Depentylation of \([^3\text{H}-\text{pentyl}]\)Methyl-\(n\)-amino-nitrosamine by Rat Esophageal and Liver Microsomes and by Rat and Human Cytochrome P450 Isoforms


Eppley Institute for Research in Cancer [S. C. C., X. W., G. X., L. Z., S. S. M.] and Departments of Pharmaceutical Sciences [J. L. V., S. S. M.] and Biochemistry and Molecular Biology [S. S. M.], University of Nebraska Medical Center, Omaha, Nebraska 68198-6805, and National Cancer Institute, Bethesda, Maryland 20892 [F. G., H. V. G.]

ABSTRACT

Methyl-\(n\)-amino-nitrosamine (MNAN) induces esophageal cancer in rats, probably involving activation by cytochromes P450. We studied the metabolic depentylation of MNAN. [\(^{3}\text{H}-\text{pentyl}]\)MNAN and [\(^{3}\text{H}-\text{2,3-pentyl}]\)MNAN were synthesized, purified, and incubated with rat esophageal microsomes (REM) or rat liver microsomes (RLM) to give [\(^{3}\text{H}\)pentyl]depepntylylhydrazone (depentylation), an indicator of MNAN activation. [\(^{3}\text{H}\)Pentyl]depepentylhydrazone was determined by high-performance liquid chromatography of its 2,4-dinitrophenylhydrazone. Adding 5 mM semicarbazide to incubations increased the observed depentylation (except that due to CYP2E1) by >60%. MNAN depentylation by REM and uninduced and induced RLM showed \(K_m\) values of 64, 610, and 170–330 \(\mu\)M, respectively (\(V_{\text{max}}\) = 20, 220, and 160–1270 pmol/mg protein/min, respectively). The depentylation of 100 \(\mu\)M MNAN by REM was inhibited 98% by CO and 65% by coumarin preincubated for 15 min with REM (\(K_i\) = 120 \(\mu\)M) but was unaffected by antibodies inhibitory to various P450s. MNAN inhibited coumarin 7-hydroxylation by REM and CYP2A6 (\(K_i\) = 3000 and 320 \(\mu\)M, respectively). REM showed slight coumarin 7-hydroxylase activity. MNAN depentylation by REM was 41% inhibited by an antibody to CYP2C11. \(K_m\) for rat CYP2E1, human CYP2E1, and human CYP2A6 was 210, 115, and 17 \(\mu\)M, respectively (\(V_{\text{max}}\) = 300, 570, and 120 pmol/mg protein/min, respectively). We conclude that MNAN activation by REM is probably due to a P450 related to CYP2A3, a rodent nasal P450.

INTRODUCTION

MNAN\(^3\) induces tumors of the esophagus and nasal cavity when injected i.p. into rats (3). Corn infected with the mold Fusarium moniliforme may produce MNAN and other unsymmetric dialkyl-NAm or the corresponding secondary amines that could be converted to these NAm in food or in the stomach. This process may contribute to the etiology of esophageal cancer in high-incidence areas of China and South Africa, where corn is the staple diet and is often infected with molds (4). Ji et al. (5) grew F. moniliforme on corn in a medium containing iso-amylamine and nitrite, and isolated 15 \(\mu\)g/kg methyl-
iso-aryl-NAm, an isomer of MNAN. NAm requires activation by cytochrome P450s for their carcinogenic action (6). \(\alpha\)-Hydroxy-
NAm-metabolites (the longest-lived intermediates in NAm activation) have half-lives of only 1–10 s; therefore, most NAm are believed to be activated in the tissues where they induce tumors (4, 7). The esophagus is the second most common site (after the liver) for tumor induction by NAm in rats (6), probably because the esophagus contains P450 isoforms that activate esophagus-specific NAm (4), although the ready diffusion of some NAm into the esophagus probably also helps determine the organ specificity (8).

Our previous studies found that RLM produced mainly 4-hydroxy-
MNAN, HCHO, and PENT from MNAN (9). Formation of HCHO and PENT should be accompanied by the formation of, respectively, a pentylation- and a methylating agent, both of which could alkylate DNA and initiate cancer (Fig. 1). Farrell and Stewart (10) reported that MNAN demethylated and depentylated MNAN with \(K_m\) values of 2.6 and 1.2 \(\mu\)M, respectively. We incubated RLM with MAbs that inhibit individual P450s and then with 6 \(\mu\)M MNAN, and determined the MNAN metabolites. The results indicated that: (a) \(\alpha\)-hydroxylation of MNAN was mainly due to CYP2C11; (b) demethylation (HCHO production) was due in about equal parts to CYP2E1, CYP2B1/2B2, and CYP2C11; and (c) 30% of depentylation (PENT production) was due to CYP2C11 (11). REM and human esophageal microsomes demethylated 6 \(\mu\)M MNAN 18–20 times more efficiently than they demethylated 5 \(\mu\)M dimethyl-NAm, whereas human liver microsomes showed similar demethylating activities for both MNAN and dimethyl-NAm (12). Esophageal and liver microsomes of both rats and humans also depentylated MNAN. Human esophageal microsomes were one-third (for MNAN demethylation) and one-tenth (for MNAN depentylation) as active as REM. These results helped explain why unsymmetrical dialkyl-NAm induces esophageal cancer in rats and suggested that such NAm could initiate this cancer in humans.

Our nonradioactive method for studying MNAN dealkylation (9, 11, 12) can be used only at millimolar concentrations of MNAN and requires microsomes with 500 \(\mu\)g protein/tube. The \(K_m\) values were 20 \(\mu\)M for demethylation of the liver carcinogen dimethyl-Nam by rat CYP2E1 and 50 \(\mu\)M for \(\alpha\)-hydroxylation by REM of \(N\)-nitrosornicotine, a tobacco-specific NAm that induces esophageal tumors in rats (13). These low \(K_m\) values suggested that we increase the sensitivity of our method by using radiolabeled MNAN. The use of \([^{3}\text{H}-\text{pentyl}]\)MNAN enabled us to examine the depentylation of 5 \(\mu\)M MNAN using microsomes with only 50–100 \(\mu\)g protein, an important factor in view of the limited supply of REM.

In the present study, we examined the dealkylation of \([^{3}\text{H}-\text{pentyl}]\)MNAN to give \([^{3}\text{H}]\)PENT rather than that of \([^{3}\text{H}-\text{methyl}]\)MNAN to give \([^{3}\text{H}]\)HCHO because methylation, but not pentylation, of DNA guanine in MNAN has been detected in the rat esophagus (14, 15) and DNA methylation is associated with MNAN depentylation (Fig. 1). The depentylation of MNAN was also linked more closely than its demethylation with its bacterial mutagenicity in the presence of RLM (16). Pentyldiazohydroxide produced by MNAN demethylating may not pentylate DNA extensively because it forms a pentyldiazonium ion that could lose a proton to yield 1-pentene, which should not alkylate DNA. Similarly, ethylene is produced during diethyl-NAm metabolism by rabbit nasal microsomes (17). Hence, depentylation is probably more relevant than demethylation of MNAN to its carcinogenicity. Accordingly, we examined the depen-
tylation of [\textsuperscript{3}H-pentyl]MNAN by REM, RLM, and certain human and rat P450s (18, 19).

MATERIALS AND METHODS

Materials. MNAN was synthesized from methylamylamine (Karl Industries Inc., Aurora, OH) with >99% purity as determined by GC-TEA (20). Because MNAN is a potent volatile carcinogen, all work was performed in a chemical hood. Esophagi of adult male Sprague Dawley rats were purchased from Harlan Bioproducts for Science (Indianapolis, IN). The company stripped the connective tissue and outer submucosa from the esophagi, which were flash-frozen in liquid N\textsubscript{2} and mailed in dry ice to us. Human CYP2E1, human CYP3A4, and all of the MAbs except the one inhibitory to CY2A6 were prepared at the National Cancer Institute (21). We obtained human CYP2E1, CYP2A6, and CYP3A4, rat CYP2A1 and CYP2E1 overexpressed in mammalian cells, and the MAb to CYP2A6 from Gentest Corporation (Waltham, MA) and organic chemicals from Aldrich Chemical Corporation (Milwaukee, WI). Synthesis of [\textsuperscript{3}H-4,5-pentyl]MNAN and [\textsuperscript{3}H-2,3-pentyl]MNAN. Aqueous methylamylamine (30 ml of 40%, 380 mmol) was added over a period of 1 h dropwise with stirring to 10 g (65 mmol) of 5-bromo-1-pentene in a flask fitted with a dry-ice condenser containing a salt-ice mixture. The reaction was completed in 2–3 h at 0°C and for 18 h at room temperature. The mixture was adjusted to pH 2 with HCl and extracted repeatedly with CH\textsubscript{2}Cl\textsubscript{2} until TLC of the aqueous phase indicated that nearly all of the methylidenpentylamine had been removed. The TLC used fluorescent silica gel plates (60-F254, Curtis-Matheson Scientific, Houston, TX), which were developed with CH\textsubscript{3}Cl\textsubscript{2}: methanol 85:15 saturated with NH\textsubscript{4}OH and showed R\textsubscript{f} 0.64 for methyln-propylamine. [\textsuperscript{3}H]MNAN was synthesized [4,5-\textsuperscript{3}H]MNAN three times and [2,3-\textsuperscript{3}H]MNAN once (batches 1–4, respectively, numbered in chronological order). Batch 1 showed 19 Ci/mol starting amine. TLC by “system 1” (silica gel 60-F254 plates developed with hexane:ether:acetic acid 50:46:4 (“system 2”). The [\textsuperscript{3}H]MNAN band (RF, 0.6) was indicated by UV detection of cold MNAN applied as spots at each side of the plate and was eluted with 20 ml of CH\textsubscript{3}Cl\textsubscript{2}. On TLC of eluate samples by system 1, the [\textsuperscript{3}H]MNAN band contained 80–90% of the eluted counts. The [\textsuperscript{3}H]MNAN contained small amounts of [\textsuperscript{3}H]PENT, which seemed to be generated during storage and was mostly removed by semicarbazide treatment. On the day of the metabolic experiment, a CH\textsubscript{3}Cl\textsubscript{2} solution of 150–200 \muCi [\textsuperscript{3}H]MNAN (unpurified batches 1 or 4, or TLC-purified batches 2 or 3) and (for batches 1 and 4) up to 25 \muCi of unlabeled MNAN were added to 1 ml of water, and the CH\textsubscript{3}Cl\textsubscript{2} was evaporated at room temperature with a N\textsubscript{2} stream over the surface of the water. The aqueous solution was mixed with 4 ml of 10% semicarbazide:HCl in 1.1 M Na acetate in water (pH 4–5) and heated at 65°C for 10 min. The [\textsuperscript{3}H]MNAN was extracted with 3×2 ml of CH\textsubscript{3}Cl\textsubscript{2}. The extract was dried over Na\textsubscript{2}SO\textsubscript{4}, concentrated to 0.7 ml, and subjected to TLC on eight 60-F254 alumina plates (65 × 50 × 0.25 mm, Curtis Matheson Scientific) developed with hexane:ether:4.6 (“system 3”). Spots of unlabeled MNAN at each side of the plates were used to indicate the [\textsuperscript{3}H]MNAN bands (RF, 0.6), which were scraped off, combined, eluted with 10 ml of CH\textsubscript{3}Cl\textsubscript{2}, and transferred (see above) to 1.0 ml of water. [\textsuperscript{3}H]MNAN and Pent semicarbazone traveled with RF 0.60 and 0.75, respectively, close to the RF of MNAN. PENT and its semicarbazone were revealed as blue spots when plates were sprayed with 5% phosphomolybdc acid in ethanol and heated for 10 min at 100°C. We determined the concentration of [\textsuperscript{3}H]MNAN by GC-TEA (22, 23) and its radioactivity in the 1-ml aqueous solution. This contained 50–150 \muCi [\textsuperscript{3}H]MNAN.

Isolation of Microsomes. Microsomes were prepared as described previously (11) from the livers of 6–8-week-old adult male Sprague Dawley rats (Sasco Inc., Omaha, NE) that were untreated or induced with PB, 3MC, or isoniazid (9, 11, 12). The livers were homogenized in 3 ml/g tissue of 100 mM potassium phosphate buffer (pH 7.4) containing 0.1 mM DTT and 0.14 mM phenylmethylsulfonyl fluoride. RLM were obtained by differential centrifugation of the homogenate in 100 mM potassium phosphate buffer (pH 7.4) containing 0.1 mM DTT and 0.14 mM phenylmethylsulfonyl fluoride. RLM were obtained by differential centrifugation in the same buffer, suspended in 100 mM potassium phosphate buffer (pH 7.4) containing 20% glycerol, analyzed for protein by the Lowry method, and stored in 1.5-ml Eppendorf tubes at −70°C.

Microsomes were prepared from rat esophagi each weighing 50–80 mg (see “Materials”) by our previous method (12) involving homogenization of the thawed esophagi in a Potter-Elvehjem homogenizer or by the following modification of Murphy’s method (13): esophagi (five or six at a time) were each cut into four or five pieces while frozen, crushed with a Bessman tissue pulverizer precooled with liquid N\textsubscript{2}, transferred to an ice-cold glass Tenbroech homogenizer, and gently homogenized with six passes of the pestle each way in 6–7 ml of 50 mM Na PP, buffer (pH 7.4), containing 1 mM EDTA, 1 mM DTT, and 5 mM phenylmethylsulfonyl fluoride. The combined homogenate from three such procedures was differentially centrifuged. The microsome fraction was analyzed for protein and stored as for liver microsomes. Three such isolations, from 17, 16, and 50 esophagi (the last combined from three batches), yielded REM with 7.2, 2.6, and 7.4 mg of protein.

DEPENTYLATION OF METHYLMYLNITROSAMINE
Use of Individual P450s. These were stored in Eppendorf tubes at −70°C. On the day of use, 1 ml of a suspension containing P450s from the National Cancer Institute was mixed with 1 ml of 100 mM potassium phosphate buffer (pH 7.4) containing 20% glycerol, ultrasonicated twice for 5 s, and centrifuged at 50,000 rpm for 30 min. The pellet was resuspended in 1.0 ml of the same buffer with a Potter-Elvehjem homogenizer. P450s from Gentest Corporation were supplied in 100 mM potassium phosphate buffer (pH 7.4) and were gently shaken by hand before use.

Metabolic Experiments. In Method A (used unless mentioned otherwise), each experiment included 12–16 tubes, each with 500 µl of medium containing 100 mM potassium phosphate buffer (pH 8.0), 10 mM MgCl₂, 5 mM semicarbazide-HCl (9, 11), [¹⁴]HNAN (3–10 × 10⁶ cpm), unlabeled MNAN (amount calculated after allowing for MNAN in the [¹⁴]HNAN sample), microsomes with 50 or 100 µg protein, and (added last to start the reaction) NADP+-generating mixture containing 2 mM NADP, 10 mM glucose-6-phosphate, and 2 units of glucose-6-phosphate dehydrogenase (final pH 7.4). The experiments used [¹⁴-4,5-penty]MNAN except for those where [¹⁴-2,3-penty]MNAN is specified. Tubes 1 and 2 were blanks with 20 µM MNAN. For K₉₅ measurements, the remaining tubes contained microsomes or a P450 and 5–7 concentrations of MNAN, each run in duplicate. The tubes were incubated for 20 min at 37°C. In Method B, incubations were performed as in Method A but for 60 min and with 15 mM semicarbazide-HCl.

The incubation mixtures were worked up as described previously (9, 11). In brief, reactions were stopped with Ba(OH)₂ and ZnSO₄. After centrifugation, the supernatants were collected as 1-min fractions from 17 to 26 min. Each fraction was evaporated to dryness with N₂ at 65°C, mixed with 4 ml of Ecolume, and subjected to the conditions of the metabolic experiment with various types of microsomes and analyzed by HPLC with detection at 355 nm (12). Results obtained from the standards of bands at 50–57 kDa depending on the P450.

Western Blots. Microsomes were solubilized in solutions of cholic acid and 2-mercaptoethanol with heating for 2 min at 100°C (26). Samples and 35–50 µg of protein/well were subjected to gel electrophoresis. The gels were developed with Mabs to individual P450s (Mabs 1-98-1 and 1-91-3 for CYP2E1, 2-66-3 and 4-7-1 for CYP2B1/2, 1-68-11 for CYP2C11/12, and 1-7-1 for CYP1A1/2; Ref. 21) and were visualized with peroxidase-labeled sheep antiamouse antibody (27). Positive results were indicated by the staining of bands at 50–57 kDa depending on the P450.

RESULTS

MNAN Metabolism by REM and RLM. We generally used REM prepared by Murphy’s method (13) because they were more active than those prepared by our previous method (Ref. 12; Table 1). Inclusion of 5 mM semicarbazide in the metabolic incubations increased PENT yield by mean values of 118% for REM and 61% for CYP2A6 and was essential when RLM were used but had no effect with rat CYP2E1 (Table 2). PENT production was linear with time for 30 min when PB-induced RLM were incubated with 100 µM [¹⁴]HNAN. Therefore, in earlier experiments of this study, reaction mixtures were incubated for 20 min in the presence of 5 mM semicarbazide (Method A). Using this method, PENT yield from 50 and 200 µM MNAN increased as the amount of REM was raised from 20 to 100 µg of protein/tube (Fig. 2A).

After much of the work had been performed, we obtained results suggesting that 15 mM semicarbazide produced higher PENT yields than the standard 5 mM level, and, hence, the experiments done by Method B used 15 mM semicarbazide. However, a more careful check found no significant differences between the effects of 5, 15, and 30 mM semicarbazide on the ability of REM to produce PENT from 100 µM MNAN in 20 min (PENT yields: 5.8 ± 0.7, 7.2 ± 1.1, and 6.1 ± 0.7 pmol PENT/mg protein/min, respectively (mean ± SE for 6–8 tests/group)). PENT yield from 100 µM MNAN using 15 mM semicarbazide and REM with 50 µg of protein/tube was nearly linear for 60 min (yield after 20, 40, 60, and 90 min was 90, 220, 410, and 460 pmol of PENT/mg, respectively). When Method B was used, PENT yield from both 50 and 200 µM MNAN increased as the amount of REM was raised from 25 to 100 µg of protein/tube (Fig. 2B).

Hence, experiments performed with Method A that showed PENT yields less than twice the background level were repeated using Method B. Method B was also used in all later studies with REM. The apparent K₉₅ for MNAN depentylation by REM was 64 µM with a V₉₃ of 20 pmol PENT/min/mg (Table 1 and Fig. 3).

Microsomes were stored as suspensions in buffer containing 20% glycerol and were normally used without removing the glycerol, giving a glycerol level in the incubation mixture of 40–130 mM. Because glycerol competitively inhibited dimethyl-NNa demethylation by CYP2E1 with a Kᵣ of 53 mM (28), we studied its effect on MNAN metabolism by REM and PB-induced RLM. The microsomes were added to the incubation mixture as suspensions in buffer with 20% glycerol or after centrifugation and resuspension in glycerol-free buffer.
buffer. Incubations were performed by Method B using [3H-2,3-penty]MNAN. PENT yields from MNAN in pmol/mg protein/min (mean ± SE for four tests/group) were 245 ± 5 with and 228 ± 13 without glycerol for PB-induced RLM, and were 16 ± 3 with and 19 ± 3 without glycerol for REM, indicating that glycerol did not affect the results.

MNAN metabolism by uninduced and PB-, 3MC-, and isoniazid-induced RLM showed classic dose-response curves for PENT yield versus MNAN concentration, with apparent \( K_m \) values of 170–610 \( \mu M \) (Table 1). When PB- and isoniazid-induced RLM were used, 100 \( \mu M \) MNAN was depentylated 7.4 and 1.6 times faster, respectively, than uninduced RLM, and the apparent \( K_m \) values were about one-half of the \( K_m \) for uninduced RLM. Although 3MC-induced and uninduced RLM depentylated 100 \( \mu M \) MNAN at similar rates, the apparent \( K_m \) was 3.6 times lower for 3MC-induced than for uninduced RLM.

We examined the effect of inhibitors on the depentylation of 100 \( \mu M \) MNAN by REM and by uninduced RLM. A 9:1 CO:air mixture inhibited MNAN metabolism by REM by a mean of 98% (Table 3). Coumarin (0.4 \( \mu M \)) produced only a 19% inhibition of MNAN de
depntylated by REM when coumarin, REM, and MNAN were added at the same time, but produced a 55% inhibition with an apparent \( K_i \) of 120 \( \mu M \) when coumarin and REM were preincubated for 15 min before adding MNAN (Tables 3 and 4). Preincubation for 30 min produced no additional effect. All of the subsequent studies with coumarin used preincubation for 15 min. Coumarin inhibition of MNAN metabolism by REM reached 65% when coumarin concentration was raised to 0.6 \( \mu M \) (Fig. 4).

We previously used MAbs that inhibit P450s 1A1/1A2, 2B1/2B2, 2C11/2C12, and 2E1 to establish the role of these P450s in the dealkylation of 6 \( \mu M \) MNAN and 6 \( \mu M \) methylbutyl-NAm by RLM (11, 29). In the present study, the depentylation of 100 \( \mu M \) MNAN by REM was not inhibited by any of these MAbs (Table 3). The depentylation of 100 \( \mu M \) MNAN was significantly inhibited (by a mean of 41%) by the MAb to CYP 2C11/2C12 and was significantly enhanced (by a mean of 24%) by the MAb to CYP2E1.

**MNAN Metabolism by Individual P450s: Rat and Human CYP2E1.** MNAN depentylation by rat CYP2E1, human CYP2E1 from the National Cancer Institute, and human CYP2E1 from Gentest showed apparent \( K_m \) values of 210, 150, and 170 \( \mu M \), respectively (Table 5). MAb 1-91-3 to rat CYP2E1 (50 \( \mu g \)) was preincubated with 10 pmol of rat CYP2E1 as in the MAb tests in Table 3, and the mixture was then incubated by Method A with 100 \( \mu M \) MNAN. The MAb inhibited MNAN production by 85, 89, and 92% in three tubes, demonstrating its strong activity. When 10, 20, and 40 pmol of human CYP2E1 from Gentest was incubated with 100 \( \mu M \) MNAN, PENT yield increased linearly with the amount of P450 (PENT yield in pmol/min: 0.41 and 0.71 for 10, 1.28 and 1.91 for 20, and 3.98 and 4.53 for 40 pmol of CYP2E1).

**Human CYP2A6.** PENT yield increased linearly with the amount of CYP2A6 incubated with 100 \( \mu M \) MNAN (PENT yield in pmol/min: 0.54 and 0.55 for 10, 0.74 and 1.44 for 20, and 3.64 and 5.03 for 40 pmol of CYP2A6). CYP2A6 showed an apparent \( K_m \) of 17 \( \mu M \) and a \( V_{max} \) of 120 pmol/nmol/min (Fig. 5; Table 5). Replacing the standard 100 \( \mu M \) phosphate buffer by 50 \( \mu M \) Tris buffer did not affect MNAN depentylation by CYP2A6, despite a contrary claim for metabolism by this P450 in the 1994 Gentest catalogue. Coumarin produced a 96% inhibition of the depentylation of 100 \( \mu M \) MNAN by 30 pmol/tube CYP2A6 (PENT yield: 68.5 and 53.9 (for CYP2A6) and 1.8 and 2.4 (for CYP2A6 preincubated with 0.6 \( \mu M \) coumarin) pmol/nmol P450/ min), with an apparent \( K_i \) of 7.5 \( \mu M \) (Table 4).

**Rat CYP2A1 and Human CYP3A4.** Rat CYP2A1 showed no activity when 10 pmol of P450/tube was incubated with 20–2000 \( \mu M \) MNAN (Table 5). Human CYP3A4 from the National Cancer Institute showed a nearly linear dose-response curve in experiments with 20 and 40 pmol of P450/tube, with no saturation even at 12 \( \mu M \) MNAN and a rate for 100 \( \mu M \) MNAN of 48 pmol of PENT/nmol CYP3A4/min (Table 5). CYP3A4 from Gentest gave similar results. This P450 might have given low results because it was not fully dispersed in the medium. However, CYP3A4 metabolism of 100 \( \mu M \) MNAN was inhibited by 86% when 0.5 mg/ml of the detergent 3 with and 228 without glycerol for REM, indicating that glycerol did not affect the results.

Fig. 2. The effect of varying amounts of REM on the metabolism of 50 (●) and 200 (○) \( \mu M \) MNAN using Method A (incubation with 5 \( \mu M \) semicarbazide for 20 min (A)) or Method B (incubation with 15 \( \mu M \) semicarbazide for 60 min (B)). Each point, the results for an individual tube. The tests in A and B were conducted at different times with different batches of REM. This may explain why A and B show similar results although Method B generally produced more PENT than Method A.
3-(3-cholamidopropyl)dimethylammonio)-1-propane sulfonate ("CHAPS") was included and was unaffected by including 1 mg/ml of BSA in the medium.

**Coumarin Metabolism and the Effect thereon of MNAN.** For coumarin 7-hydroxylation, Table 4 and Fig. 6 demonstrate a rapid metabolism of coumarin with an apparent $K_m$ of 50 mM for CYP2A6, a slower metabolism with a higher apparent $K_m$ for RLM, and low but still measurable activity for REM. MNAN inhibited coumarin 7-hydroxylation by RLM and CYP2A6 with apparent $K_i$ values of 3000 and 320 mM, respectively but did not seem to inhibit the low activity of REM for this reaction (Table 4). Table 4 also shows whether each inhibition of MNAN and coumarin metabolism seemed to be competitive, uncompetitive, or noncompetitive (30, 31).

**Detection of P450s on Western Blots.** Immunoblots of solubilized REM and RLM were developed with MAbs to several P450s. The results (data not shown) indicated that REM did not contain any CYP1A1/1A2, CYP2C11, or CYP2E1, but did show a trace of CYP2B1/2B2. In contrast, Ahn et al. (32) detected CYP1A1 in REM.

The results for RLM indicated, in addition to well-established effects of PB, 3MC, and isoniazid on the induction of P450s 1A1/1A2, 2B1/2B2, and 2E1, that isoniazid induced CYP2B1/2B2 (confirming our previous finding based on MAb inhibition of MNAN metabolism; Ref. 11) and that our uninduced RLM contained a little CYP2B1/2B2.

### DISCUSSION

**Comments on Methods.** In the measurements of $[^{3}H]PENT$ production from $[^{3}H]MNAN$, the experimental/background ratio of counts was $>2$–3 when up to 200 mM MNAN was used but fell below 2 when $>2000$ mM MNAN was used. We think this occurred because the background radioactivity was due to impurities in the $[^{3}H]MNAN$ and hence was a constant percentage of the added $[^{3}H]MNAN$ irrespective of MNAN concentration, whereas the absolute PENT yield reached a maximum when the enzyme became saturated and then stayed constant as the MNAN level was raised. Hence, only $K_m$ values $<500$ mM could be measured accurately. Fortunately, we are mainly interested in NAm metabolism at low concentrations to which people might be exposed.

We used $[^{3}H,4,5-pent-yl]MNAN$ for most of the studies and $[^{3}H-2,3-pent-yl]MNAN$ for the more recent experiments. The results should not depend on which MNAN isomer was used because MNAN activation does not involve a compound with labile hydrogen at C-2 and because PENT, which could enolize and exchange T for H at C-2 under alkaline conditions, was kept at neutral or acidic pH or was combined with semicarbazide or dinitrophenylhydrazine. The two isomers of $[^{3}H]MNAN$ seemed to give similar results but the $[^{3}H,2,3-pentyl]$ isomer seemed to be more readily synthesized and more stable than the $[^{3}H,4,5-pentyl]$ isomer.

Semicarbazide was included in all of the incubations with MNAN. It increased the yield of HCHO from dimethyl-NAm 2.5-fold in a 1979 study on mouse liver microsomes (33) and has since been used in many similar investigations, e.g., those in references (9, 11, 12, 29). Presumably, semicarbazide acts because it forms unstable semicarbazones of aldehydes that protect them from oxidation to carboxylic acids, a reaction catalyzed by rodent liver microsomes (34). After the microsomal incubation, the semicarbazone is converted to a more stable dinitrophenylhydrazone or other derivative for determination (33). Although semicarbazide competitively inhibited dimethyl-NAm demethylation by rat CYP2E1 in RLM (28), it did not affect MNAN

### Table 4 Summary of enzyme kinetics for coumarin 7-hydroxylation, the effect of MNAN inhibition of coumarin 7-hydroxylation for REM, RLM, and human CYP2A6

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#### Table 3 The effect of CO, coumarin, and MAbs to individual P450s on the demethylation of 100 μM $[^{3}H]MNAN$ by REM and uninduced RLM

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Preincubation</th>
<th>Incubation method</th>
<th>Percent mean control value for PENT yield$^a$ [mean ± SE (no. of tests)]</th>
</tr>
</thead>
<tbody>
<tr>
<td>CO$^b$</td>
<td>5</td>
<td>B</td>
<td>REM</td>
</tr>
<tr>
<td>Coumarin</td>
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<td>B</td>
<td>REM</td>
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<td>15</td>
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<td>REM</td>
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<td>Coumarin</td>
<td>30</td>
<td>B</td>
<td>REM</td>
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<tr>
<td>MA 1-7-1 to CYP1A1/1A2$^b$</td>
<td>15</td>
<td>B</td>
<td>REM</td>
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<tr>
<td>MA 1-91-3 to CYP2E1</td>
<td>15</td>
<td>A and B</td>
<td>REM</td>
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<tr>
<td>MA 4-7-1 to CYP2B1/2B2</td>
<td>15</td>
<td>B</td>
<td>REM</td>
</tr>
<tr>
<td>MA 1-6B-11 to CYP2C11/2C12$^b$</td>
<td>15</td>
<td>B</td>
<td>REM</td>
</tr>
<tr>
<td>MA to CYP2A6$^d$</td>
<td>15</td>
<td>B</td>
<td>REM</td>
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</tbody>
</table>

$^a$ Results are shown for "treated" microsomes with inhibitors preincubated with the microsomes and for "control" microsomes preincubated without added inhibitors.

$^b$ A 9:1 CO-air mixture was bubbled for 5 min at 1 bubble/s into the standard incubation mixture containing REM with 100 μg of protein, the NADPH-generating system was added, and the tubes were stopped and incubated. The control tubes were not treated with CO.

$^c$ Significantly different from control values; $P < 0.01$. The Wilcoxon rank order test was used to determine significance here and in footnotes $f$ and $g$.

$^d$ Not examined

$^e$ Coumarin (0.4 mM) was added to the standard incubation mixture containing REM at the same time as, or 15 or 30 min before, adding $[^{3}H,2,3-pent-yl]MNAN$ and the NADPH-generating system. The mixture was then incubated for 20 min, and PENT yield was determined.

$^f$ Significantly different from the results in row 2 (coumarin added without preincubation); $P < 0.01$.

$^g$ Significantly different from control values; $P = 0.03$.

$^h$ Each experiment included 2 blank tubes with MNAN but without microsomes, 2 tubes with microsomes, and 2 each with microsomes plus various MAbs, totaling up to 18 tubes. Microsomes with 50 or 100 μg of protein were preincubated for 15 min with 50 or 100 μg, respectively, of the listed MAbs; 100 μM MNAN and the cofactors were added; the mixtures were incubated; and PENT yield was determined. MAb results were expressed relative to those for the tubes without MAb. Some of these tests used $[^{3}H,2,3-pent-yl]MNAN$.
depentylation by overexpressed rat CYP2E1 (Table 2), perhaps because semicarbazide inhibition of CYP2E1 activity was counterbalanced by an inhibition of PENT oxidation. The addition of 5 mM semicarbazide increased by 60% the depentylation of MNAN by REM, RLM, and CYP2A6 (Table 2). The use of higher levels of semicarbazide did not have an additional effect (see “Results, MNAN Metabolism by REM and RLM”). Therefore, semicarbazide should continue to be used in dealkylation studies not involving CYP2E1.

MNAN Metabolism by REM and RLM. REM showed an apparent $K_m$ of 64 μM for MNAN depentylation, with 10% of the $K_m$ for uninduced RLM (Table 1). Although this high-affinity activity of REM showed a $V_{max}$ that was only 9% of that for RLM, the low $K_m$ for MNAN metabolism by REM supports the view that NAm carcinogenesis in the rat esophagus is due to tissue-specific activation of these NAms. One reason for the low $V_{max}$ for REM is presumably that MNAN is mainly metabolized by basal cells of the esophageal mucosa (14), but REM were prepared from the entire mucosa and part of the submucosa. For comparison, methylbenzyl-NAm (a more potent esophageal carcinogen than MNAN on a molar basis) is debenzylated by REM with a $K_m$ of $10^{-4} M$ (35).

The finding that MNAN activation by REM was 98% inhibited by CO (Table 3) demonstrates that the reaction involved P450s. Coumarin (0.4 mM) inhibited CYP2A5 in mouse liver microsomes (36) but this P450 apparently does not occur in rat liver, although rat nasal mucosa contains a P450, probably CYP2A3, that metabolizes dimethyl-NAm and NNK and is inhibited by coumarin (37). The observation that coumarin inhibited REM metabolism of MNAN by up to 65% with a $K_i$ of 120 μM (Fig. 4; Tables 3 and 4) suggests that a CYP2A5-like enzyme makes a major contribution to the esophageal metabolism of NAms that induce esophageal cancer. The weak activity of REM for the 7-hydroxylation of coumarin (Table 4) confirms a similar observation by Murphy et al. (35). Findings that none of the test MAbS inhibited MNAN metabolism by REM (Table 3) and that immunobLOTS did not reveal any P450s other than traces of CYP2B1/2B in REM (50 μg/tube, see “Results”) indicate that MNAN depentylation in the esophagus did not involve P450s 1A1, 1A2, 2C11, 2E1, or (probably) 2B1 or 2B2. The lack of inhibition of REM activity by the MAb to human CYP2A6 (Table 3) may have occurred because rat CYP2A5 or the analogous rat esophageal P450 is not inhibited by this MAb (we found no information on this point).

![Fig. 4](image-url) The effect of coumarin concentration on the depentylation of 100 μM [3H-2,3-pentyl]MNAN by REM. Coumarin was preincubated for 15 min with the REM (50 μg protein/tube) before adding MNAN (see Table 3). Each point, the mean results for two tubes. Results are combined from two experiments.

![Fig. 5](image-url) Kinetics of MNAN depentylation by human CYP2A6 (40 pmol/tube). A shows the substrate concentration curve, and B shows the double reciprocal plot of the results (1/S versus 1/V, where $S$ = substrate concentration and $V$ = rate of reaction). Each point, the results for an individual tube.

![Fig. 6](image-url) Kinetics of 7-hydroxycoumarin formation from coumarin by RLM and REM (each with 50 μg protein/tube). A shows the substrate concentration curve, and B shows the double reciprocal plot of the results (1/S versus 1/V, where $S$ = substrate concentration and $V$ = rate of reaction). Each point, the results for an individual tube.
The observation that MNAN depentylation by uninduced RLM was enhanced 24% by MAb 1-91-3 to CYP2E1 (Table 3) indicates that CYP2E1 catalyzed a pathway of MNAN metabolism other than depentylation. This other pathway is presumably demethylation, one-third of which was due to CYP2E1 at a MNAN level of 6 mM (11). The finding that 41% of the depentylation of 100 μM MNAN by RLM was inhibited by the MAbs to the constitutive male P450, CYP2C11, indicates that about 41% of this metabolism was due to CYP2C11 (Table 3), similar to the figure of 30% found for the depentylation of 6 mM MNAN (11). It is not known which enzymes catalyze the remaining 50–60% of MNAN depentylation by RLM.

The effect of P450 inducers was examined for RLM but not REM because of the large number of induced rats that would be needed to prepare sufficient REM for such a study. The $K_m$ values for MNAN depentylation by PB-, 3MC-, and isoniazid-induced RLM were 28–54% of the $K_m$ for uninduced RLM, and $V_{max}$ for PB-induced RLM was 5.8 times that for uninduced RLM (Table 1). These results indicate that CYP2B1 or CYP2B2 (induced by PB), CYP1A1 or CYP1A2 (induced by 3MC), and CYP2E1 (induced by isoniazid; Ref. 11) can all depentylate MNAN. The rates for the depentylation of 100 μM MNAN were 7.4 and 1.6 times faster for PB- and isoniazid-induced RLM, respectively, than for uninduced RLM (Table 1), similar to the corresponding relative rates of 5.9 and 1.7 for the depentylation of 6 mM MNAN (11).

**MNAN Metabolism by Individual P450s.** The rat liver enzyme, CYP2E1, showed a $K_m$ of 210 μM for MNAN depentylation (Table 5). This relatively high $K_m$ value suggests that large, but not small, doses of MNAN methyleate rat liver DNA (14, 15) because of activation by CYP2E1. This $K_m$ value was higher than those of 15–40 μM for the dealkylation of dimethyl- and diethyl-NAm by CYP2E1 (38). Rat CYP2E1 also demethylates and debutylates methylbutyl-NAm by CYP2E1 (38). Rat CYP2E1 also demethylates and debutylates methylbutyl-NAm by CYP2E1 (38). Rat CYP2E1 also demethylates and debutylates methylbutyl-NAm by CYP2E1 (38). Rat CYP2E1 also demethylates and debutylates methylbutyl-NAm by CYP2E1 (38). Rat CYP2E1 also demethylates and debutylates methylbutyl-NAm by CYP2E1 (38). Rat CYP2E1 also demethylates and debutylates methylbutyl-NAm by CYP2E1 (38). Rat CYP2E1 also demethylates and debutylates methylbutyl-NAm by CYP2E1 (38). Rat CYP2E1 also demethylates and debutylates methylbutyl-NAm by CYP2E1 (38).

**CONCLUSIONS.** Our results demonstrate that REM and RLM can depentylate low concentrations of MNAN. REM, rat CYP2E1, human CYP2E1, and human CYP2A6 activated MNAN with $K_m$ values of 210 μM (Table 5). Our inhibition and metabolism studies indicate that an enzyme resembling CYP2A3 catalyzes most MNAN depentylation by REM and confirm our finding (11) that CYP2C11 contributes to MNAN depentylation by RLM. Identification of the major NAm-metabolizing P450 in the rat esophagus and extension of this study to microsomes obtained from various human tissues (we are currently completing the latter project; Ref. 45) should help disclose how MNAN and certain other NAmides induce esophageal cancer in rats and help indicate whether NAm activation could be involved in the etiology of human esophageal cancer.

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**REFERENCES.**


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Depentylation of [3H-pentyl]Methyl-n-amylNitrosamine by Rat Esophageal and Liver Microsomes and by Rat and Human Cytochrome P450 Isoforms

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