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Altered Microsome Function during Acute Thioacetamide Poisoning

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SUMMARY

The effects of the hepatotoxin and carcinogen, thioacetamide, on several hepatic mixed-function oxidases and the microsomal cytochrome content were examined following a single challenge with the toxin. Most of the metabolic pathways tested were deranged early in the course of the intoxication, and remained thus during the entire week of observation. The hepatic content of cytochromes P-450 and b_5 decreased; the activities of aryl hydrocarbon hydroxylase, *N*-demethylase, *N*-oxidase, and lipid peroxidase also declined. $NADPH_2$-neotetrazolium reductase activity diminished later, and to a lesser degree, than the above activities. Comparison of these changes with those observed following either carbon tetrachloride administration or partial surgical hepatectomy revealed several differences in the patterns of the responses. Results were obtained which suggest that the formation of formaldehyde and *N*,*N*-dimethylaniline *N*-oxide from *N*,*N*-dimethylaniline may proceed by separate and distinct pathways.

INTRODUCTION

The endoplasmic reticulum of mammalian hepatocytes is altered by a variety of exogenous substances (1). The complex of enzymes contained in these membranes metabolizes many of these agents which produce changes in the ergastoplasm, and in some instances converts innocuous substances to proximate toxins [a form of the "lethal synthesis" concept of Peters (2)]. Thioacetamide is a compound which modifies the morphology of the endoplasmic reticulum (3), and for this reason we set about to assess whether or not functional alterations accompany this modification.

Thioacetamide poisoning resulted in decreased activity in microsomal mixed-function oxygenases at or beyond the level of the NADPH–cytochrome *c* reductase. This change differs in some respects from the alterations noted after CCl_4 administration or partial surgical hepatectomy. The changes in the relative levels of formaldehyde release and *N*-oxide formation from *N*,*N*-dimethylaniline suggested that the *N*-oxide may not be an intermediate in this demethylation.

MATERIALS AND METHODS

Male Sprague-Dawley rats weighing 200–300 g were maintained on Purina laboratory chow in bare, wire-bottom cages and permitted water ad libitum. They were fasted for 16 hr prior to administration of the toxin. Thioacetamide was given as an aqueous solution by stomach tube (without anesthesia) in a dose of 20 mg/100 g of body

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weight; control animals received tap water. After administration of the toxin, the animals were permitted access to the Purina chow. In experiments in which recovery from thioacetamide exceeded 24 hr, the animals were again fasted prior to death. All animals were killed by cerebral concussion between 6 and 9 a.m. in order to minimize diurnal variation. The thoracic and abdominal cavities were opened, and the livers were perfused *in situ* with ice-cold 0.85% NaCl via the thoracic aorta. The livers were removed, placed in tared glass beakers containing ice-cold 0.2 M potassium phosphate buffer, pH 7.5, and minced with sharp scissors. All subsequent operations were carried out at 4° unless otherwise specified.

Preparation of microsomes. Livers were disintegrated in glass homogenizers with Teflon pestles in 3 volumes of 0.2 M phosphate buffer, pH 7.5. The crude homogenate was centrifuged at 15,000 × *g* for 10 min, and the supernatant fluid was centrifuged at 105,000 × *g* for 60 min. The resulting microsomal pellets were resuspended in 5–10 ml of 0.2 M phosphate buffer by gentle homogenization, and were used directly or recentrifuged. The protein concentration of the microsomal suspension was measured by the method of Lowry *et al.* (4), using crystalline bovine serum albumin as a standard.

NADPH-dependent N-demethylation and DMA-N-oxide[2] *formation.* Measurement of *N*-demethylation and *N*-oxide formation was carried out in an incubation system containing the following components in a final volume of 2.0 ml: 0.8 mg of microsomal protein, 0.15 M potassium phosphate buffer (pH 7.5), 60 mM nicotinamide, 5 mM *dl*-isocitrate, 0.2 mM $MnCl_2$, 0.05 mM NADP, 2.5 mM *N*,*N*-dimethylaniline, and enough isocitrate dehydrogenase to reduce 0.32 μmole of NADP per minute (5). The incubation was continued aerobically for 20 min at 37°. At the end of this period, the proteins were precipitated with 1.0 ml of 20% trichloracetic acid. The mixtures were agitated with a Vortex mixer and allowed to stand in an ice bath for 15 min, then centrifuged for 10 min at 2000 × *g*; 2-ml volumes of the supernatant fluid were transferred to separate tubes. Formaldehyde produced by the demethylation reaction was assayed after addition of 0.6 ml of Nash's reagent (6) to the supernatant fluid. Color development during incubation at 37° for 30 min was measured at 415 nm against the blank obtained from microsomes incubated without the addition of NADP. Similar analysis was carried out with a standard formaldehyde solution which was prepared daily from a 37% formaldehyde solution. The results are expressed as nanomoles of formaldehyde formed per milligram of microsomal protein per 20 min of incubation at 37°. Similar analyses were carried out using *N*,*N*-dimethylaniline *N*-oxide as the substrate. In addition, similar reactions were carried out after the addition of thioacetamide to the tubes, in final concentrations of 0.13, 1.3, 13, and 65 mM.

The formation of *N*,*N*-dimethylaniline *N*-oxide was determined by a modification of the method of Pettit and Ziegler (5). Duplicate samples were prepared as described for *N*-demethylation. Incubation was carried out for 20 min at 37°, the tubes were chilled, protein was precipitated by the addition of 1.0 ml of 1 M $HClO_4$, and the mixture centrifuged at 2000 × *g* for 10 min; 2 ml of the supernatant fluid were adjusted to pH 9.4 by the dropwise addition of 2 M NaOH, after which the samples were extracted three times with 3 ml of ether. Following the last extraction, 0.5 ml of 1 M glycine was added, and the pH of the aqueous phase was adjusted to 2.4 by addition of 3 M trichloracetic acid. To this, 0.1 ml of 2% $NaNO_2$ was added, and the solution was heated at 60° for 5 min. The pH was raised to 6, and the resulting yellow color was measured at 420 nm against a blank prepared from the same microsomal source and incubated without the addition of NADP. Similar analysis was carried out with a standard solution of *N*,*N*-dimethylaniline *N*-oxide (obtained from K & K Laboratories). The results are expressed as nanomoles of *N*,*N*-dimethylaniline *N*-oxide formed per milligram of microsomal protein per 20 min of incubation at 37°, or as a percentage of the control value. Similar incubations were

[2] The abbreviation used is: DMA: *N*,*N*-dimethylaniline.

carried out after the addition of thioacetamide to the tubes, in final concentrations of 0.13, 1.3, 13, and 65 mM.

NADPH-dependent oxidative p-hydroxylation of aniline. The microsomal suspension was adjusted to 2 mg of protein per milliliter. *p*-Hydroxylation was measured following incubation of 1, 2, 3, and 4 mg of microsomal protein with 0.15 M phosphate buffer (pH 7.5), 0.1 mM NADP, 4 mM *dl*-isocitrate, 44 mM nicotinamide, 0.01 mM $MnCl_2$, 8 mM aniline, and enough isocitrate dehydrogenase to reduce 0.32 μmole of NADP per minute in a final volume of 2.6 ml. The incubation was carried out at 37° for 30 min aerobically, and was stopped by the addition of 0.25 ml of 25% $ZnCl_2$ and 0.25 ml of saturated $Ba(OH)_2$. The *p*-aminophenol content in 3 ml of the supernatant solution after centrifugation at 800 × *g* for 30 min was measured by the indophenol reaction (7), with the addition of 2 ml of 0.01 N HCl, 1 ml of 1% phenol, and 1 ml of sodium hydrobromide solution. The resulting blue color was measured at 620 nm against a blank prepared from the same microsomal source and incubated without the addition of NADP. Similar color development was carried out with a standard *p*-aminophenol solution. The results were calculated as micrograms of *p*-aminophenol formed per milligram of microsomal protein per 30 min, and converted to a percentage of the control value.

NADPH-dependent neotetrazolium reductase activity. The microsome suspension was adjusted to a protein concentration of 0.75 mg/ml, and neotetrazolium reduction was assayed in an incubation system containing the following components in a final volume of 1.55 ml: 0.65 mM NADPH, 0.5 mg of bovine serum albumin, 0.5 mM neotetrazolium salt, 75 μg of microsomal protein, and 0.15 M potassium phosphate, pH 7.5 (8). The tubes were incubated for 10 min at 37°, and the reaction was stopped by the addition of 1.5 ml of a mixture containing 0.26 M formate buffer (pH 3.5), 9 mg/ml of Triton X-100, and 52.8 mg/ml of formaldehyde. The resultant purple color was measured at 505 nm against the blank obtained from microsomes incubated without the addition of NADPH. The results are expressed as a percentage of the control value.

NADPH-dependent lipid peroxidation. The microsomal suspension was adjusted to a protein concentration of 0.75 mg/ml. Analysis of lipid peroxidation was undertaken in an incubation system containing the following components in a final volume of 2.6 ml: 0.75 mg of microsomal protein, 0.15 M phosphate buffer (pH 7.5), 0.1 mM NADP, 4 mM *dl*-isocitrate, 44 mM nicotinamide, 0.4 mM ADP, 0.01 mM $MnCl_2$, and enough isocitrate dehydrogenase to reduce 0.32 μmole of NADP per minute (9). The tubes were incubated for 20 min at 37°, and the reaction was stopped by the addition of 0.5 ml of 40% trichloracetic acid and 0.25 ml of 5 N HCl. After centrifugation at 2000 × *g* for 10 min, 0.5 ml of 1% thiobarbituric acid was added to 2 ml of the supernatant fluid. The tubes were heated in a boiling water bath for 10 min, and the resulting color was measured at 550 nm against the blank obtained from the same microsomal source and incubated without the addition of NADP. The results are expressed as a percentage of the control value.

Cytochrome b_5 determination. A 1-ml portion of microsomal suspension (15 mg of microsomal protein) was mixed with 0.3 ml of 10% sodium deoxycholate and 4.7 ml of 0.2 M phosphate buffer, pH 7.5. The suspension was divided into 3-ml aliquots. The difference spectrum was measured in a Cary model 15 spectrophotometer from 600 to 375 nm at room temperature (8). Sufficient solid sodium dithionite was added to one cell to achieve complete reduction, and the difference spectrum was again recorded. The differences in absorbance at 427 and 410 nm were taken as an arbitrary measure of the amount of cytochrome b_5, and expressed as change in absorbance per milligram of protein or as a percentage of the control value.

Cytochrome P-450 determination. A 0.5-ml portion of the microsomal suspension (7.5 mg of microsomal protein) was added to 5.5 ml of 0.2 M phosphate buffer, pH 7.5, and enough solid sodium dithionite was added to achieve maximum reduction. The mixture was divided into two aliquots, and the difference spectrum was recorded in a Cary

model 15 spectrophotometer from 500 to 375 nm at room temperature (8). Subsequently one cell was removed, and carbon monoxide was bubbled through the suspension for 2 min. The difference spectrum was again recorded, and the amount of cytochrome P-450 was determined from the changes in absorbance between 450 and 490 nm after gassing with CO. This value was expressed as change in absorbance from 450 to 490 nm per milligram of protein or as a percentage of the control value.

Reduction of DMA-N-oxide. Measurement of reduction of DMA-*N*-oxide to DMA was carried out in an incubation system containing the following components in a volume of 2.0 ml: 3.9 mg of microsomal protein, 0.15 M potassium phosphate buffer (pH 7.5), 60 mM nicotinamide, 5 mM *dl*-isocitrate, 0.2 mM $MnCl_2$, 0.05 mM NADP, enough isocitrate dehydrogenase to reduce 0.32 μmole of NADP per minute, and approximately 5 mM DMA-*N*-oxide. The reactions were incubated at 37° aerobically for 20 min and then chilled, and 1 ml of cold 1 M $HClO_4$ was added. After centrifugation the supernatant fluid was adjusted to pH 9.4 by the addition of NaOH and extracted three times with 3 ml of ether. The extracts were pooled, and 2-ml aliquots were added to 8 ml of 50% ethanol in water and adjusted to pH 2.6 with HCl. Then 1 ml of 2% $NaNO_2$ was added, and the color was developed by incubation for 5 min at 60°. The color was measured at 420 nm.

RESULTS

The microsomal content of cytochrome b_5 or P-450 was not altered for 16 hr after administration of thioacetamide. Following this period cytochrome b_5 declined to 42% of the control value at 4 days, and remained there for the duration of these experiments. Cytochrome P-450 declined somewhat more precipitously, reaching 32% of the control value at 2–4 days, and also remained depressed up to 7 days after intoxication (Fig. 1). In order to assess the functional state of the cytochrome P-450, enzymatic reactions which are believed to utilize this hemoprotein were measured (Fig. 2 and Table 1). Ring hydroxylation of aniline (Fig. 3) and *N*-demethylation of *N*,*N*-dimethylaniline (Fig. 2) were depressed, and these changes preceded changes in microsomal cytochrome concentration. Furthermore, *N*-demethylation was not detectable at 2 days, but returned to 30% of the control value by 7 days; this is to be contrasted with the pattern of response noted for the cytochromes. The response of aniline hydroxylation was similar to (though less striking than) that of *N*-demethylation. This distinction is underscored in Table 1, where the enzyme activities are expressed in terms of ratios to cytochrome P-450 content in the microsomal fraction. Production of formaldehyde from *N*,*N*-dimethylaniline *N*-oxide was depressed during the entire course of observation, but to a smaller degree than the other two reactions. No change occurred in this activity between days 4 and 7, while demethylation of DMA began to recover.

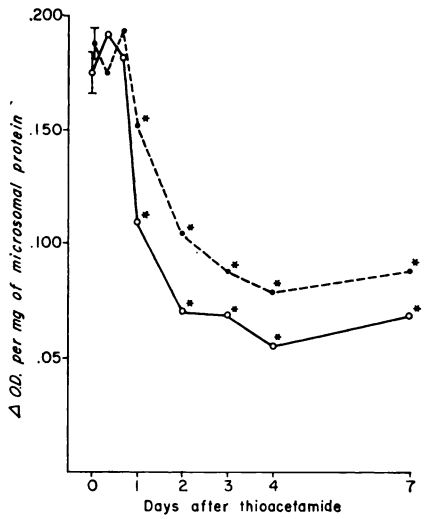

FIG. 1. *Effect of thioacetamide on microsomal cytochromes*

Thioacetamide, 20 mg/100 g of body weight, was given by stomach tube at zero time, and the amounts of microsomal cytochrome pigments were determined as described in MATERIALS AND METHODS. ●- - -●, amount of cytochrome b_5/per milligram of microsomal protein; O——O, amount of cytochrome P-450 per milligram of microsomal protein. Asterisks indicate data points which are significantly different from controls ($p < 0.05$). The bars around control points denote standard errors of the mean.

Two reactions which are believed not to

require the participation of the cytochrome, i.e., lipid peroxidation and formation of DMA-*N*-oxide, showed early depressions of their activities and began to recover toward the end of the course of observation (Figs. 2 and 3). Measurement of electron transport, as far as the neotetrazolium acceptor point, believed to be $NADPH_2$–cytochrome *c* reductase, did not reveal changes of a magnitude similar to those of the above reactions (Fig. 3). This suggests that the previously described functional changes are related to enzyme-substrate or cytochrome-substrate interaction. These data confirm and extend those of Sladek and Mannering

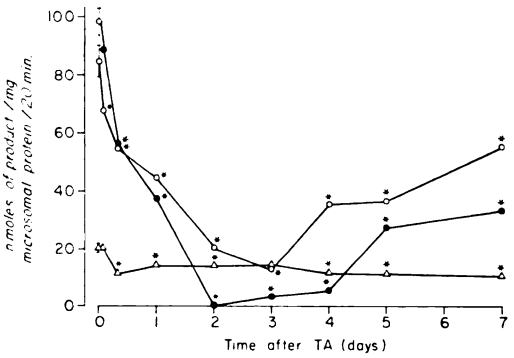

FIG. 2. *Effect of thioacetamide on N-oxidation and N-demethylation of dimethylaniline*

Thioacetamide (TA), 20 mg/100 g of body weight, was given by stomach tube at zero time, and microsomes were incubated as described in the text. ●——●, production of formaldehyde from DMA; ○——○, production of DMA-*N*-oxide; △——△, production of formaldehyde from *N*-oxide. Asterisks indicate data points which are significantly different from controls ($p < 0.05$). The bars around the control points denote standard errors of the mean.

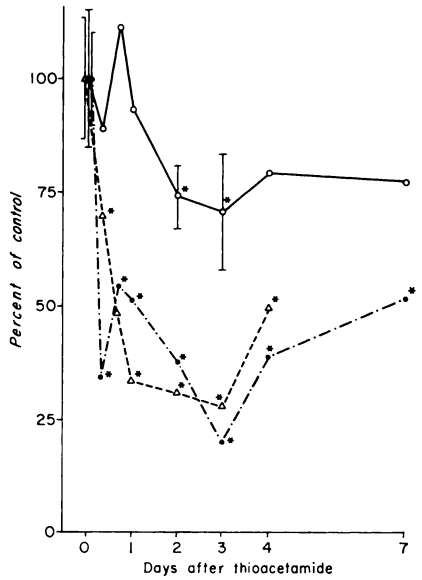

FIG. 3. *Effects of thioacetamide on NADPH-neotetrazolium reduction, lipid peroxidation, and aromatic hydroxylation*

Thioacetamide, 20 mg/100 g of body weight, was given by stomach tube at zero time, and microsomes were incubated as described in MATERIALS AND METHODS. ○——○, NADPH-neotetrazolium reductase activity; ●-·-·●, lipid peroxidation; △- - -△, *p*-hydroxylation of aniline. Asterisks indicate data points which are significantly different from controls ($p < 0.05$). The bars denote standard errors of the means.

TABLE 1

Ratio of enzyme activities to microsomal cytochrome P-450 content after thioacetamide administration

Time	Formaldehyde production		*N*-Oxide formation		Aromatic hydroxylation		Lipid peroxidation		Neotetrazolium reduction	
hr	*nmoles/ $A_{450-490}$ unit*	*% control*	*nmoles/ $A_{450-490}$ unit*	*% control*	*μg p-amino-phenol/ $A_{450-490}$ unit*	*% control*	*A_{650}/ $A_{450-490}$ unit*	*% control*	*A_{505}/ $A_{450-490}$ unit*	*% control*
0	571	100	493	100	13.5	100	4.5	100	45.3	100
8	296	52	288	58	8.6	64	1.4	31	36.5	81
16			266	54	6.3	47	2.3	51	48.3	107
24	351	61	415	84	7.3	54	3.7	82	67.8	150
48	3	<1	294	60	10.3	76	4.1	91	82.9	183
72	53	9	184	37	9.3	69	2.3	51	79.0	177
96	106	19	647	131	20.7	153	5.5	122	112.2	248
168	500	88	825	167			6.1	136	88.0	194

TABLE 2

Effect of thioacetamide on products of dimethylanaline oxidation by hepatic microsomes in vitro

Thioacetamide	Product formed	
	Formaldehyde	*N*-Oxide
mM	*% control*	
0	100	100
0.13	111	75
1.3	93	60
13.0	73	—[a]
65.0	54	—[a]

[a] *N*-Oxide determination was prevented by turbidity upon addition of $NaNO_2$.

(10), who reported a decline in the level of cytochrome P-450 and in *N*-demethylation (of ethylmorphine) after four daily (smaller) doses of thioacetamide.

Addition of thioacetamide directly to assays for the production of formaldehyde or of *N*-oxide from DMA resulted in progressive dose-related depression of the product (Table 2). The interpretation of these data must be conservative, since a precipitate formed in the tubes containing larger concentrations of thioacetamide when DMA-*N*-oxide was the substance being measured. Thus one cannot be sure whether the enzymatic reaction or the assay for product is the component which was depressed. The latter is more likely, since the precipitate did not form until addition of $NaNO_2$. The amount of thioacetamide added was calculated to be similar to that assumed to be present in liver shortly after intragastric administration of the toxin. This assumption rests on data published by Nygaard *et al.* (11), indicating that the hepatic concentration of thioacetamide is about 0.16 mM 85 min after subcutaneous administration of 2.5 mg/100 g (our calculations).

Attempts to measure the reduction of DMA-*N*-oxide to DMA were only partially successful. Reduction occurred readily in the presence of ferrous ion and cysteine (Table 3). However, when that reducing couple was omitted, little more reduction occurred than in microsomal incubations from which DMA-*N*-oxide was omitted.

TABLE 3

Reduction of N-oxide to dimethylaniline

Microsomes were incubated as described in MATERIALS AND METHODS. Numbers in parentheses indicate the number of animals used. Treated animals were used 4 days after thioacetamide administration.

System	Control	Treated
	A_{420}/mg microsomal protein	
Complete	0.028 ± 0.005 (7)	0.027 ± 0.008 (3)
−*N*-Oxide	0.010 ± 0.005 (7)	0 (3)
$+Fe^{++}$, cysteine	0.715 ± 0.057 (7)	0.553 ± 0.055 (2)

We conclude that DMA-*N*-oxide reduction is not significantly altered in animals which have been given thioacetamide.

DISCUSSION

Administration of thioacetamide to mature rodents is associated with structural and functional modification of the endoplasmic reticulum (12, 13). During the first day, dispersion of polysomes and alterations in the integrity of membrane-ribosome interaction occur, accompanied by decreased protein formation, measured *in vivo* or *in vitro* (14). In addition, a series of changes in membrane-related functions arise. The distorted microsomal function is not due merely to general destruction of the smooth endoplasmic reticulum, since some membrane-associated functions remained either relatively unchanged or altered to a much smaller degree than others. It is known that significant membrane damage, as seen by the electron microscope (3), occurs later than 12 hr after a large dose of thioacetamide. It is only after this time period that the levels of the membrane-associated cytochromes begin to decline, as though their integrity (at least as reflected by spectroscopic analysis) is maintained by association with the membrane. The biochemical functions associated with cytochrome P-450 decrease much sooner than either the decline in P-450 levels or the visible change in the smooth endoplasmic reticulum. These biochemical abnormalities also occur several hours prior to alterations in protein synthesis and are the earliest

alterations yet detected after thioacetamide administration. This may represent direct inhibition by thioacetamide, since addition of the toxin to microsomal suspensions apparently decreases the formation of both formaldehyde and DMA-*N*-oxide, proportional to the amount added (Table 2).

Two reactions which are thought to involve the electron transport system prior to the terminal hemoprotein, formation of *N*,*N*-dimethylaniline *N*-oxide and lipid peroxidation, are markedly depressed early in the course of intoxication, while a third, NADPH-neotetrazolium reduction, is affected to a much smaller degree, and then only much later. Similarly, two reactions requiring the participation of cytochrome P-450, i.e., the production of formaldehyde from *N*,*N*-dimethylaniline and aromatic hydroxylation of aniline, are markedly depressed, while production of formaldehyde from *N*,*N*-dimethylaniline-*N*-oxide is decreased much less. These results are difficult to reconcile with present concepts of the organization of the microsomal electron transport system, unless one assumes that the toxin affects more than one locus of the system.

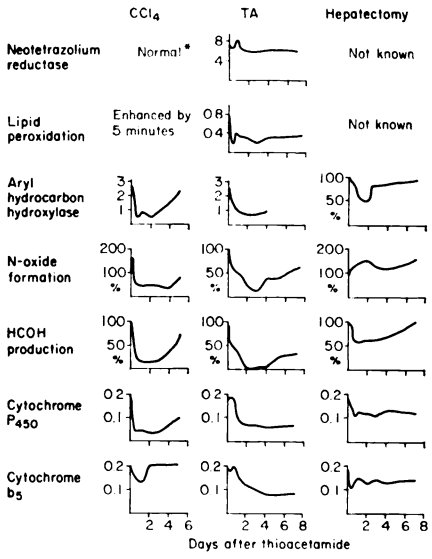

FIG. 4. *Comparison of effects of carbon tetrachloride (7, 15), thioacetamide (TA), and hepatectomy (15, 16) on microsomal functions*

The units on the ordinate for DMA-*N*-oxide and formaldehyde production are percentages of the control. The others correspond to units used in other figures in this paper. The asterisk denotes enzyme activity 2 hr after administration of the toxin; later values are not available. These values and patterns were observed at the noted times after a single injurious stimulus.

The pattern of injury induced by thioacetamide is different from that resulting from partial surgical hepatectomy or CCl_4 intoxication (Fig. 4). The fact that the pattern differs from that seen after partial hepatectomy indicates that the thioacetamide-related response in surviving liver cells is more complex than regeneration after removal of hepatocytes. These differences may be related to specific toxic effects on metabolic pathways.

It has been suggested that *N*-demethylation of aromatic compounds occurs via the formation of an *N*-oxide intermediate (5). The formation of the *N*-oxide is thought to be enzymatic and dependent on $NADPH_2$ and O_2, but not to require cytochrome P-450. The second step in the pathway is dependent on the participation of cytochrome P-450 and does not require molecular O_2 (Fig. 5) (5, 17, 18). Several observations favor such a formulation: (a) the *N*-oxide is formed at a rate equal to or greater than the rate of the over-all reaction (5); (b) incubation of microsomes in the presence of CO inhibits formaldehyde production but enhances the formation of the *N*-oxide (19); (c) aging or treatment of microsomes with detergent results in *N*-oxide accumulation and inhibition of formaldehyde production (1); and (d) in the early phase of CCl_4 poisoning formaldehyde production is depressed while DMA-*N*-oxide formation is enhanced, concomitantly with loss of cytochrome P-450 (7).

If *N*,*N*-dimethylaniline *N*-oxide is an obligate intermediate in the oxidative demethylation of *N*,*N*-dimethylaniline (Fig.

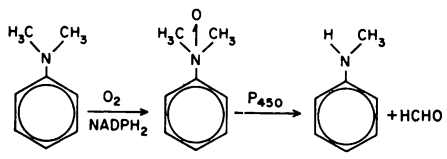

FIG. 5. *Hypothetical sequential steps in N-demethylation*

5), then, following thioacetamide treatment, the *N*-oxide should either accumulate as formaldehyde production is diminished, commensurate with diminished activity of the second step, or decrease as formaldehyde production is diminished, commensurate with destruction of the first step or both steps. The latter was observed after thioacetamide intoxication. The data indicate that both steps of this hypothetical scheme are inhibited, since formaldehyde production from DMA-*N*-oxide and *N*-oxide production from DMA were depressed. Late in the course of intoxication, formaldehyde production from DMA seemed to recover while that from DMA-*N*-oxide remained depressed, suggesting that formaldehyde production from DMA did not proceed through the *N*-oxide intermediate, at least at that point in time.

If pathways B and D in Fig. 6 (see Fig. 5) represent an actual path of formaldehyde formation, the *N*-oxide may still serve as an intermediate in the production of formaldehyde; however, an alternative pathway (C) exists for disposal of the *N*-oxide (Fig. 6). DMA-*N*-oxide may accumulate after thioacetamide intoxication, owing to its decreased conversion to formaldehyde, unless the reduction of *N*-oxide to DMA is enhanced. In fact, this reductive step was not increased 4 days after thioacetamide administration (Table 3).

Several observations suggest that the formation of *N*-oxides and *N*-demethylation of tertiary amines are separate and distinct reactions. (a) The K_m for demethylation of *N*,*N*-dimethylaniline *N*-oxide is quite high, suggesting that little *N*-oxide would be demethylated when low concentrations of *N*-oxide are present (20). (b) The patterns of differentiation of *N*-oxidation and *N*-demethylation are different in developing rat liver (21). (c) There are immunological differences between the *N*-oxidation and *N*-demethylation systems for *N*,*N*-dimethylaniline 22). (d) *N*-Oxides of certain other tertiary amines are demethylated much more slowly than the corresponding tertiary amines (23) (these results should be interpreted with caution, since differences in substrate specificity are known to exist in microsomal systems). (e) Separation of the

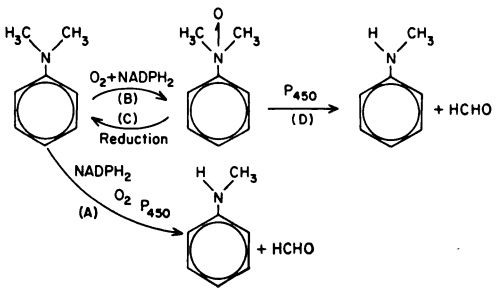

FIG. 6. *Alternative pathways of N-demethylation* Steps B and D are represented in Fig. 5.

endoplasmic reticulum into smooth and rough fractions results in partial separation of *N*-demethylation and *N*-oxidation activities (24). Alterations in *N*-oxide levels in toxic liver injury could arise from differential inhibition of separate paths of *N*-oxide formation and methyl carbon release. Thus it seems likely that the formation of *N*,*N*-dimethylaniline *N*-oxide and the production of formaldehyde from DMA proceed by separate pathways, and that the *N*-oxide is not an intermediate in the latter reaction.

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