SUPPRESSION OF PHENACETIN-INDUCED METHEMOGLOBINEMIA BY DIETHYLDITHIOCARBAMATE AND CARBON DISULFIDE AND ITS RELATION TO PHENACETIN METABOLISM IN MICE

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Oral pretreatment with diethyldithiocarbamate (DTC) and carbon disulfide (CS₂) prevented mice from methemoglobinemia induced by phenacetin. This treatment resulted in marked elevation of plasma *p*-phenetidine concentrations, prolongation of phenacetin levels, and lowering of *N*-acetyl-*p*-aminophenol and *p*-aminophenol levels. Both DTC and CS₂ also suppressed *p*-phenetidine-induced methemoglobinemia with a delay in plasma *p*-phenetidine disappearance. *In vitro*, methemoglobin formation by *p*-phenetidine was decreased in liver microsomes isolated from DTC- or CS₂-treated mice. The liver microsomal phenacetin and *p*-phenetidine O-deethylation activities and *p*-phenetidine N-hydroxylation activity decreased 1 h after administration of DTC or CS₂, whereas deacetylation of phenacetin and *N*-acetyl-*p*-aminophenol by microsomes and acetylation of *p*-phenetidine by a soluble fraction from a liver homogenate were scarcely affected. The suppression of methemoglobinemia by DTC and CS₂ may result from an inhibition of metabolic conversion of *p*-phenetidine to methemoglobin-forming substances such as *N*-hydroxy-*p*-phenetidine which is of most importance, *p*-aminophenol and 2-hydroxy-*p*-phenetidine by the microsomal cytochrome P-450-containing monooxygenase system in the liver.

Keywords — phenacetin methemoglobinemia; diethyldithiocarbamate; carbon disulfide; methemoglobinemia prevention; phenacetin metabolism inhibition

INTRODUCTION

Phenacetin, an antipyretic analgesic, because of the serious toxic side effects such as methemoglobinemia, renal papillary necrosis and pelvic carcinomas,¹⁾ has long been replaced by acetaminophen (*N*-acetyl-*p*-aminophenol, NAPA). The mechanism of these phenacetin toxicities has been studied in terms of its toxic metabolites.^{2–5)} The major initial pathways of phenacetin metabolism include deethylation to NAPA catalyzed by the microsomal cytochrome P-450-containing monooxygenase⁶⁾ and deacetylation to *p*-phenetidine by a carboxyesterase.^{7,8)} *p*-Phenetidine is responsible for the methemoglobin formation^{9,10)} after being metabolized to reactive compounds.^{11–13)}

As reported previously,¹⁴⁾ diethyldithiocarba-

mate (DTC) and carbon disulfide (CS₂) prevented mice from liver injury induced by a variety of structurally different hepatotoxicants that require metabolic activation by the liver microsomal monooxygenase system. Since both agents suppressed various liver microsomal drug metabolizing enzyme activities, we suggested that the preventive action might be due to an inhibition of bioactivation of the toxic agents. Both agents also affected pharmacologic actions of some drugs, *e.g.* prolongation of hexobarbital sleeping time and zoxazolamine paralysis time.

Thus, as an extension of our previous study, we tested the possible preventive action of DTC and CS₂ on phenacetin-induced methemoglobinemia and their effects on phenacetin metabolism.

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MATERIALS AND METHODS

Chemicals — Purity and sources of the test compounds used were as follows: sodium diethyldithiocarbamate trihydrate (DTC) (99.5%, nonaqueous titration), CS₂ (>99%, gas chromatography (GC)), and phenacetin (>95%), from Wako Pure Chemicals; *p*-phenetidine HCl (>99%), from Nakarai Chemicals Ltd. *p*-Nitrosophenetol was synthesized by etherification of *p*-nitrosophenol according to the method of Hays *et al.*¹⁵⁾ and the crystals obtained were determined pure by gas chromatography-mass spectrometry (GC-MS). (The authors are grateful to Drs. Okabayashi and Fujiwara, Niigata College of Pharmacy, for their advice in preparing this compound and for GC-MS analysis). The other chemicals were of guaranteed reagent grade and used without further purification.

Animals and Treatments — SPF-grade-male mice of ddY strain, 6 weeks old, were used with food and water given *ad libitum* throughout the experimental period. Phenacetin (500 mg/kg) suspended in 0.5% carboxymethyl cellulose sodium (CMC-Na) solution was given *p.o.*; *p*-phenetidine HCl (242 mg/kg) dissolved in saline was given *i.p.*; DTC (30–300 mg/kg) and CS₂ (30 mg/kg) dissolved in distilled water and olive oil respectively, were given *p. o.* 1 h before administration of phenacetin or *p*-phenetidine. All solutions were freshly prepared, and control animals received the vehicles alone.

Determination of Methemoglobin, Plasma Phenacetin and Its Metabolites — At various hours after administration of phenacetin or *p*-phenetidine, mice were decapitated and the blood immediately collected in heparinized syringes. Methemoglobin was determined as percent of total hemoglobin according to the method of Van Assendelft.¹⁶⁾ Plasma phenacetin and *p*-phenetidine concentrations were determined by the method of Brodie and Axelrod.⁹⁾ *p*-Aminophenol (PAP) and NAPA were determined by using the phenol reagent of Frings and Saloom¹⁷⁾ before and after hydrolysis of the deproteinized plasma.

Preparation of Liver Microsomes — Mice

were killed by exsanguination 1 h after administration of DTC (30–300 mg/kg) or CS₂ (30 mg/kg). The liver was homogenized in 4 volumes of 0.15 M KCl–20 mM potassium phosphate buffer (pH 7.4) containing 0.1 mM ethylenediaminetetraacetic acid (EDTA) and the homogenate was centrifuged at 9000 *g* for 20 min. The supernatant fraction, without the fluffy layer, was further centrifuged at 105000 *g* for 60 min. The microsomal pellet was resuspended in the same medium at a protein concentration of 20 mg/ml and used while fresh. Protein was determined by the method of Lowry *et al.*¹⁸⁾

Methemoglobin Formation in Vitro — The method of Uehleke¹³⁾ was slightly modified. The regular complete incubation mixture contained 10% of washed mouse red blood cells, 1 mg protein of microsomes, 200 μ M nicotinamide adenine dinucleotide phosphate (NADPH), a NADPH generating system (2.5 mM nicotinamide, 10 mM sodium isocitrate, 5 mM MgCl₂ and 0.5 units/ml of isocitrate dehydrogenase, Oriental Yeast Co., Ltd.) and 1 mM *p*-phenetidine in a final volume of 1.0 ml of calcium- and glucose-free Krebs–Ringer-phosphate solution (pH 7.4). The reaction was started by addition of *p*-phenetidine after preincubation for 3 min at 37 °C, and 0.2 ml-aliquots were determined for methemoglobin concentration at 5, 15, 30 and 60 min. Omission of microsomes, NADPH or *p*-phenetidine from the complete mixture produced no methemoglobin.

Assays for Drug Metabolizing Enzyme Activities — **O-Deethylation of Phenacetin:** The complete reaction mixture containing 1 mg/ml of microsomes, 200 μ M NADPH, a NADPH generating system, 3 mM phenacetin, 0.1 M potassium phosphate buffer (pH 7.4) and 0.1 mM EDTA in a final volume of 2.0 ml was incubated at 37 °C for 20 min. The reaction was stopped by addition of 1.0 ml of 20% trichloroacetic acid (TCA), and NAPA in the supernatant fluid was determined according to the method of Frings and Saloom.¹⁷⁾

O-Deethylation of *p*-Phenetidine: The same procedure as described for phenacetin deethyla-

tion was used except that phenacetin was substituted for *p*-phenetidine (3 mM). PAP in the TCA supernatant fluid was directly reacted with the phenol reagent.¹⁷⁾

Deacetylation of Phenacetin and NAPA: The complete reaction mixture containing 1 mg of microsomes, 3 mM phenacetin or NAPA and 0.1 M sodium phosphate buffer (pH 7.2) in a final volume of 1.0 ml was incubated at 37 °C for 30 min. The reaction was stopped by addition of 0.5 ml of 10% TCA and an aliquot of the supernatant fluid was determined for *p*-phenetidine⁹⁾ or PAP.¹⁷⁾

Acetylation of *p*-Phenetidine: The liver homogenate (20%) in 0.1 M sodium phosphate buffer (pH 7.2) was centrifuged at 15000 *g* for 30 min. The supernatant fraction was used as an enzyme source. The reaction mixture containing 0.05 ml of the supernatant fraction, 6 mM *p*-phenetidine, 6 mM acetyl CoA and 0.1 M sodium phosphate buffer (pH 6.8) in a final volume of 0.5 ml was incubated at 37 °C for 30 min. The reaction was stopped by addition of 0.25 ml of 10% TCA and the unreacted *p*-phenetidine in the TCA supernatant was determined.⁹⁾

N-Hydroxylation of *p*-Phenetidine: The reaction mixture contained 1 mg/ml of microsomes, 400 μ M NADPH, a NADPH generating system, 1 mM *p*-phenetidine and 1 mM dithiothreitol in a final volume of 2.0 ml of Krebs-Ringer-phosphate solution (pH 7.4) without calcium. The reaction was started by the addition of *p*-phenetidine and the mixture was incubated at 37 °C for 15 min. N-Hydroxylated product was determined as *p*-nitrosophenetol according to the method of Uehleke.¹³⁾ All reactions were conducted in air with shaking, and appropriate blanks were run in parallel in each assay.

Statistics — Statistical analysis was made by the method of Bonferoni after one-way analysis of variance¹⁹⁾ and $p < 0.05$ was considered statistically significant.

RESULTS

Effects on Phenacetin Methemoglobinemia

As shown in Fig. 1, phenacetin (500 mg/kg, *p. o.*) produced marked methemoglobinemia in ddY mice, which lasted for 2 to 3 h. Pretreatment with 300 mg/kg of DTC almost completely prevented methemoglobin formation. Suppression by 100 mg/kg of DTC was also evident, although slightly higher methemoglobin levels than those in controls were retained in later hours. With 30 mg/kg of DTC, only prolongation of methemoglobinemia was observed. Pretreatment with 30 mg/kg of CS₂, nearly equimolar to 100 mg/kg of DTC, was similarly effective.

Effects on Plasma Levels of Phenacetin and Its Metabolites

In the control group given phenacetin (500 mg/kg, *p. o.*) alone, plasma phenacetin concentrations rapidly increased, reaching a peak in 15 min, and then decreased to very low levels at 3 h, indicating rapid absorption and disposition of phenacetin. The plasma disappearance of phenacetin was significantly delayed by a pretreat-

FIG. 1. Effects of DTC and CS₂ on *in Vivo* Methemoglobin Formation Induced by 500 mg/kg of Phenacetin

DTC and CS₂ were given *p.o.* 1 h before administration of phenacetin (*p.o.*). Methemoglobin (MetHb) concentrations were determined as percent of the total hemoglobin in the blood. Each point represents the mean $\pm$ S.E. ($n=5$).

●—●, phenacetin (Ph) 500 mg/kg alone; ○—○, DTC 30 mg/kg + Ph; △—△, DTC 100 mg/kg + Ph; □—□, DTC 300 mg/kg + Ph; ▽—▽, CS₂ 30 mg/kg + Ph.

a, b) Significantly different from the control values at a) $p < 0.01$ or b) $p < 0.05$.

ment with either DTC (100 mg/kg, *p. o.*) or with an equimolar dose of CS₂ (Fig. 2 (A)). NAPA concentrations in the control mice reached a peak at 0.5–1 h and decreased more slowly than phenacetin. DTC and CS₂ suppressed NAPA production although complete disappearance of NAPA was slightly delayed (Fig. 2 (B)). *p*-Phenetidine level in the control was elevated in parallel with NAPA with a peak concentration comparable to that of NAPA. DTC and CS₂ markedly enhanced *p*-phenetidine production and delayed its disappearance (Fig. 2 (C)). PAP levels, about one tenth the NAPA levels, were also decreased by both agents (Fig. 2 (D)).

Suppression of *p*-Phenetidine-Induced Methemoglobin Formation

In confirmation of the observations with

dogs,⁹⁾ in control mice, *p*-phenetidine HCl (242 mg/kg, *i. p.*) produced even a greater degree of methemoglobinemia than that observed with twice the molar dose (500 mg/kg) of phenacetin. In the control mice, the high methemoglobin levels were maintained for 2 h and then rapidly decreased, whereas the plasma *p*-phenetidine concentrations began to fall much earlier and reached a considerably low level at 2 h (Fig. 3 A and B). DTC- or CS₂-pretreatment suppressed *p*-phenetidine-induced methemoglobinemia, to a similar extent as that observed in the case of phenacetin and significantly delayed the disappearance of plasma *p*-phenetidine.

On the other hand, Uehleke¹³⁾ reported that methemoglobin formation occurred when red blood cells were incubated with *p*-phenetidine in the presence of liver microsomes and

FIG. 2. *In Vivo* Effects of DTC and CS₂ on Plasma Concentrations of Phenacetin and Some of Its Metabolites Following 500 mg/kg of Phenacetin

DTC and CS₂ were given *p.o.* 1 h before administration of phenacetin. Each point represents the mean $\pm$ S.E. ($n=4-5$). ●—●, phenacetin (Ph) 500 mg/kg; Δ — Δ , DTC 100 mg/kg + Ph; ∇ — ∇ , CS₂ 30 mg/kg + Ph. a, b) Significantly different from the control values at a) $p < 0.01$ or b) $p < 0.05$.

NADPH. In a similar experiment using mouse red blood cells and microsomes, the *in vitro* methemoglobin formation by *p*-phenetidine was evidently suppressed when microsomes isolated from DTC- or CS₂-treated mice were used (Fig. 4).

Effects on Some Related Enzyme Activities

Some liver enzyme activities involved in phenacetin metabolism were determined 1 h after administration of 30–300 mg/kg of DTC or CS₂. Microsomal O-deethylation of phenacetin and *p*-phenetidine and N-hydroxylation of *p*-phenetidine were dose-dependently suppressed in DTC-treated mice (Fig. 5 A and B). However, deacetylation of phenacetin by microsomal esterase, the activity of which was fairly high as

compared with phenacetin O-deethylase activity, and deacetylation of NAPA, were not significantly affected by DTC (Fig. 5-C). CS₂ (30 mg/kg) produced a degree of change similar to that observed with 100 mg/kg of DTC in all of these enzyme activities. Acetylation of *p*-phenetidine by a soluble fraction of liver was almost unaffected by 300 mg/kg of DTC or 100 mg/kg of CS₂ (Fig. 5-D). Thus, both agents rather specifically inhibited the microsomal cytochrome P-450-dependent enzyme activities in the metabolism of phenacetin.

DISCUSSION

Brodie and Axelrod⁹⁾ first suggested that *p*-phenetidine is the precursor of a substance that

FIG. 3. *In Vivo* Effects of DTC and CS₂ on Methemoglobin Formation (A) and Plasma *p*-Phenetidine Levels (B) Following Administration of *p*-Phenetidine

DTC and CS₂ were given *p.o.* 1 h before administration of *p*-phenetidine HCl (242 mg/kg, *i. p.*). Each point represents the mean $\pm$ S.E. ($n=4-5$). $\bullet$ — $\bullet$, *p*-phenetidine HCl (*p*-Ph) 242 mg/kg; $\triangle$ — $\triangle$, DTC 100 mg/kg + *p*-Ph; $\square$ — $\square$, DTC 300 mg/kg + *p*-Ph; ∇ — ∇ , CS₂ 30 mg/kg + *p*-Ph. a, b) Significantly different from the control values at a) $p < 0.01$ or b) $p < 0.05$.

forms methemoglobin following phenacetin administration. They observed that the amount of methemoglobin in dogs paralleled the plasma *p*-phenetidine and a dose of *p*-phenetidine 3 times lower than phenacetin was equally effective, and that blood incubated with either drug showed no accumulation of methemoglobin. Similar results were obtained in the present study using mice. In addition, we also observed that following *p*-phenetidine administration, recovery from methemoglobinemia was much delayed as compared to the decrease in plasma *p*-phenetidine level. This may also indicate that metabolites of *p*-phenetidine accumulated in the blood, rather than *p*-phenetidine itself, cause methemoglobinemia. Heymann *et al.*,¹⁰ in another line of experiments, demonstrated that the formation of methemoglobin caused by phenacetin in rats was inhibited by simultaneous treatment with bis-(*p*-nitrophenyl) phosphate, a carboxyesterase inhibitor. Thus, both the hydrolysis of the aceto-amide bond of phenacetin to *p*-phenetidine by the esterase and the subsequent metabolism of *p*-phenetidine to methemoglobin forming substances are required for methemoglobin formation

DTC and CS₂ suppressed methemoglobinemia induced by phenacetin, which was accompanied by a delay in plasma phenacetin disappearance, lowering of NAPA levels and marked elevation and prolongation of *p*-phenetidine levels. The increased plasma *p*-phenetidine levels may be due mainly to an increase in the rate of phenacetin metabolism *via* deacetylation pathway which is a result of suppression of phenacetin deethylation to NAPA and consequent accumulation of phenacetin. DTC and CS₂ suppressed liver microsomal phenacetin O-deethylase activity with no significant effect on the deacetylase activity. Suppression of further metabolism of *p*-phenetidine may also be considered. The reason for the suppression of methemoglobinemia under such high plasma *p*-phenetidine levels may be an inhibition of further metabolism of *p*-phenetidine to more toxic substances. In support of this, DTC and CS₂ prevented the *in*

vivo *p*-phenetidine-induced methemoglobinemia accompanying a significant delay in plasma *p*-phenetidine disappearance. The *in vitro* methemoglobin formation by *p*-phenetidine, which requires the presence of liver microsomes and NADPH, was suppressed by microsomes isolated from DTC- or CS₂-pretreated mice.

Methemoglobin formation by various aromatic amines is proposed to be caused mainly by their N-hydroxylated metabolites.^{2,20} In addition, Uehleke¹³ showed that *p*-phenetidine was metabolized to *N*-hydroxy-*p*-phenetidine both *in vivo* and *in vitro* and that the methemoglobin formation correlated well with N-hydroxylation activity of liver microsomes. In the present experiment, N-hydroxylation of *p*-phenetidine, determined as *p*-nitrosophenetol, was indeed suppressed in microsomes isolated

FIG. 4. Effect of *in Vivo* Treatment of DTC and CS₂ on *in Vitro* *p*-Phenetidine-Induced Methemoglobin Formation

The liver microsomes were isolated 1 h after *in vivo* administration of DTC or CS₂ and incubated with red blood cells in the presence of NADPH and *p*-phenetidine as described in the Methods. Bars on the right indicate the methemoglobin (MetHb) formation by different concentrations of the control microsomes at 60 min of incubation. Each point represents the mean $\pm$ S.E. ($n=4$ for the control and $n=3$ for treated groups).

●—●, control; ○—○, DTC 30 mg/kg; △—△, DTC 100 mg/kg; □—□, DTC 300 mg/kg; ▽—▽, CS₂ 30 mg/kg.

a) Except for this point, values of all treated groups are significantly lower than the controls at $p < 0.01$.

from DTC- and CS₂-treated mice. Another methemoglobin-forming metabolite proposed is 2-hydroxy-*p*-phenetidine,^{4,12,21)} which may be formed by direct oxidation of the ring or by a rearrangement of *N*-hydroxy-*p*-phenetidine. Since both DTC and CS₂ inhibited various cytochrome P-450-dependent drug biotransformation reactions including hydroxylation,¹⁴⁾ it is possible that the direct ring hydroxylation of *p*-phenetidine by the monooxygenase could also be suppressed by these agents. PAP, which may be produced by deethylation of *p*-phenetidine and deacetylation of NAPA, can not completely be neglected as a methemoglobin-forming

metabolite even though it may have minor importance.^{2,20)} Pretreatment with DTC and CS₂ lowered plasma PAP concentrations after phenacetin administration and is consistent with suppression of microsomal O-deethylase activity.

Thus, the suppression of phenacetin-methemoglobinemia by DTC and CS₂ may be due to an inhibition of the conversion of *p*-phenetidine to methemoglobin-forming substances such as *N*-hydroxy-*p*-phenetidine (being most important), 2-hydroxy-*p*-phenetidine and PAP by the microsomal cytochrome P-450-containing monooxygenase

FIG. 5. *In Vivo* Effects of DTC and CS₂ on Some *In Vitro* Enzyme Activities Related to Phenacetin Metabolism

Enzyme activities were measured 1 h after administration of DTC or CS₂. Experimental details are described in the Methods. Each point represents the mean $\pm$ S.E. ($n=3-4$ in A, B and C, and $n=5$ in D). ○, DTC; ▽, CS₂ 30 mg/kg; △, CS₂ 100 mg/kg. 'a)' Significantly lower than the control values at $p < 0.01$.

system.

It remains unknown, however, why lower doses of DTC tended to delay the recovery from methemoglobinemia (Fig. 1). This tendency was more evident with lower doses of phenacetin (data not shown.)

Finally, actions of oral doses of DTC may be mediated through CS₂ produced in the stomach as discussed previously.¹⁴⁾ Depression of liver microsomal drug metabolizing enzyme activities both *in vivo* and *in vitro* has also been reported by other investigators with CS₂²²⁻²⁴⁾ and DTC.^{25,26)} The mechanism of the inhibition by CS₂ may involve the covalent binding of the sulfur atom to microsomal proteins after bioactivation by microsomal monooxygenase and the resultant loss of cytochrome P-450 activity.^{27,28)} A high dose of CS₂ is reported to cause hepatic necrosis in phenobarbital-treated, starved rats,^{29,30)} and in our preliminary experiments, 1.0 ml/kg, *i. p.* of CS₂ produced only a slight to moderate increase in plasma alanine amino transferase activity in normal male ddY mice. From the present and previous studies,¹⁴⁾ CS₂ and CS₂-producing agents, such as DTC may serve as extensive inhibitors of microsomal drug metabolizing enzyme activities at doses that produce no observable histopathologic changes in the liver. Such agents may be useful, for example, in detecting a possible involvement of microsomal bioactivation mechanism in the development of toxicity. Further studies on the disturbance of the microsomal cytochrome P-450 system by CS₂ are currently being conducted.

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