Alterations in Microsomal Electron Transport, Oxidative *N*-Demethylation and Azo-Dye Cleavage in Carbon Tetrachloride and Dimethylnitrosamine-Induced Liver Injury

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The effect of administration of carbon tetrachloride and dimethylnitrosamine *in vivo* on hepatic microsomal function related to drug metabolism was measured. It was found that the capacity of isolated microsomes to demethylate dimethylaniline was diminished during the first hour after carbon tetrachloride poisoning and during the second hour after dimethylnitrosamine poisoning. Thereafter the microsomes from carbon tetrachloride-poisoned livers showed a continuous decline in activity so that at 24hr. there was little residual capacity to undertake demethylation. Microsomes from dimethylnitrosamine-poisoned animals were not different from controls at 24hr. During the first 3hr. there was a transient rise in the accumulation of the *N*-oxide intermediate in carbon tetrachloride-poisoned livers, with a subsequent fall to below control values. In dimethylnitrosamine poisoning there was a parallel decrease in *N*-oxide accumulation with decreased demethylation. In the latter part of the first 24hr. the ratio of *N*-oxide accumulation to demethylation was increased in both instances. At 2hr. after poisoning with either compound there was no evidence of altered $NADPH_2$-dependent neotetrazolium reduction or lipid peroxidation. $NADPH_2$-dependent azo-dye cleavage was decreased. There was no difference in microsomal cytochrome b_5 content, but there was a decrease in the amount of cytochrome *P*-450. This latter change was correlated with the decreased capacity for $NADPH_2$-dependent oxidative demethylation. It is suggested that dimethylnitrosamine is associated with a defect in microsomal $NADPH_2$-dependent electron transport at the level of cytochrome *P*-450. In addition to affecting cytochrome *P*-450, carbon tetrachloride is associated with a second severe block involving the release of formaldehyde from the *N*-oxide intermediate.

Mammalian hepatic cells possess a group of enzymes, classified as mixed-function oxidases (Mason, 1957), which modify various compounds of exogenous and endogenous origin not readily handled by the general 'metabolic network' (Mason, North & Vanneste, 1965). These enzymes are associated with the endoplasmic reticulum, and after differential centrifugation appear in the microsomal fraction. Some are $NADPH_2$-dependent and are thereby linked with one of the microsomal electron-transport pathways. One or more of these enzymes carry out the dealkylation of *N*-substituted amines. This process involves the formation of an *N*-oxide intermediate, which may accumulate in the system. The accumulation is markedly increased if the microsomes have been pretreated by aging or with potassium cholate (Ziegler & Pettit, 1964).

The hepatotoxins carbon tetrachloride and dimethylnitrosamine are known to alter the morphology and function of the endoplasmic reticulum *in vivo* (Bassi, 1960; Hultin, Arrhenius, Löw & Magee, 1960; Emmelot & Benedetti, 1960; Oberling & Rouiller, 1956; Recknagel & Lombardi, 1961; Smuckler, Iseri & Benditt, 1962; Reynolds, 1963; Smuckler & Benditt, 1965). It has been shown that late in the development of the carbon tetrachloride-produced lesion the capacity of the liver to demethylate aminopyrine oxidatively is significantly diminished (Neubert & Maibauer, 1959). In the experiments described below we have followed the capacity of liver microsomes from carbon tetrachloride- and dimethylnitrosamine-poisoned rats to undertake oxidative *N*-demethylation of dimethylaniline. We also determined whether alteration occurred in the reaction sequence of microsomal electron transport. We found that both carbon tetrachloride and dimethylnitrosamine

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decreased the capacity of isolated liver microsomes to demethylate dimethylaniline, but early during the poisoning there were differences in the points of attack. Both poisons led to a decrease in the quantity of cytochrome *P*-450 in the isolated microsomes, and the extent of the decrease of this component paralleled the altered capacity of the particles to undertake oxidative demethylation.

A preliminary report of this work has been made (Smuckler, Arrhenius & Hultin, 1966).

MATERIALS AND METHODS

Treatment of animals. Male Sprague–Dawley rats were starved for 16–18hr. before treatment but permitted water *ad libitum.* CCl_4 (0·25ml./100g. body wt., dissolved in an equal volume of mineral oil) and dimethylnitrosamine (2mg./100g. body wt., dissolved in water) were administered by stomach tube without anaesthesia. Control animals received equivalent amounts of mineral oil or water. Water but no food was provided until the animals were killed. At intervals from 1 to 24hr. after poisoning the animals were killed by ether narcosis and exsanguination. The livers first were perfused via the aorta *in situ* with ice-cold 0·9% NaCl and then removed. All subsequent preparative operations were carried out at 0–4° unless otherwise specified.

Determination of demethylation and N*-oxide formation.* The perfused livers were immersed in ice-cold 0·2M-potassium phosphate buffer, pH7·5 (20°), weighed and homogenized in 4vol. of this same medium. The brei was centrifuged at 15000*g* for 10min. and the supernatant was recentrifuged at 105000*g* for 60min. The resulting sediment was suspended in 0·2M-phosphate buffer, pH7·5, by gentle homogenization and used directly or after recentrifuging at 105000*g* for 45min. followed by resuspension. The protein concentration of the suspensions was measured by the method of Lowry, Rosebrough, Farr & Randall (1951), with crystalline bovine albumin as a standard, and was adjusted to 2·6mg./ml. The incubation mixture contained 3·9mg. of microsomal protein with 0·1μmole of NADP, 10μmoles of glucose 6-phosphate, 0·25 Kornberg unit (Kornberg, 1950) of glucose 6-phosphate dehydrogenase, 120μmoles of nicotinamide, 1·0μmole of ADP and 10μmoles of the substrate in a total volume of 2ml. The incubation was performed aerobically at 35°.

N-Oxide was determined by a modification of the procedure described by Ziegler & Pettit (1964) in the supernatant after the addition of 0·5vol. of 0·9N-$HClO_4$ followed by centrifugation at 1000*g* for 15min. Then 2ml. of the supernatant was adjusted to pH9·4 with 2N-NaOH, after which it was extracted three times with 10ml. of ether. After the last extraction the pH of the aqueous phase was adjusted to 2·4 with 3N-trichloroacetic acid. To this, 0·1vol. of 0·1M-$NaNO_2$ was added and the solution was heated at 60° for 5min. The yellow colour was measured in a Beckman DB spectrophotometer at 420mμ against a blank prepared from the same microsomal source and incubated without addition of NADP. In other experiments the extinction coefficient for the coloured product, *NN*-dimethyl-*p*-nitrosoaniline, was found to agree with that given by Ziegler & Pettit (1964). Also, in preliminary experiments it was found that the concentrations of added cofactors were optimum for the accumulation of *N*-oxide in liver systems from normal or poisoned animals. The results are presented as mμmoles of *N*-oxide formed/mg. of microsomal protein/20min. incubation at 35°.

For determination of formaldehyde the proteins were precipitated with 0·5vol. of 20% (w/v) trichloroacetic acid followed by centrifugation for 15min. Then 2ml. of the supernatant was mixed with 0·6ml. of Nash reagent (Nash, 1953) and incubated at 35° for 30min. The resulting colour was read in a Beckman DB spectrophotometer at 415mμ against a blank obtained from microsomes incubated without the addition of NADP, and was compared with a standard prepared from formaldehyde solution. The results are expressed as mμmoles of formaldehyde formed/mg. of microsomal protein/20min. incubation at 35°. As with the *N*-oxide, these conditions gave optimum results.

In a separate set of experiments microsomes were prepared from control animals and were incubated with and without the addition of CCl_4 and dimethylnitrosamine to the incubation medium. Determinations of the *N*-oxide and the formaldehyde were carried out as described above.

Determination of enzymic lipid peroxidation. Pieces of the livers used for the analysis of demethylation were homogenized in 4vol. of 0·175M-KCl in 0·035M-tris–HCl buffer, pH7·5. Microsomes were prepared as indicated above and were washed by recentrifugation at 105000*g* for 60min. The particles were resuspended in the tris–KCl medium and the protein content was adjusted to 1·5mg./ml. This suspension was used in an incubation medium of the same composition as that used for demethylation but without *NN*-dimethylaniline or Pyramidon (4-dimethylamino-2,3-dimethyl-1-phenyl-3-pyrazolin-2-one). After 20min. incubation at 35° the reaction was stopped by the addition of 0·25vol. of 40% (w/v) trichloroacetic acid and 0·125vol. of 5N-HCl. After 30min., 2ml. of the supernatant obtained by centrifugation was mixed with 0·5ml. of 1% thiobarbituric acid and placed in a boiling-water bath for 10min. The resulting coloured solutions were diluted with 10ml. of water and the colour was measured in a Beckman DB spectrophotometer at 535mμ (Kohn & Liversedge, 1944; Hunter, Gebicki, Hoffsten, Weinstein & Scott, 1963; Hultin & Arrhenius, 1965*a*). The colour was measured against a blank prepared from microsomes incubated without the addition of NADP, and this in turn against a water blank. The values are expressed as ΔE_{535}/mg. of protein/20min. The conditions used resulted in optimum production of thiobarbituric acid-positive material, and the addition of more of one or all the cofactors did not alter the result.

Determination of NADPH–neotetrazolium reductase. Microsomes were prepared from perfused livers in a medium composed of 0·155M-KCl in 0·035M-tris–HCl buffer, pH7·5. The microsomal sediments were resuspended in the original volume of buffer and recentrifuged at 105000*g* for 45min. The protein concentration of the resuspended washed microsomes was adjusted to 1·5mg./ml. The following system was used for measuring neotetrazolium reduction: 0·15mg. of microsomal protein, 100μmoles of $NADPH_2$, 5mg. of bovine serum albumin and 110μmoles of neotetrazolium in a total volume of 1·5ml. The incubations were carried out at 35° for 0, 5, 10 and 15min., at which times 1·5ml. of a formalin–Triton mixture was added. The formazan colour was measured at 505mμ with a Beckman DB spectrophotometer, with a blank prepared without the addition of $NADPH_2$ (Lester & Smith, 1961; Dallner, 1963). The results are expressed as ΔE_{505}/0·15mg. of protein/10min. incubation.

Determination of cytochromes b_5 *and* P-450. The perfused livers were homogenized in 4vol. of 0·155M-KCl in 0·035M-tris–HCl buffer, pH7·5. This brei was centrifuged at 15000*g* for 15min. and the supernatant recentrifuged at 105000*g* for 60min. The 105000*g* pellets were suspended by gentle homogenization in the original volume of medium and recentrifuged at 105000*g* for 45min. The final pellets were resuspended in 0·02M-potassium phosphate buffer, pH7·5. The protein concentration of these suspensions were adjusted to 15mg./ml.

(*a*) Cytochrome b_5. A 1ml. sample of the microsomal suspension was mixed with 0·3ml. of 10% (w/v) sodium deoxycholate and 4·7ml. of 0·02M-potassium phosphate buffer. The suspension was divided into 3ml. portions. The difference spectrum (base line) over the range 380–600mμ was measured in a Beckman DK-2 spectrophotometer at 30°. To one cell sufficient solid $Na_2S_2O_4$ or $NADH_2$ was added to achieve complete reduction and the difference spectrum was recorded. The difference $E_{427}-E_{410}$ was taken as an arbitrary measure of the amount of cytochrome b_5 (Ernster, Siekevitz & Palade, 1962). Measurement of the 557mμ peak was made as a control and gave the same general results (Strittmatter & Ball, 1952).

(*b*) Cytochrome *P*-450. A 0·5ml. sample of the microsomal suspension was added to 5·5ml. of the 0·02M-potassium phosphate buffer, and to the mixture sufficient solid $Na_2S_2O_4$ was added to achieve maximum reduction. The mixture was divided and the difference spectrum over the range 380–600mμ was recorded in a Beckman DK-2 spectrophotometer at 30°. Subsequently one of the cells was removed and CO bubbled through the suspension for 2min. The difference spectrum was again recorded and the amount of CO-binding pigment determined from the change in $E_{450}-E_{500}$ after gassing with CO. This value is expressed as $\Delta(E_{450}-E_{500})$/mg. of protein (Klingenberg, 1958).

The effects of dimethylnitrosamine and CCl_4 on cytochrome *P*-450 were tested in the following manner. Microsomes were prepared as indicated and diluted to 10mg. of protein/ml. Portions of the sample were placed in silica cells for the Beckman DK-2 spectrophotometer and after dithionite reduction a base-line difference spectrum was measured. To both cells either CCl_4 or dimethylnitrosamine was added in amounts of 1, 5, 10, 20, 30 and 50μl. in the former case and 5, 25, 50, 100 and 150μg. in the latter case before or after gassing with CO for 2min. The difference spectra were recorded.

Arachidonic acid was peroxidized with u.v. light (Wilbur, Bernheim & Shapiro, 1949) and the effect of irradiation measured with the thiobarbituric acid reaction. The suspension of the fatty acid with or without previous peroxidation was added to a control microsomal suspension at a concentration of 50μM before and after gassing with CO. The difference spectra were recorded as described above. In a similar experiment H_2O_2 was added to a control suspension in final concentrations of 0·01 and 0·1% and the difference spectra were measured.

Determination of $NADPH_2$*-dependent reductive cleavage of azo-dye.* Microsomes were prepared and washed in 0·175M-KCl–0·01M-$MgCl_2$ in 0·035M-tris–HCl buffer, pH7·5. The washed microsomes were resuspended in the same buffer and the protein concentration was adjusted to 1mg./ml. Then 1mg. of microsomal protein was incubated aerobically at 35° with 0·1μmole of NADP, 10μmoles of glucose 6-phosphate, 0·25 Kornberg unit of glucose 6-phosphate dehydrogenase, 120μmoles of nicotinamide and 250μg. of monomethylamino azobenzene in a total volume of 2ml. The reaction was stopped at 0, 5, 10, 15 and 20min. with the addition of an equal volume of 20% (w/v) trichloroacetic acid in acetone–ethanol (1:1, v/v). After 20min. the supernatant was obtained by centrifuging at 1000*g* for 15min. The remaining azo-dye colour, in acid solution, was measured at 520mμ in a Beckman DB spectrophotometer (Mueller & Miller, 1953).

RESULTS

Within 1hr. after the administration of carbon tetrachloride and by the second hour after dimethylnitrosamine administration the capacity of isolated microsomes to demethylate dimethylaniline was significantly diminished (Fig. 1). At the dosages used, the decrease in activity was more marked with carbon tetrachloride. The subsequent course of carbon tetrachloride poisoning was associated with a continuous decline in activity so that by 24hr. the residual capacity to form formaldehyde was small. The decreased capacity for demethylation was not substrate-specific. Another *N*-substituted compound, Pyramidon, was also

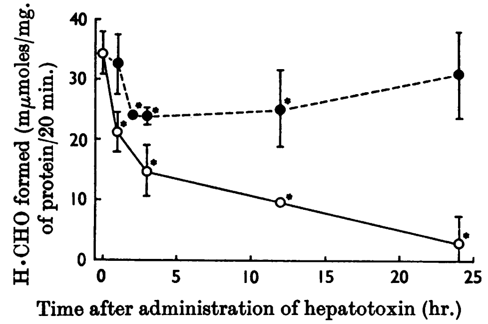


Fig. 1. Effects of poisoning with CCl_4 (0·25ml./100g. body wt.) or dimethylnitrosamine (2mg./100g. body wt.) on microsomal $NADPH_2$-dependent oxidative demethylation of dimethylaniline. Microsomes were prepared and washed in 0·2M-phosphate buffer and incubated in a total volume of 2ml. with 0·1μmole of NADP, 10μmoles of glucose 6-phosphate, 120μmoles of nicotinamide, 1·0μmole of ADP, 0·25 Kornberg unit of glucose 6-phosphate dehydrogenase and 10μmoles of dimethylaniline. The content of microsomal protein was 3·9mg. The reaction was carried out aerobically for 20min. at 35°. The formaldehyde produced was determined by the Nash (1953) method. Each point represents the mean of three or more experiments, the vertical lines indicating S.D. Points statistically different from the controls are indicated by asterisks (*). ○, CCl_4-treated; ●, dimethylnitrosamine-treated.

handled less efficiently by microsomes from carbon tetrachloride-treated animals (activities of microsomes from control and carbon tetrachloride-treated rats were 67·8 ± 14·7 and 35·7 ± 4·1 mμmoles of formaldehyde/mg. of protein/20min. respectively, each result being the mean of three experiments). Demethylation of dimethylaniline after dimethylnitrosamine treatment showed an initial decrease that remained for about 12hr. The results obtained at this time had a larger spread, the trend appearing to be towards a restoration of the control values. By 24hr. there was no difference between the preparations from treated and control animals.

A striking difference in the accumulation of *N*-oxide was apparent (Fig. 2). In carbon tetrachloride poisoning there was an early increase in the amount of the intermediate at a time of decreased demethylation. The rise was significant at 1hr. and remained above control values until 3hr. At 8 and 12hr. *N*-oxide formation was lowered below control values and remained so for the next 12hr. With microsomes isolated from dimethylnitrosamine-poisoned animals the *N*-oxide accumulation was decreased in a manner similar to the demethylation and then increased at 12hr. above control values. This elevation was present up to 24hr.

Addition of either hepatotoxin to the cell-free preparations at concentrations approximating those found in the livers after 2hr. treatment *in vivo* (Recknagel & Litteria, 1960) did not alter the activity of the microsomes in demethylation of *N*-oxide accumulation (Table 1 and Fig. 3). Increased concentrations of the hepatotoxins slightly decreased the activity, and this decrease was proportionally similar to the small decrease in the amount of cytochrome *P*-450 under the same conditions. Preincubation of microsomal preparations with carbon tetrachloride gave similar results, except when 10μl. of carbon tetrachloride or more was added/ml. These amounts produced an approximately 50% decrease (Fig. 3).

During the first 3hr. the capacity of microsomes isolated from either dimethylnitrosamine- or carbon tetrachloride-treated animals to undertake enzymic lipid peroxidation was not different from control values. [This is in contrast with previous reports of increased peroxidation *in vitro* after carbon tetrachloride treatment *in vivo*. The difference may be due to the techniques employed (Ghoshal & Recknagel, 1965; Comporti, Saccocci & Dianzani, 1965). In the present experiments great care was used in removing the supernatant fraction from the microsomes, since soluble cell components have been shown to interfere with the lipid-peroxidation assay (E. Arrhenius, unpublished work). Moreover, microsomes were prepared in the absence of phosphate, and ADP was added to act

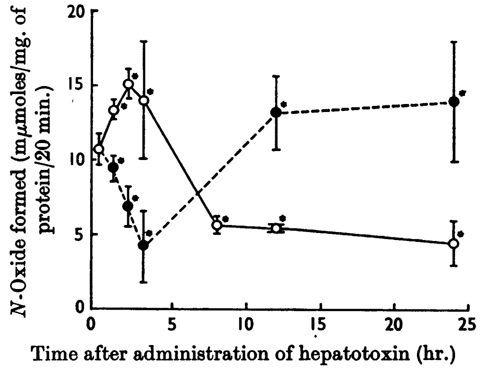


Fig. 2. Effects of poisoning with CCl_4 (0·25ml./100g. body wt.) or dimethylnitrosamine (2mg./100g. body wt.) on microsomal $NADPH_2$-dependent *N*-oxide formation. Isolation of microsomes and incubation were performed as described in Fig. 1. *N*-Oxide was determined by the method of Ziegler & Pettit (1964). Each point represents the mean of three or more experiments, the vertical lines indicating S.D. (except at 8 and 12hr. in CCl_4 poisoning, where the vertical lines indicate the range of values). Points statistically different from controls are indicated by asterisks (*). ○, CCl_4-treated; ●, dimethylnitrosamine-treated.

Table 1. *Effects of poisoning with carbon tetrachloride or dimethylnitrosamine on microsomal* $NADPH_2$*-dependent* N*-oxide formation and demethylation of dimethylaniline*

Microsomes were prepared as described in Fig. 1. The washed microsomes (3·9mg. of protein) were incubated in a final volume of 2·0ml. with 0·1μmole of NADP, 10μmoles of glucose 6-phosphate, 120μmoles of nicotinamide, 1·0μmole of ADP, 0·25 Kornberg unit of glucose 6-phosphate dehydrogenase, 10μmoles of dimethylaniline and the indicated amounts of either CCl_4 or dimethylnitrosamine. The reaction was carried out aerobically for 20min. at 35°. *N*-Oxide formation was measured by the method of Ziegler & Pettit (1964) and formaldehyde by the Nash (1953) method. Each value represents the mean of two or more experiments, and is expressed as mμmoles formed/mg. of protein/20min.

CCl_4 (μl.)	Dimethylnitrosamine (μg.)	*N*-Oxide formed	Formaldehyde formed
0	0	11·2	40·5
1·5	0	10·6	43·4
7·5	0	10·5	41·4
15·0	0	9·9	37·8
0	0	—	35·9
0	7·5	—	33·1
0	75	—	32·3
0	750	—	32·2

as a chelator (Hochstein & Ernster, 1963; E. Arrhenius, unpublished work). Addition of carbon tetrachloride *in vitro* did result in increased formation of thiobarbituric acid-positive materials, confirming the results of others. The similarity of activity of microsomal enzymic lipid peroxidation in control and treated animals neither supports nor denies the role proposed for peroxidation in the pathogenesis of the lesion. The mechanism for peroxidation in carbon tetrachloride poisoning has been suggested to be the result of free-radical formation and to be self-perpetuating, and not dependent on enzymic lipid peroxidation (see El-Khatib, Chenau, Carpenter, Trucco & Caputto, 1964).] Measurement of neotetrazolium-reductase activity at 2hr. after either carbon tetrachloride or dimethylnitrosamine poisoning failed to reveal any differences (Table 2).

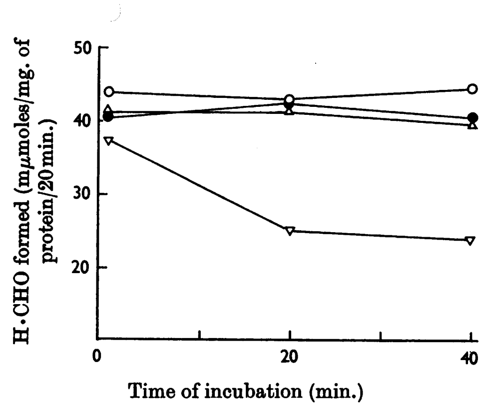


Fig. 3. Effect of preincubation of microsomes with CCl_4 on $NADPH_2$-dependent demethylation of dimethylaniline. Microsomes were prepared from control animals and as described in Fig. 1. They were incubated with the indicated amounts of CCl_4, and at 0, 20 and 40min. of preincubation at 35° 0·1 μmole of NADP, 10 μmoles of glucose 6-phosphate, 120 μmoles of nicotinamide, 1·0 μmole of ADP, 0·25 Kornberg unit of glucose 6-phosphate dehydrogenase and 10 μmoles of dimethylaniline were added. Incubation was continued aerobically for 20min. and then stopped. Formaldehyde formation was measured by the Nash (1953) method. ●, Control; ○, 1 μl. of CCl_4; △, 5 μl. of CCl_4; ▽, 10 μl. of CCl_4.

The microsomes isolated from carbon tetrachloride and dimethylnitrosamine-treated animals had the same quantity of cytochrome b_5 as the controls when measured 2hr. after the administration of the hepatotoxins. The spectrum of the pigment was the same in all three animal groups (Table 2). On the other hand, the quantity of cytochrome *P*-450 was decreased in both carbon tetrachloride- and dimethylnitrosamine-poisoned animals, more so in the former. The difference spectra in the treated animals were similar to the ones from control animals, and there was no significant increase in the 420mμ shoulder. In some instances there appeared to be an increase in the curve height at 429mμ, but this was not a constant finding. Additions of deoxycholate to all preparations resulted in the appearance of a strong band at 420mμ and a compensating decrease at 450mμ. Addition of carbon tetrachloride in amounts up to 25μl./ml. and of dimethylnitrosamine in amounts up to 83μg./ml. either before or after gassing with carbon monoxide resulted in a small diminution of the height of the 450mμ peak and a small increase in the shoulder at 420mμ, but in no way were these changes comparable with those seen in the intact animal.

The addition of 50μM-peroxidized arachidonic acid was without effect on the difference spectrum

Table 2. *Effects of poisoning with carbon tetrachloride or dimethylnitrosamine on microsomal cytochrome* b5, *cytochrome* P-450, *neotetrazolium reductase and lipid peroxidation*

Microsomes were isolated from rat liver 2hr. after the administration of 0·25ml. of CCl_4 or 2mg. of dimethylnitrosamine/100g. body wt. The results are given as means ± S.D., with the numbers of determinations in parentheses. Values that are statistically different from the appropriate control as determined by Student's *t* test are indicated ($P < 0·05$). No absorption in the 420mμ region was found after gassing with CO, indicating no contamination with haemoglobin.

	Control	CCl_4-treated	Dimethylnitrosamine-treated
Cytochrome b_5 [$\Delta(E_{427}-E_{410})$/mg. of protein]	0·0385 ± 0·0009 (4)	0·0365 ± 0·0026 (4)	0·0366 ± 0·0060 (4)
Cytochrome *P*-450 [$\Delta(E_{450}-E_{500})$/mg. of protein]	0·0227 ± 0·0037 (6)	0·0113 ± 0·003 (5) ($P < 0·05$)	0·0185 ± 0·0025 (5) ($P < 0·05$)
Neotetrazolium reductase (ΔE_{505}/0·15mg. of protein)	0·369 ± 0·050 (4)	0·391 ± 0·062 (4)	0·373 ± 0·039 (4)
$NADPH_2$-dependent lipid peroxidation (ΔE_{535}/0·3mg. of protein)	0·352 ± 0·036 (4)	0·304 ± 0·123 (4)	0·363 ± 0·035 (4)

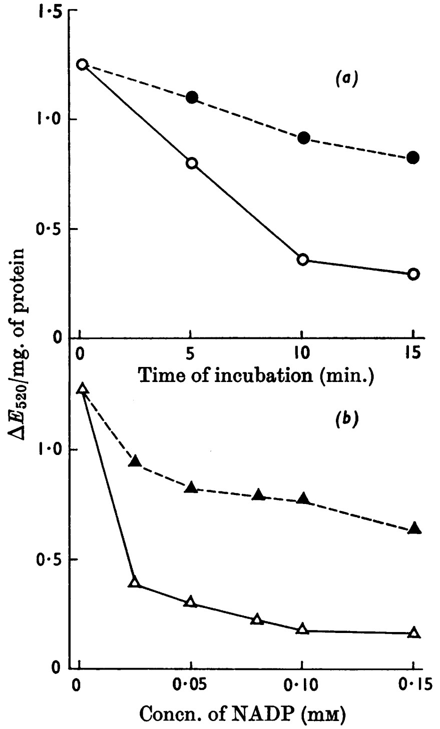
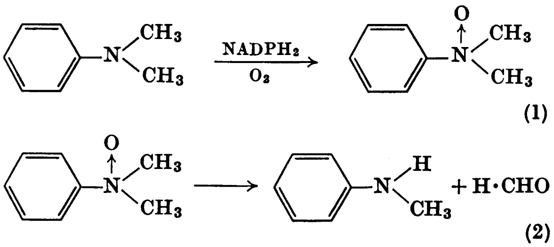


Fig. 4. Effect of CCl_4 poisoning on the capacity of isolated microsomes to undertake $NADPH_2$-dependent azo-dye cleavage. (*a*) Microsomes were prepared as described in Fig. 1, and 1 mg. of microsomal protein was incubated at 35° aerobically with 0·1 mole of NADP, 10 μmoles of glucose 6-phosphate, 0·25 Kornberg unit of glucose 6-phosphate dehydrogenase, 120 μmoles of nicotinamide and 250 μg. of monomethylaminoazobenzene in a total volume of 2 ml. The reaction was stopped at 0, 5, 10 and 20 min. by the addition of an equal volume of 2% (w/v) trichloroacetic acid in ethanol–acetone. The colour of the supernatant was read at 520 mμ. The rate of disappearance of colour in preparations of control microsomes (○) is more rapid than in treated ones (●). (*b*) In a separate experiment the effect of increased NADP concentration was measured, and the reaction stopped at 15 min. The control (△) and treated (▲) materials have parallel curves. Increased amounts of NADP do not restore activity to treated microsomes.

of control microsomes. Addition of 0·01% hydrogen peroxide was without effect, but at a concentration of 0·1% there was a decrease in the absorption.

At 1 and 2 hr. after the administration of carbon tetrachloride the capacity of microsomes isolated from treated animals to undertake reductive cleavage of monomethylaminoazobenzene was decreased by about 55%. A representative experiment is illustrated in Fig. 4. Addition of one or more of the cofactors did not restore the activity of treated microsomes to control values.



Scheme 1. Representation of the two-step process of $NADPH_2$-dependent oxidative demethylation of dimethylaniline (Ziegler & Pettit, 1964). The *N*-oxide is formed in the first step, requiring $NADPH_2$ and oxygen. The second step is also enzymic but does not depend on $NADPH_2$.

DISCUSSION

Microsomes derived from mammalian livers possess a complement of mixed-function oxidases that participate in the modification of various endogenous and exogenous substances (Mason *et al.* 1965). Among these reactions the oxidative demethylation of *N*-substituted amines has been investigated in particular detail (Mueller & Miller, 1953; La Du, Gaudette, Trousof & Brodie, 1955; Gillette, Brodie & La Du, 1957; Pettit & Ziegler, 1963; Ziegler & Pettit, 1964). It has been shown that the demethylation of dimethylaniline proceeds by a two-step process, the first part of which is the formation of an *N*-oxide intermediate (Scheme 1). This demethylation, and specifically the formation of the *N*-oxide, requires the presence of molecular oxygen and $NADPH_2$. The proposed mechanism of interaction indicates that the $NADPH_2$ is the electron donor, and is linked to the oxygen by means of the microsomal electron-transport chain. The resulting 'activated' oxygen reacts with the nitrogen atom with the formation of the *N*-oxide intermediate. Then a rearrangement occurs with the transfer of the oxygen to one methyl carbon atom and the release of formaldehyde. This step is thought to be enzymic and carbon monoxide-sensitive, but to be not $NADPH_2$-dependent (Ziegler & Pettit, 1966).

Microsomal injury *in vitro* by 'aging' or addition of potassium cholate results in an accumulation of the *N*-oxide intermediate (Ziegler & Pettit, 1964). It was of interest to us to see whether injury *in vivo* was associated with an altered ability of the microsomes to form and further modify the *N*-oxide intermediate, and, if so, whether related alterations could be observed in the microsomal electron-transport pathway, linking $NADPH_2$ to the demethylation. Evidence for *N*-oxide accumulation in vitamin E deficiency and after aminofluorene treatment has been given (Hultin & Arrhenius, 1965*b*).

It is well established that both dimethylnitrosamine and carbon tetrachloride give rise to structural and functional alterations of the endoplasmic reticulum of liver cells (Bassi, 1960; Hultin *et al.* 1960; Emmelot & Benedetti, 1960; Oberling & Rouiller, 1956; Recknagel & Lombardi, 1961; Smuckler *et al.* 1962; Reynolds, 1963; Smuckler & Benditt, 1965). It has been shown that the capacity of isolated liver microsomes to demethylate aminopyrine is depressed 24hr. after carbon tetrachloride administration *in vivo* (Neubert & Maibauer, 1959). In the present experiments the decreased demethylation was observed as soon as 1hr. after administration, and at the end of the first day there was little activity remaining. During the first 3hr. of carbon tetrachloride poisoning there was an accumulation of *N*-oxide, but not stoicheiometrically related to the decrease in formaldehyde formation. After the first 3hr. *N*-oxide formation fell and remained below the control value for the remainder of the first day. However, *N*-oxide formation was never decreased as much as formaldehyde production, and the ratio of *N*-oxide to formaldehyde remained above control values.

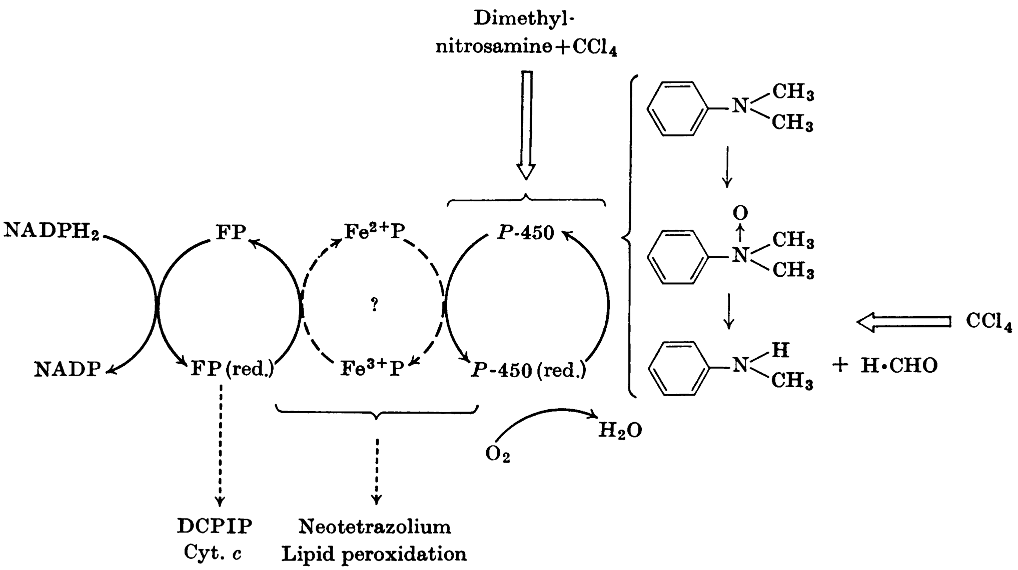
After dimethylnitrosamine poisoning there was also an initial decrease in the capacity of the microsomes to demethylate dimethylaniline. At the dosage investigated (2mg./100g. body wt.), the time of onset of this effect was later than with carbon tetrachloride and the decrease more modest. By the end of the first day the demethylation activity was not different from control values. The formation of *N*-oxide decreased concomitantly with formaldehyde production, but during the recovery phase the accumulation of *N*-oxide increased more rapidly and exceeded that of control microsomes. The ratio of *N*-oxide to formaldehyde also exceeded control values.

In neither poisoning was there evidence that these changes were due to an alteration in the cofactor requirements for the assay of demethylation and *N*-oxide formation. Maximal yields of formaldehyde and of the *N*-oxide were obtained with identical systems for control and treated microsomes. The possibility that the presence of the hepatotoxins themselves interfered with the assay systems was measured, and it was found that addition of carbon tetrachloride or dimethylnitrosamine to the isolated systems did not alter the yields in a manner analogous to the administration of either hepatotoxin *in vivo*.

Reductive cleavage of azo-dyes is also carried out by one or more microsomal enzymes that have, in common with *N*-demethylation, a requirement for $NADPH_2$. Unlike oxidative demethylation these reactions are not oxygen-dependent. The pathway from $NADPH_2$ to the azo-dye is not known, but by analogy it might be expected that similar steps are involved. After carbon tetrachloride poisoning there was a decrease in the activity of this enzyme that could not be restored by increasing the NADPH concentration. Considering these findings we were prompted to examine the pathway linking $NADPH_2$ and oxidative demethylation.

Current evidence suggests that the sequence of microsomal electron transport proceeds by the steps indicated in Scheme 2, and that the electrons may be donated to various added acceptors at the points indicated (Mason *et al.* 1965; Omura, Sato, Cooper, Rosenthal & Estabrook, 1965; Orrenius, 1965). In previous experiments the initial transfer of electrons to the flavoprotein was measured by the capacity of this intermediate to donate electrons from $NADPH_2$ to cytochrome *c* or 2,6-dichlorophenol-indophenol (Cleveland & Smuckler, 1965; E. A. Smuckler, unpublished work). No difference was found between microsomes from control, carbon tetrachloride- or dimethylnitrosamine-treated animals. The next carrier in the electron-transport chain in liver microsomes has not been characterized, but by analogy with adrenal microsomal electron transport it is supposed to contain an iron–protein complex. It has been assumed that this carrier can transfer electrons to neotetrazolium and can be utilized for enzymic lipid peroxidation. These two functions were not significantly altered after carbon tetrachloride or dimethylnitrosamine poisoning. Measurement of $NADPH_2$-dependent formation of thiobarbituric acid-positive material by microsomes from carbon tetrachloride-treated rats indicated that there was a slight decrease in the total activity, but no significant difference from the control value was verified by statistical analysis (see above). These findings indicated that at 2hr. after poisoning the electron transport by this pathway was intact this far along the chain.

It has been suggested that cytochrome *P*-450 has a role in oxidative demethylation. Poisoning with dimethylnitrosamine and especially with carbon tetrachloride was associated with a marked decrease in the amount of this pigment in the liver microsomes. The assay of cytochrome *P*-450 depends on the formation of the absorption band at 450mμ in the presence of carbon monoxide (Omura & Sato, 1964*a*; Klingenberg, 1958; Garfinkel, 1958). The decreased absorption could result from loss of the pigment or a functional alteration *in vivo* in such a way that it became unable to react with carbon monoxide. To test whether the hepatotoxins acted directly on the pigment, rendering it unable to bind, these substances were added to microsomes both before and after gassing with carbon monoxide. In neither instance was there an alteration in the production



Scheme 2. Proposed scheme illustrating the steps in microsomal $NADPH_2$-dependent electron transport. Electrons from $NADPH_2$ are carried by a flavoprotein (FP), an iron–protein complex ($Fe^{2+}P$) and cytochrome *P*-450 (*P*-450) to molecular oxygen. The inclusion of the iron–protein complex $Fe^{2+}P$ is based on the similarity of the liver system to the adrenal microsomal one. However, the presence of this material in liver microsomes has not been ascertained, as indicated by the question mark. The interaction of the oxygen, cytochrome *P*-450 and in this case the amine is not clear. 2,6-Dichlorophenol-indophenol (DCPIP) or cytochrome *c* (Cyt. *c*) can act as acceptors of electrons from the first step, the flavoprotein. It has been suggested that neotetrazolium and enzymic lipid peroxidation accept electrons from the same point between the flavoprotein and cytochrome *P*-450. We suggest that at an early stage of poisoning the rate-limiting block produced by dimethylnitrosamine is at or before the cytochrome *P*-450, whereas CCl_4 preferentially blocks the *N*-oxide rearrangement and formaldehyde release.

of the difference spectra. In experiments in which large amounts of the hepatotoxins were added small increments of the extinction at 420mμ occurred, but these were less than 10% and did not resemble the changes produced *in vivo*. To see whether the 450mμ-absorbing pigment remaining after treatment with carbon tetrachloride or dimethylnitrosamine *in vivo* or *in vitro* had altered capacity to be transformed into the 420mμ-absorbing pigment, deoxycholate was added (Omura & Sato, 1964*b*). The amount of 420mμ-absorbing material that formed in these experiments was proportional to the loss of 450mμ-absorbing material. It also seemed possible that the decrease in the amount of the microsomal cytochrome *P*-450 might be the result of its loss through solubilization or relocation during the isolation procedure or both. To test this possibility dimethylnitrosamine or carbon tetrachloride was added to liver homogenates during the process of isolation of the microsomes. No difference was found in the quantity of cytochrome *P*-450 in the prepared material. Further support for the lack of solubilization comes indirectly from the fact that other enzymes of the electron-transport chain and another pigment, cytochrome b_5, were not altered. We conclude that there must be a direct alteration of the carbon monoxide-binding pigment by active substances formed from these hepatotoxins.

It has been postulated that carbon tetrachloride and dimethylnitrosamine may give rise to peroxides or free radicals within the cells, and that this is the basis of the various alterations that are associated with their administration (Brouwers & Emmelot, 1960; Kriek & Emmelot, 1963; Ghoshal & Recknagel, 1965; Recknagel & Ghoshal, 1966; Slater, 1966). It has also been suggested that peroxides will destroy protohaems of the carbon monoxide-binding type (Tappel & Zalkin, 1960; Omura & Sato, 1964*a*,*b*). The addition of peroxidized arachidonic acid to preparations of control microsomes before or after gassing with carbon monoxide did not alter the capacity of the microsomal cytochrome *P*-450 to react with carbon monoxide. The difference spectra were identical with those of control microsomes in the presence or absence of non-peroxidized arachidonic acid. Addition of hydrogen peroxide resulted in loss of the spectrum when added at a final concentration of 0·1% but was without effect at lower concentrations (0·01%). It appears that the presence of these peroxides in the test *in vitro* does not mimic the changes observed *in vivo*.

The evidence presented indicates that during the early period after dimethylnitrosamine poisoning there is a single major defect in the link between microsomal electron transport and oxidative demethylation. The correlation between the decreases in formaldehyde and *N*-oxide formation suggests that a block occurs before the formation of the *N*-oxide intermediate. The facts that transport to the flavoprotein is unaltered and that there is a decrease in the amount of cytochrome *P*-450 indicate that dimethylnitrosamine disrupts the chain in this area; however, neither the means of the effect nor the relationship between cytochrome *P*-450 and demethylation are clear (see above).

The increased accumulation of *N*-oxide intermediate in the early phase of carbon tetrachloride poisoning and during the recovery phase supports the notion that the transfer of oxygen from the nitrogen to the methyl carbon atom is a separate step (Ziegler & Pettit, 1964). It appears that in the early phase of carbon tetrachloride poisoning the formation of formaldehyde from *N*-oxide is more severely blocked. During the subsequent development of the lesion, this second step remains more affected than the first, since there is always an increased amount of the intermediate present even when the formation of the formaldehyde is decreased to very low values.

The overcompensation in *N*-oxide accumulation during the recovery phase after dimethylnitrosamine poisoning may be a direct consequence of the previous injury to the membranes, or it may be the result of stress mediated by the adrenal gland. It has been shown that dimethylnitrosamine poisoning gives rise to an increased activity of the adrenal cortex (G. E. Bauer, E. A. Smuckler & T. Hultin, unpublished work) and that adrenal-mediated stress or the administration of glucocorticoids results in an increased accumulation of the *N*-oxide intermediate in the demethylation of dimethylaniline *in vitro* (Hultin & Arrhenius, 1965*a*,*b*). In the latter phase of the carbon tetrachloride injury it is possible that the combination of effects of the injury itself and the adrenal-mediated stress results in the maintenance of the high ratio of *N*-oxide to formaldehyde formation.

It has been suggested that cytochrome *P*-450 plays a role in steroid hydroxylation (Cooper, Estabrook & Rosenthal, 1964) and in *N*-demethylation (Orrenius, Dallner & Ernster, 1964; Ernster & Orrenius, 1965; Ziegler & Pettit, 1966; Machinist, Orme-Johnson & Ziegler, 1966). The particular role that this pigment plays is not clear, but it may be the ultimate oxygen donor in steroid hydroxylation (Cooper *et al.* 1964). It was postulated to serve a similar function in *N*-demethylation (Ernster & Orrenius, 1965; S. Orrenius, personal communication); however, evidence presented by Ziegler & Pettit (1966) suggests that it serves in the demethylation step of the *N*-oxide intermediate. Support for this observation comes from the carbon tetrachloride experiments. In these instances a correlation of diminished demethylation of dimethylaniline and decreased cytochrome *P*-450 concentrations is apparent (Table 3) at times of *N*-oxide accumulation. How the alteration in cytochrome *P*-450 occurs, the specific means of action of cytochrome *P*-450 in *N*-demethylation and the means of alteration in this process produced by carbon tetrachloride and dimethylnitrosamine remain to be shown.

Table 3. *Comparison of the effects of carbon tetrachloride and dimethylnitrosamine on microsomal NADPH$_2$-dependent demethylation of dimethylaniline and cytochrome* P-450

For experimental details, including control values, see Tables 1 and 2. The results are given as means ± S.D. of 4 or more determinations.

	Demethylation (% of control)	Cytochrome *P*-450 (% of control)
Control	(100)	(100)
CCl_4-treated	55 ± 11	46 ± 26
Dimethylnitrosamine-treated	74 ± 15	82 ± 13

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