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Dimethylnitrosamine Demethylation by Reconstituted Liver Microsomal Cytochrome *P*-450 Enzyme System

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Oxidative demethylation of dimethylnitrosamine was studied with both reconstituted and unresolved liver microsomal cytochrome *P*-450 enzyme systems from rats and hamsters. Proteinase treatment of liver microsomal preparations yielded cytochrome *P*-450 particulate fractions. Both cytochrome *P*-450 and NADPH–cytochrome *c* reductase fractions were required for optimum demethylation activity. Particulate cytochrome *P*-450 fractions were more efficient than either Triton X-100- or cholate-solubilized preparations of these particles in demethylation studies. Differences in demethylation activity with rat and hamster liver preparations appear to be due to differences in specificity in their cytochrome *P*-450 fractions.

Several nitrosamines including dimethylnitrosamine are toxic and carcinogenic to a wide variety of animal species including primates (Druckrey *et al.*, 1967; Magee & Barnes, 1967). It has been suggested that oxidative demethylation of dimethylnitrosamine is an activation step in its carcinogenic process (Druckrey *et al.*, 1967; Magee & Barnes, 1967; Miller, 1970). Recent studies have shown that such an oxidative demethylation of dimethylnitrosamine is mediated via a cytochrome *P*-450 enzyme system (Czygan *et al.*, 1973). Since mammalian liver microsomal cytochrome *P*-450 enzyme system has been solubilized and resolved, it has been shown that all three resolved fractions, cytochrome *P*-450, NADPH–cytochrome *P*-450 reductase and phosphatidylcholine, are required for oxidation of several physiological substrates, such as steroids and fatty acids, and a variety of foreign compounds, including drugs and some carcinogens (Lu *et al.*, 1969, 1973).

In the present study, oxidative demethylation of dimethylnitrosamine is studied with both reconstituted and unresolved liver microsomal cytochrome *P*-450 enzyme systems from rats and hamsters.

Materials and methods

Horse heart cytochrome *c* type III, bacterial proteinase VII, NADPH and Triton X-100 were purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A. Sodium cholate was obtained from Schwarz–Mann Co., Orangeburg, N.Y., U.S.A. Dimethylnitrosamine and di[^{14}C]methylnitrosamine (sp. radioactivity 4.8 mCi/mmol) were obtained from Eastman Kodak Co., Rochester, N.Y., U.S.A., and New England Nuclear Corp., Boston, Mass., U.S.A., respectively. All other chemicals were of reagent grade.

Adult male Sprague–Dawley rats (200–250 g body wt.) and Syrian golden hamsters (100–105 g body wt.) were used for these studies. Immediately after the animals had been killed by decapitation, livers were removed and placed in ice-cold 0.25 M-sucrose solution. Preparation of liver microsomal fractions, proteinase treatment and isolation of particles after proteinase treatment were as described previously (Lotlikar *et al.*, 1974). In brief, for proteinase treatment, liver microsomal fractions (10 mg of protein/ml) suspended in 0.1 M-potassium phosphate buffer, pH 7.0, containing 25% glycerol were incubated with bacterial proteinase (10 μg of proteinase/mg of protein) for 1 h at 4°C. After incubation, samples were centrifuged at 105000*g* for 1 h. The pellet was washed with glycerol–phosphate buffer, re-centrifuged and suspended in 0.25 M-sucrose. Where indicated, these particles were solubilized with either sodium cholate or Triton X-100 (1 mg of detergent/mg of protein) in the presence of 0.1 M-phosphate buffer, pH 7.8, containing 10% glycerol (5 mg of protein/ml). After being stirred for 30 min at 4°C, the sample was centrifuged at 105000*g* for 1 h. The supernatant was dialysed overnight against 200 vol. of 0.01 M-phosphate buffer, pH 7.8, containing 20% glycerol and 0.1 M-dithiothreitol.

Cytochrome *P*-450 content and NADPH–cytochrome *c* reductase activity were assayed by the methods of Omura & Sato (1964) and Masters *et al.* (1971) respectively. Microsomal preparations after proteinase treatment contained about 1.5–

2.0nmol of cytochrome *P*-450/mg of protein and less than 5nmol/min per mg of protein of NADPH–cytochrome *c* reductase activity. Liver microsomal NADPH–cytochrome *c* reductase fractions from rats and hamsters were prepared by the method of Lu & West (1972). Such preparations did not have any detectable amount of cytochrome *P*-450; reductase activity of these fractions was about 1500–2000nmol/min per mg of protein. Protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as standard.

The incubation medium for dimethylnitrosamine demethylation contained 0.1M-potassium phosphate buffer, pH6.5, 2mM-NADPH, 0.01M-semicarbazide, 2mM-di[^{14}C]methylnitrosamine (sp. radioactivity 0.25mCi/mmol) and liver microsomal fractions as indicated to a total volume of 1.0ml. After incubation in air for 30min at 37°C, the reaction was terminated by the addition of 1.5ml of 50% trichloroacetic acid. Blank samples were prepared by adding NADPH after the addition of trichloroacetic acid. Oxidative demethylation of dimethylnitrosamine was determined by measuring the formation of H^{14}CHO, which was precipitated as formaldemethone by the procedure of Frisell & Mackenzie (1958) as modified by Paik & Kim (1974). Precipitated formaldemethone was weighed, put in vials and dissolved in 10ml of toluene containing 0.4% (w/v) 2,5-diphenyloxazole and 0.01% (w/v) 1,4-bis-(5-phenyloxazol-2-yl)benzene. Radioactivity was measured by liquid-scintillation spectrometry. Radioactivity data obtained with blank samples were deducted from radioactivity data obtained with incubated samples. Enzyme activity is expressed in terms of nmol of H^{14}CHO formed/30min per nmol of cytochrome *P*-450.

Results and discussion

Preliminary studies standardized optimum conditions for oxidative demethylation of dimethylnitrosamine by rat and hamster liver microsomal preparations. Previous investigators (Czygan *et al.*, 1973; Venkatesan *et al.*, 1968) used pH7.4 phosphate buffer system to study demethylation of dimethylnitrosamine with mouse and rat liver microsomal fractions in an NADPH-generating system. In our present studies, pH6.5 was found to be an optimum pH for both rat and hamster liver microsomal fractions. Enzyme activity with hamster liver microsomal fractions at pH7.4 was about 50% of that at pH6.5; activity with rat preparations at pH7.4 was about 35% of that at pH6.5. Demethylation activity was about equal with either NADPH or NADPH-generating system at pH6.5. Study of enzyme concentration and period of incubation indicated that the reaction was linear up to 1nmol of cytochrome *P*-450 concentration and for a period of 40min. Under such optimum conditions, adult male rat and hamster liver microsomal fractions showed different dimethylnitrosamine demethylation activities (Table 1). Hamster preparations were severalfold more active than those from rat when specific activities were compared on the basis of either mg of protein or nmol of cytochrome *P*-450. These differences in enzyme activities were observed over a wide range of dimethylnitrosamine (0.25–3mM) and NADPH (0.25–3mM) concentrations. Montesano & Magee (1971) studied di[^{14}C]methylnitrosamine metabolism by rat and hamster liver tissue slices *in vitro* and also obtained higher $^{14}CO_2$ production with hamster liver slices than with rat liver slices.

Table 1. *Liver microsomal dimethylnitrosamine demethylation activity*

Microsomal protein containing 1nmol of cytochrome *P*-450 was used for incubation. All other details are described under 'Materials and methods'. Livers from three animals were pooled for each analysis. Results are given as means±S.E.M. of analyses.

Species	H^{14}CHO formed (nmol/30min per mg of protein)	H^{14}CHO formed (nmol/30min per nmol of cytochrome *P*-450)
Rat	16± 4	21± 4
Hamster	79±14	92±20

There was a possibility that lower enzyme activities in rat liver microsomal preparations might be due to the presence of an inhibitor in such preparations. Our dimethylnitrosamine demethylation results obtained from mixing liver microsomal preparations from rats and hamsters rule out any possibility of the presence of an inhibitor in rat liver preparations (P. D. Lotlikar, W. J. Baldy, Jr. & E. N. Dwyer, unpublished work).

Further differences in microsomal preparations from these two species were obtained when these microsomal fractions were treated with a bacterial proteinase. After a short period (1h) of proteinase treatment, microsomal particles lose more than 95% of their NADPH–cytochrome *c* reductase activity without an appreciable loss in their cytochrome *P*-450 content (Comai & Gaylor, 1973; Lotlikar *et al.*, 1974; Lotlikar & Zaleski, 1975; Mitani *et al.*, 1971). In earlier studies, no catalytic activities were shown with such proteinase-treated microsomal fractions either before or after detergent solubilization (Comai & Gaylor, 1973; Mitani *et al.*, 1971), but, in our recent studies, we have demonstrated catalysis of *N*- and ring-hydroxylation of a carcinogenic aromatic amide with Triton X-100-solubilized proteinase-treated liver microsomal fractions in the presence of a reductase fraction from rats or hamsters (Lotlikar *et al.*, 1974; Lotlikar & Zaleski, 1975). It might be mentioned here that the hydroxylation activities in these proteinase-treated particles were much lower

Table 2. *Requirements of microsomal fractions for oxidative demethylation of dimethylnitrosamine*

Livers from 16 hamsters and eight rats were pooled separately for isolation of microsomal fractions. Other details are described under 'Materials and methods'. Cytochrome *P*-450 fractions obtained after proteinase treatment of liver microsomal fractions from rats (R) and hamsters (H) are designated unsolubilized cytochrome *P*-450 fractions. Where indicated, cytochrome *P*-450 fraction containing 1.0nmol of cytochrome *P*-450 and reductase fraction containing 400nmol/min of NADPH–cytochrome *c* reductase were added. Oxidative dimethylnitrosamine demethylation activity of whole microsomal preparations from livers of rats and hamsters were 23.0 and 102nmol of $H^{14}CHO$ formed/30min per nmol of cytochrome *P*-450 respectively. Reductase fraction alone (400nmol/min of reductase activity) from either rat or hamster formed only 0.2nmol of $H^{14}CHO$/30min.

Microsomal fractions added	$H^{14}CHO$ formed with the cytochrome *P*-450 fraction (nmol/30min per nmol of cytochrome *P*-450)		
	Unsolubilized	Cholate-solubilized	Triton X-100-solubilized
Cytochrome *P*-450 (R)	2.0	0.5	0.4
Cytochrome *P*-450 (R)+reductase (R)	12.1	6.1	11.0
Cytochrome *P*-450 (R)+reductase (H)	12.2	5.9	14.9
Cytochrome *P*-450 (H)	4.7	2.2	1.7
Cytochrome *P*-450 (H)+reductase (H)	38.6	13.2	20.6
Cytochrome *P*-450 (H)+reductase (R)	38.5	13.1	18.8

than those after Triton X-100 solubilization (P. D. Lotlikar & K. Zaleski, unpublished work). When such proteinase-treated particles, designated unsolubilized cytochrome *P*-450 fractions, were tested for dimethylnitrosamine demethylation activity it was found that such particles from rat or hamster alone had very low activity (Table 2). Similarly, reductase from neither rat nor hamster alone was able to demethylate dimethylnitrosamine. However, in the presence of both unsolubilized cytochrome *P*-450 fraction and reductase fraction from hamster, an appreciable amount of formaldehyde, about 40% of that formed with whole microsomal preparations, was formed. In such studies, cytochrome *P*-450 fraction from hamster could use the reductase fraction from either hamster or rat very efficiently. Similar results were obtained with unsolubilized cytochrome *P*-450 fraction from rat. However, activity with unsolubilized cytochrome *P*-450 fraction from rat was much lower than that from hamster. These studies indicated that, even though both cytochrome *P*-450 and reductase fractions are required for optimum demethylation activity, the degree of dimethylnitrosamine demethylation is determined by the source of the cytochrome *P*-450 fraction and not by the source of the reductase fraction.

Cholate- and Triton X-100-solubilized cytochrome *P*-450 preparations from these two species also showed differences in their catalytic activities in the presence of the reductase fraction (Table 2). Addition of either synthetic phospholipid, dilauroyl phosphatidylcholine or total microsomal lipid extract to the incubation medium did not increase the enzyme activity of any of the unsolubilized or detergent-solubilized cytochrome *P*-450 fractions.

Narasimhulu (1974) reported catalytic activity in the particulate cytochrome *P*-450 fraction from bovine adrenal cortex in the presence of reductase fraction and a non-ionic detergent. However, our present paper appears to be the first report demonstrating the presence of an appreciable amount of catalytic activity in the unsolubilized microsomal cytochrome *P*-450 fraction from a mammalian liver in the presence of reductase fraction.

The present data show differences in catalytic properties between whole microsomal preparations from livers of rats and hamsters. Reconstitution studies with unsolubilized cytochrome *P*-450 fractions also demonstrated similar differences in catalytic activity between these two species. These results suggest that oxidative demethylation of dimethylnitrosamine mediated via the cytochrome *P*-450 enzyme system is catalysed by different cytochrome *P*-450 molecules in these two species. Kawalek & Lu (1975) demonstrated different catalytic properties between rat and rabbit liver microsomal cytochrome *P*-448. On the basis of several lines of evidence, they concluded that rat cytochrome *P*-448 and rabbit cytochrome *P*-448 represent two different proteins. Definitive proof for the existence of such different proteins in the livers of rats and hamsters in the present study would require further purification of the cytochrome *P*-450 fractions.

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Comai, K. & Gaylor, J. L. (1973) *J. Biol. Chem.* **248**, 4847–4855

Czygan, P., Greim, H., Garro, A. J., Hutterer, F., Schaffner, F., Popper, H., Rosenthal, O. & Cooper, D. Y. (1973) *Cancer Res.* **33**, 2983–2986

Druckrey, H., Preussman, R., Ivankovic, S. & Schmahl, D. (1967) *Z. Krebsforch.* **69**, 103–201

Frisell, W. R. & Mackenzie, C. G. (1958) *Methods Biochem. Anal.* **6**, 63–77
Kawalek, J. C. & Lu, A. Y. H. (1975) *Mol. Pharmacol.* **11**, 201–210
Lotlikar, P. D. & Zaleski, K. (1975) *Biochem. J.* **150**, 561–564
Lotlikar, P. D., Luha, L. & Zaleski, K. (1974) *Biochem. Biophys. Res. Commun.* **59**, 1349–1355
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
Lu, A. Y. H. & West, S. (1972) *Mol. Pharmacol.* **8**, 490–500
Lu, A. Y. H., Junk, K. W. & Coon, M. J. (1969) *J. Biol. Chem.* **244**, 3714–3721
Lu, A. Y. H., Levin, W., West, S., Jacobson, M., Ryan, D., Kuntzman, R. & Conney, A. H. (1973) *Ann. N.Y. Acad. Sci.* **212**, 156–174
Magee, P. N. & Barnes, J. M. (1967) *Adv. Cancer Res.* **10**, 163–246
Masters, B. S. S., Baron, J., Taylor, W. E., Isaacson, E. L. & LoSpalluto, J. (1971) *J. Biol. Chem.* **246**, 4143–4150
Miller, J. A. (1970) *Cancer Res.* **30**, 559–576
Mitani, F., Alvares, A. P., Sassa, S. & Kappas, A. (1971) *Mol. Pharmacol.* **7**, 280–292
Montesano, R. & Magee, P. N. (1971) *Proc. Am. Ass. Cancer Res.* **12**, 14
Narasimhulu, S. (1974) *Drug Metab. Dispos.* **2**, 573–576
Omura, T. & Sato, R. (1964) *J. Biol. Chem.* **239**, 2370–2378
Paik, W. K. & Kim, S. (1974) *Arch. Biochem. Biophys.* **165**, 369–378
Venkatesan, N., Arcos, J. C. & Argus, M. F. (1968) *Life Sci.* **7**, 1111–1119