Effect of Acute Treatment with Dimethylnitrosamine on the Contents of Cytochromes and on the Activities of δ -Aminolevulinic Acid Synthetase, Heme Oxygenase and Drug-Metabolizing Enzymes in the Liver of Rats

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The effects of acute treatment with dimethylnitrosamine (DMN) on the contents of cytochromes and on the activities of key enzymes in heme metabolic pathways were investigated in male Wistar rats.

Oral administration of DMN at a single dose of 30 mg/kg markedly decreased the contents of microsomal cytochrome P-450 and b_5 , and this depression remained during 7 days after treatment. On the other hand, the activity of heme oxygenase was enhanced by 140% at 1 day, reached a peak (about 335% of the control) at 2 days, and returned to the initial level at 7 days after treatment. However, no significant change was observed in the activity of δ -aminolevulinic acid synthetase throughout these experiments.

The activities of aminopyrine demethylase, aniline hydroxylase, bilirubin UDP-glucuronyltransferase, NADPH-cytochrome c reductase and NADH-cytochrome b_5 reductase were all markedly reduced at 2 or 3 days after DMN treatment. In contrast, the activities of serum GPT and GOT and the content of serum bilirubin were significantly increased at 2 or 3 days after treatment.

In the case of phenobarbital pretreatment (80 mg/kg/day for 3 days), no remarkable differences were seen in the activities of bilirubin UDP-glucuronyltransferase and of key enzymes in the heme metabolic pathways between groups given simultaneous treatment with phenobarbital and DMN and DMN treatment alone, although phenobarbital apparently potentiated the activities of serum GPT and GOT induced by DMN treatment.

Key words: dimethylnitrosamine; heme oxygenase; δ -aminolevulinic acid synthetase; cytochrome P-450; cytochrome b_5 ; NADPH-cytochrome c reductase; NADH-cytochrome b_5 reductase; bilirubin UDP-glucuronyltransferase; serum bilirubin; serum transaminase

Introduction

Many workers have postulated that dimethylnitrosamine (DMN) is converted into toxic, carcinogenic and mutagenic intermediates by the cytochrome P-450 dependent mixed function oxidase system^{1)~6)}. However, some authors reported that the metabolism of DMN did not appear to be wholly mediated by cytochrome P-450 and DMN demethylase, and suggested that other enzyme systems

may play an important role, because the cytochrome P-450 dependent mixed function oxidase involved in the metabolism of DMN was found to differ from some well established mixed function oxidases in its inducibility and susceptibility to inducers or inhibitors^{7)~10)}.

Although many studies have appeared on DMN demethylase^{11)~20)}, there is little information available concerning the influence of acute administration of DMN on the contents of cytochromes and on the activities of key

enzymes which regulate the heme content in the heme metabolic pathways. Therefore, the purpose of this study was to determine the time course of the effects of DMN (single dose) on the contents of cytochrome P-450 and b_5 and on the activities of δ -aminolevulinic acid (ALA) synthetase and heme oxygenase, which are the initial and rate-limiting enzymes in the biosynthesis and degradation of heme, respectively. In addition, we investigated whether any alteration occurred in the reaction sequence of microsomal electron transport systems or in the drug-metabolizing enzyme activities.

Materials and Methods

Chemicals.

Uridine-5'-diphosphoglucuronic acid trisodium salt was obtained from Nakarai Chemicals Ltd., Kyoto, Japan. Cytochrome c was purchased from Sigma Chemicals Co., St. Louis, Mo., USA. NADP, NADH, glucose 6-phosphate disodium salt and glucose 6-phosphate dehydrogenase were obtained from Boehringer Mannheim Yamanouchi Co., Ltd. Tokyo, Japan. Hemin, bilirubin and DMN were obtained from Tokyo Kasei Kogyo Co., Ltd., Tokyo, Japan. Other chemicals of reagent grade were obtained from commercial sources and used without further purification.

Animals.

Five-week-old male Wistar rats were pur-

chased from Kyudo Co., Ltd., Tosu, Saga, Japan, and maintained on commercial rat chow (Clea CE-2, Nippon Clea Co., Ltd., Tokyo, Japan) and tap water *ad libitum* in the animal room for 1 week, then used for experiments.

Assay procedures.

Animals were given DMN orally at a single dose of 30 mg/kg in 0.9% saline solution at 5 ml per kg by gastric intubation. Pretreatment of the animals involved intraperitoneal injections of phenobarbital (PB, 80 mg/kg in 0.9% saline solution at 2 ml per kg) at 72, 48 and 24 hrs before sacrifice. After treatment with DMN on schedule animals were sacrificed by decapitation; blood was collected for the determination of serum transaminase activities, and the livers were perfused *in situ* with cold 0.9% saline solution. The livers were removed and homogenized with 4 volumes of 0.25 M sucrose containing 1 mM EDTA in a Potter-Elvehjem homogenizer with a Teflon pestle. Microsomes were prepared by the method described previously²¹⁾. Protein concentration was determined according to Lowry *et al.*²²⁾ using bovine serum albumin as a standard. ALA synthetase activity of liver homogenates was determined by the method of Marver *et al.*²³⁾. Heme oxygenase activity of microsomes was measured by the method of Maines and Kappas²⁴⁾. Cytochrome P-450 and b_5 contents were estimated

Table 1. Effect of Dimethylnitrosamine on the Microsomal Cytochrome Contents and on the Activities of δ -Aminolevulinic Acid Synthetase and Heme Oxygenase

	After treatment (d)	Rat No.	Cytochrome content ^{a)}		Enzyme activity	
			P-450	b_5	δ -ALA synthetase ^{b)}	Heme oxygenase ^{c)}
Control	—	8	0.90 $\pm$ 0.04	0.38 $\pm$ 0.01	35.8 $\pm$ 2.8	1.45 $\pm$ 0.16
Dimethyl-	1	8	0.50 $\pm$ 0.03****	0.31 $\pm$ 0.01****	46.5 $\pm$ 3.0	2.06 $\pm$ 0.17**
nitrosamine	2	8	0.45 $\pm$ 0.02****	0.23 $\pm$ 0.01****	31.9 $\pm$ 1.5	4.85 $\pm$ 0.36****
Control	—	4	0.99 $\pm$ 0.08	0.36 $\pm$ 0.01	29.9 $\pm$ 2.3	1.44 $\pm$ 0.18
Dimethyl-	3	4	0.56 $\pm$ 0.04***	0.20 $\pm$ 0.01****	36.9 $\pm$ 3.0	3.04 $\pm$ 0.43*
nitrosamine	7	4	0.66 $\pm$ 0.03***	0.28 $\pm$ 0.02***	26.7 $\pm$ 1.5	1.47 $\pm$ 0.7

Dimethylnitrosamine at a single dose of 30 mg/kg was administered to animals by gastric intubation, and animals were sacrificed 1, 2, 3 or 7 days after the treatment with DMN. Each value represents the mean $\pm$ S.E. of 4 to 8 rats. ^{a)}: nmol/mg protein; ^{b)}: nmol δ -ALA/g liver/hr; ^{c)}: nmol bilirubin/mg protein/hr. Significantly different from the corresponding mean of the control, * $P < 0.05$, ** $P < 0.02$, *** $P < 0.01$ and **** $P < 0.001$.

as described by Omura and Sato²⁵⁾. Methods of determination of other enzyme activities were as follows—NADPH-cytochrome c reductase according to Omura and Takesue²⁶⁾; NADH-cytochrome b₅ reductase according to Takesue and Omura²⁷⁾; aminopyrine demethylase by means of the Nash reaction²⁸⁾; aniline hydroxylase according to Imai *et al.*²⁹⁾; glucuronyltransferase according to Heirwegh *et al.*³⁰⁾; glutamic oxaloacetic and glutamic pyruvic transaminase according to Reitman and Henkel³¹⁾.

Results and Discussion

The results of the time course experiments after single oral administration of DMN at a dose of 30 mg/kg on the contents of cytochrome P-450 and b₅ as well as on the activities of ALA synthetase and heme oxygenase are summarized in Table 1.

Numerous workers have reported that various chemicals, such as PB^{7), 11), 15), 19), 35), 38), 41)}, 3-methylcholanthrene^{6), 7), 12), 15)~17), 32), 38), 41)}, β -naphthoflavone^{6), 14), 38), 40)}, pregnenolone-16 α -carbonitrile^{6), 34), 38), 40)}, and so on^{13), 20), 33), 36)~39)}, and various factors^{18), 35), 42)} affect the metabolism and hepatotoxicity of DMN. However, no detailed study has been reported

of the effects on the contents of hepatic cytochromes and on the activities of enzymes of heme biosynthesis and degradation following acute intake of DMN. Smuckler *et al.*⁴⁴⁾ observed that DMN at a dose of 20 mg/kg led to a decrease in the amount of cytochrome P-450 at 2 hrs after oral administration, and Skaare *et al.*³⁹⁾ reported that DMN at a dose of 30 mg/kg caused a 42% decrease in cytochrome P-450 concentration at 24 hrs after intraperitoneal injection. However, Smuckler *et al.*⁴⁴⁾ noted no difference in cytochrome b₅ content and Skaare *et al.*³⁹⁾ did not report on cytochrome b₅ content after DMN treatment. In this study, we found that DMN produced maximum reduction in the cytochrome P-450 content at 2 days but gave maximum reduction in the cytochrome b₅ content at 3 days after treatment. Subsequently, the contents of both cytochromes recovered somewhat, but were still low at 7 days after treatment (67% and 78% of control levels, respectively). These findings suggest that such prolonged depression in the amount of cytochromes is due to loss of heme through an increase of heme degradation or a reduction of heme synthesis. Heme oxygenase activity was enhanced by 140% when meas-

Table 2. Effect of Dimethylnitrosamine on the Liver Constituents, Serum Bilirubin Content and on the Activities of Serum Transaminase and Hepatic Microsomal Enzymes

	Control	DMN-treatment	
		after 1d	after 2d
Body weight initial (g)	123 $\pm$ 4	125 $\pm$ 5	126 $\pm$ 4
final (g)	141 $\pm$ 5	135 $\pm$ 5	133 $\pm$ 5
Liver weight (g/100 g body weight)	4.52 $\pm$ 0.12	4.44 $\pm$ 0.14	4.52 $\pm$ 0.09
Liver glycogen (mg/g)	65.3 $\pm$ 4.5	35.0 $\pm$ 2.2****	16.6 $\pm$ 2.0****
Liver microsomal protein (mg/g)	27.0 $\pm$ 0.9	23.7 $\pm$ 1.0**	21.8 $\pm$ 1.1***
Serum GOT (Karmen unit)	88 $\pm$ 2	98 $\pm$ 7	151 $\pm$ 10***
Serum GPT (Karmen unit)	43 $\pm$ 5	65 $\pm$ 9	138 $\pm$ 15***
Serum bilirubin (mg/100 ml)	0.16 $\pm$ 0.03	0.21 $\pm$ 0.01	0.56 $\pm$ 0.09***
NADPH-cytochrome c reductase ^{a)}	40.0 $\pm$ 3.8	36.4 $\pm$ 2.1	32.5 $\pm$ 1.5
NADH-cytochrome b ₅ reductase ^{b)}	2.21 $\pm$ 0.11	2.03 $\pm$ 0.12	1.77 $\pm$ 0.06**
Aminopyrine demethylase ^{a)}	4.26 $\pm$ 0.26	3.41 $\pm$ 0.38	2.16 $\pm$ 0.25****
Aniline hydroxylase ^{a)}	0.71 $\pm$ 0.03	0.42 $\pm$ 0.02****	0.44 $\pm$ 0.02****
Bilirubin UDP-glucuronyltransferase ^{c)}	18.9 $\pm$ 1.1	16.5 $\pm$ 0.9	14.3 $\pm$ 0.5***

Dimethylnitrosamine (DMN, 30 mg/kg, po) was administered to animals 1 or 2 days prior to sacrifice. Each value represents the mean $\pm$ S.E. of 8 rats. ^{a)}: nmol/mg protein/min; ^{b)}: μ mol/mg protein/min; ^{c)}: nmol bilirubin conjugated/mg protein/10 min. Significantly different from the corresponding mean of the control, ** P<0.02, *** P<0.01 or **** P<0.001.

Table 3. Effect of Dimethylnitrosamine on the Liver Constituents and the Activities of Serum Transaminase and Hepatic Microsomal Enzymes

	Control	DMN-treatment	
		after 3d	after 7d
Body weight initial (g)	110±4	108±2	111±3
final (g)	142±4	134±5	147±4
Liver weight (g/100 g body weight)	5.07±0.03	5.00±0.16	5.25±0.20
Liver glycogen (mg/g)	61.6±2.7	29.7±8.8**	53.8±7.6
Liver microsomal protein (mg/g)	26.8±0.6	22.3±0.4***	26.5±1.6
Serum GPT (Karmen unit)	38±7	84±9***	30±3
NADPH-cytochrome c reductase ^{a)}	35.9±1.7	25.4±1.9***	32.4±1.6
NADH-cytochrome b ₅ reductase ^{b)}	2.28±0.16	1.55±0.10***	2.05±0.13
Aminopyrine demethylase ^{a)}	3.44±0.16	1.43±0.14***	2.28±0.28*
Aniline hydroxylase ^{a)}	0.79±0.06	0.53±0.06*	0.72±0.08
Bilirubin UDP-glucuronyltransferase ^{c)}	17.1±0.5	12.3±0.9***	16.1±0.5

Dimethylnitrosamine (DMN, 30 mg/kg, po) was administered to animals 3 or 7 days prior to sacrifice. Each value represents the mean±S.E. of 4 rats. ^{a)}: nmol/mg protein/min; ^{b)}: μ mol/mg protein/min; ^{c)}: nmol bilirubin conjugated/mg protein/10 min. Significantly different from the corresponding mean of the control, * $P<0.05$, ** $P<0.02$ or *** $P<0.01$.

ured 1 day after treatment with DMN, reached a peak (about 335% of the control) at 2 days, and returned to the initial level at 7 days after treatment at the dose used, as shown in Table 1. On the other hand, no significant alteration was observed in ALA synthetase activity throughout this experiment. Recently, many papers have demonstrated that heme or hemoprotein⁴⁵⁾, heavy metals^{24), 46), 48)} and halogenated compounds^{47), 49)} markedly enhance the hepatic heme oxygenase activity and also accelerate the degradation of the heme moiety of cellular hemoprotein, cytochrome P-450. From these studies and our findings, we speculate that the enhancement of hepatic heme oxygenase activity by acute intake of DMN is associated with the destruction of hemoprotein, cytochrome P-450 and b₅. However, it is uncertain whether the observed depression of hemoprotein is a direct cause of heme depletion, or a consequence of enhancement of heme oxygenase activity.

As shown in Tables 2 and 3, the enzyme activities in the reaction sequence of microsomal electron transport systems, NADPH-cytochrome c reductase and NADH-cytochrome b₅ reductase, were also appreciably decreased at 2 or 3 days after DMN treatment. Maximum reduction in these enzyme activi-

ties was seen at 3 days after treatment. However, this reduction seems noteworthy, because prolonged depressions in cytochrome contents and reductase activities in the reaction sequence of the microsomal electron transport system may to some extent induce the modification of cytochrome P-450 dependent mixed function oxidase, DMN demethylase, and DMN-mediated mutagenesis, even if the relationship between DMN demethylase activity and DMN-mediated mutagenesis is not linear^{19), 41)}.

In addition, serum glutamic pyruvic transaminase (GPT) and glutamic oxaloacetic transaminase (GOT), which were measured as indicators of liver damage, were significantly enhanced at 2 or 3 days after DMN treatment. Maduagwu and Bassir⁴²⁾ observed high values of serum GPT and GOT at 30 hrs after oral administration of DMN at a single dose of 50 mg/kg. High values of serum transaminase indicate severe damage to liver cells. As shown in Table 2 or 3, DMN markedly decreased the contents of liver glycogen and microsomal protein, whereas it increased the content of serum bilirubin. Maduagwu and Bassir⁴²⁾ also reported a marked increase of serum bilirubin in rats after administration of DMN at a single dose of 50 mg/kg, or at a daily oral dose

Table 4. Alterations of Dimethylnitrosamine Effect on the Hepatic Constituents and on the Activities of Serum and Hepatic Enzymes by Phenobarbital

	Control	PB	DMN	PB+DMN
Body weight initial (g)	94±3(3)	92±3(3)	93±6(4)	91±3(4)
final (g)	119±3	115±4	115±2	94±3*
Liver weight (g/100 g body weight)	4.64±0.36	5.33±0.13	4.50±0.51	5.71±0.28
Liver glycogen (mg/g)	65.5±1.0	46.4±3.7**	16.8±5.8***	19.5±1.0****
Liver microsomal protein (mg/g)	28.1±1.9	32.1±0.4	20.6±0.1***	22.9±0.5*
Serum GOT (Karmen unit)	89±9	81±7	269±48**	450±56***
Serum GPT (Karmen unit)	22±5	20±3	59±16*	157±18****
NADPH-cytochrome c reductase ^{a)}	37.2±1.3	59.3±2.0****	31.1±0.9*	47.1±0.9***
NADH-cytochrome b ₅ reductase ^{b)}	2.52±0.13	2.67±0.12	1.78±0.01**	1.64±0.06****
Cytochrome P-450 ^{c)}	0.86±0.07	1.87±0.14***	0.45±0.08***	1.02±0.07
Cytochrome b ₅ ^{c)}	0.36±0.03	0.49±0.02**	0.21±0.01***	0.31±0.01
Aminopyrine demethylase ^{a)}	3.35±0.33	8.71±0.68***	2.20±0.25*	6.34±0.46****
Aniline hydroxylase ^{a)}	0.72±0.08	1.42±0.05***	0.38±0.05*	0.78±0.08
Bilirubin UDP-glucuronyltransferase ^{d)}	18.7±0.6	19.6±2.1	13.5±1.8**	14.3±0.9***
δ-ALA synthetase ^{e)}	37.0±4.7	25.3±1.4	30.6±2.1	35.0±3.8
Heme oxygenase ^{f)}	1.74±0.16	1.09±0.02**	3.84±0.13**	4.03±0.17**

Phenobarbital (PB, 80 mg/kg, ip) pretreated to rats daily for 3 days, and at 4th day animals were given a single dose of dimethylnitrosamine (DMN, 30 mg/kg, po) and then sacrificed 48 hr after DMN treatment. Each value represents the mean±S.E. of 3 to 4 rats. ^{a)}: nmol/mg protein/min; ^{b)}: μmol/mg protein/min; ^{c)}: nmol/mg protein; ^{d)}: nmol bilirubin conjugated/mg protein/10 min; ^{e)}: nmol δ-ALA/g liver/hr; ^{f)}: nmol bilirubin/mg protein/hr. Significantly different from the corresponding mean of the control, * P<0.05, ** P<0.02, *** P<0.01 or **** P<0.001.

of 5 mg/kg for 5 to 11 days or of 1 mg/kg for 30 days. They suggested that the high values of serum bilirubin might be due to intrahepatic cell damage. In this study, we observed that the single administration of DMN produced considerable depression of bilirubin UDP-glucuronyltransferase in the phase II reaction as well as of aminopyrine demethylase and aniline hydroxylase in the phase I reaction. These findings also suggest that acute intake of DMN may alter the metabolism of DMN itself and enhance DMN-mediated toxicity or mutagenicity. Furthermore, the depression of UDP-glucuronyltransferase activity for bilirubin would lead to an increase in the amount of unconjugated bilirubin in serum with an increase of heme oxygenase activity as shown in Table 1. This would appear to present a serious risk of hyperbilirubinemia to the fetus and newborn as a result of direct or maternal acute intake of DMN.

Table 4 shows the influence of PB pretreatment on the above effects of DMN. Some investigators reported that PB modified DMN

demethylase or the rate of metabolism of DMN^{(7), (11), (15), (19), (35), (38), (41)}. In this study, PB partially enhanced the toxic effects of DMN, namely concomitant treatment with PB and DMN significantly reduced the average body weight gains by rats and potentiated the enhanced activities of serum GPT and GOT induced by DMN as compared to the activities in animals treated with DMN alone. However, no significant differences were seen in the activities of key enzymes of the heme metabolic pathways, of bilirubin UDP-glucuronyltransferase or of NADH-cytochrome b₅ reductase between groups given simultaneous treatment with PB and DMN and DMN treatment alone. Interestingly, PB reduced the activity of heme oxygenase compared to the control. This may suggest an increase of heme content in the hepatic heme pool *in vivo*. Striking differences in the contents of cytochromes and in the activities of aminopyrine demethylase, aniline hydroxylase and NADPH-cytochrome c reductase were apparent between combined treatment with PB and DMN and DMN treatment alone. On

the other hand, the cytochrome contents and enzyme activities in the case of simultaneous administration of PB and DMN were similar to or slightly larger than those of non-treated controls, as shown in Table 4. It is toxicologically important that DMN demethylase activities, as well as the rate of metabolism of DMN and its biological effects in living animals, are susceptible to modification by various environmental chemicals and factors, as has been demonstrated in many studies.

Various biological effects of DMN at chronic and very low dose levels are being investigated and further study on the interaction effects of DMN with common chemicals, such as alcohol, tobacco smoke constituents and essential metals, is required.

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