CHAPTER XIX

EFFECT OF ACROLEIN AND CROTONEALDEHYDE ON HEPATIC
AND EXTRA-HEPATIC MIXED FUNCTION OXIDASE SYSTEM
OF ADULT MALE RATS,
**INTRODUCTION**

Acrolein a highly reactive and toxic aldehyde is used in the manufacture of glycerine (430) chemicals of textiles, rubbers, plasticizers, disinfectants and as a military lacrimatory agent. Similarly crotonaldehyde an analog of acrolein is used in the manufacture of various industrial products like butyl alcohol, aldehydes, quinolines, insecticides, and malic acid. Crotonaldehyde is also used in the locating brake and leaks, in purification of mineral oil, and as a warning agent. Both acrolein and crotonaldehyde are detected in foods (431-433), cigarette smoke (434,435) heavy and light vehicle engine exhaust (436-438) cool flames (439-440) iron foundries, varnishes (441) etc.

Cyclophosphamide itself is inactive per se, and requires activation by microsomal enzymes, CP is metabolised to various metabolites like 4-hydroxycyclophosphamide, 4-ketocyclophosphamide, aldophosphamide, carboxyphosphamide, phosphoramid mustard, and acrolein. Acrolein a highly toxic metabolite of CP has been shown to be mutagenic, cytotoxic and teratogenic. Various side effects including urinary bladder damage, urotoxicity, and hemorrhagic cystitis of CP therapy are attributed to acrolein toxicity.

Crotonaldehyde an analog of acrolein is generated during microsomal MFO dependent metabolism of 6-methylcyclophosphamide (442-444) which is an analog of cyclophosphamide.
6-methyl cyclophosphamide is claimed to be an equally potent and powerful immunosuppressive and antineoplastic agent (445,446) and crotonaldehyde has been found to be less toxic than that of acrolein (442).

In vitro acrolein has been found to inhibit drug metabolising enzymes activities, and causes destruction of cytochrome P 450 to its inactive form cytochrome P 420 (295). Various sulphhydril groups have been known to offer protection against in vitro toxicities of acrolein and in vivo cyclophosphamide mediated depression of microsomal NFOs (447). Cysteine and N-acetyl cysteine also offered protection against urinary bladder damage and other toxicities of cyclophosphamide. However data pertaining to in vivo and in vitro effect of crotonaldehyde and comparative toxicity with acrolein on microsomal NFOs is not available. The present studies were therefore planned to investigate in vivo and in vitro effects of crotonaldehyde and acrolein on hepatic and renal microsomal NFOs of adult male rats, so as to understand mechanism of action of cyclophosphamide and acrolein, and possibility of use of 6-methylcyclophosphamide in chemotherapy.
ANIMALS AND TREATMENT

Adult male wistar rats (200-250 gms) obtained from National Institute of Nutrition, Hyderabad were used in present investigations. Rats were acclimatized for 2 weeks before experiments and randomly distributed in groups of 6, were maintained identically at 24°C ± 1, and received commercially available rat pellet diet (Hindustan Lever, Bombay) and water ad libitum.

For the survival time studies, animals were intraperitoneally treated as follows:

Group 1: received Acrolein 5.04 mg/kg b.wt.
Group 2: received CP 100 mg/kg b.wt.
Group 3: received Acrolein 5.04 mg/kg plus Cysteine 100 mg/kg b.wt.
Group 4: received Crotonaldehyde 20.94 mg/kg b.wt.
Group 5: received Crotonaldehyde 20.94 mg/kg plus Cysteine 100 mg/kg b.wt.

For in vivo and in vitro toxicity studies animals were intraperitoneally treated as follows:

Control: received 0.9% saline
Group 1: received Cysteine 100 mg/kg b.wt.
Group 2: received cyclophosphamide 100 mg/kg b.wt.
Group 3: received CP 100 mg/kg plus Cysteine 100 mg/kg b.wt.
Group 4: received Acrolein 2.00 mg/kg b.wt.
Group 5: received Acrolein 2.00 mg/kg plus Cysteine 100 mg/kg b.wt.
Group 6: received crotonaldehyde 5.04 mg/kg b.wt.

Group 7: received Crotonaldehyde 5.04 mg plus Cysteine 100 mg/kg b.wt.

Group 8: received PB 80 mg/kg b.wt. for 3 days

Group 9: received 3-MC 20 mg/kg b.wt. for 2 days.

Animals were treated daily early in the morning between 8.00 to 9.00 a.m., and were observed every three hours for the mortality during survival time studies.

Material and Methods: As described earlier.
RESULTS

Survival time -

When animals were treated with acrolein and crotonaldehyde it was found that the acrolein was more toxic than that of the crotonaldehyde (Table - 1). The mean survival time of $1.62 \pm 0.4$ D was observed with 5.04 mg/kg b.wt. dose of acrolein, whereas at the same dose level crotonaldehyde had no effect. The mean survival time of $1.42 \pm 0.2$ D was observed when crotonaldehyde dose was raised to 20.94 mg/kg b.wt. Pretreatment of cysteine offered protection by increasing the mean survival time by almost 50% i.e. from $1.62 \pm 0.4$ to $3.20 \pm 0.4$ in acrolein treated and by 65% in crotonaldehyde treated animals, whereas administration of CP and cysteine alone had no effect on the survival time of the adult male rats.

Body weight -

CP, AC and CR treated rats were active and no significant change in the dietary intake was observed. However, the administration of CP (100 mg/kg), AC (2.00 mg/kg) and CR (3.04 mg/kg) caused 8, 21 and 17% decrease respectively in the body weight of adult rats over the period of 7 days (Figure 1). Pretreatment of cysteine prior to administration of CP, AC or CR protected against the loss in body weight.

Hepatic glutathione depletion -

Administration of CP, acrolein and crotonaldehyde caused hepatic glutathione depletion, however the magnitude of
depletion of glutathione due to CP, AC and CR differed at various time intervals (Figure 2). At the end of 12 hours CP caused 26% decrease in glutathione levels as compared to 86% and 66% decrease due to AC and CR administration respectively. At the end of 36 hours hepatic glutathione levels remained almost constant, in acrolein treated group of rats, whereas in cyclophosphamide and crotonaldehyde treated group of rats, the hepatic glutathione levels started to return towards normal.

Pretreatment of cysteine caused significant protection against GSH depletion in CP, AC and CR treated rats. At the end of 24 hours of cysteine pretreatment the hepatic GSH levels were 1240, 805 and 170 µgms as compared to 960, 410 and 300 µgms/gm liver in CP, AC and CR treated group of rats respectively.

Drug Metabolising Enzymes -

(1) Aminopyrine N-demethylase -

Administration of CP, AC and CR caused significant decrease in the hepatic and renal drug metabolising enzyme activities (Figure 3). The magnitude of inhibition was highest due to acrolein administration as compared to that of CP and crotonaldehyde. At the end of 6 hours of CP administration, CP did not cause any significant decrease, whereas AC caused 19 and 16% and crotonaldehyde caused 14 and 13% decrease in hepatic and renal aminopyrine N-demethylase activity respectively (Figure 3a).
The magnitude of inhibition of hepatic aminopyrine N-demethylase was 14, 29 and 34% at the end of 24 hours due to CP, AC and CR administration respectively. Cysteine administration caused significant protection against inhibitory effects of CP, AC and CR administration. Cysteine pretreatment caused 14, 25 and 32% protection in hepatic and 31, 23 and 29% protection in renal aminopyrine N-demethylase in CP, AC and CR treated group of rats respectively, at the end of 24 hours.

Acetanilide hydroxylase

CP, AC and CR administration caused significant decrease in hepatic and renal acetanilide hydroxylase activity in adult male rats (Figure 3b). At the end of 6 hours, CP caused only marginal decrease in the hepatic, whereas 12% decrease in the renal acetanilide hydroxylase activity was observed. At the end of 6 hour, acrolein and crotonaldehyde administration caused 15 and 17% decrease in hepatic and 12 and 14% decrease in renal acetanilide hydroxylase activity respectively.

At the end of 24 hours, administration of CP caused 14 and 16% decrease, AC caused 40 and 33% decrease, and CR and caused 26 and 23% decrease in hepatic and renal acetanilide hydroxylase activity respectively. Cysteine pretreatment offered significant protection against the toxicity. The magnitude of protection of hepatic acetanilide hydroxylase, at the end of 24 hours was 19% in CP treated, 26% in AC treated and 32% in CR treated rats.
Lipid peroxidation

Administration of CP, AC and CR exhibited increase in hepatic and renal lipid peroxidation (Figure 5). At the end of 6 hours increase in lipid peroxidation due to CP was least in kidney but was highest at the end of 24 hours. AC lead to 140 and 212 % increase, whereas CR lead to 126% and 181% increase in hepatic and renal lipid peroxidation respectively.

Cysteine administration exhibited significant protection against CP, AC and CR mediated increase in hepatic and renal lipid peroxidation. At the end of 24 hours, in cysteine pretreated rats the percent of protection in hepatic lipid peroxidation was 10 in CP, 14 in AC, and 7 in CR treated rats.

ELECTRON TRANSPORT COMPONENTS

Cytochrome P-450

A significant decrease in the hepatic and renal cytochrome P 450 levels was observed due to administration of CP, AC and crotonaldehyde (Figure 4a). The magnitude of decrease in cytochrome P-450 levels was more due to AC as compared to CP and CR. At the end of 6 hours CP did not cause any significant change, whereas AC caused 17 and 16% decrease, and CR caused 15 and 16% decrease in hepatic and renal cytochrome P-450 levels respectively. At the end of 24 hours the magnitude of decrease in the P-450 contents was highest. At the end of 24 hours CP caused 16 and 21% decrease, AC caused 40 and 33% decrease and CR caused 33 and 30% decrease in hepatic and renal cytochrome P-450 contents respectively.
Cysteine pretreatment caused significant protection against CP, AC and CR toxicity. At the end of 24 hours the percent decrease in cysteine pretreated rats was 13, 26 and 29 in CP, AC and CR treated rats respectively.

**NADPH-cytochrome c reductase**

The hepatic and renal NADPH-Cytochrome c reductase activity was significantly decreased due to CP, AC and CR administration (Figure 4b). At the end of 6 hours CP did not cause any significant change, whereas 14 and 16% decrease in hepatic and 16 and 17% decrease in renal NADPH-cytochrome c reductase activity was observed due to AC and CR administration respectively. At the end of 24 hours CP caused 17 and 20% decrease, AC caused 28 and 30% decrease and CR caused 25 and 27% decrease in hepatic and renal NADPH-cytochrome c reductase activity respectively.

Cysteine pretreatment offered protection in the CP, AC and CR treated rats. At the end of 24 hours in cysteine pretreated rats, only 7, 21 and 24% decrease in renal NADPH-cytochrome c reductase activity was observed due to CP, AC and CR administration respectively.

**In vitro studies**

In *vivo* addition of acrolein and crotonaldehyde (0.0 to 2.0 mM) to hepatic and renal microsomal preparations from control, PB and 3-MC treated rats caused significant inhibition of drug metabolizing enzymes (Figure 6).
inhibition was found to be concentration dependent. The magnitude of inhibition was more due to acrolein addition as compared to crotonaldehyde. The inhibition of aminopyrine N-demethylase due to acrolein addition (2.00 mM) ranged from 87 to 92% and due to crotonaldehyde ranged from 65 to 73% in hepatic microsomal preparation from control, PB and 3-MC treated rats (Table 2). In vitro addition of Acrolein (2.00 mM) caused 80, 76 and 75% inhibition of acetonilide hydroxylase, and 68, 59 and 57% inhibition of NADPH-cytochrome c reductase activity in microsomes respectively from control, PB and 3-MC treated rats. However, In vitro addition of crotonaldehyde (2.00 mM) caused 60, 51 and 53% inhibition of acetonilide hydroxylase and 54, 47 and 49% inhibition of NADPH-cytochrome c reductase activity in hepatic microsomal preparations from control, PB and 3-MC treated rats, respectively.

Both In vitro addition of acrolein and crotonaldehyde, caused concentration dependent destruction of cytochrome P-450 was more due to acrolein as compared to that of crotonaldehyde. In vitro addition of acrolein caused 60% destruction, and crotonaldehyde caused 43% destruction of P-450 in control hepatic microsomal preparations.

In vitro addition of cysteine offered significant protection against acrolein and crotonaldehyde mediated destruction of cytochrome P-450 and inhibition of enzyme activities. In vitro addition of acrolein and crotonaldehyde
caused 54 and 34% destruction of P-450, 77 and 65% inhibition of aminopyrine \( N \)-demethylase and 65 and 50% inhibition of acetanilide hydroxylase respectively in microsomal preparations from control rats containing 100 mM cysteine (Figure 6).

**In vitro** CP did not cause any significant alterations in acetanilide hydroxylase, whereas 45% inhibition of aminopyrine \( N \)-demethylase was observed. CP addition did not cause destruction of cytochrome P-450 to cytochrome P-420.

**In vitro** inhibition of drug metabolising enzymes and destruction of cytochrome P-450 was also studied in renal microsomes (Figure 8).

**In vitro** addition of AC and CR both caused destruction of renal cytochrome P-450 to its inactive form P-420 (Table 3). At 1.00 mM concentration acrolein caused 56% destruction, whereas CR caused 46% destruction of renal cytochrome P-450. Cysteine offered 11% and 9% protection against AC and CR mediated destruction of cytochrome P-450 whereas CP had no effect on P-450.

Acrolein and crotonaldehyde at 1.00 mM concentration caused 65% and 54% inhibition of aminopyrine \( N \)-demethylase and 61% and 51% inhibition of acetanilide hydroxylase activity of renal microsomes of control rats. Cysteine also offered protection against AC and CR mediated inhibition of drug metabolising enzymes activities. CP, however had no significant effect on drug metabolising enzymes.
Table - 1 : Effect of cystein pretreatment on Acrolein and crotonaldehyde toxicity in adult male rats.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>Survival time(days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1) Cyclophosphamide (100 mg/kg)</td>
<td>(4)</td>
<td>&gt; 5.00</td>
</tr>
<tr>
<td>2) Acrolein (5.04 mg/kg)</td>
<td>(6)</td>
<td>1.62 ± 0.4</td>
</tr>
<tr>
<td>3) Acrolein (5.04 mg/kg) + cystein</td>
<td>(6)</td>
<td>3.20 ± 0.4</td>
</tr>
<tr>
<td>4) Crotonaldehyde (5.04 mg/kg) (6)</td>
<td></td>
<td>&gt; 5.00</td>
</tr>
<tr>
<td>5) Crotonaldehyde (20.94 mg/kg)</td>
<td>(8)</td>
<td>1.48</td>
</tr>
<tr>
<td>6) Crotonaldehyde (20.94 mg/kg) + Cystein</td>
<td>(8)</td>
<td>3.93</td>
</tr>
</tbody>
</table>

Acrolein and Crotonaldehyde were injected i.p., and cystein was administered 15 min earlier to Acrolein or Crotonaldehyde.

p \leq 0.01, Significantly different from Acrolein or crotonaldehyde alone Mean ± S.E.

n = indicates number of animals used.
Table 2: *In vitro* effect of cyclophosphamide, acrolein and crotonaldehyde on hepatic drug metabolising enzymes.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Control</th>
<th>Phenobarbital treated</th>
<th>3-Methylcholanthrene treated</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Aminopyrine N-demethylase</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(nmoles of formaldehyde formed/min/mg protein)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Microsomes</td>
<td>5.15 (±0.12)</td>
<td>6.75 (±0.17)</td>
<td>6.25 (±0.15)</td>
</tr>
<tr>
<td>Microsomes + Acrolein</td>
<td>0.67 (±0.08)</td>
<td>1.61 (±0.15)</td>
<td>0.88 (±0.12)</td>
</tr>
<tr>
<td>Microsomes + crotonaldehyde</td>
<td>2.22 (±0.10)</td>
<td>2.80 (±0.11)</td>
<td>0.93 (±0.07)</td>
</tr>
<tr>
<td><strong>Acetanilide hydroxylase</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(nmoles of p-hydroxy acetanilide formed/min/mg/protein)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Microsomes</td>
<td>5.25 (±0.05)</td>
<td>6.50 (±0.09)</td>
<td>7.80 (±0.05)</td>
</tr>
<tr>
<td>Microsomes + Acrolein</td>
<td>1.05 (±0.09)</td>
<td>1.90 (±0.10)</td>
<td>1.95 (±0.15)</td>
</tr>
<tr>
<td>Microsomes + crotonaldehyde</td>
<td>2.10 (±0.07)</td>
<td>4.15 (±0.05)</td>
<td>3.66 (±0.12)</td>
</tr>
<tr>
<td><strong>NADPH-cytochrome c reductase</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(nmoles of cytochrome c reduced/min/mg)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Microsomes</td>
<td>25.40 (±1.20)</td>
<td>36.80 (±1.80)</td>
<td>35.05 (±1.50)</td>
</tr>
<tr>
<td>Microsomes + Acrolein</td>
<td>8.12 (±0.60)</td>
<td>15.00 (±0.80)</td>
<td>15.07 (±1.10)</td>
</tr>
<tr>
<td>Microsomes + crotonaldehyde</td>
<td>11.68 (±1.50)</td>
<td>19.50 (±1.25)</td>
<td>17.85 (±1.30)</td>
</tr>
</tbody>
</table>
Table 3. Effect of Cyclophosphamide, acrolein and crotonaldehyde on renal drug metabolising enzymes and electron transport components.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Untreated</th>
<th>Cyclophosphamide treated</th>
<th>Acrolein treated</th>
<th>Crotonaldehyde treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMINOPYRINE N-DEMETHYLASE *</td>
<td>0.98</td>
<td>0.90(0.93)</td>
<td>0.34(0.52)</td>
<td>0.45(0.63)</td>
</tr>
<tr>
<td>ACETANILIDE HYDROXYLASE **</td>
<td>0.65</td>
<td>0.54(0.59)</td>
<td>0.25(0.34)</td>
<td>0.33(0.43)</td>
</tr>
<tr>
<td>CYTOCHROME P-450 ***</td>
<td>0.93</td>
<td>0.92(0.92)</td>
<td>0.39(0.58)</td>
<td>0.48(0.67)</td>
</tr>
<tr>
<td>NADPH-CYTOCHROME C REDUCTASE ****</td>
<td>28.80</td>
<td>27.6(27.6)</td>
<td>4.35(10.5)</td>
<td>7.45(13.9)</td>
</tr>
</tbody>
</table>

* n moles of formaldehyde formed/min/mg protein

** n moles of p-hydroxy acetanilide formed/min/mg protein

*** n moles of cytochrome P-450/mg protein

**** n moles of cytochrome c reduced/min/mg of protein

Values are mean of three sets of experiments, and experimental details are described in text.
FIGURE 1. EFFECT OF CYCLOPHOSPHAMIDE, ACROLEIN, CROTONALDEHYDE AND THEIR COMBINATIONS WITH CYSTEINE ON BODY WEIGHT OF ADULT MALE RATS.

ADULT MALE RATS RECEIVED 100 mg/kg b.wt. CYCLOPHOSPHAMIDE, 2.0 mg/kg b.wt. ACROLEIN, 5.04 mg/kg b.wt. CROTONALDEHYDE, AND 100 mg/kg b.wt CYSTEINE. DAILY WEIGHT GAIN OVER THE PERIOD OF 7 DAYS WAS RECORDED FOR EACH ANIMAL. C = CONTROL, CP = CYCLOPHOSPHAMIDE, AC = ACROLEIN, CR = CROTONALDEHYDE, CYS = CYSTEINE. EACH POINT IS A MEAN OF 6 ANIMALS. INDIVIDUAL VALUES IN EACH GROUP VARIED LESS THAN 10% FROM THE MEAN VALUE.
FIGURE 2. TIME DEPENDENT DEPLETION OF HEPATIC GLUTATHIONE BY CYCLOPHOSPHAMIDE, ACROLEIN, AND CROTONALDEHYDE.

ADULT MALE RATS RECEIVED 100 mg/kg b.wt. CYCLOPHOSPHAMIDE, OR 2.0 mg/kg b.wt. ACROLEIN, OR 5.04 mg/kg b.wt. CROTONALDEHYDE. CYSTEINE WAS ADMINISTERED AT DOSE LEVEL OF 100 mg/kg b.wt. ANIMALS WERE KILLED AT VARIOUS TIME INTERVALS (6, 12, 18, 24, and 36 hours). LIVERS WERE QUICKLY REMOVED AND ASSAYED INDIVIDUAL -LY FOR THE LEVELS OF GLUTATHIONE, AS DESCRIBED IN TEXT. EACH POINT IS A MEAN OF 6 ANIMALS. INDIVIDUAL VALUES VARIED LESS THAN 10% FROM THE MEAN VALUE.
Figure 3a. Effect of cyclophosphamide, acrolein and crotonaldehyde on hepatic and renal electron transport components of adult male rats and protection by cysteine.

Effect of single i.p. administration of cyclophosphamide (CP) 100 mg/kg, acrolein (AC) 2.0 mg/kg, crotonaldehyde (CR) 5.04 mg/kg, cysteine (CYS) 100 mg/kg was investigated at various time intervals (6, 12, 18, and 24 hours) in adult male rats. Experimental details are described in text. Each point is a mean of 6 animals, individual values 1

Figure 3b. Effect of cyclophosphamide, acrolein and crotonaldehyde on hepatic and renal electron transport components of adult male rats and protection by cysteine.

Effect of single i.p. administration of cyclophosphamide (CP) 100 mg/kg, acrolein (AC) 2.0 mg/kg, crotonaldehyde (CR) 5.04 mg/kg, cysteine (CYS) 100 mg/kg was investigated at various time intervals (6, 12, 18, and 24 hours) in adult male rats. Experimental details are described in text. Each point is a mean of 6 animals, individual values 1

a group varied less than 10% from the mean value.
Figure 4. Effect of cyclophosphamide, acrolein and crotonaldehyde on hepatic and renal drug metabolising enzymes of adult male rats and protection by cysteine.

Effect of single i.p. administration of cyclophosphamide (CP) 100 mg/kg, acrolein (AC) 2.5 mg/kg, crotonaldehyde (CR) 5.04 mg/kg, cysteine (CYS) 100 mg/kg was investigated at various time intervals (6, 12, 18, and 24 hours) in adult male rats. Experimental details are described in text. Each point is a mean of 5 animals, individual values in a less than 10% from the mean value.
Figure 5.

EFFECT OF CYCLOPHOSPHAMIDE, ACROLEIN AND CROTONALDEHYDE ON HEPATIC AND RENAL LIPID PEROXIDATION IN ADULT MALE RATS.

EFFECT OF SINGLE I.P. ADMINISTRATION OF CYCLOPHOSPHAMIDE (CP) 100mg/kg, ACROLEIN (AC) 2.0mg/kg, CROTONALDEHYDE (CR) 5.04 mg/kg and CYSTEINE (CYS) 100 mg/kg was investigated at various time intervals (6, 12, 18, and 24 hours) in adult male rats. Experimental details are described in text. Each point is a mean of 6 animals. Individual values in a group varied less than 10% from mean value.
Figure 6. In-Vitro inhibition of drug metabolising enzymes by cyclophosphamide, acrolein, crotonaldehyde and protective role of cysteine.

Rat hepatic microsomes were prepared and incubated with various concentrations (0.4, 0.8, 1.2, 1.6 and 2.0 mM) of cyclophosphamide (CP), acrolein (AC), and crotonaldehyde (CR) in absence or presence of cysteine at 37°C for 30 minutes. These microsomes were used for determination of drug metabolising enzymes activities. Experimental details are described in text.
Figure 7. In-Vitro denaturation of cytochrome P-450 and inhibition of NADPH-cytochrome c reductase activity by cyclophosphamide, acrolein and crotonaldehyde and protection by cysteine. Rat hepatic microsomes were prepared and incubated with various concentrations of (0.4, 0.8, 1.2 1.6, and 2.0 mM) cyclophosphamide (CP), acrolein (AC), crotonaldehyde (CR) in absence or presence of cysteine for 37 °C for 30 minutes. These microsomes were used for the spectrophotometric measurement of cytochrome P-450 and NADPH-cytochrome c reductase activity. Experimental details are described in text.
FIGURE 8. IN-VITRO EFFECT OF ACROLEIN AND CROTONALDEHYDE ON RENAL MICROSOMAL CYTOCHROME P-450, DRUG METABOLISING ENZYMES, AND NADPH CYTOCHROME C REDUCTASE ACTIVITY OF ADULT MALE RATS.

MICROSOMES FROM UNTREATED KIDNEY OF RATS WERE PREPARED, AND IN VITRO EFFECT OF ACROLEIN (1.0 mM) AND CROTONALDEHYDE (1.0 mM) ON DESTRUCTION OF CYTOCHROME P-450, N-DEMETHYLATION OF AMINOPYRINE, HYDROXYLATION OF ACETANILIDE, AND CYTOCHROME C REDUCTASE WERE INVESTIGATED. CONTROL RAT RENAL MICROSOMES = a, ACROLEIN TREATED = b, ACROLEIN + CYSTEINE TREATED = c, CROTONALDEHYDE TREATED = d, CROTONALDEHYDE + CYSTEINE TREATED = e. EXPERIMENTAL DETAILS ARE DESCRIBED IN TEXT. VALUES ARE EXPRESSED AS MEAN ± S.E.
Figure 3. In Vitro acrolein and crotonaldehyde mediated denaturation of cytochrome P-450 and protection by cysteine.
Rat hepatic microsomes were incubated with varying concentrations of acrolein and crotonaldehyde in the absence or presence of cysteine. This microsomal suspensions were used for the spectral measurement of cytochrome P-450. Control -a, acrolein treated -b, acrolein treated, in presence of cysteine -c, crotonaldehyde treated d, crotonaldehyde treated in presence of cysteine -e. Experimental details are described in text.
DISCUSSION

Extensive investigations were carried out in recent years to evaluate toxic and chemotherapeutic properties of Cyclophosphamide. Acrolein, a highly reactive and toxic aldehyde is well established as a metabolite generated from microsomal MFOs dependent metabolism of CP (449). Similarly, crotonaldehyde, an analog of Acrolein, is generated from the metabolism of 6-methylcyclophosphamide, an analog of CP. This 6-Methyl-CP is claimed as an equally potent and powerful immuno-suppressive and anticancerous drug, with reduced toxicity (442-446).

It has been reported that the CP administration causes depression of hepatic cytochrome P-450, and drug metabolizing enzyme and NADPH-cytochrome c reductase activity (294,361). Acrolein has been shown to cause destruction of cytochrome P-450 (379,295) and our results are quite in agreement with the earlier reports.

The toxicity of CP, AC and CR were well reflected by decrease in electron transport components, drug metabolizing enzyme activities, body wt. profiles and glutathione depletion. The magnitude of inhibition was more due to acrolein and least due to CP. These results suggest that the metabolites (s) are more toxic than that of the parent compound. It has been demonstrated that AC is causative agent in production of hemorrhagic cystitis in CP therapy (308,309). Similarly
the \textit{in vivo} depression of MFOs due to CP administration could be attributed to acrolein toxicity.

Various modulators of MFOs have been shown to act differently in hepatic and extrahepatic tissues. 3-MC, TCDD induces hepatic and extrahepatic MFOs whereas PB induces hepatic predominantly (227, 232). CCl\textsubscript{4} inhibits hepatic without affecting renal MFOs (360). Similarly BHT, FCC, DBCP have biphasic actions (449, 450, 345). Administration of CP caused inhibition of hepatic and renal both, the magnitude of inhibition was higher in kidney as compared to liver. This differential effect of CP on hepatic and extrahepatic microsomal MFOs could be explained as follows:

As shown in Figure 2 (General Introduction) most of the CP administered is metabolised by microsomal MFOs to form various active and inactive metabolites like 4-OH-CP, PM, aldophosphamide and acrolein. Some of the 4-OH-CP and acrolein binds to liver proteins. The unmetabolised CP and remaining metabolites are liberated in circulation and reaches extrahepatic tissues, mainly kidney. The CP and its metabolites have been identified in blood by Hipken \textit{et al.} (307). These metabolites and some of the CP binds to micromolecules. The metabolites of CP in kidney binds to protein and predominantly to nucleic acid and remaining are excreted through urine. Some of the metabolites of CP are also identified in urine (302).
These bindings of CP and its metabolites to protein and nucleic acid leads to depression of hepatic and renal MFOS. Since kidney cells receive fairly high concentration of toxic metabolites from CP metabolism in liver as well as from kidney, the toxic effects in kidney are bound to be more. This hypothesis assumes that most of the CP is metabolised in liver and most of the metabolites of liberated in circulation, on the basis of available data.

It has been demonstrated that CP mediated depression of hepatic microsomal MFOS, cystitis, urinary bladder damage and toxicity is reversed by sulphhydryl containing compounds like cysteine, N-acetyl cysteine etc. In vitro destruction of cytochrome P-450 and inhibition of drug metabolising enzymes by acrolein and protection offered by cysteine is also documented (295). Our results are in agreement with the earlier reports and reflects the protective role of cysteine by increase in survival time, body wt. gain profile, glutathione depletion and repletion, in acrolein and CP treated animals. These results suggest possible biochemical protective role of cysteine could be due to,

(1) Stimulation of glutathione repletion; (2) Stimulation of glutathione biosynthesis, (3) Formation of adducts with acrolein and (4) Direct or indirect trapping of acrolein. Various different compounds like carbon tetrachloride \((\text{CCL}_4)\), sulfur compounds, and substances containing double or
triple bonds have been reported to cause suicidal inactivation of cytochrome P-450 on metabolism. Some of the suggested mechanisms include:

1. Generation of free radicals initiating peroxidative destruction of cytochrome P-450 as in case of CCl₄ (451, 452);
2. Exoaxial formations, leading to production of green pigment via alkylation of heme moieties of P-450 (453-456);
3. Chelation of heme iron by metal chelators (32), and

Our results suggest another mechanism: where the sulphydryl groups in active site as well as at other places in apo-protein forms addition product with the double bond of acrolein. This interaction leads to the destruction of cytochrome P-450 as in the case of P-hydroxymerscury benzoate. This further suggest that metabolism of CP is obligatory requirement for the this destruction of P 450, and acrolein does not interact with porphyrine nucleus or iron of heme moiety.

The depression of hepatic and renal microsomal MFOs due to CP, AC and CR was accompanied with significant increase in lipid peroxidation. Formation of lipid peroxides causes degradation of phospholipids, loss of capacity to carry out hydroxylation reactions conversion of cytochrome P-450 to its inactive form P-420 (253-255, 248, 249). Thus the depression of microsomal MFOs due to CP, AC and CR
through the formation of lipid peroxides can not be overruled. The CP is shown to be strong alkylating agent, thus certain amount of \textit{in situ} alkylation of cytochrome P-450 and denaturation may be another factor affecting microsomal NFOs.

The 6-Methyl-Cyclophosphamide an analog of CP generates CR during metabolism. Our results suggest that CR is also highly toxic and reactive aldehyde, which possess double bond similar to AC. We observed that AC and CR both causes depression of NFOs and this depression was reversed significantly by cysteine. It seems that both AC and CR acts in similar way and have common mode of action. However, the magnitude of depression due to AC was higher than that due to CR. The feasibility of using 6-Me-CP instead of CP in chemotherapy of neoplastic diseases does not seems to be much appreciable.
SUMMARY

1) **In vivo** and **in vitro** effect of cyclophosphamide, acrolein and crotohaldehyde on hepatic and renal MFOs, and role of cysteine in CP, AC and CR mediated toxicities of various time intervals (6, 12, 18 and 24 hrs) were investigated in adult male rats.

2) In the mean survival time studies acrolein and crotohaldehyde were found to be very toxic and cysteine increased the mean survival time in adult male rats.

3) Acrolein, crotohaldehyde and cyclophosphamide caused hepatic glutathione depletion. The magnitude of depletion was higher due to Acrolein and least due to cyclophosphamide. Simultaneous administration of cysteine along with acrolein, crotohaldehyde and cyclophosphamide lead to faster glutathione repletion.

4) **In vitro** effect of acrolein, crotohaldehyde and cyclophosphamide on electron transport components and drug metabolising enzymes of microsomal preparations from liver and kidney of control, phenobarbital and 3-methylcholanthrene treated rats were investigated.

5) Acrolein and crotohaldehyde both caused destruction of cytochrome P-450 and inhibition of NADPH cytochrome c reductase activity, however, cyclophosphamide had no effect. The magnitude of destruction of P-450 and inhibition of NADPH-cytochrome c reductase was higher due to acrolein than that of the crotohaldehyde. The NADPH cytochrome c reductase was susceptible than that of the cytochrome P-450.
6) Acrolein and crotonaldehyde both caused inhibition of aminopyrine N-demethylase and acetanilide hydroxylase activity however cyclophosphamides caused inhibition of aminopyrine N-demethylase without affecting acetanilide hydroxylase.

7) \textit{In vivo} and \textit{in vitro} cysteine offered significant protection against destruction of cytochrome P-450 inhibition of drug metabolising enzymes and NADPH cytochrome \(c\) reductase activity due to cyclophosphamide, acrolein and crotonaldehyde.