The Stimulatory Effects of Carbon Tetrachloride on Peroxidative Reactions in Rat Liver Fractions in vitro

INTERACTION SITES IN THE ENDOPLASMIC RETICULUM

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1. The actions of various inhibitors of the microsomal NADPH-cytochrome P-450 electron-transport chain have been studied on the stimulatory effect of carbon tetrachloride on malonaldehyde production. 2. Carbon monoxide, p-chloromercuribenzoate, β-diethylaminoethyl-3,3′-diphenylpropyl acetate (SKF 526A) and nicotinamide did not decrease the stimulatory action of carbon tetrachloride on malonaldehyde production when present in concentrations shown to be capable of strongly inhibiting the demethylation of aminopyrine. 3. In contrast with the effects of the substances mentioned above, low concentrations of cytochrome c strongly depressed the stimulatory action of carbon tetrachloride on malonaldehyde production while increasing the endogenous rate of peroxidation. 4. Aging the microsomal suspensions at 0°C caused a rapid decrease in aminopyrine demethylation activity and in lipid peroxidation catalysed by ADP and Fe2+. The stimulation of malonaldehyde production by carbon tetrachloride was relatively unaffected, however, by aging the microsomes at 0°C for 3 days; during this period cytochrome P-450 decreased by more than 30%. 5. The conclusion is reached that the interaction between carbon tetrachloride and the NADPH–cytochrome P-450 electron-transport chain necessary for the stimulation of malonaldehyde production involves a locus near to if not identical with the NADPH–cytochrome c reductase flavoprotein.

General features of the enzyme reaction responsible for the stimulation of malonaldehyde production by carbon tetrachloride in suspensions of rat liver endoplasmic reticulum were described in the preceding paper (Slater & Sawyer, 1971a).

The components of the endoplasmic reticulum required for the stimulation of malonaldehyde production by carbon tetrachloride have been studied and evidence is presented here that the major interaction occurs in the vicinity of the NADPH–cytochrome c reductase flavoprotein. Differences in the pathways involved in the stimulatory action of carbon tetrachloride on lipid peroxidation or in the endogenous peroxidation that is dependent on NADPH in the absence of carbon tetrachloride have also been studied; it has been found possible to affect selectively either of these two routes independently of the other. Some of these results have been reported in abbreviated form (Slater, 1967a,b).

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METHODS

The sources of the rats used and the preparation of liver microsomal suspensions are as described in the previous paper (Slater & Sawyer, 1971a).

Rate from source (C) [Carworth (Europe) Ltd., Alconbury, Hunts., U.K.] were used in almost all the experiments reported here; it is mentioned at the appropriate position in the text when rats of a different source were used. For details concerning preparation of microsome–standard stock suspension, incubation conditions and the determination of malonaldehyde see Slater & Sawyer (1971a).

Cytochrome P-450 was determined in microsomal suspensions by the method of Omura & Sato (1962) by using a Unicam SP. 800 recording spectrophotometer. The final concentration of microsomal protein in the cuvettes was approx. 1 mg/ml in 0.1 M-NaH2PO4-Na2HPO4 buffer, pH 7.4. The extinction difference (E450–E500) was converted into nmol/mg of protein by using the millimolar extinction value 91 mm−1·cm−1 (Omura & Sato, 1964).

NADPH–cytochrome c reductase was determined by the method given by Slater & Sawyer (1969), by using a
Unicam SP. 800 recording spectrophotometer. The milli-
molar extinction value for reduced-minus-oxidized cyto-
chrome c used was 18.5 mm$^{-1}$ cm$^{-1}$ (Margoliash, 1954).

The uptake of oxygen coupled to the process of lipid
peroxidation in microsomes in the presence of NADPH,
ADP and Fe$^{2+}$ was measured as described by Slater (1968).
The results are given as nmol of oxygen taken up/min per
mg of microsomal protein.

The demethylation of aminopyrine was measured by a
modification of the procedure of Orrenius (1965) in which
isocitrate, MnCl$_2$, isocitrate dehydrogenase and nicotin-
amide were replaced by glucose 6-phosphate (5 mm),
glucose 6-phosphate dehydrogenase (5.6 i.u.) and acet-
amide (105 mm). Incubations were for 15 or 20 min at
37°C in the dark; formaldehyde production was deter-
mined by the method of Nash (1955).

Protein was determined by the method of Lowry,
Rosebrough, Farr & Randall (1961); a calibration curve
was constructed by using crystalline bovine plasma albumin
as a standard.

Microsomal suspensions in 0.15 M-KCl were aged at 0°C
by keeping the suspensions in stoppered tubes immersed
in crushed ice.

The effects of CO on malonaldehyde production and on
aminopyrine demethylation were studied as follows: microsomes–stock suspensions were gassed for 5 min at
0°C with either O$_2$ + CO$_2$ (95:5) or with CO. Samples of
the gassed suspensions were then quickly transferred to
Warburg flasks for malonaldehyde incubations or were
used to study the rate of aminopyrine demethylation.

Chemicals were obtained as follows. β-Diethylamino-
ethyl-3,3′-diphenyl propyl acetate (SKF 525A) was a
generous gift from Smith, Kline and French Ltd., Welwyn
Garden City, Herts., U.K. Malonaldehyde bis-diethyl
acetate was obtained from Schuchardt, München, Germany;
crystalline cytochrome c was purchased from Boehringer
und Soehne G.m.b.H., Mannheim, Germany; CCl$_4$ was
microanalytical grade from Hopkin and Williams Ltd.,
Chadwell Heath, Essex, U.K.

RESULTS AND DISCUSSION

Interaction of carbon tetrachloride with the endo-
plasmic reticulum results in a stimulation of the
production of malonaldehyde and this is associated
with an increased lipid peroxidation (Recknagel,
1967). The evidence for the suggestion that a free
radical metabolite of carbon tetrachloride is re-
sponsible for initiating the increased lipid per-
oxidation was discussed in the preceding paper
(Slater & Sawyer, 1971a). An important question is:
with which component of the endoplasmic reticulum
does carbon tetrachloride react to yield the homo-
lytic products responsible for the increased
peroxidation?

Chemical considerations suggest that the inter-
action of carbon tetrachloride with the endoplasmic
reticulum involves an endogenous radical species
in the endoplasmic reticulum that, in a relatively
non-polar medium, could readily react with carbon
tetrachloride to yield trichloromethyl radicals. There
are two main candidates for such an endo-
genous radical species: the flavin component of
NADPH–cytochrome c reductase, and cytochrome
P-450. The flavin is known to oscillate between the
fully reduced and half-reduced semiquinone radical
(Kamin, Masters, Gibson & Williams, 1965; Masters,
Kamin, Gibson & Williams, 1965). The cytochrome
P-450 site in drug-hydroxylating reactions involves an interaction with a radical
oxygen species (possibly the hydroxyl radical or the
superoxide radical anion); moreover, electron spin
resonance of liver endoplasmic reticulum has shown
the existence of a radical iron component closely
associated with cytochrome P-450 (Ichikawa &

\[ \text{NADPH} \rightarrow \text{FP} \rightarrow (-\text{SH}) \rightarrow \text{X}_1 \rightarrow (\text{B.S.})(P-450) \rightarrow \text{Products} \]

\[ \text{Drug} + \text{O}_2 \rightarrow \text{Products} \]

\[ \text{CCl}_3 \text{ CCl}_4 \]

Scheme 1. Diagrammatic representation of the NADPH–cytochrome P-450 chain. Abbreviations: FP, flavoprotein; X$_1$, rate-limiting component for drug metabolism (see Dallner et al. 1965); pCMB, p-chloro-
mercuribenzoate, 100 μM; SKF, β-diethylaminoethyl-3,3′-diphenylpropyl acetate, 100 μM; B.S., drug-
binding site. Lipid peroxidation in the presence of ADP and Fe$^{2+}$ is shown diverging from the main chain
at the drug-binding site (see Orrenius et al. 1964); the stimulating action of CCl$_4$ on lipid peroxidation
is shown arising from the formation of trichloromethyl radicals at the NADPH flavoprotein locus
(see the Discussion section and Slater & Sawyer, 1971a). The concentrations indicated are those required
for selective inhibition of the corresponding terminal regions of the chain.
Yamano, 1967). These potential sites of interaction between the endoplasmic reticulum and carbon tetrachloride are at widely differing sites on the NADPH–cytochrome P-450 electron-transport chain. It is possible to reach a decision as to which part of the NADPH–cytochrome P-450 chain is involved in the stimulatory action of carbon tetrachloride on malonaldehyde production by the use of selective chain inhibitors. The interpretation of such inhibitor studies is far easier if microsomes–standard stock mixtures are used rather than the more complex microsomes–plus-cell supernatant–standard stock mixtures. For this reason the studies reported here were restricted to experiments involving microsomes–standard stock suspensions although higher stimulations of malonaldehyde production by carbon tetrachloride are found with the more complex mixture (see Slater & Sawyer, 1971a). A representation of the sequence of carriers in the NADPH–cytochrome P-450 electron-transport chain is shown in Scheme 1 together with the interaction sites of inhibitors used in this study. The rate-limiting factor for drug metabolism is involved in the coupling of the reduced NADPH–flavoprotein to the cytochrome P-450 region. The precise nature of the rate-limiting interaction or factor is unknown and it is indicated in Scheme 1 by the symbol X_1 (see Dallner, Siekevitz & Palade, 1965). It is important to note the concentrations indicated for selective inhibitions of parts of the chain; if much higher concentrations are used then unselective membrane damage may result. For example, 100 μM-SKF 525A is sufficient to decrease strongly drug metabolism in microsomal suspension since SKF 525A has a low K_m for the cytochrome P-450 site (0.5–1 μM, Schenkman, Remmer & Estabrook, 1967); higher concentrations of SKF 525A may cause anomalous effects on membrane systems indicative of surface-active changes or micelle formation (Lee, Yamamura & Dixon, 1968).

Relatively low concentrations (50–100 μM) of

Table 1. Effects of p-chloromercuribenzoate (pCMB) and β-diethylaminoethyl-3,3′-diphenylpropyl acetate (SKF 525A) on the stimulation in malonaldehyde production by carbon tetrachloride in rat liver suspensions

<table>
<thead>
<tr>
<th>Expt.</th>
<th>Incubation mixture</th>
<th>Incubation time (min)</th>
<th>Concentration (μM)</th>
<th>Malonaldehyde production (nmol/ml of suspension)</th>
<th>Stimulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>Control</td>
<td>15</td>
<td>—</td>
<td>5.18 (3)</td>
<td>0.75*</td>
</tr>
<tr>
<td></td>
<td>Control+CCl_4</td>
<td>15</td>
<td>100</td>
<td>5.93 (3)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SKF 525A</td>
<td>15</td>
<td>100</td>
<td>4.02 (3)**</td>
<td>1.00†</td>
</tr>
<tr>
<td></td>
<td>SKF 525A+CCl_4</td>
<td>15</td>
<td>100</td>
<td>5.02 (3)</td>
<td></td>
</tr>
<tr>
<td>b</td>
<td>Control</td>
<td>15</td>
<td>—</td>
<td>6.05 (6)</td>
<td>0.82*</td>
</tr>
<tr>
<td></td>
<td>Control+CCl_4</td>
<td>15</td>
<td>420</td>
<td>6.87 (6)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SKF 525A</td>
<td>15</td>
<td>420</td>
<td>3.97 (6)</td>
<td>0.24*</td>
</tr>
<tr>
<td></td>
<td>SKF 525A+CCl_4</td>
<td>15</td>
<td>420</td>
<td>4.21 (6)</td>
<td></td>
</tr>
<tr>
<td>c</td>
<td>Control</td>
<td>10</td>
<td>—</td>
<td>5.20 (4)</td>
<td>0.73*</td>
</tr>
<tr>
<td></td>
<td>Control+CCl_4</td>
<td>10</td>
<td>94</td>
<td>5.93 (4)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>pCMB</td>
<td>10</td>
<td>94</td>
<td>6.30 (4)††</td>
<td>1.35††</td>
</tr>
<tr>
<td></td>
<td>pCMB+CCl_4</td>
<td>10</td>
<td>94</td>
<td>7.65 (4)</td>
<td></td>
</tr>
<tr>
<td>d</td>
<td>Control</td>
<td>15</td>
<td>—</td>
<td>4.38 (6)††</td>
<td>2.19††</td>
</tr>
<tr>
<td></td>
<td>Control+CCl_4</td>
<td>15</td>
<td>—</td>
<td>6.57 (6)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>pCMB</td>
<td>15</td>
<td>6.57 (6)††</td>
<td>5.68 (4)††</td>
<td>3.22††</td>
</tr>
<tr>
<td></td>
<td>pCMB+CCl_4</td>
<td>15</td>
<td>100</td>
<td>8.80 (4)</td>
<td></td>
</tr>
</tbody>
</table>

* P<0.01 for difference between control and CCl_4 values, or between drug and CCl_4 values.
† P<0.001 for difference between control and CCl_4 values, or between drug and CCl_4 values.
‡ Not significantly different from control stimulation.
§ P<0.001 for difference between the stimulations.
∥ P<0.05 for difference between the stimulations.
†† P<0.01 for difference between the stimulations.
** P<0.01 for differences between control and drug values.
††† P<0.001 for differences between control and drug values.
p-chloromercuribenzoate also strongly inhibit drug metabolism in microsomal suspensions and have no inhibitory action on NADPH-cytochrome c reductase. At higher concentrations, however, p-chloromercuribenzoate decreases the activity of purified NADPH-cytochrome c reductase as well as drug metabolism (Kamin et al. 1965).

The effects of p-chloromercuribenzoate and SKF 525A on malonaldehyde production in rat liver suspensions are shown in Table 1. p-Chloromercuribenzoate (94 μM) produced a small increase in endogenous malonaldehyde production (i.e. in the presence of NADPH but in the absence of carbon tetrachloride); SKF 525A (100 μM), however, decreased the extent of endogenous peroxidation.

Table 1 shows that 94 μM-p-chloromercuribenzoate increased the stimulation of malonaldehyde production due to carbon tetrachloride in contrast with its effect on aminopyrine demethylation under the same incubation conditions: with an incubation time of 15 min at 37°C, 94 μM-p-chloromercuribenzoate decreased aminopyrine demethylation in a microsome-standard stock suspension by 61%.

A prior period of food deprivation for 24 h increases the effect of carbon tetrachloride on malonaldehyde production in microsomes-standard stock suspensions (Slater & Sawyer, 1971a). p-Chloromercuribenzoate (100 μM) produced a further stimulation of the carbon tetrachloride effect even under these conditions of prior starvation (Table 1); under identical conditions p-chloromercuribenzoate (100 μM) inhibited aminopyrine demethylation by 59%. The increase in the stimulatory action of carbon tetrachloride in the presence of p-chloromercuribenzoate is possibly the result of directing electron flow from the terminal stage of the cytochrome P-450 chain towards the formation of trichloromethyl radicals (see Scheme 1). Thus, despite a strong inhibition of overall drug metabolism by p-chloromercuribenzoate there is an increase in the stimulatory effect of carbon tetrachloride on malonaldehyde production.

The effects of SKF 525A on the stimulatory action of carbon tetrachloride on malonaldehyde production are also shown in Table 1. In experiments with three different concentrations of SKF 525A (85, 100, and 126 μM) there was a tendency for the stimulatory action of carbon tetrachloride to be increased (as with p-chloromercuribenzoate) but the increases were not statistically significant. The important result of our experiments, however, was that SKF 525A did not decrease the stimulatory effect of carbon tetrachloride when present in concentrations that severely depressed drug metabolism. Under identical conditions SKF 525A (100 μM) inhibited aminopyrine demethylation by 58%. Further experiments with SKF 525A (420 μM, Table 1) showed that this substance will eventually inhibit the stimulatory action of carbon tetrachloride as well as overall drug metabolism if the concentration is substantially increased. The effect of such high concentrations of SKF 525A may reflect unspecific damage to the microsomal membranes as referred to earlier in the discussion or to an antioxidant activity as demonstrated by Hochstein & Ernster (1963). Our results with high

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Table 2. Effect of cytochrome c on the stimulation of malonaldehyde production by carbon tetrachloride in a rat liver microsome-stock suspension

Incubation was for 10 min at 37°C with and without 2 μl of CCl₄-liquid paraffin (1:1, v/v) in the side arm. Cytochrome c was added to the central compartment in the amounts given. Malonaldehyde production was determined by the thiobarbituric acid reaction.

<table>
<thead>
<tr>
<th>Mixture</th>
<th>Concen. of cytochrome c (mg/100 ml)</th>
<th>Malonaldehyde production (nmol/ml of suspension)</th>
<th>Stimulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>—</td>
<td>4.75</td>
<td>+0.95*</td>
</tr>
<tr>
<td>Control + CCl₄</td>
<td>—</td>
<td>5.70</td>
<td>+0.63†</td>
</tr>
<tr>
<td>Cytochrome c</td>
<td>2</td>
<td>5.00</td>
<td></td>
</tr>
<tr>
<td>Cytochrome c + CCl₄</td>
<td>2</td>
<td>5.00</td>
<td></td>
</tr>
<tr>
<td>Cytochrome c</td>
<td>50</td>
<td>7.78§</td>
<td></td>
</tr>
<tr>
<td>Cytochrome c + CCl₄</td>
<td>50</td>
<td>7.50</td>
<td></td>
</tr>
</tbody>
</table>

* P < 0.05 for difference between control and CCl₄ values.
† P < 0.01 for difference between cytochrome c and CCl₄ values.
‡ Difference not significant.
§ P < 0.001 for difference between cytochrome c and control values.
Table 3. Effect of pre-treatment with carbon monoxide on the stimulation of malonaldehyde production due to carbon tetrachloride and on aminopyrine demethylation by rat liver microsomes

In the experiment with malonaldehyde, microsomes (1 g equiv. of liver/ml of 0.15M-KCl) were mixed with standard stock solution; a 15 ml sample was gassed for 5 min at 0°C with O2 + CO2 (95:5) and a similar sample was gassed for 5 min at 0°C with CO. After gassing, the samples were kept in stoppered flasks until 2.5 ml portions were quickly withdrawn for transfer to Warburg flasks. Where necessary 2 µl of CCl4-liquid paraffin (1:1, v/v) was added to the side arms of the Warburg flasks which were incubated for 15 min at 37°C in the dark. Malonaldehyde was determined by the thiobarbituric acid reaction. For the aminopyrine demethylation experiment, the microsome–stock suspension described above was mixed with the aminopyrine stock mixture described in the Methods section but with the omission of aminopyrine itself. The microsomal mixture was then divided into two samples which were gassed with O2 + CO2 or CO at 0°C as described above. Samples (2.0 ml) were then placed into tubes containing 10 µmol of aminopyrine, quickly stoppered, and incubated for 20 min at 37°C. Malonaldehyde production was measured by the method of Nash (1952). For other details see the Methods section.

<table>
<thead>
<tr>
<th>Gas passed</th>
<th>Endogenous malonaldehyde production (nmol/ml of suspension)</th>
<th>Additional malonaldehyde production due to CCl4 (nmol/ml of suspension)</th>
<th>Aminopyrine demethylation (µmol of formaldehyde/min per ml of microsomal suspension)</th>
</tr>
</thead>
<tbody>
<tr>
<td>O2 + CO2</td>
<td>14.3</td>
<td>4.75</td>
<td>0.115</td>
</tr>
<tr>
<td>CO</td>
<td>9.3*</td>
<td>7.35*</td>
<td>0.061†</td>
</tr>
</tbody>
</table>

* P < 0.001 for difference between CO and O2 + CO2 values.
† P < 0.01 for difference between CO and O2 + CO2 values.

Table 4. Effects of aging microsomal suspensions at 0°C on the activities of various enzymes

Microsomal suspensions in 0.15M-KCl (equivalent to 1 g of liver/ml of suspension) were prepared as described in the Methods section and determinations of aminopyrine demethylation activity, NADPH-cytochrome c reductase activity, oxygen uptake coupled to lipid peroxidation in the presence of NADPH and Fe2++ADP, malonaldehyde production in the presence or absence of CCl4, and cytochrome P-450 were immediately carried out. The microsomal suspensions were kept at 0°C for 1, 2 and 3 days and measurements were repeated at these intervals. The results are expressed as percentage changes ± S.E.M. from the values obtained with fresh suspensions. The number of animals used are in parentheses. Results for the stimulation of malonaldehyde production by CCl4 were not included where the endogenous production in the absence of CCl4 exceeded 15 nmol/ml of suspension (see Slater & Sawyer, 1971a, Results section). For other details see the Methods section.

<table>
<thead>
<tr>
<th>Aging period (days)</th>
<th>Aminopyrine demethylation</th>
<th>Cytochrome P-450</th>
<th>NADPH-cytochrome c reductase</th>
<th>Malonaldehyde stimulation by CCl4</th>
<th>Oxygen uptake (NADPH + Fe2++ADP)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>−33 ± 4 (4)</td>
<td>+22 ± 3 (3)</td>
<td>−16 ± 8 (3)</td>
<td>−14 ± 6* (5)</td>
<td>−42 (2)</td>
</tr>
<tr>
<td>2</td>
<td>−41 ± 16 (3)</td>
<td>+7 ± 15 (3)</td>
<td>+60 (2)</td>
<td>+6 ± 6* (3)</td>
<td>−62 (2)</td>
</tr>
<tr>
<td>3</td>
<td>−40 ± 8 (4)</td>
<td>−32 ± 8 (5)</td>
<td>+37 ± 33 (4)</td>
<td>−15 (2)</td>
<td>−31 (2)</td>
</tr>
</tbody>
</table>

* P < 0.01 for difference between aminopyrine demethylation change and the change in malonaldehyde stimulation by using values for determinations carried out on the same preparations.
† P = 0.05 for difference between aminopyrine demethylation change and the change in malonaldehyde stimulation by using values obtained with different preparations.

concentrations of SKF 525A resemble those reported by Rao, Glende & Recknagel (1970) with 100 µM-SKF 525A; this difference may reflect the use of rats from different sources and with different dietary backgrounds.

Low concentrations of cytochrome c increased the endogenous production of malonaldehyde in microsome–stock solutions and, by contrast, strongly decreased the stimulation due to carbon tetrachloride (Table 2). This result is similar to that observed with 94 µM-p-chloromercuribenzoate (Table 1; Expt. c) and strongly suggests that the pathway responsible for endogenous peroxidation can be affected independently of the pathway involved in the stimulatory effect of carbon tetrachloride on malonaldehyde production. A similar conclusion arises from the experiments with CO (see below).
Table 5. Effect of nicotinamide on the stimulation in malonaldehyde production due to carbon tetrachloride

A suspension of microsomes in standard stock was incubated for 15 min at 37°C in the dark with or without 82 mM-nicotinamide, and with and without 2 μl of CCl₄-liquid paraffin (1:1, v/v) in the side arms of Warburg flasks. At the end of the incubation period samples of the suspension were assayed for malonaldehyde by the thiobarbituric acid reaction. For other details see the Methods section.

<table>
<thead>
<tr>
<th>Additions</th>
<th>Malonaldehyde production (nmol/ml of stock)</th>
<th>Stimulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5.54</td>
<td>0.81*</td>
</tr>
<tr>
<td>Control+CCl₄</td>
<td>6.35</td>
<td></td>
</tr>
<tr>
<td>Control+nicotinamide</td>
<td>6.48‡</td>
<td>0.92†</td>
</tr>
<tr>
<td>Control+nicotinamide+CCl₄</td>
<td>7.40</td>
<td></td>
</tr>
</tbody>
</table>

* P<0.01 for difference between control and CCl₄ values.
‡ P<0.001 for difference between control and CCl₄ values.
† P<0.001 for difference between control and control+nicotinamide values.

Table 6. Comparison of the effects of various substances or treatments on aminopyrine demethylation and, under similar conditions, on the stimulatory effect of carbon tetrachloride on malonaldehyde production in rat liver microsomes—standard stock suspensions

The values are from Tables 1–5 and from Slater & Sawyer (1971b). N.S., no significant change. For further details and other abbreviations see the text.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Conen.</th>
<th>Malonaldehyde stimulation by CCl₄</th>
<th>Aminopyrine demethylation</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-Chloromercuribenzoate</td>
<td>100μM</td>
<td>+52%</td>
<td>-58%</td>
</tr>
<tr>
<td>SKF 525A</td>
<td>100μM</td>
<td>N.S.</td>
<td>-59%</td>
</tr>
<tr>
<td>Carbon monoxide</td>
<td>82mM</td>
<td>+55%</td>
<td>-47%</td>
</tr>
<tr>
<td>Nicotinamide</td>
<td>82mM</td>
<td>+14%</td>
<td>-50%</td>
</tr>
<tr>
<td>Aging, 2 days</td>
<td>1μM</td>
<td>-56%</td>
<td>N.S.</td>
</tr>
<tr>
<td>Promethazine</td>
<td>5.5μM</td>
<td>-92%</td>
<td>N.S.</td>
</tr>
</tbody>
</table>

Table 3 gives the results obtained after gassing microsome–stock solutions with either carbon monoxide or an oxygen–carbon dioxide mixture. Carbon monoxide treatment decreases endogenous peroxidation relative to the treatment with oxygen and carbon dioxide, but increases the stimulation due to carbon tetrachloride in comparison with the effects of gassing with oxygen–carbon dioxide. Under the same conditions the treatment with carbon monoxide strongly inhibited aminopyrine demethylation (Table 3).

Table 4 gives results obtained after aging microsome suspensions at 0°C; this procedure has been reported by Orrenius, Dallner & Ernster (1964) to cause a rapid loss of aminopyrine demethylation activity and their finding was confirmed here (Table 4). Although aminopyrine demethylation activity had decreased significantly after 24 h aging there were no significant decreases either in the concentration of cytochrome P-450, or in the activities of NADPH-cytochrome c reductase and the enzyme system responsible for the stimulatory action of carbon tetrachloride on malonaldehyde production; similar results were obtained after 48 h aging. Statistical analysis showed that the changes in aminopyrine demethylation activity were significantly different from those found for the stimulatory activity of carbon tetrachloride on malonaldehyde production, the change in cytochrome P-450 content, or the change in NADPH-cytochrome c reductase activity. This indicates that the decrease in aminopyrine demethylation activity results from a change in a component that is not directly involved in the measurements of the other entities; from these results the most likely change on aging is a decrease in the activity of the rate-limiting component (designated X₁ in Scheme 1).

Table 5 gives further evidence that the terminal region of the NADPH-cytochrome P-450 chain is
not involved in the stimulatory action of carbon tetrachloride on malonaldehyde production. Nicotinamide (82 mM) had no significant effect on the stimulatory action of carbon tetrachloride and yet caused an appreciable inhibition of aminopyrine demethylation (30% inhibition at a final concentration of 21 mM and 50% inhibition at a final concentration of 82 mM) presumably by competing for the cytochrome P-450 site (Schenkman et al. 1967).

Table 6 summarizes the changes reported here, and includes some results from the following paper (Slater & Sawyer, 1971b), in relation to effects on drug metabolism and on the stimulatory effect of carbon tetrachloride on malonaldehyde production. It can be seen that the effects of the various treatments on the metabolism of aminopyrine (this requires the participation of the entire NADPH-cytochrome P-450 chain) show no direct correlation with the effects on the stimulatory action of carbon tetrachloride. In general, drug metabolism may be strongly inhibited without affecting the carbon tetrachloride effect. Conversely, with low concentrations of free-radical scavengers, the carbon tetrachloride effect on malonaldehyde production can be inhibited in the absence of significant depression of drug metabolism (e.g. with promethazine, Table 6). These results are consistent with the suggestion that the stimulatory action of carbon tetrachloride on malonaldehyde production involves an interaction between carbon tetrachloride and the proximal region only of the NADPH-cytochrome P-450 electron-transport chain. For reasons outlined earlier it is possible that the actual site of interaction is with the NADPH-flavoprotein.

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REFERENCES