activity

		Liver* % Activity	Lung* % Activity
Control†		100	100
0.1 mM	$NiCl_2$	100	100
0.5 mM	$NiCl_2$	74	42
1.0 mM	$NiCl_2$	66	16
5.0 mM	$NiCl_2$	50	0
10.0 mM	$NiCl_2$	18	0
20.0 mM	$NiCl_2$	18	
0.1 mM	$CdCl_2$	44	100
0.5 mM	$CdCl_2$	3	6
1.0 mM	$CdCl_2$	0	0
0.05 mM	$ZnSO_4$	100	66
0.10 mM	$ZnSO_4$	100	60
0.25 mM	$ZnSO_4$	45	0
0.50 mM	$ZnSO_4$	0	0

*Each value represents the averages of two different sets of data which was determined in duplicate. Microsomes containing *in vitro* added metal ions in the reaction medium were centrifuged twice at 7500 *g* following the addition of 0.75 N perchloric acid.
†In the presence of generating system.

Table 3. Effects of ethylalcohol, acetone and PMSF added *in vitro* on sheep liver and lung microsomal ethylmorphine *N*-demethylase activity

		Liver* % Activity	Lung** % Activity
Control†		100.0	100.0
Ethylalcohol‡	5%‡	20.0	0.0
Ethylalcohol‡	10%‡	0.0	0.0
Acetone	5%	110.0	0.0
Acetone	10%	108.0	0.0
Acetone	12.5%	109.0	0.0
Acetone	15%	108.0	0.0
PMSF	0.1 mM	100.0	65.0
PMSF	0.25 mM	100.0	65.0

*Each value represents the averages of two different sets of data which was determined in duplicate.
†In the presence of generating system.
‡No effect on Nash reaction.

the graphs given in Fig. 6 and Fig. 7 up to 2 mM ethylmorphine concentration is in agreement with those reported for rat liver microsomes (Alvares and Mannering, 1970; Hildebrandt and Estabrook, 1971; Holtzman and Rumack, 1973; Thompson and Holtzman, 1977).

Holtzman and Rumack (1973) found that addition of the substrate, ethylmorphine, to rat liver microsomes stimulated the activity of cytochrome P-450 reductase. Double reciprocal plot of stimulation of the reductase activity by ethylmorphine vs the ethylmorphine concentration was curvilinear, indicating the presence of two activation sites for liver microsomes from male rats. Bend *et al.* (1972) reported that Lineweaver–Burk plots of substrate–activity relationships of rabbit liver and lung microsomal aniline 4-hydroxylase were curvilinear. Arınç and İşcan (1983) found similar curving in Lineweaver–Burk and Eadie–Hofstee plots of sheep liver microsomal aniline 4-hydroxylase and calculated two K_m values.

In contrast to the sheep liver ethylmorphine *N*-demethylase, lung enzyme was inhibited by its subtrate, ethylmorphine, when its concentration was 25 mM and above, reaching to 67% inhibition at 50 mM concentration (Figs 4, 5). The K_m of the sheep

Table 4. The reaction rates of ethylmorphine N-demethylase and aniline 4-hydroxylase in sheep lung and liver reconstituted systems

Fractions*	Ethylmorphine *N*-demethylation nmol HCHO/ml/min	% Maximum	Aniline 4-hydroxylation† nmol pAP/ml/min	% Maximum
$P\text{-}450_{Lu}$ (Lung)	0	0	0	0
$Reductase_{Lu}$ (Lung)	0	0	0	0
Lipid	0	0	0	0
$P\text{-}450_{Lu} + Reductase_{Lu}$	1.68	80	0.20	100
$P\text{-}450_{Lu} + Reductase_{Lu} + Lipid$	2.10	100	0.20	100
$P\text{-}450_{Li}$ (Liver)	0	0	0	0
$P\text{-}450_{Li} + Reductase_{Li}$	0	0	0	0
$P\text{-}450_{Lu} + Reductase_{Li}$	0	0	0	0
$P\text{-}450_{Li} + Reductase_{Lu}$	2.24	80	0.35	50
$P\text{-}450_{Li} + Reductase_{Lu} + Lipid$	2.80	100	0.70	100

*In a final vol. 1 ml of the reaction medium contained 360 units of lung reductase (210 units/mg protein) or liver reductase (800 units/mg protein), 0.42 nmol lung P-450 (0.8 nmol/mg protein) or 0.72 nmol liver P-450 (1.20 nmol/mg protein) and 0.1 mg phosphatidylcholine dilauroyl.

†Determined as described by Arınç (1980).

lung microsomal enzyme was calculated to be 4.84 mM ethylmorphine from its Lineweaver–Burk graph (Fig. 5). Similar substrate–activity relationships were obtained for sheep lung aniline 4-hydroxylase. However, in that case the substrate inhibition of the enzyme activity was only 35% at 50 mM aniline concentration (Arınç and İşcan, 1983).

To further characterize sheep lung and liver ethylmorphine *N*-demethylase enzymes, the effects of some inhibitors (i.e. divalent metal ions and ethylalcohol) and stimulators (acetone) on the activities of the enzymes obtained from both tissues were examined.

Ethylmorphine *N*-demethylase activities of both liver and lung were inhibited by $NiCl_2$, $CdCl_2$ and $ZnSO_4$ (Table 2). $NiCl_2$ and $ZnSO_4$ were found to be more inhibitory to the lung enzyme. $CdCl_2$ at 0.1 mM concentration inhibited the activity of the liver enzyme by 56%, whereas $CdCl_2$ at this concentration had no effect on lung ethylmorphine *N*-demethylase activity. However, 1mM $CdCl_2$ inhibited the lung and liver *N*-demethylase activity completely (Table 2). Aniline 4-hydroxylase activity of sheep lung and liver microsomes was also inhibited by the addition of $NiCl_2$ and $CdCl_2$ into the incubation medium (Arınç and İşcan, 1983). A relatively stronger inhibition of sheep aniline 4-hydroxylase activity by $NiCl_2$ and $CdCl_2$ in liver than in lung was observed (Arınç and İşcan, 1983). Inhibitory effects of cadmium ions on rat liver microsomal mixed-function oxidase enzymes were shown by Lui and Lucier (1981). *In vivo* treatment of adult male rats with 2mg/kg of $CdCl_2$ resulted in marked reduction in liver microsomal cytochrome P-450 levels and ethylmorphine *N*-demethylation activity. The rates of ethylmorphine *N*-demethylation and ethoxyresofurin *O*-deethylation of rat liver microsomes were reduced by 95 and 70% respectively in the *in vitro* presence of 0.1 mM cadmium (Lui and Lucier, 1981).

Ethylalcohol inhibited *N*-demethylation of ethylmorphine in sheep lung and in liver microsomes. (Table 3). When 5% ethylalcohol was added *in vitro* only 20% of the control activity was observed in the liver microsomes whereas the same concentration of ethylalcohol was sufficient to inhibit ethylmorphine *N*-demethylase activity completely in lung. Ethylalcohol has long been known to effect the metabolism of a variety of drugs. *In vitro* inhibition of drug metabolism by ethylalcohol may reflect the competition between ethylalcohol and the drug for oxidation by the mixed-function oxidase system (Rubin and Lieber, 1968; Teschke *et al.*, 1975; Koop *et al.*, 1982).

A distinct difference was observed in the *N*-demethylation activity of ethylmorphine in lung and liver microsomes in response to acetone. As seen in Table 3, 5–15% acetone slightly stimulated ethylmorphine *N*-demethylase activity of liver enzyme, whereas 5% acetone completely inhibited the activity of the lung enzyme. Anders (1968) has showed that *in vitro* addition of acetone enhanced hydroxylation of aniline in rat, mouse, rabbit and dog liver microsomes. Kitada *et al.* (1983) found that acetone stimulated NADPH-dependent aniline hydroxylation in a reconstituted system containing cytochrome P-450 purified from phenobarbital-treated rats, whereas aniline hydroxylation catalysed by cytochrome P-448 purified from 3-methylcholanthrene-treated rats was inhibited with increasing concentrations of acetone. It is possible that the qualitative and quantitative differences observed for ethylmorphine *N*-demethylation of sheep liver and lung microsomes may be due to different forms of cytochrome P-450 present in sheep liver and lung microsomes. Some spectral differences observed in the solubilized cytochrome P-450s of lung and liver microsomes support this hypothesis. As seen in Fig. 9, CO-difference spectrum of dithionite-reduced solubilized lung cytochrome P-450 gave a peak at 451 nm, while the similar spectrum of solubilized liver microsomal cytochrome P-450 showed a maximum absorbance at 450 nm when the spectra were recorded at the same sheet.

Further purification of sheep lung and liver microsomal cytochrome P-450 and NADPH–cytochrome *c* reductase is required in order to find out how the components of the systems differ and which fractions are responsible for some of the differences observed in

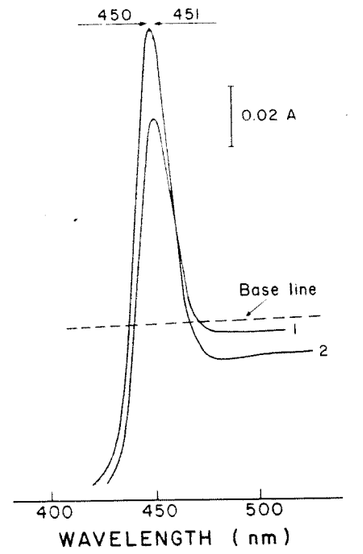

Fig. 9. The CO-difference spectra of dithionite-reduced cytochrome P-450s solubilized from sheep lung (1) and liver microsomes (2).

drug metabolism between liver and lung microsomal systems. Studies are now being undertaken in our laboratory to purify cytochrome P-450 and reductase from sheep liver and lung microsomes.

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