ALTERED MICROSOMAL FUNCTION IN ACETAMINOPHEN-INDUCED

HEPATIC NECROSIS: PROTECTIVE ROLE OF CYSTEAMINE

by

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ABSTRACT

Massive overdosage of acetaminophen, a common anti-
pyretic analgesic drug, has recently been found to in-
duce acute liver necrosis and liver failure in humans.
The specific biochemical lesion, however, whereby cova-
lent binding of the toxic metabolite of acetaminophen
with macromolecules precipitates cellular death, remains
incompletely understood. The therapeutic efficacy of
cysteamine in reversing acetaminophen hepatotoxicity has
not been established. In an attempt to elucidate the
biochemical mechanism of acetaminophen-induced hepatic
necrosis, the present study was undertaken to evaluate
the possible changes in lipid peroxidation and the con-
comitant alterations in microsomal enzymes. The protect-
ive action of cysteamine was likewise assessed in the light
of these biochemical parameters, and electron microscopic
study was performed concurrently to correlate the changes
in the fine structural features of the liver with the
changes in the microsomal function.

In the present investigation, male hamsters were di-
vided into four groups: 1) saline-control group; 2) cys-
teamine-control group (treated with cysteamine 200 mg/kg
i.p.); 3) non-protected acetaminophen-poisoned group
(treated with acetaminophen 600 mg/kg i.p.); 4) cystea-
mine-protected acetaminophen-poisoned group (treated with

acetaminophen 600 mg/kg i.p., followed one hour later by cysteamine 200 mg/kg i.p.). At six, twelve, eighteen and twenty-four hours, the animals were sacrificed, liver sections were prepared for electron microscopy, and microsomal fractions were isolated for biochemical assay.

Results showed that significant enhancement of lipid peroxidation occurred in the non-protected acetaminophen-poisoned group, as contrasted to the cysteamine-protected group. Glucose 6-phosphatase activity was markedly suppressed at six, twelve and eighteen hours after acetaminophen overdosage. Cysteamine treatment completely prevented the curtailment of NADPH-cytochrome c reductase and glucose 6-phosphatase activities in the protected group, and partially maintained aniline hydroxylase activity. Cytochrome P-450 level was unaltered in both the non-protected and cysteamine-protected groups at the respective time intervals.

Electron microscopic examination revealed dilation of the cisternae of the granular endoplasmic reticulum (GER) associated with partial loss of membrane-bound ribosomes, at six hours after acetaminophen overdosage. At twelve hours, vesiculation of GER and hyperplasia of agranular endoplasmic reticulum (AGER) occurred, poly-

somal disintegration became accentuated, and fatty in-

filtration and vacuolation were evident. At eighteen

and twenty-four hours, increased numbers of myeloid

figures appeared, and sinusoidal congestion and vacuo-

lation were prominent.

In the hepatocytes from cysteamine-protected animals,

slight distention of the GER occurred at six hours. At

twelve hours, partial ribosomal disaggregation and limit-

ed lipid accumulation were observed. At eighteen hours,

polysomes reassembled around the GER.

Evidence is presented in this study that lipid pero-

xide formed in vivo may facilitate the microsomal oxidation

of acetaminophen to the toxic metabolite. NADPH-cyto-

chrome c reductase is likely to be the locus within the

NADPH-cytochrome P-450 electron transport chain suscep-

tiable to lipoperoxidation. The free-radical-related

lipoperoxidation may mediate the impairment of in vitro

drug metabolism, as reflected by the depressed aniline

hydroxylase activity. Cysteamine treatment attenuates

lipid peroxidation and the consequent derangement of micro-

somal enzymes.

The histopathological consequences of abnormal phos-

pholipid metabolism and distribution are manifested by

the extensive accumulation of lipolysosomes and myeloid

bodies, as well as marked vacuolation, at the advanced stage of necrosis. The occurrence of myeloid bodies constitutes a secondary lysosomal response to the acute pathogenic stimuli, suggesting that the toxic metabolite of acetaminophen may be sequestered within the vesiculated membraneous inclusions. Cysteamine treatment prevents any significant derangement of phospholipid metabolism, as evident from the absence of these morphological correlates of acetaminophen-induced secondary phospholipidosis. The maintenance of the normal polysomal profile by cysteamine administration explains the concomitant enhancement of microsomal enzyme activities.

It is concluded that the facilitatory interaction of lipid peroxidation and microsomal oxidation of acetaminophen may be the primary event of acetaminophen hepatotoxicity. Cysteamine protects against acetaminophen-induced liver necrosis by inhibiting the formation of the toxic metabolite.

This thesis is dedicated to
my parents and my sister,
without whose encouragement
and inspiration I would not
have accomplished anything
worthwhile.

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CHAPTER I

INTRODUCTION

Acetaminophen *(paracetamol, N-acetyl-p-aminophenol,
p-hydroxyacetanilide), a common antipyretic analgesic, appears
to be quite safe within the normal therapeutic dosage, even
though some toxic actions including agranulocytosis (Lloyd,
1961) and hypersensitivity dermatitis (Henriques, 1970) have been
observed. However, suicidal and accidental overdosage with
acetaminophen causes acute liver damage leading to fulminant
centrilobular hepatic necrosis and liver failure (Davidson
and Eastham, 1966; Prescott et al, 1971; Clark et al)and in
some cases, renal papillary necrosis and renal failure as well
(Khrishler,1967: Boyer and Rouff, 1971). In the United Kingdom,
acetaminophen poisoning has become critical over the last de-
cade and hospital admissions for acetaminophen overdose have
been estimated to be 7,000 per annum and the mortality rate to
be about 1 per cent   (Volans, 1976). The apparent increase in
the abuse of the analgesic, as reflected in the higher inci-
dence of acetaminophen poisoning, warrants a more vigorous
appraisal of possible hepatotoxic and nephrotoxic effects of
prolonged use of the drug. Moreover, the development of better
emergency therapeutic measures in preventing hepatic failure in
severely poisoned patients is considered mandatory. This, in

* Acetaminophen is marketed in Canada under the proprie-
tary name of Atasol (Horner) etc.

turn, necessitates more extensive clinical trials to evaluate the relative therapeutic potencies of various antidotes, as well as more experimental animal studies to elucidate the biochemical mechanism of acetaminophen hepatotoxicity.

It is controversial whether renal failure occurs concomitantly with acetaminophen-induced hepatic necrosis, and more significantly, whether acetaminophen, the active metabolite of its precursor, phenacetin, plays any role in the pathogenesis of phenacetin-induced analgesic nephropathy. Chronic administration of phenacetin has been associated with renal papillary necrosis (Levin et al, 1961; Koutsaimanis and Wardener 1970), which invariably precipitates carcinoma of the renal pelvis (Jacobs, 1962). Granted that the analgesic properties of phenacetin depend upon its metabolic conversion to acetaminophen in the liver, this would not exclude the possibility that acetaminophen may also mediate the nephrotoxic effects ascribed to the parent compound. Krishler (1967) cited one clinical case of renal papillary necrosis substained in a patient who had taken the analgesic for six months at a daily consumption rate of 11 to 18 grams per day. Although the studies of Schnitzer and Smith (1966) in rats failed to demonstrate any renal lesions caused by acetaminophen, Boyd & Breczyk (1966) observed congestive and degenerative changes in the renal tubules after acetaminophen and phenacetin treatment. Hence caution should be exercised in the distribution of the drug with respect to the nephrotoxic potential for chronic use and abuse.

The validity and reliability of various biochemical data in determining the prognosis of hepatic failure and coma has been discussed in quite a number of studies. Liver function tests revealed that elevation of serum levels of glutamic-oxaloacetic and glutamic-pyruvic transaminases occurred maximally 3 to 5 days after ingestion and therefore proved to be of limited value in predicting the likelihood of liver damage (James et al, 1975). By contrast, Prescott et al (1976) found that plasma paracetamol level correlated well with severity of poisoning in man. Even though individual variability in susceptibility to acetaminophen hepatotoxicity exists, plasma concentration of the free drug in excess of 250-300 mg per l at four hours after acetaminophen ingestion (Prescott et al, 1971), as well as bilirubin level and prothrombin time ratio greater than 4 mg % and 2.2 respectively (Clark et al, 1973), were invariably associated with liver damage. Unconjugated hyperbilirubinemia, showing that the hepatic capacity to form sulfate and glucuronide conjugates is impaired, has been observed in man following an acute hepatotoxic dose of acetaminophen (Davis et al, 1975). This may be explained by the decreased bilirubin uptake, depression of UDP-glucuronyl transferase or depletion of glucuronide itself. Histological examination of liver biopsy specimens during the recovery phase revealed centrilobular necrosis of all lobes and fibrosis, but no cirrhosis was evident (Clark et al, 1973). Likewise, massive and confluent necrosis of all lobules was observed upon necropsy in patients who died of hepatic encephalopathy and fulminant failure.

None of the measures of therapy advocated in the treatment of acetaminophen poisoning have been demonstrated unequivocally to reverse the incidence of liver failure. Although Farid (1972) reported that hemodialysis enhanced the plasma clearance of the drug, it is not certain if the rate of elimination is related directly to the severity of hepatic failure. The cumulative clearance of acetaminophen is apparently limited by its distribution into the total body water (Gwilt et al, 1963).

Activated charcoal hemoperfusion depends for its ultimate success upon early referral of patients, since acetaminophen could only be removed effectively from the plasma while freely circulating (Wilson et al, 1973a). Activated charcoal or cholestyramine ingestion failed to reduce the amount of absorption of the drug even when administered as early as one hr. after a small dose of acetaminophen. However, intestinal absorption of the analgesic was delayed following massive overdosage (Dordoni et al, 1973). Forced diuresis may precipitate acute pulmonary edema (McLean et al, 1968) as acetaminophen itself has an antidiuretic action (Nusynowitz and Forsham, 1966). The apparent uncertainties and complexities manifested in the treatment of acetaminophen poisoning necessitates a fundamental review of the biochemical mechanism of its heptatoxicity to provide a more rational basis for the pursuit of appropriate and effective therapeutic measures.

Research in the area of chemical carcinogenesis has implicated the formation of chemically active arylating metabolites

as the causative factor responsible for malignant transformation
and tumour development (Miller and Miller, 1966; Magees and
Barnes, 1967). It soon becomes increasingly evident that normal
nontoxic therapeutic agents may also evoke their toxicological
responses and produce pathological lesions by converting to
highly reactive intermediates which may combine covalently with
various tissue macromolecules, including nueleic acids and pro-
teins (Gillette, Mitchell and Brodie, 1974). Mitchell and his
colleagues (1973a), in a series of experiments, presented evi-
dence that acetaminophen-induced hepatic necrosis is caused by
the conversion of the drug to a highly reactive toxic metabolite
catalysed by a cytochrome P-450-dependent microsomal mixed func-
tion oxidase. It is conceivable that irreversible covalent
linkage of the toxic metabolite with vital cellular constituents
may lead to loss of functional integrity of various organelles
and perturb the regulation and control of the various metabolic
activities and bioenergetics. Preferential localization of
radioactive acetaminophen in the endoplasmic reticulum may well
reflect direct injury to the cell organelle (Jollow et al, 1973).
Parallelism between the magnitude of covalent binding with micro-
somal proteins in vivo and in vitro and the degree of hepatic
necrosis further supports the theory of toxic metabolite forma-
tion (Potter et al, 1973, Jollow et al, 1973). The kinetics
of covalent binding revealed that the biochemical lesion pre-
ceded the cytopathological events by two hours. Upon electron
microscopic examination, acetaminophen-induced hepatic necrosis

is characterized by ribosomal disaggregation and vacuolation in centrilobular hepatocytes (Dixon et al, 1971). That the microsomal cytochrome P-450-requiring enzyme system mediates the covalent binding is evidenced from the observation that in mice, pretreatment with phenobarbital and 3-methylcholanthrene, specific inducers of microsomal drug-metabolizing enzymes, potentiated the degree of hepatotoxcity and covalent binding (Mitchell et al, 1973a; Jollow et al, 1973). Conversely, inhibitors of the synthesis of cytochrome P-450, the terminal oxidase of microsomal electron chain, for example, cobaltous chloride and piperonyl butoxide, alleviated the hepatic injury and reduced the covalent binding.

The hepatic metabolism of therapeutic doses of acetaminophen occurs by cytochrome P-450 catalysed-sulfation and glucuronidation of the p-hydroxyl group (Brodie and Auxelrod, 1948). Davis et al 1975) found that unconjugated hyperbilirubinemia attributed to impaired conjugation of acetaminophen and bilirubin preceded the covalent binding of the toxic metabolite and further exacerbated hepatic paranchymal cellular necrosis. Transient hypoglycemia, indicating disturbance in carbohydrate metabolism, has also been noted in man (Record et al, 1975).

In addition to hepatic glucuronidation and sulfation of acetaminophen and urinary excretion of glucuronide and sulfate conjugates, the alternative detoxifying pathway for the active metabolite appears to be related to glutathione metabolism. Hepatic glutathione has been shown to conjugate with exogenous

compounds and mercapturic acid conjugates have been identified in the urine of some animal species (Jagenburg and Toezko, 1964). De novo synthesis of glutathione tends to maintain the glutathione pool. It is evident that glutathione will conjugate with the toxic metabolite of acetaminophen and renders the latter harmless. Mitchell et al (1973 b) showed that when the glutathione pool was depleted to 70% in mice, arylation of the toxic metabolite with the microsomal proteins occurred. A summary of the metabolic pathways of acetaminophen is given in Fig. (1). His hypothesis of the glutathione-related detoxification mechanism is supported by the effects of modulators of glutathione synthesis on covalent binding and hepatic necrosis. Accordingly, pretreatment with cysteine a precursor of glutathione, decreased the extent of covalent binding in vivo and liver damage. On the other hand, prior administration with diethyl maleate, an inhibitor of glutathione biosynthesis, enhanced the hepatotoxic effects of acetaminophen and the accompanying covalent binding. In vitro studies further confirmed his findings. However, it is not clear whether glutathione S-transferase mediated its conjugation with the toxic metabolite (Boyland and Chasseaud, 1969). It is perhaps relevant to note that glutathione depletion reflects a loss of total glutathione (GSH and GSSG) rather than an increase in the glutathione peroxidase-catalysed metabolic conversion to the oxidized species (Mitchell et al, 1973).

In summary, it is evident that the magnitude of covalent binding and the severity of hepatic necrosis depends on the

Figure 1: Pathways of biotransformation of acetaminophen (adapted from Mitchell et al, 1974). Modes of cysteamine protection are indicated by: *I) inhibition of microsomal oxidation: *II) conjugation with the toxic metabolite; *III) replenishing glutathione stores.

level of glutathione (GSH), which in turn is determined by the
relative rates of de novo synthesis of glutathione from cysteine
and the formation of glutathione conjugate with the toxic pro-
duct. Hence Potter and his colleagues (1973) proposed that the
fundamental role of glutathione is to protect important nucleo-
philic sites, especially membrane-bound thiol groups of proteins,
against electrophilic attack by alkylating metabolites of ace-
taminophen and other drugs. The therapeutic implications of
the central hypothesis of glutathione availability of acetamino-
phen-induced hepatic necrosis are obvious, since thiol nucleo-
philic agents such as cysteine, glutathione and methionine, may
protect against liver damage by replenishing the glutathione
pool. McLean and Day (1974) found that dietary supplementation
with the sulphur-containing amino acid, methionine, offered
protection against acetaminophen-incuded liver injury, and the
susceptibilities to acetaminophen poisoning in animals were re-
lated to the nutritional status, since yeast-fed rats exhibited
a thirteen-fold decrease in the $L D_{50}$ of paracetamol. Although
methionine may exert its protective effects by stimulating the
γ-glutamyl cycle of glutathione metabolism at the membrane
level, the extent of variation of liver damage seen in rats would
render any critical evaluation of McLean's data difficult, if
not impossible. Furthermore, the relative ineffectiveness of
glutathione itself in reversing liver damage (Gazzard et al,
1974) unless given in massive doses (Benedetti et al, 1975)
suggests that some other regulatory factor may contribute

equally towards the pathogenesis of acetaminophen poisoning.

Cysteamine (β-mercaptoethylamine), another SH-containing compound, has been shown to be more effective than cysteine in preserving liver function in mice, although it only brought about a partial repletion of glutathione (Strubelt et al, 1974; Gazzard et al, 1974). Prescott and his colleagues (1974) reported the first successful treatment of acetaminophen overdosage in man with intravenous cystamine and, more recently (1976), claimed that cysteamine was more effective than methionine and penicillamine. The mechanism of protection afforded by cysteamine is not certain, although isolated liver perfusion studies in rats demonstrated that it inhibited microsomal cytochrome P-450-catalysed oxidation of the drug to the toxic metabolite (Harvey and Levitt, 1976). More extensive clinical studies with methionine and cysteamine are required to establish the therapeutic efficacy of these SH-compounds and possible toxic side-effects.

RATIONAL AND OBJECTIVES
OF THE STUDY

Although the data of Mitchell and his coworkers (1973 a and b) compel one to conclude that the biochemical mechanism of acetaminophen-induced hepatic necrosis consists of covalent binding of the toxic metabolite with vital cellular macromolecules, when the hepatic glutathione stores are depleted, the toxic metabolite hypothesis fails to explain the specificity of biochemical processes whereby the irreversible binding leads to

cellular necrosis and death. If the covalent binding theory is indeed valid in explaining acetaminophen heptatoxicity, it is expected that the plasma concentration of free drug would not correlate with the degree of liver injury. In support of this view, Mitchell and his colleagues (1973) noted that pretreatment with phenobarbital potentiated the hepatic necrosis and enhanced the clearance of the drug from the plasma. However, clinical studies revealed that the plasma paracetamol level at four hours after acetaminophen overdosage predicted quite accurately the prognosis of hepatic failure (Prescott et al, 1974). It is diffi- cult, if not impossible, to reconcile these discrepancies other than by invoking species differences in the relative enzymatic activities of metabolic pathways in the liver. Successful clinical studies with cysteamine (Prescott et al, 1976) and methionine (Crome et al) make it unlikely that the mechanism of acetaminophen detoxification in human differs qualitatively from that in certain species of animals.

Circumstantial evidence tends to suggest that lipid peroxi- dation in vivo of the membranes of the endoplasmic reticulum may mediate the hepatotoxicity consequent upon acetaminophen overdosage. Walker and his associates (1974) found that die- tary supplementation with vitamin E (α-tocopherol) seemed to protect acetaminophen-intoxicated rats from liver damage and the antioxidant properties of vitamin-E against lipid peroxi- dation are documented in quite a number of studies (as reviewed by Tappel et al, 1972). Moreover, peroxidative decomposition

of the membranes of the endoplasmic reticulum has been proposed by quite a number of investigators as the causative factor responsible for hepatotoxicity of a variety of chemical agents, such as carbon tetrachloride , ethanol, hydrazine and phosphorus (Recknagel, 1967). It is worthwhile, therefore, to delineate the mechanism of lipid peroxidation _in vivo_ and to evaluate the relative significance of the lipid peroxidation hypothesis and covalent binding theory in acetaminophen hepatotoxicity.

The importance of phospholipid in maintaining the functional integrity of the microsomal drug hydroxylating enzymes is emphasized in a number of studies (Jones and Wakil, 1967; Strobel et al, 1970). Lipid peroxidation and drug hydroxylation share the same components of hepatic microsomal NADPH-cytochrome P-450 electron transport chain (Lu et al, 1969). NADPH-cytochrome c reductase (hemoprotein cytochrome P-450 reductase), has been shown to participate in both the lipid peroxidation as well as microsomal hydroxylation of steroids, drugs and fatty acids (Pederson et al, 1973). Likewise, cytochrome P-450, the terminal oxidase of the electron transport chain, depends for its reactivity upon its intimate association with membrane phospholipids (Chaplain and Mannering, 1970). In view of the relationship between lipid peroxidation and drug metabolism, it will be interesting to determine if these processes occur simultaneously in acetaminophen-induced hepatic necrosis, since Potter et al (1973) suggested that the toxic metabolite of acetaminophen may be an N-hydroxyl derivative. Furthermore, acetaminophen may be acti-

vated at NADPH-cytochrome c reductase site or the cytochrome
P-450 site to produce free radical-like reactive intermediate
which may affect oxidation of the drug itself. Attempts are
made in this study to localise the sites of activation and lipid
peroxide formation induced by acetaminophen and to define more
vigorously the relationship between microsomal lipid peroxida-
tion in vivo and oxidation of the drug in the liver.

Glende (1972) furnished evidence to support the contention
that carbon tetrachloride-induced liver damage and microsomal
lipid peroxidation in vivo mediated inhibition of microsomal
drug-metabolising activities in vitro towards lipid-requiring
substrates, including aniline and aminopyrine. Castro et al
(1968) claimed that lipoperoxidative process could be dissociated
from the curtailment of in vitro drug metabolism. If acetamino-
phen-induced hepatic necrosis involves peroxidative damage to
the membranes of the endoplasmic reticulum, it will be pertinent
to investigate the relationship between the extent of lipid
peroxidation in vivo and the depression of in vitro drug meta-
bolism. It is even possible to determine the potentiating effect,
if any, of acetaminophen on NADPH-dependent or ascorbate-linked
lipid peroxidation in vitro and this may throw some light on the
mechanism of possible acetaminophen-induced lipoperoxidative
process.

It remains to be established that cysteamine protects
against acetaminophen hepatotoxicity by virtue of its inhibitory
effect on the microsomal cytochrome P-450-catalysed oxidation of

the analgesic to the toxic metabolites (Havey and Levitt, 1976).
Alternatively, cysteamine and cystamine (with which cysteamine
exists in equilibrium in vivo) may protect the organism from the
deleterous effect of free radicals produced during lipid peroxi-
dative damage to the liver microsome, since in carbon tetra-
chloride-induced hepatic necrosis it has been demonstrated that
cystamine acts as free radical scavenger in decomposing $CCl_3 \cdot$
generated at the NADPH-cytochrome c reductase site and maintain-
ing the integrity of the sulfhydryl and disulfide groups of
membrane proteins (Castro et al, 1973). It is anticipated that
the study will elucidate the protective mechanism of cysteamine.

For the sake of clarity, specific objectives of this study
are summarized as follows:

1) to evaluate the possible role of microsomal lipid peroxi-
dation in vivo and in vitro as the determinant in acetaminophen
hepatotoxicity, as reflected by the conjugate diene absorption
(Srinivasan and Recknagel, 1973) and malonaldehyde formation
according to the thiobarbituric acid method (Bidlack and Tappel,
1972);

2) to delineate the relationship between possible acetamino-
phen-induced lipid peroxidation in the liver and hydroxylation
of acetaminophen within the NADPH-cytochrome P-450 electron
transport complex;

3) to investigate whether acetaminophen-induced lipoperoxi-
dative decomposition of the membranes of the endoplasmic reticu-
lum is related to the inhibition of in vitro drug metabolism;

4) to evaluate the antioxidant properties of cysteamine in preventing acetaminophen-induced liver injury;

5) to correlate the alterations in microsomal function with concomitant morphologic changes involving the endoplasmic reticulum as examined by electron microscopy.

IMPORTANCE OF THE STUDY

The incidence of hepatic necrosis and fulminant hepatic failure and death from acetaminophen overdosage has increased exponentially over the last decade in the United Kingdom (Gazzard et al, 1976). In sharp contrast to the acute and rapid development of loss of consciousness upon massive overdosage with sedatives and antidepressants, hepatic encephalopathy and failure would not occur until 2 to 3 days after ingestion of the analgesic. Clinical management of patients with severe acetaminophen poisoning is further complicated by the consideration that some psychiatric patients simultaneously consumed alcohol, aspirin, D-propoxyphene, barbiturates and benzodiazepines which are known inducers of hepatic microsomal enzymes (Prescott, 1976) and hence the likelihood of potentiation of the acute toxic effects of acetaminophen is very real. Although it is difficult to predict the occurrence of acetaminophen overdosage in Canada in the future, the ever-increasing popularity of the drug necessitates a more careful assessment of the fundamental mechanism of liver injury caused by toxic dosage of the analgesic, in an attempt to develop a rational and

specific therapy towards alleviating the risk of liver failure.

The biochemical mechanism underlying acetaminophen-induced liver necrosis has been elucidated by Mitchell and his co-workers (1973) that acetaminophen is converted to a chemically reactive metabolite via the cytochrome P-450-dependent mixed function oxidase of the liver. Pharmacokinetic analysis of the disposition of the drug in human revealed that therapeutic doses of acetaminophen is extensively metabolized to the glucuronide and sulfate conjugates and only a small proportion is excreted in the urine as the unchanged form (Levy & Yamada, 1971). In addition, urinary metabolite of acetaminophen formed by its conjugation with the endogenous glutathione pool in the liver was identified to be acetyl-cysteine conjugate (mercapturic acid) of the drug by mass spectrometry (Mitchell et al, 1974). This reinforces the view that arylation of vital hepatocellular macromolecules by electrophilic toxic metabolite of acetamino-phen upon exhaustion of glutathione stores constitutes the causa-tive factor in acetaminophen hepatotoxicity, and that the hepa-totoxic pathway of acetaminophen in the liver in humans is similar to that observed in other animal species (mice, rats and hamsters). Accordingly, the activity of the hepatotoxic route for the covalent binding of the reactive intermediate with the microsomal proteins reflects directly the extent of conjuga-tion of acetaminophen with mercapturic acid (Jollow et al, 1974). These findings may be relevant in extrapolating the results obtained in animal studies to assessing the probability of

liver failure in patients with acetaminophen overdosage.

However, the molecular mechanism of the interaction of this toxic metabolite with vital cellular constituents remains to be determined. If the essential function of glutathione in humans and animals is to protect important nucleophilic sites from electrophilic attack by the highly chemically reactive inter- mediate, it is difficult to conceive the apparent failure of glutathione itself to prevent effectively the development of hepatic necrosis (Gazzard et al, 1974). Sulfhydryl protecting agents such as thioctic acid likewise did not significantly reduce the extent of hepatic necrosis caused by acetaminophen (Gazzard et al, 1974). As a natural extension of the "toxic metabolite" hypothesis expounded by Mitchell and his colleagues, the funda- mental biochemical mechanism of acetaminophen-induced hepatic necrosis may be similar to that postulated for carbon tetra- chloride poisoning and ionizing radiation injury, in that free radicals formed in situ may contribute significantly towards the pathogenesis of cellular necrosis and death. It is perhaps more than fortuitous that cysteamine, an established radiopro- tective agent against radiation-induced damage, also protects against acetaminophen hepatotoxicity (Prescott et al, 1974). Lipoperoxidative damage to the membranes of the endoplasmic reticulum of the liver is likely the sequel to the covalent binding of the toxic metabolite. Hence this study is specifi- cally designed to evaluate critically the mechanism of microsomal lipid peroxidation underlying the cytopathological sequence of

events of acetaminophen poisoning. It is anticipated that know-ledge derived from understanding the specific biochemical lesion responsible for acetaminophen-induced hepatic necrosis will help clarify some of the apparent discrepancies occurred in animal studies.

Clinicians have not agreed on the minimum effective dose of cysteamine in treating acutely acetaminophen-poisoned patients. Although cysteamine was effective in preventing hepatic failure within ten hours of poisoning (Prescott, 1976), Hughes et al (1976) and Douglas et al (1976) reported no difference either in morbidity in mortality between cysteamine-treated patients and control groups; besides, severe gastrointestinal and central nervous system symptoms were observed to occur after cysteamine administration. Accordingly, correlation of the histopathological alterations of the liver with the biochemical changes involving the microsomes may explain the inconsistencies identified in clinical trials of cysteamine in treating acutely poisoned pat-ients.

CHAPTER II

LITERATURE REVIEW

Since the primary objective of the project is to determine if lipid peroxidation plays any role in acetaminophen-induced hepatic necrosis, it seems relevant to review the recent literature on the mechanism of lipid peroxidation, both at the cellular and molecular level; the implications of peroxidative damage with respect to the functional integrity and viability of different subcellular organelles, especially the mitochondria, the endoplasmic reticulum, and to a lesser extent, the lysosomes; and finally, the function of phospholipids in hepatic microsomal drug-metabolizing enzyme systems, with special reference to the relationship between microsomal lipid peroxidation and drug hydroxylation.

I)   INTRODUCTION

Lipid peroxidation in vivo has been implicated in a variety of pathological disorders including cellular aging (Tappel, 1968) hepatic damage due to ethanol (Di Luzio, 1969), and chlorinated hydrocarbons (Recknagel, 1967), and more recently, carcinogenesis (Shamberger, 1972). It is evident that peroxidative damage to biological membranes of mitochondria, endoplasmic reticulum and lysosomes as well as erythrocytes will interfere drastically with the regulatory and modulating function of membrane in cellular transport phenomenon, bioenergetics of intermediary metabolism and the molecular lipid-protein interaction.

Mitochondria and the endoplasmic reticulum are the most susceptible to oxidative chain cleavage by virtue of high content of polyunsaturated fatty acids and phospholipids. The electron availability of the various phosphoryl bases, as well as the chain length and degree of unsaturation of the fatty acid moiety, may determine the extent of peroxidative damage. Advances in this area of cellular pathology are derived from model reactions of organic hydroperoxides with olefins in the presence of molecular oxygen. In these model systems, metal ion-catalysed homolytic scission of peroxides leads to autocatalysis which proceeds by self-propagating mechanism. Although the significance of free radical formation as the fundamental biochemical lesion underlying chemical-induced hepatotoxicity has not yet been unequivocably established, it is highly likely that the propagative sequence of events incurred by free radicals, mainly the peroxy free radicals LOO·, once they are produced in biochemical systems in _vitro_ and _vivo_, consists of hydrogen abstraction, group transfer and addition (Pryor, 1973). Sulfhydryl and non-sulfhydryl enzymes were observed to be inactivated by lipid peroxidation in mitochondrial suspensions _in vitro_ (McKnight and Hunter, 1966). The highly reactive intermediate, malonaldehyde, is probably the agent responsible for inter- and intra-molecular cross linking of protein-protein polymers. Interestingly enough, enzyme inactivation caused by lipoperoxidation correlated closely with the process of radiation-induced enzyme efflux (Dawes & Wills,1970),indicating that peroxides may

mediate the enzyme release. Malonaldehyde tends to react with
ϵ-amino groups of lysine in proteins. Chio and Tappel (1969)
showed that malonaldehyde-inactivated ribonuclease A exhibited
similar fluorescence characteristics to that obtained from per-
oxidizing polyunsaturated lipids in that the fluorescence maxi-
mum occurred at 470 nm and excitation at 395 nm.

Hunter and his coworkers (1963) observed that dilute sus-
pensions of mitochondrial preparations, when incubated in the
presence of low concentrations of ascorbate, underwent tripha-
sic permeability changes: swelling, lysis and ultimately mem-
brane disintegration. Although phosphate-dependent and ascor-
bate-induced mitochondrial swelling both require the presence
of molecular oxygen and the activation of ferrous ion, and exhi-
bit sensitivity to inhibition by chelating agents such as EDTA
(ethylenediaminetetracetate), and known inhibitors of electron
transport chain including cyanide, a longer lag period occurred
during ascorbate-induced swelling. Mellors et al (1966) and
Hunter (1963) concluded that peroxidation of mitochondrial lipid
as assayed by the thiobarbituric acid method is directly related
to ascorbate-promoted membrane alterations, since antioxidants,
for example, alpha-tocopherol and ubiquinol-6(reduced coenzyme
Q_6), effectively inhibited the stable free radical diphenyl-p-
picrylhydrazyl in situ and the concomitant heme-catalysed peroxi-
dation of arachidonic acid emulsions. However, the lipid peroxi-
dation hypothesis fails to account for the bivalent ion efflux
and the resultant permeability changes in the case of cystamine

(Skrede and Christophersen, 1966), as a definite lag period
existed between the release of mitochondrial protein employed
as an index of membrane disintegration and the formation of
malonaldehyde. In contrast, cysteamine markedly stimulated per-
oxide formation but exerted little effect on the ionic fluxes
and protein loss. It appears more probable that cystamine
alters the membrane organization by virtue of the disulfide-thiol
interchange reactions at the hydrophobic-polar membrane inter-
face. Furthermore, cysteamine, if prevented from auto-oxidation
by thiolated Sephadex, afforded a complete protection against
ascorbate-induced mitochondrial lysis and the attendant peroxi-
dation. This suggests that thiol reactive intermediate may
mediate directly the peroxidation of membrane-bound lipids. In
this respect it is noteworthy to evaluate the relative impor-
tance of two types of proposed mechanisms responsible for the
decomposition of intracellular peroxides. The intramitochon-
drial and cytosol-localised glutathione peroxidase appears to
exert no effect on the hydroperoxides from the inner mitochon-
drial membrane, since no significant amount of glutathione could
be identified in the matrix (Green and O'Brien, 1970). The
microsomal cytochrome P-450-dependent pathway seems to prevail
in the overall scheme of cellular defence against the deleter-
ious effects of peroxidative products (Hrycay and O'Brien,
1971a and b).

The lysosomes are relatively resistant to peroxidative
damage, though the release of hydrolytic enzymes such as phos-
phatases and cathepsins has been found to accompany ascorbate-

dependent lipid peroxidation (Dingle et al, 1969) and irradia-
tion (Dawes and Wills, 1972). By contrast, glucose 6-phos-
phate dehydrogenase-deficient erythrocytes are extremely suscep-
tible to peroxide attack and patients with hereditary haemolytic
anemia exhibited abnormal malonaldehyde curve (Stocks et al,
1971). It appears from current literature that antioxidants
such as alpha-tocopherol confer some degree of protection against
the lability of erythrocyte membrane to hemolysis. Shimasaki
and Privett (1975) presented evidence that the destruction of
erythrocytes by hydroperoxides prepared from autoxidized methyl
linoleate was attributed to the concomitant loss of the antioxy-
genic potential of vitamin E in vivo. It is noteworthy that
oxyhemoglobin predisposes the erythrocyte to lipid peroxidation
formation, and that methemoglobin reverses both the lipid peroxi-
dation and the resultant hemolysis induced by hyperbaric stress
and deficiency of α-tocopherol status of mice (Osborne et al,
1973).

The ramifications of microsomal lipid peroxidation in vitro
and in vivo will be dealt in some length in the section "Mechan-
ism of microsomal lipid peroxidation".

In summary, the possible role of lipid peroxidation in the
disruption of cellular economy and metabolism is implicated in
many studies. Although the ultimate status of lipid peroxida-
tion theory remains to be seen, the simplicity and scientific
elegance of the hypothesis in explaining the multiplicity of
pathological disorders at the molecular level prove to be fruit-

ful in guiding future research in molecular and cellular patho-
logy.

II) LIPID PEROXIDATION AS A DETERMINANT OF CHEMICAL-INDUCED
     HEPTATOTOXICITY

Peroxidation of intracellular lipids in vivo has been pro-
posed by a number of investigators as the underlying mechanism
of hepatotoxicity induced by a variety of exogenously adminis-
tered chemical agents, such as carbon tetrachloride, ethanol,
hydrazine and phosphorus   (Recknagel, 1967; Di Luzio, 1973).
The role of microsomal lipid peroxidation in the pathogenesis
of a fatty liver as reflected in the accumulation of triglycerides,
which can ultimately lead to cellular necrosis, is clearly de-
monstrated in ethanol-induced liver injury   (Di Luzio, 1973).
Although the plasma from patients suffering from acute and
chronic alcoholic hepatitis failed to exhibit the conjugated
diene ultraviolet spectrum   typical of in vivo lipid peroxidation,
prior administration of lipid antioxidants such as alpha-toco-
pherol significantly reduced the plasma conjugated diene absorp-
tion, accompanied by an increase in endogenous lipid-soluble
antioxidants. It is clear from the data of Di Luzio (1973) that
ethanol-induced lipid peroxidation is primarily a mitochondrial
event, and that interaction between tissue lipid-soluble antioxi-
dants including alpha-tocopherol and water-soluble antioxidant,
for example, ascorbate, determines the course of peroxidative
damage to vital cellular organelles. In this respect, the
contribution of lipid peroxidation towards fatty infiltration

in the liver is further confirmed in phenobarbital-induced fatty liver. Hahn and his colleagues (1975) demonstrated that the enhancement of lipoperoxidation as reflected in the observed malonaldehyde production and the conjugate diene absorption of microsomal lipids paralleled quite closely the triglyceride accumulation, both processes being abolished by an antioxidant, N,N'-diphenyl-p-phenylenediamine.

Whereas ethanol-induced conjugated diene formation is mitochondrial, carbon tetrachloride-induced lipid peroxidation is primarily microsomal in nature. Phospholipids are very important in maintaining the functional integrity of the membranes of the endoplasmic reticulum and reactivity of hepatic and adrenal mixed function oxidases. In fact, NADPH-cytochrome c reductase is involved in both the lipid peroxidation and the microsomal drug-metabolizing system (Lu, 1969; Pederson and Aust, 1972). In addition, glucose 6-phosphatase has been observed to be subject to the peroxidative decomposition of the membranes of the endoplasmic reticulum (Wills, 1969; Ghoshal and Recknagel, 1965).

The classic model of chemical-induced hepatotoxicity consequent of lipoperoxidation is carbon tetrachloride-induced hepatic necrosis (Recknagel, 1967). Although the detailed molecular sequence of events are at best speculative, it is conceivable that highly reactive paramagnetic molecular oxygen and chloromethyl free radicals are very effective in attacking the methylene bridges separating the double bonds of polyenic fatty

acids, notably linolenic and arachidonic acids. Peroxyl free
radicals may be expected to undergo resonance shift to yield a
variety of complex degenerate lipid products principally carbonyl
and aldehyde moieties. In view of the close correlation of mal-
onaldehyde formation with the oxidative scission of lipids from
membrane preparations (Tam and McCay, 1970), the thiobarbituric
acid (TBA) method has been used to assess lipid peroxidation
potential in vitro (Di Luzio, 1973; Ghoshal and Recknagel, 1965).
The specificity of the TBA method has been criticized by Bloom
and Westerfield (1972)who showed that in the liver homogenates
from acute ethanol-intoxicated rats, increased amounts of malonal-
dehyde reflected the development of fatty infiltration rather than
the extent of peroxidative lesion per se. Any alterations of
malonaldehyde formation varied with the amounts of polyenic fatty
acids deposited in the liver. In this respect, the conjugated
diene ultraviolet absorption provides a better index of in vivo
lipid peroxidative damage (Recknagel and Ghoshal, 1966), since
the complex of degenerate lipids were probably produced by the
free radical attack upon the methylene carbons of polyunsatura-
ted phospholipids.

That the microsomal drug metabolizing enzymes are involved
in carbon tetrachloride toxicity is shown by the observation that
pretreatment with phenobarbital potentiated the hepatotoxic
effects, as reflected by the rise in serum glutamic oxaloacetate
transaminase, curtailment of ethylmorphine demethylase and cyto-
chrome P-450, vesiculation of cisternae of the rough endoplasmic
reticulum and dilation of double membranes of the nuclear enve-

lope (Reynolds et al, 1972). On the other hand, 3 methylcholan-
threne tended to counteract certain parameters of CCl_4-induced
hepatic necrosis, including centrilobular necrosis, structural
de rangement of membrane-bound organelles (Reid et al, 1971),
the depression of ethylmorphine demethylase and benzopyrene
hydroxylase (Stripp et al, 1972), as well as the rise in serum
enzymes (Swarez et al, 1972). However, it is difficult, if not
impossible, to reconcile these changes with the potentiating
effect of 3-methylcholanthrene on hepatic ribosomal disaggrega-
tion and the decrease in the incorporation of ^{14}C L-leucine into
microsomal protein (Shah and Carlson, 1974). Consequently,
Shah and Carlson (1974) formulated the two-site activation hypo-
thesis to explain the apparent paradox of simultaneous potentia-
tion and inhibition of CCl_4-induced hepatic necrosis (Gardner
and McLean (1969) by 3-methylcholanthrene. Phenobarbital induction
of microsomal enzymes and the resultant formation of toxic meta-
bolites can be correlated directly with the increase in NADPH-
cytochrome C-reductase and cytochrome P-450. It appears that if
indeed alkoxyl and peroxyl free radicals are generated during
CCl_4 poisoning, they would interact with the flavoprotein as
expressed by the NADPH-cytochrome c reductase activity and ini-
tiate the lipid peroxidative process. Alternatively, if the
toxic metabolite preferentially binds at the cytochrome P-450
locus, inactivation of neighbouring sulfhydryl enzymes and mem-
brane-bound thiol groups would finally cause polyribosomal dis-
aggregation. The difficulty in the two site-activation hypothe-

sis lies in the observation that cytochrome P-450 has recently been shown to be the intracellular site of hydroperoxide decomposition and free radical generation; the peroxidase activity of cytochrome P-450 is largely responsible for the decomposition of any lipid hydroperoxide generated by various metabolic processes (Hrycay and O'Brien, 1971). On the other hand, Bidlack and Tappel (1972) proposed that autocatalytic ferrous ion-dependent lipid peroxidation was associated with the inhibition of NADPH-cytochrome c reductase activity.

There has been considerable controversy concerning the relationship of microsomal lipid peroxidation and drug-metabolizing activities in vitro. Castro and his coworkers (1968) held that it is possible to dissociate the microsomal peroxidative decomposition of membranes from the impairment of oxidative metabolizing functions of the smooth endoplasmic reticulum, although both processes share the same electron transport complex. N,N'-diphenyl-p-phenylenediamine, an antioxidant, for example, reduced the peroxidative process without affecting the diminution in the drug-metabolizing activities elaborated at the terminal oxidase site, the cytochrome P-450 site. Nevertheless, in an elegant series of experiments, Glende (1972) presented evidence that depressed drug metabolism in vitro induced by CCl_4 is linked directly to the peroxidative damage of the membranes of the endoplasmic reticulum, since it was observed that no change occurred in demethylase activities when in vitro lipid peroxidation induced by ascorbate was prevented by addition of EDTA.

In summary, available evidence suggests that hepatic micro-
somal lipid peroxidation associated with alterations in micro-
somal drug oxidation is implicated in the toxic effects exerted
by some chemical agents, especially carbon tetrachloride. It is
the purpose of next section to delineate the molecular events
underlying microsomal lipid peroxidation.

III) MECHANISM OF MICROSOMAL LIPID PEROXIDATION

NADPH-dependent peroxidation of endogenous microsomal lipids
has been shown to cause alterations in the composition and quan-
tity of phospholipids, as well as lipid-protein interactions in
the microsomal membranes (Tam & McCay, 1970; Poyer et al, 1971).
Tam and McCay, (1969) noted that peroxide formation was associa-
ted with the oxidative chain cleavage of the β position of poly-
unsaturated fatty acids and of phosphatidylcholine and phospha-
tidylethanolamine, producing carbonyl-containing residues and
malonaldehyde. The process was accompanied by considerable
oxygen uptake and the release of degenerate polyunsaturated fatty
acids to the surrounding medium. NADPH-oxidase-catalysed lipid
peroxidation appears to be distinguished from auto-catalytic
oxidation of unsaturated lipids in that the former process de-
pends upon the continuous oxidation of NADPH (May and McCay,
1968), only limited by the antioxidant properties of other micro-
somal components, such as phosphoryl bases, as manifested in
the availability of free electrons of the amine moiety (Tsai
and Smith, 1971). The mechanistically "tight" coupling between

NADPH oxidation and functional alterations in membrane bound
lipids serves to illustrate the stringent structural require-
ments of the microsomal membrane. It could be envisaged that
the insertion of polar groups, principally peroxyl and carbonyl
groups, into the hydrophobic environment of the membrane, would
impose severe perturbations in the molecular orientation and
structural requirements, as shown in changes in turbidity measure-
ments in brain microsomes (Robinson, 1965). Recently, the role
of phospholipids as the integral component of microsomal electron
transport complex responsible for the hydroxylation of steroids
and drugs as well as the oxidative metabolism of xenobiotic sub-
stances has been recognized (Chaplain and Mannering, 1970;
Strobel et al, 1970), and there exists a definite relationship
between microsomal lipid peroxidation and microsomal mixed func-
tion oxidase system. For example, cytochrome P-450, the terminal
oxidase in the hepatic and adrenal microsomal system, depends
for its physicochemical properties upon the intimate association
with the membrane-bound phospholipid and treatment with phospho-
lipase C resulted in the loss of hydrophobicity of type I-bind-
ing site on the hemoprotein and the concomitant ability to oxi-
dize ethylmorphine and hexobarbital (Chaplain and Mannering,
1970). The biochemical conversion of cytochrome P-450 to an
inactive species referred to as cytochrome P-420 is thought to
be responsible for this change.

The fundamental molecular mechanism of hepatic microsomal
lipid peroxidation is the area of intense research. In this

respect the isolation, purification and identification of an enzyme named erythrocuprein from bovine and human erythrocytes (Morkowitz et al, 1959) shown to catalyse the dismutation of superoxide free radical anion (McCord and Fridovich, 1969) represents somewhat an achievement in the peroxide theory. The postulated reaction sequence is as follows:

$$O_2^{\cdot -} + O_2^{\cdot -} + 2H^+ \longrightarrow O_2 + H_2O_2$$
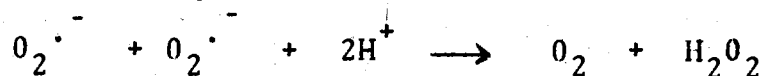
It has been observed that the oxidation of epinephrine to adrenochrome by xanthine oxidase was mediated by the membrane-bound superoxide radical and the reduction of cytochrome c by superoxide anion could be competitively inhibited by superoxide dismutase. Although conclusive evidence is scanty at the present moment, the ubiquitous occurrence of superoxide dismutase in animal tissues suggests that it may play an important role in protecting the organism against the deleterious effects of superoxide anion. Yamazaki and Piatte (1963), in a study of the peroxidase-oxidase reaction, proposed that the superoxide free radical is the active intermediate in the reaction.

Fong and his associates (1971) postulated that the hydroxyl free radical, produced by the reaction of superoxide with hydrogen peroxide formed from the dismutation of previously generated superoxide during flavin oxidation, constitutes the initiator for the peroxidation process. Although no free radical has yet been identified in vitro and vivo, Pfeifer and McCay (1971) provided evidence for the generation of a component possessing the properties of a free radical during the oxidation of NADPH by liver

microsomes. The component caused hemolysis of erythrocytes in
the incubation medium, the phenomenon being reversible by free
radical trapping agents and inhibitors of NADPH oxidation.

However, the direct intervention of superoxide or hydroxyl
free radical has not been universally accepted. Pederson and
Aust (1973) suggested the possible involvement of singlet oxygen
as the reactive intermediate responsible for the oxidation of
lipid hydroperoxides. Previously, it was reported (Pederson
et al, 1973; Aust et al, 1972) that NADPH-cytochrome c reductase,
in the presence of $FeCl_3$ and EDTA, catalysed the microsomal
lipid peroxidation in the absence of substrates with the produc-
tion of superoxide anion. The capacity to reduce cytochrome-c
via the superoxide anion mechanism correlated with the degree
of homogeneity of the solubilized enzyme. In addition, the sensi-
tivity of NADPH-cytochrome c reductase to inhibition of super-
oxide yield by superoxide dismutase activity of erythrocuprein
was maximal at the higher pH. In this regard superoxide dis-
mutase has recently been reported to reverse glutathione-induced
peroxidation of mitochondrial membrane (Zimmerman et al 1973)
as well as dialuric acid-induced peroxidative damage of erythro-
cyte membrane (Fee, 1972). Pederson and Aust (1975) showed that
xanthine oxidase-promoted peroxidative oxidation of extracted
hepatic microsomal lipids, in the presence of $FeCl_3$ and EDTA,
was inhibited by the superoxide dismutase activity of erythro-
cuprein, as well as by the singlet oxygen trapping agent, 1,3-
diphenylisobenzoylfuran. This tends to support the view that

superoxide anion would non-enzymatically dismutate to evolve singlet oxygen which react with unsaturated fatty acids to produce hydroperoxides which initiate the peroxidative chain cleavage of lipids.

The evidence for the mechanism of microsomal lipid peroxidation is far from conclusive, however. Poyer and McCay demonstrated (1970) Fe^{3+} to be an absolute requirement for the enzyme-catalyzed peroxidative process, since the activity of NADPH oxidase was depressed significantly by repeated washing of the microsomal particles in iron-free media, and restored by addition of ferric ion to the assay system. The rate of NADPH oxidation was enhanced by including ADP-Fe to the intact microsomes, implying that ferric ion serves as the electron acceptor. Recently, the purified bromelain-solubilized (Pederson et al, 1973) NADPH-cytochrome c reductase was observed to promote NADPH-dependent peroxidation only if the ferric ion was chelated by EDTA, indicating that the NADPH-requiring system is specific for a yet unidentified molecular species of iron. Furthermore, NADH-dependent peroxidation of microsomal lipids could be achieved by yet another microsomal flavoprotein, NADH-cytochrome b_5-reductase which also reduced Fe-EDTA. It appears that NADPH requiring pathway operates by a mechanism which may not involve either the superoxide anion, singlet oxygen, hydrogen peroxide or hydroxyl radical, since the singlet oxygen scavenger, 1,3-diphenylisobenzoylfuran and hydroxyl radical trapping agent, did not affect the peroxidation process. Likewise, the system was less sensitive to inhibition by superoxide dismutase (Pederson

and Aust, 1975). It was therefore concluded that the reduction
of ferric ion and the oxidation of ferrous by molecular oxygen
to form perferryl ion, FeO_2^{2+}, might be the initial step in
the decomposition of hydroperoxide. However, Pederson and Aust's
conclusion may not be entirely valid as the extremely short-
lived superoxide produced by ferrous ion would coincide with
the equally rapid rate at which ferric ion was reduced.

IV) PHOSPHOLIPID REQUIREMENT IN MICROSOMAL DRUG HYDROXYLATION

As discussed in the previous section, NADPH-dependent pero-
xidation of extracted microsomal lipids can be promoted by the
activity of the microsomal flavoprotein, NADPH-cytochrome c-
reductase, as well as some additional component of the micro-
somal electron transport chain complex whose function could be
substituted by ferric ion ch lated by EDTA. The NADPH-cyto-
chrome c reductase has been shown to participate in both the
lipid peroxidation as well as the hydroxylation reaction of
steroids and drugs (Lu et al, 1969a). Recently the role of
phospholipid as an integral requirement for microsomal mixed
function oxidase systems has been demonstrated in quite a number
of studies. Jones and Wakil (1967) found a requirement for
phospholipids, possibly phosphatidylcholine and phosphatidyl-
ethanolamine by the microsomal membrane-bound NADPH-cytochrome
c reductase, and that phosphatidylcholine and phosphatidylethanol-
amine completely restored the activity of the enzyme in acetone-
treated microsomes. His data suggest that the lipid probably
functioned in the transfer of electrons beyond the flavoprotein

of the NADH-cytochrome c reductase system of the intact micro-
somes, possibly at the site of cytochrome b_5, since the lipid
requirement could be replaced by ferricyanide as the electron
acceptor. On the other hand NADPH-cytochrome c reductase did
not depend entirely upon phospholipid for the hydroxylation
function in acetone-extracted microsomes (Lu et al, 1969b)
However, the reconstituted enzyme system for oxidative metabo-
lism of drugs of which NADPH-cytochrome c reductase forms an
integral component requires phospholipid for its function and
this aspect will be expounded in greater detail later in this
section.

Attempts have been made from several sources to define
more vigorously the essential components required for the drug
hydroxylation system and establish the optimum conditions for
the catalytic function of the mixed function oxidase in the
liver, as well as to study the kinetics of electron transfer from
NADPH to hemoprotein P-450. Lu and Coon (1968) reported that
a deoxycholate-solubilized enzyme system, identified as hemo-
protein P-450, namely, the carbon monoxide-binding pigment of
microsomes, hemoprotein P-450 reductase (NADPH-cytochrome c
reductase), and a heat-stable factor, in the presence of NADPH
and molecular oxygen, supported the ω-hydroxylation of lauric
acid. Subsequently, the heat stable factor required for the
oxidation of fatty acid, hydrocarbon and drugs in a reconstitu-
ted liver microsomal enzyme system consisting of cytochrome
P-450, was shown to be phosphatidylcholine (Strobel, Lu and

Coon, 1970). Moreover, the electron transfer from NADPH to hemoprotein cytochrome P-450 was investigated in a series of fast-kinetics experiments. It was found from stopped-flow measurements that the rate of electron transfer appeared to be biphasic, the fast component of which being attributed to the presence of phospholipid. Interestingly enough, the reconstituted enzyme system revealed some degree of substrate specificity, since Lu, Strobel and Coon (1970) found that alpha-benzphetamine, hexane and cyclohexane proved to the most active substrate for hydroxylation. More recently, the molecular weight of deoxycholate-solubilized, partially purified, cytochrome P-450 from rabbit liver microsomes was determined to be 280,000 by sucrose density gradient centrifugation (Hoeven and Coon, 1974), and polyacrylamide gel electrophoresis revealed two major peptides corresponding to two sub-units with molecular weights of 47,000 and 52,000 daltons. Although Hoeven claimed that the larger of the two fractions represented the pharmacologically active species induced by phenobarbital, more definitive evidence is required to substantiate his hypothesis. Furthermore, the stoichiometry of the reduction of cytochrome P-450 by dithionite seemed to indicate that some unidentified electron acceptor other than the ferric ion may be involved in the drug hydroxylation (Guengerich, et al, 1975). This unknown component has been named factor C by Hoeven (1974).

If the phospholipids impose vigorous requirement for the microsomal function, it is expected that highly organized struc-

tural orientation best describes the enzyme complex. Preliminary evidence, however, seems to indicate otherwise. Autor and his coworkers (1972), in a study of the sedimentation properties of the reconstituted system, showed that upon gel exclusion chromatography, the NADPH-cytochrome c reductase, cytochrome P-450 and phosphatidylcholine behaved as individual molecular species rather than a tightly associated complex. This phenomenon was further confirmed by electron microscopic examination, suggesting that aggregation is not necessary for the reconstituted microsomal enzyme system to express its catalytic and oxidative function. However, Showman et al (1969) observed that the presence of phospholipid caused the cytochrome P-450 to undergo aggregation and form tubular elements; upon centrifugation the level of N-demethylase activity was correlated quite closely with cytochrome P-450 content, indicating that highly structured architecture of the complex conferred its hydroxylating activity. It is difficult to reconcile these apparent differences in the data obtained. Suffice to say that the fundamental relationship between drug metabolism and phospholipid-related membrane phenomena remains unsettled.

It is even possible that different isoenzymes differing only in their amino acid sequence or different allosteric sites of the same oligomeric protein, exist for hemoprotein cytochrome P-450. The importance of the intimate association of hemoprotein cytochrome P-450 with phospholipid is emphasized in a number of studies. Treatment of cytochrome P-450 with a

variety of agents such as deoxycholate, phospholipase c,urea
and mercurial sulfhydryl binding agents, for example, p-chloro-
mercuribenzoate (Chaplain and Mannering, 1970), disrupted the
functional integrity of the hemoprotein and caused the conver-
sion of cytochrome P-450 to a spectrally different inactive
cytochrome P-420 which is incapable of supporting drug oxidation.
The kinetics of binding of substrate with the hemoprotein para-
llels sufficiently closely that of enzyme-substrate interaction
and therefore any spectral change can be adduced as an altera-
tion in the binding of the enzyme-substrate complex and the re-
sultant oxidation rate of the substrate. Chaplain and Mannering
(1970) noted phospholipase C treatment inactivated the hydropho-
bicity of type-I binding site capable of effecting the metabo-
lism of hexobarbital and ethylmorphine. Liebman and Estabrook
(1970) observed that isooctane-treated microsomes failed to pro-
duce type-I difference spectrum upon addition of hexobarbital,
since the loss of drug-metabolizing activities during the extrac-
tion process with nonpolar solvent resulted in the dissociation
of membrane-bound phospholipids. It may be concluded tenta-
tively that type-I binding site is hydrophobic and phospholipid-
associated, and type-II binding site is specific for carbon
monoxide. Recently, Philpot and Aric (1975) succeeded in iso-
lating and partially purifying two forms of hepatic cytochrome
P-450 which exhibit different reduced-, oxidized-, and carbon
monoxide- combined spectral characteristics. Cytochrome P-450
in fraction A was twice as active as cytochrome P-450 in frac-
tion B in activating ethylmorphine N-demethylase and benzopy-

rene hydroxylase when combined with NADPH-cytochrome c reductase and the heat stable factor. Additional data are required to ascertain the pharmacological significance of these forms of cytochrome P-450 with different substrate affinities in vivo.

In view of the apparent complexities concerning the relationship between microsomal lipid peroxidation and drug hydroxylation, studies performed hitherto in this area are open to a variety of interpretations. It has been established that two processes share a common microsomal electron transport system which supports cytochrome P-450 catalysed drug hydroxylation. Wills (1969) reported that aminopyrine oxidation was accompanied by a marked inhibition of NADPH-induced lipid peroxidation, and, to a lesser extent, ascorbate-induced system, and that deoxycholate and p-chloromercuribenzoate treatment destroyed both the aminopyrine demethylase activity and the lipid peroxide formation in vitro. It is highly likely that differential sensitivity of various substrates may be explained by the enzymatic and non-enzymatic mechanisms. In this respect, it is difficult, if not impossible, to reconcile the marked microsomal lipid peroxidation in vivo induced by overdosage of carbon tetrachloride as observed by Recknagel (1967) with the inhibitory effect of carbon tetrachloride on lipid peroxidation in vitro. (Wills, 1969). It should be noted that the concentration of carbon tetrachloride in isolated microsomal suspensions is much higher than that administered to the intact organism. Furthermore, the thiobarbituric acid method employed to determine the

extent of lipid peroxidation potential in vitro quantitated the
amount of malonaldehyde, the degradatory product of oxidative
chain scission of unsaturated fatty acids, and not peroxide per
se. It is not certain whether lipid peroxidation necessarily
occurs during drug hydroxylation.

Alternatively, the inhibition of lipid peroxidation by drug
hydroxylation may be explained by the hypothesis that these two
processes are competing for reducing equivalents of NADPH.
However, phenobarbitone was observed to be readily oxidized by
the hepatic microsomes and yet exerted no effect on in vitro
lipid peroxidation (Wills, 1969). The inhibitory effect of
aromatic amines and phenols on lipid peroxidation may be explain-
ed by their antioxidant properties of the compound itself or
the metabolite rather than competition for reducing equivalents.
In a brief communication, Pederson and Aust (1974) showed that
3,4-bezopyrene inhibited NADPH-dependent lipid peroxidation,
but not the ascorbate-induced system, only in the presence of
NADPH. However, their view would not embrace those substrates
which are neither antioxidants nor metabolized to antioxidant-
like compounds, and would not exclude the possibility that both
processes are indeed competing for reducing equivalents of NADPH.

On the contrary, increasing evidence has accumulated that
lipid peroxidation in vitro may be associated with the break-
down of cytochrome P-450, the terminal oxidase mediating the
formation of the active metabolite from its precursor. Levin
et al (1973) reported that, when rat liver microsomes was incu-

bated with NADPH, significant amounts of malonaldehyde were
formed accompanied by the loss of cytochrome P-450. By the
use of radiolabelled delta-H^3-aminolevulinic acid to label the
heme moiety of cytochrome P-450 in vivo, the loss of cytochrome
P-450 was demonstrated to be due to the degradation of hemopro-
tein, probably via the fission of one methene bridge in the
tetrapyrole ring, though no attempts have been made to identify
the radioactive products of hemoprotein catabolism. In addition
the destruction of cytochrome P-450 during lipid peroxidation
explains release of carbon monoxide in a 1:1 molar ratio with
respect to each mole of heme lost (Schacter et al, 1972), and
the reversal of CO generation by adding EDTA to the incubation
medium.

It is perhaps more than fortuitious that microsomal lipid
peroxidation is associated with the breakdown of cytochrome
P-450. Hrycay and O'Brien (1971a and b) furnished evidence
that cytochrome P-450 functioned as microsomal peroxidase cata-
lysing intracellular hydroperoxide decomposition, since peroxi-
dase activity was inhibited by both type-I and type-II ligands
which bind to cytochrome P-450, and that the increase in cyto-
chrome P-450 content upon phenobarbital treatment correlated
with the enhanced peroxidase activity. Whereas cystosol-located
glutathione peroxidase is responsible for catalysing the decomposition
of hydroperoxides formed from membrane-bound phospholipids (Little
et al, 1970) and ADP-Fe induced lipid peroxidation is associated
with decrease in intracellular glutathione in isolated hepato-

cytes (Hogberg et al, 1975) membrane-bound cytochrome P-450 peroxidase may serve as the site for free radical production and hydrogen donor oxidation. Hrycay and O'Brien (1971 a and b) postulated a mechanism for the reaction of linoleic acid hydroperoxide with the thiol ligand of cytochrome P-450 to produce a high-spin cytochrome P-420. Free radical species are implicated and during the process, glutathione, alpha-tocopherol, ascorbate and cysteine are probably oxidized. In addition, hydroperoxide intermediates probably mediate the hydroxylation of steriods to hydroxysteroids via cytochrome P-450 (Jellinck and Fletcher, 1970).

In summary, drug hydroxylation exhibits an absolute phospholipid requirement within the microsomal electron transport chain. Further investigations are required to delineate more precisely the relative importance of the individual components of the reconstituted microsomal enzyme system in supporting hydroxylation of steroids, drugs and fatty acids. The exact relationship between microsomal lipid peroxidation and drug hydroxylation remains to be determined.

CHAPTER III

MICROSOMAL FUNCTIONAL CHANGES DURING ACETAMINOPHEN

POISONING: CYSTEAMINE PROTECTION

Despite the wealth of studies on the covalent binding of
toxic metabolite of acetaminophen with microsomal proteins in
vitro and in vivo (Mitchell et al 1973a and b; Potter et al,
1973; Jollow et al, 1973), very few reports have been published
on the specific biochemical changes following acetaminophen
poisoning. Although the two short abstracts (Thorgeirsson et al,
1973; Racz and McDonald, 1976) agreed that loss of microsomal
function correlated directly with the loss of microsomal pro-
tein, discrepancy was observed with respect to the effect of
acute hepatotoxic dose of acetaminophen on in vitro drug meta-
bolism. In contrast to the results of Thorgeisrsson et al
(1973), Racz and McDonald (1976) found that aryl hydrocarbon
hydroxylase and N-demethylase activities were drastically depress-
ed twelve hours after various hepatotoxic doses of acetamino-
phen. Furthermore, although the data of Walker et al (1974)
indicated that lipid peroxidation in vivo might play a role in
the pathogenesis of acetaminophen hepatotoxicity, in vivo lipid
peroxidation was not quantitated.

As a corollary to the toxic metabolite theory, attempts to
develop a rational and efficacious therapeutic regimen in revers-
ing liver damage in humans have been directed towards replen-
ishing the glutathione pool (Strubelt et al, 1974; Mitchell et
al, 1973b), as well as interfering with microsomal oxidation

(Harvey and Levitt, 1976; Prescott et al, 1974). There has been considerable controversy regarding the relative effectiveness of cysteamine and methionine in treating patients with severe acetaminophen poisoning. Although Prescott (1974 and 1976) claimed that cysteamine was more effective in preventing liver and renal failure in patients with 4-hour acetaminophen concentrations above 300 mg per 1, Hughes et al (1976) and Douglas et al(1976) showed that methionine treatment achieved better results than cysteamine in clinical trials. Some of the apparent discrepancies may be explained by such factors as the crucial interval elapsed between the ingestion of the analgesic and the initiation of treatment, the potentiating effects of prior self-administration of barbiturates and other anti-psychotic drugs, and the variability observed in the absorption and half-life curves. It should be borne in mind that the minimum effective dose of cysteamine and methionine has not been unanimously established.

The present study was therefore undertaken to determine:1) the possible changes in in vivo and in vitro lipid peroxidation by the conjugate diene ultraviolet absorption (Srinivasan and Recknagel, 1973) and the thiobarbituric acid method (Bidlack and Tappel, 1972)respectively; 2)microsomal glucose 6-phosphatase activity; 3) NADPH-cytochrome reductase activity; 4)cytochrome P-450 level; 5) in vitro

drug metabolizing capacity as evaluated by aniline hydroxylase activity. Morever, in an attempt to clarify some of the inconsistencies reported in clinical trials (Prescott et al, 1976; Douglas et al, 1976), the protective action of cysteamine was evaluated by examining the possible alterations in in vivo and in vitro lipid peroxidation and microsomal enzymes at various time intervals after acetaminophen overdosage. It is considered that model animal studies in hamsters will simulate the clinical condition, since Jollow et al (1974) and Mitchell et al (1974) have shown conclusively that the metabolic disposition of toxic and nontoxic doses of acetaminophen in certain species of animals and humans are similar, if not identical.

The histopathological features of acetaminophen hepatotoxicity upon electron microscopy are correlated with the biochemical changes, and treated in some detail in Chapter IV: "Protection by cysteamine against Acetaminophen-induced hepatic Necrosis: an Electron Microscopic study".

MATERIALS AND METHODS

Animals and Chemicals

Male golden Syrian hamsters (Canada Breeding Farm Lab., Quebec) weighing between 120-130 g upon arrival were housed in separate wire-floored cages and allowed free access to food (Purina Lab Chow) and water.

Acetaminophen was obtained from Sigma Chem. Co. (St. Louis, MO, USA); cysteamine hydrochloride from Calbiochem (San Diego,

CA, USA). All other reagents were of the best analytical grade or the best available commercial grade.

Drug Treatment

Acetaminophen was prepared freshly as a 0.25 M supersaturated solution at 40^OC. Cysteamine hydrochloride was dissolved in isotonic saline solution. Hamsters were divided into four groups: 1) Saline-control; 2) Non-protected acetaminophen-treated; 3) Cysteamine-protected acetaminophen-treated; 4) Cysteamine-control. The non-protected acetaminophen-treated group was administered a toxic dose of acetaminophen (600 mg/kg i.p.) in isotonic saline. Saline-control was treated with 0.9 per cent isotonic saline solution as a placebo. Cysteamine-control group was dosed with 200 mg/kg of cysteamine i.p. while cysteamine-protected acetaminophen-treated group was administered 600 mg/kg i.p. of acetaminophen, followed 1 hr. later by cysteamine treatment (200 mg/kg i.p.). The animals in the various groups were sacrificed at 6, 12 and 18 hr. after treatment, by vertical abdominal incision and thoracotomy under ether anaesthesia and the liver were quickly excised and immersed in ice-cold 0.01 M Tris-1.15 per cent KCl-buffer, pH 7.4. Small portions of the liver were simultaneously placed in 3 percent glutaraldehyde solution, buffered with sodium cacodylate pH 7.4, followed by post-fixation in osmium tetroxide for electron microscopy*.

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*   The details of the procedure of transmission electron microscopic preparation of hepatic tissues are described in Chapter IV.

Hepatic microsomal preparation

The livers were rinsed twice with 0.1 M Tris-1.15 per cent KCl buffer, pH 7.4, and weighed. They were then homogenized with a motor-driven glass Teflon Potter-Elvehjen homogenizer in ice-cold measured volumes of Tris-KCl buffer to prepare 20 per cent homogenates. The homogenates were centrifuged for 15 minutes at 9,000 x g at 0°C. The microsomal pellet was then layered with 1 ml of Tris-KCl buffer and recentrifuged for 1 hour at 105,000 x g at 0°C. The microsomal pellet was then layered with 1 ml of Tris-KCl buffer and stored frozen at -15°C, prior to performing enzyme assays, as recommended by Levin et al (1969), to prevent any drastic loss of microsomal enzyme activities. All enzyme assays were carried out within three days after the animals were sacrificed. Microsomal protein concentration was determined by the method of Lowry (1951).

Lipid peroxide analysis in vivo and in vitro

Estimation of diene conjugation of microsomal lipids in the liver has been used by many investigators to determine the extent of lipid peroxidation in vivo (Srinivasan et al, 1973; Wilson et al, 1973b). The microsomal pellet was rehomogenized in 30 ml of methanol-chloroform mixture (2:1 by volume). After 30 min., 10 ml of water was added to the mixture which was thoroughly agitated, and recentrifuged at 1,000 x g for 15 min. After the upper methanol-water phase was removed, the lower chloroform phase was evaporated to dryness at 40-50°C under a stream of oxygen-free nitrogen. The extracted lipid was re-

dissolved in 3 ml of cyclohexane and the absorbance was measured against a cyclohexane blank in a Beckman DB-GT spectrophotometer in cuvettes with a 1-cm path length over the range of 290-220 nm. Lipid phosphorus of the same sample was estimated according to Rahaje et al (1973) using L-phosphatidylcholine (obtained from Sigma Chem. Co., MO, USA) as the standard. A conversion factor of 25 was used to convert the lipid phosphorus to phospholipid (Bauer et al, 1968). The results of diene conjugation were expressed as absorbance 232 nm per mg phospholipid dissolved in 1 ml of cyclohexane.

Lipid peroxidation in vitro was assessed by the thiobarbituric acid method (TBA) (Bidlack and Tappel, 1972). Aliquot of the microsomal suspension was added to 2 ml of 30% of trichloroacetic acid and, after mixing, the mixture was centrifuged at 3,000 x g for 15 min. To the clear supernatant obtained after filtering through Whatman filter paper, was added 2 ml of an aqueous solution of 0.67 percent thiobarbituric acid. The resulting mixture was then heated in a boiling water bath for 15 min. and allowed to cool to room temperature. The absorbance at 532 nm was measured against a tissue blank with no TBA added. Malonaldehyde production (peroxide value) was expressed as μmol per gram of 9,000 x g supernatant protein, using the molar extinction coefficient of 1.56×10^{-5} M^{-1} cm^{-1} as determined by Sinnhuber et al (1958).

Microsomal enzyme assays

All microsomal enzyme assays were performed on freshly

isolated microsomal fractions within 3 days after sacrificing the animals.

1) <u>NADPH-cytochrome c reductase</u>: NADPH-cytochrome c reductase was assayed by following the change in absorbance at 550 nm caused by the reduction of cytochrome c (Sigma, type IV) in a Beckman DB-GT Spectrophotometer fitted with Philips model PM 8100 flat-bed recorder, as described by Glende (1972). The volume of the final reaction mixture was 3.0 ml and contained the following (NADPH being omitted in the blank): 1.0 mol KCN, 0.1 mmol Tris-buffer, 0.08 mmole nicotinamide, 0.3 μmol NADPH (from Sigma), 0.1 μmol oxidized cytochrome C (Sigma, type IV) and microsomal material containing approximately 40 mg of protein. The reaction was started by the addition of NADPH to the reaction mixture at 25°C and followed for 3-5 minutes. The rate of reduction of cytochrome c was linear for 5 min. and the enzymatic activity was linear with respect to microsomal protein concentration. Under standard assay conditions, NADPH-cytochrome c reductase activity (in units) is defined as the change in absorbance at 550 nm per min. mg^{-1} of microsomal protein.

2) <u>Glucose 6-phosphatase activity</u> in microsomal preparations was determined according to Harper (1963). Glucose 6-P-ase activity was expressed as micrograms of inorganic phosphorus released in 15 min. per mg of microsomal protein at 37° C.

3) <u>Aniline Hydroxylase</u>: Drug metabolism <u>in vitro</u> was measured by determining aniline hydroxylase as described by Kato

(1965). Flask$_S$ containing 5 μmole of aniline hydrochloride and microsomal material containing approximately 6 mg protein were incubated at 37° C for 15 min. with a NADPH-generating system of 1.5 μmol NADP, 20 μmol nicotinamide, 48 μmole isocitrate and 8 mg isocitrate dehydrogenase (Sigma, type I). The total incubation volume was 4.0 ml. After exactly 10 min. the reaction was stopped by adding 2.0 ml of 20 percent trichloroacetic acid to the incubation mixtures. After centrifugation, phenol was added to the clear supernatant to form the phenol-indophenol complex which was measured spectrophotometrically at 640 nm. Enzyme activity was expressed as $_{mg}$ of p-aminophenol formed per $_g$ of microsomal protein in 10 min.

Cytochrome P-450 determination

Cytochrome P-450 differential spectrum was recorded in a Beckman DB-GT Spectrophotometer equipped with zero-suppression control fully active so as to allow full scale expansion on a Philips model PM 8100 flat-bed recorder to correspond to any absorbance unit desired. The absorption spectrum of the two species of cytochrome P-450, namely, cytochrome P-450 Fe^{2+} : CO exhibiting absorption maximum at 450 nm and cytochrome P-450 Fe^{2+} with absorption maximum at 407-408 nm, could be measured after reduction of all cytochromes by adding sodium dithionite and aerating the sample in the cuvette with carbon monoxide, according to the method of Omura and Sato (1964). However, the differential spectrum of hemoglobin Fe^{2+} : CO and hemoglobin Fe^{2+} overlaps that of cytochrome P-450 to such an extent that accur-

acy would be sacrificed. The method was therefore modified by
Schoene et al (1972) to record the differential spectrum of
cytochrome P-450 Fe^{2+}: CO and cytochrome P-450 Fe^{3+}. Both sam-
ples of microsomal suspensions were aerated with carbon monoxide
for sixty seconds, but to the sample cuvette was added approxi-
mately 2 mg sodium dithionite. The spectrum was scanned over the
range of 490-400 nm and revealed two separate absorption peaks,
one occurring at 450 nm due to cytochrome P-450 and the other
at 420 nm due to cytochrome b_5. Using the molar extinction co-
efficient of 91 mM^{-1} cm^{-1} as found by Omura and Sato (1964)
cytochrome P-450 activity was expressed as μmol of cytochrome
P-450 per g of microsomal protein.

Statistical analysis of data

For the purpose of comparison between the experimental and
control groups of animals, the conventional Student's T-test was
regarded as inadequate, since it will not permit simultaneous
multiple comparisons of the means from various groups sacrificed
at different times after drug treatment. Hence the Scheffe's
multiple range test for multiple comparison of means for uneven
sample size was chosen to evaluate the significant difference
among the different groups with respect to the biochemical var-
iables (Scheffe, 1959). In each case the data were subjected
to the F-test for an analysis of variance between and within the
groups to determine if any significant difference occurred in
the population variances (Dixon and Massay 1957). The computer
program for Scheffe's multiple range test and F-test for analysis

of variance was contained in Simon Fraser University's Program
Index which is available from the Computing Centre (1976).

RESULTS

Although the lethality of acetaminophen in hamsters was not quantitated in the present study, most deaths from acetaminophen overdosage (600 mg/kg i.p.) occurred within 10-24 hours of poisoning and were consist ntly accompanied by hemorrhage, featuring spotted purplish-red patches in all lobes of the liver. Similar observations were reported by McLean and Day (1972) in rats. The morphological changes of the liver at various time intervals after treatment are described in Chapter IV.

As can be seen in Table 1 and Figure 2, the toxic dose of acetaminophen (600 mg/kg i.p.) significantly stimulated lipid peroxidation in vivo, as reflected by a two-fold increase in diene conjugation over that observed in the saline- and cysteamine-control at 6 and 12 hr. The maximal increase in lipid peroxidation in vivo occurred at 18 hr. and tended to correspond to the severity and incidence of liver damage. A similar trend was found in in vitro lipid peroxidation, as indicated by amount of malonaldehyde formed; indeed, the malonaldehyde concentration increased linearly as a function of time (Table II and Figure 3).

The functional state of the microsomal NADPH-cytochrome P-450 electron transport chain was evaluated by determining the activities of various enzymes. Glucose 6-phosphatase activity was significantly ($P < 0.01$) depressed at 6, 12 and 18 hr. (Table III and Figure 4). NADPH-cytochrome c reductase activity was also significantly ($P < 0.01$) diminished at 12 and 18 hr.

(Table IV and Figure 5). On the other hand, in vitro drug metabolism as reflected by aniline hydroxylase activity was severely curtailed at 6, 12 and 18 hr. (Table V, Figure 6), despite the fact that the content of cytochrome P-450 was relatively unchanged throughout the course of acetaminophen poisoning (Table VI). No diurnal variation was observed in the microsomal function in the saline- and cysteamine-controls (Table I-VI).

To evaluate the protective effects of cysteamine, it will be more informative to make multiple comparisions of the measured biochemical parameters among saline-control, cysteamine-control, non-protected acetaminophen-treated and cysteamine-protected acetaminophen-treated groups, at the corresponding time intervals.

As can be seen in Table I and II, cysteamine treatment did not significantly ($P < 0.01$) reduce lipid peroxidation in vitro and in vivo at twelve and eighteen hours when compared to the respective saline- and cysteamine-control. However, in vivo and in vitro lipid peroxidation were greatly diminished in the cysteamine-protected group, in comparision with the non-protected acetaminophen-poisoned group (Table VIIa). On the other hand, cysteamine treatment maintained normal activity of NADPH-cytochrome c reductase activity in the protected group (Table IV and VIIb). Although glucose 6-phosphatase activity was lower in the cysteamine-protected

group than in the corresponding saline- and cysteamine-
control, the difference was not significant ($P < 0.01$)
(Table III). Although cysteamine did not completely pre-
vent the inhibition of aniline hydroxylase activity at
all time intervals, with respect to the saline- and cys-
teamine-control groups (Table V), the catalytic function
of aniline hydroxylase was significantly better in the
cysteamine-protected group when compared with the non-
protected group (Table VIIb). However, no change was
observed in cytochrome P-450 level in all four groups at
the given time intervals (Table VI).

TABLE I

EFFECT OF VARIOUS DRUG TREATMENTS ON in vivo MICROSOMAL
PEROXIDATION

Time after treatment (Hr.)	Treatment	No. of animals in each group	Lipid peroxidation in vivo[1]	Percent change from control
6	Saline-control	5	0.27+0.03	
	AAMP[2]	4	0.89+0.03*	229
	AAMP+CYS[3]	4	0.35∓0.04	29
	CYS[4]	4	0.28∓0.05	3
12	Saline-control	4	0.26+0.04	
	AAMP[2]	4	0.91+0.09*	250
	AAMP+CYS[3]	4	0.64∓0.09*	146
	CYS[4]	4	0.25∓0.02	- 3
18	Saline-control	4	0.28+0.02	
	AAMP[2]	4	1.71+0.09*	510
	AAMP+CYS[3]	4	0.63∓0.07*	126
	CYS[4]	4	0.28∓0.04	0

[1] Hepatic microsomal lipid peroxidation in vivo is expressed as absorbance at 232 nm per mg phospholipid dissolved in 1 ml of cyclohexane. Values represent the mean±S. E.

[2] AAMP: Acetaminophen. Acetaminophen was administered at 600 mg/kg i.p.

[3] AAMP+CYS: Acetaminophen + cysteamine. Acetaminophen was administered at 600 mg/kg i.p., followed 1 hr. later by cysteamine treatment (200 mg/kg i.p.).

[4] CYS: cysteamine. Cysteamine was administered at 200 mg/kg i.p.

* Significantly different from the saline-control and CYS groups ($p < 0.01$) by Scheffe's multiple range test.
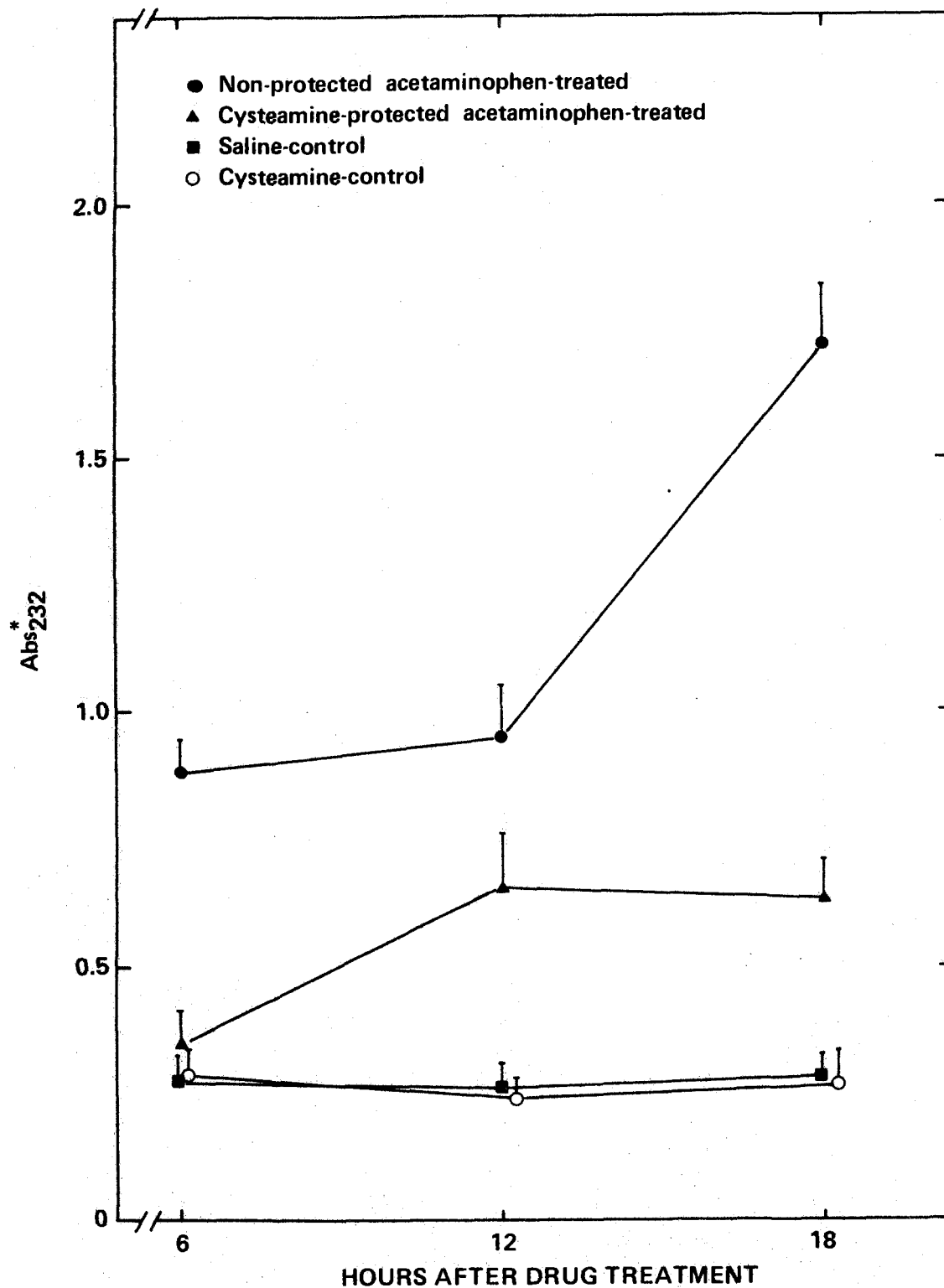
Figure 2: Diene conjugation at various time intervals after drug treatments. *Abs_{232} indicates absorbance at 232 nm. per mg of phospholipid dissolved in 1 ml cyclohexane.

TABLE II

EFFECT OF VARIOUS DRUG TREATMENTS ON in vitro
PEROXIDE FORMATION

Time after treatment (Hr.)	Treatment	No. of animals in each group	Lipid Peroxide formed in vitro[1]	Percent change from control
6	Saline-control	5	0.21+0.04	
	AAMP[2]	4	0.52+0.07*	147
	AAMP+CYS[3]	4	0.48+0.06*	130
	CYS[4]	4	0.20+0.01	- 4
12	Saline-control	4	0.22+0.08	
	AAMP[2]	4	1.17+0.07*	431
	AAMP+CYS[3]	4	0.60+0.05*	172
	CYS[4]	4	0.21+0.07	- 4
18	Saline-control	4	0.20+0.06	
	AAMP[2]	4	1.83+0.03*	815
	AAMP+CYS[3]	4	0.59+0.05*	195
	CYS[4]	4	0.21+0.09	5

[1] Lipid peroxide formed in vitro is expressed as μmol of malonaldehyde formed per g of 9000xg supernatant protein. Value represents the mean $\pm$ S.E. in each group of animals.

[2] AAMP: Acetaminophen. Acetaminophen was administered at 600 mg/kg i.p.

[3] AAMP+CYS: Acetaminophen+cysteamine. Acetaminophen was administered at 600 mg/kg i.p., followed 1 hr. later by cysteamine injection (200 mg/kg i.p.).

[4] CYS: Cysteamine. Cysteamine was administered at 200 mg/kg i.p.

* Significantly different from the saline-control and CYS groups ($p < 0.01$) by Scheffe's multiple range test.
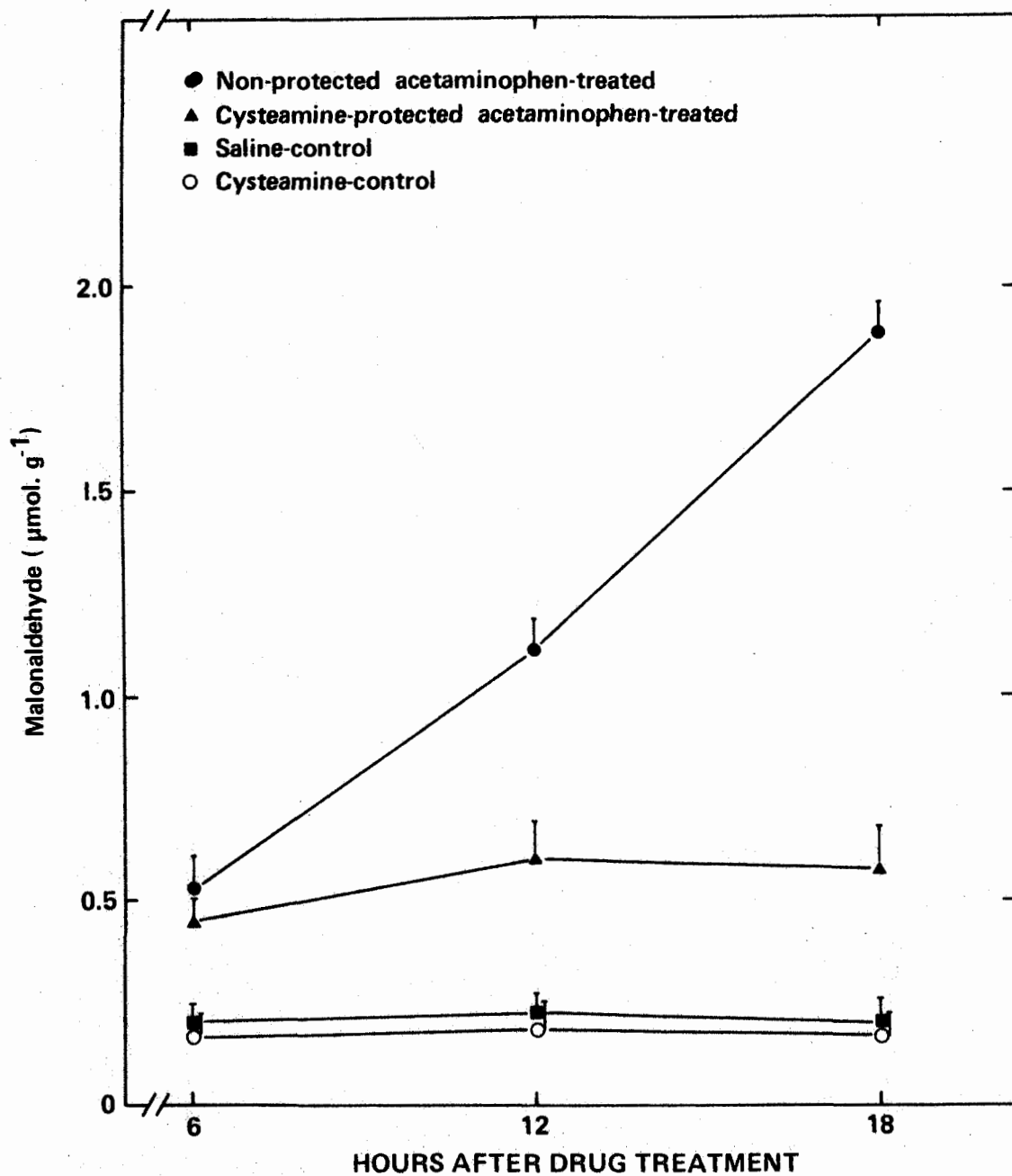
Figure 3: Peroxide level measured as malonaldehyde formed at various time intervals after drug treatments.

TABLE III

EFFECT OF VARIOUS DRUG TREATMENTS ON GLUCOSE
6-PHOSPHATASE ACTIVITY

Time after treatment (Hr.)	Treatment	No. of animals in each group	Glucose 6-phosphatase activity[1]	Percent change from control
6	Saline-control	5	3.02+0.38	
	AAMP[2]	4	1.81+0.22*	-40
	AAMP+CYS[3]	4	2.47+0.13	-18
	CYS[4]	4	3.21+0.07	6
12	Saline-control	4	3.10+0.27	
	AAMP[2]	4	1.57+0.17*	-46
	AAMP+CYS[3]	4	2.34+0.08	-24
	CYS[4]	4	3.18+0.16	2
18	Saline-control	4	3.09+0.09	
	AAMP[2]	4	0.77+0.11*	-54
	AAMP+CYS[3]	4	2.22+0.13	-28
	CYS[4]	4	3.00+0.10	- 3

[1] Glucose 6-phosphatase activity is expressed as microgram of inorganic phosphorus formed in 15 min. per mg. of microsomal protein. The value represents the mean± S.E. in each group of animals.

[2] AAMP: Acetaminophen. Acetaminophen was administered at 600 mg/kg i.p.

[3] AAMP+CYS: Acetaminophen+cysteamine. Acetaminophen was administered at 600 mg/kg i.p., followed 1 hr. later by cysteamine treatment (200 mg/kg i.p.).

[4] CYS: Cysteamine. Cysteamine was administered at 200 mg/kg i.p.

* Significantly different from the saline-control and CYS groups ($P < 0.01$) by Scheffe's multiple range test.
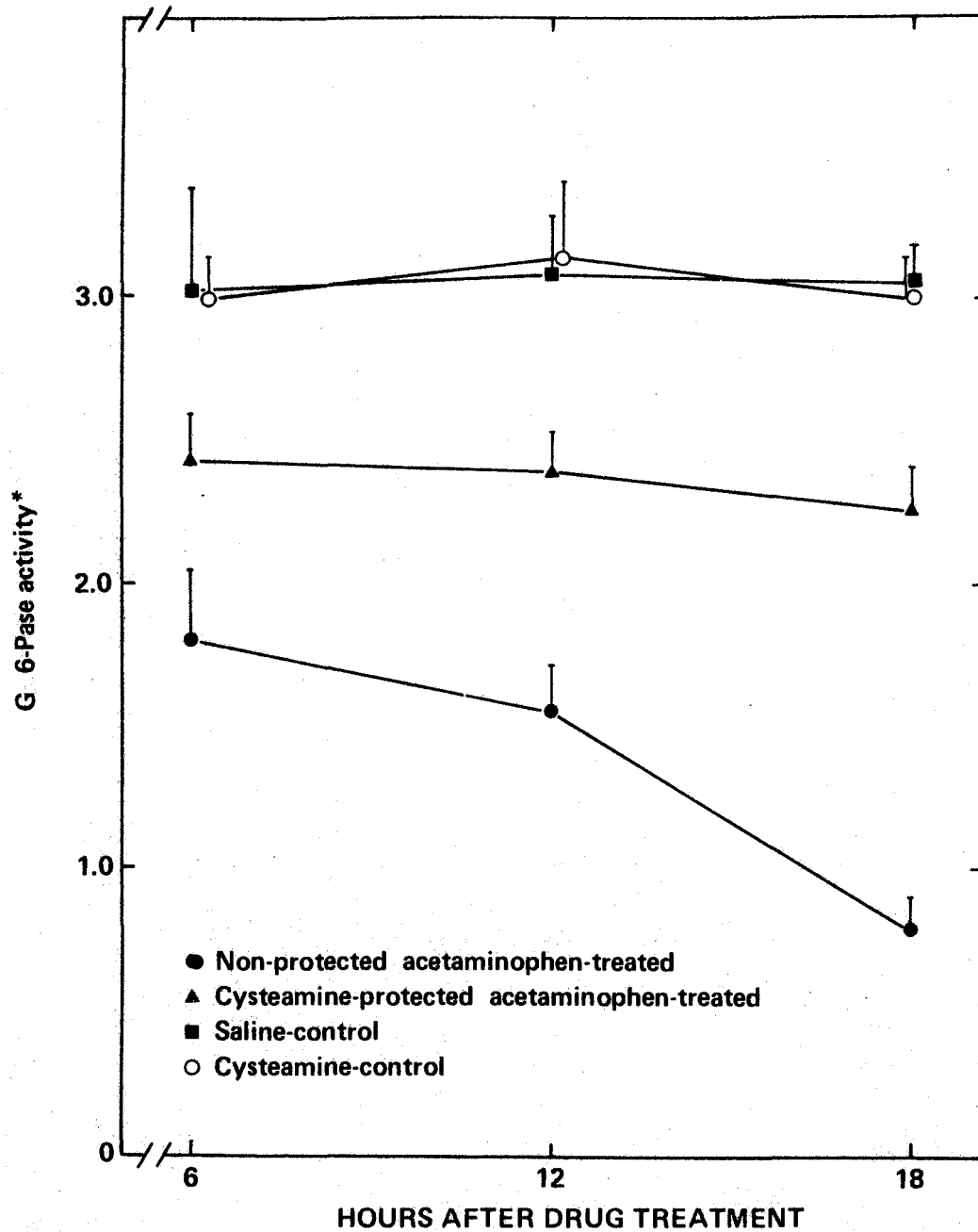
Figure 4: Glucose 6-phosphatase activity at various time intervals after drug treatments. G 6-Pase activity* is expressed as μg inorganic phosphorus released in 15 min per mg of microsomal protein.

TABLE IV

EFFECT OF VARIOUS DRUG TREATMENTS ON NADPH-CYTOCHROME
C REDUCTASE ACTIVITY

Time after treatment (Hr.)	Treatment	No. of animals in each group	NADPH-cytochrome c reductase activity	Percent change from control
6	Saline-control	5	0.55+0.05	
	AAMP[2]	4	0.34+0.04	-39
	AAMP+CYS[3]	4	0.58+0.03	6
	CYS[4]	4	0.54+0.03	- 1
12	Saline-control	4	0.58+0.04	
	AAMP[2]	4	0.16+0.01*	-72
	AAMP+CYS[3]	4	0.44+0.04	-24
	CYS[4]	4	0.59+0.07	1
18	Saline-control	4	0.53+0.05	
	AAMP[2]	4	0.15+0.03*	-71
	AAMP+CYS[3]	4	0.48+0.05	- 8
	CYS[4]	4	0.51+0.05	- 3

[1] NADPH-cytochrome c reductase activity is expressed as change in absorbance at 550 nm per min per mg of microsomal protein. The value represents the mean + S.E. in each group of animals.

[2] AAMP: Acetaminophen. Acetaminophen was administered at 600 mg/kg i.p.

[3] AAMP+CYS: Acetaminophen+cysteamine. Acetaminophen was administered at 600 mg/kg i.p., followed 1 hr. later by cysteamine treatment (200 mg/kg i.p.).

[4] CYS: cysteamine. Cysteamine was administered at 200 mg/kg i.p.

* Significantly different from the saline-control and CYS groups ($p < 0.01$) by Scheffe's multiple range test.

Figure 5: NADPH— cytochrome c reductase activity at various time intervals after drug treatments. NHDPH - cytochrome c reductase activity is expressed as $\triangle$absorbance $_{550}$ per min. mg^{-1} protein.

TABLE V

EFFECT OF VARIOUS DRUG TREATMENTS ON ANILINE
HYDROXYLASE ACTIVITY

Time after treatment (Hr.)	Treatment	No. of animals in each group	Aniline Hydroxylase activity[1]	Percent change from control
	Saline-control	5	8.26+0.24	
6	AAMP[2]	4	4.28+0.35*	-49
	AAMP+CYS[3]	4	6.04+0.35*	-26
	CYS[4]	4	8.09+0.17	- 2
	Saline-control	4	8.15+0.27	
12	AAMP[2]	4	4.46+0.21*	-45
	AAMP+CYS[3]	4	5.71+0.30*	-29
	CYS[4]	4	8.21+0.09	1
	Saline-control	4	8.36+0.30	
18	AAMP[2]	4	3.38+0.18*	-59
	AAMP+CYS[3]	4	5.71+0.18*	-31
	CYS[4]	4	8.30+0.69	- 1

[1] Aniline hydroxylase activity is expressed as mg p-amino-phenol formed per g of microsomal protein per 10 min interval. The value represents the mean ± S.E. in each group of animals.

[2] AAMP: Acetaminophen. Acetaminophen was administered at 600 mg/ kg i.p.

[3] AAMP+CYS: Acetaminophen+cysteamine. Acetaminophen was administered at 600 mg/kg i.p., followed 1 hr. later by cysteamine treatment (200 mg/kg i.p.).

[4] CYS: cysteamine. Cysteamine was administered at 200 mg/kg i.p.

* Significantly different from the saline-control and CYS groups ($P < 0.01$) by Scheffe's multiple range test.
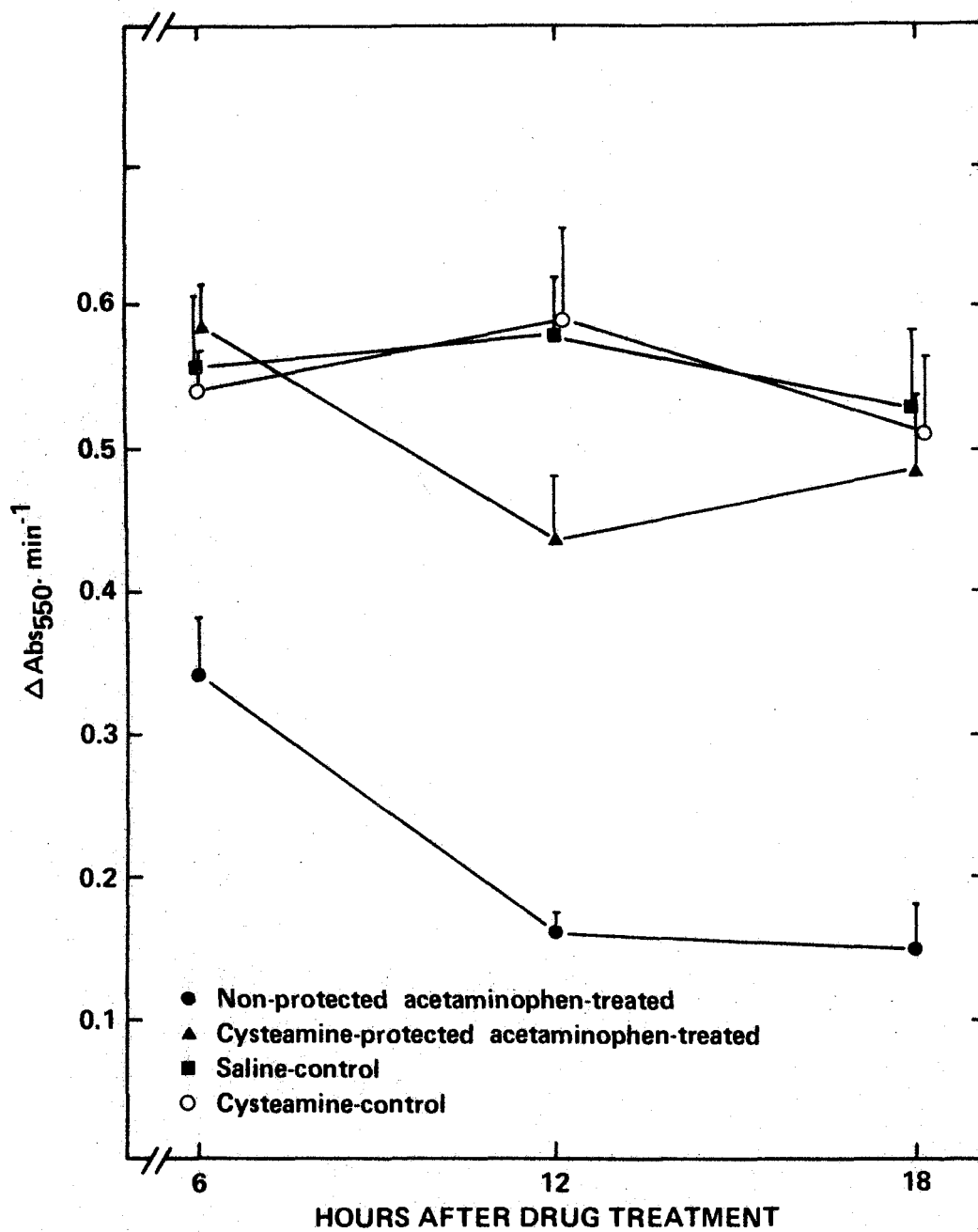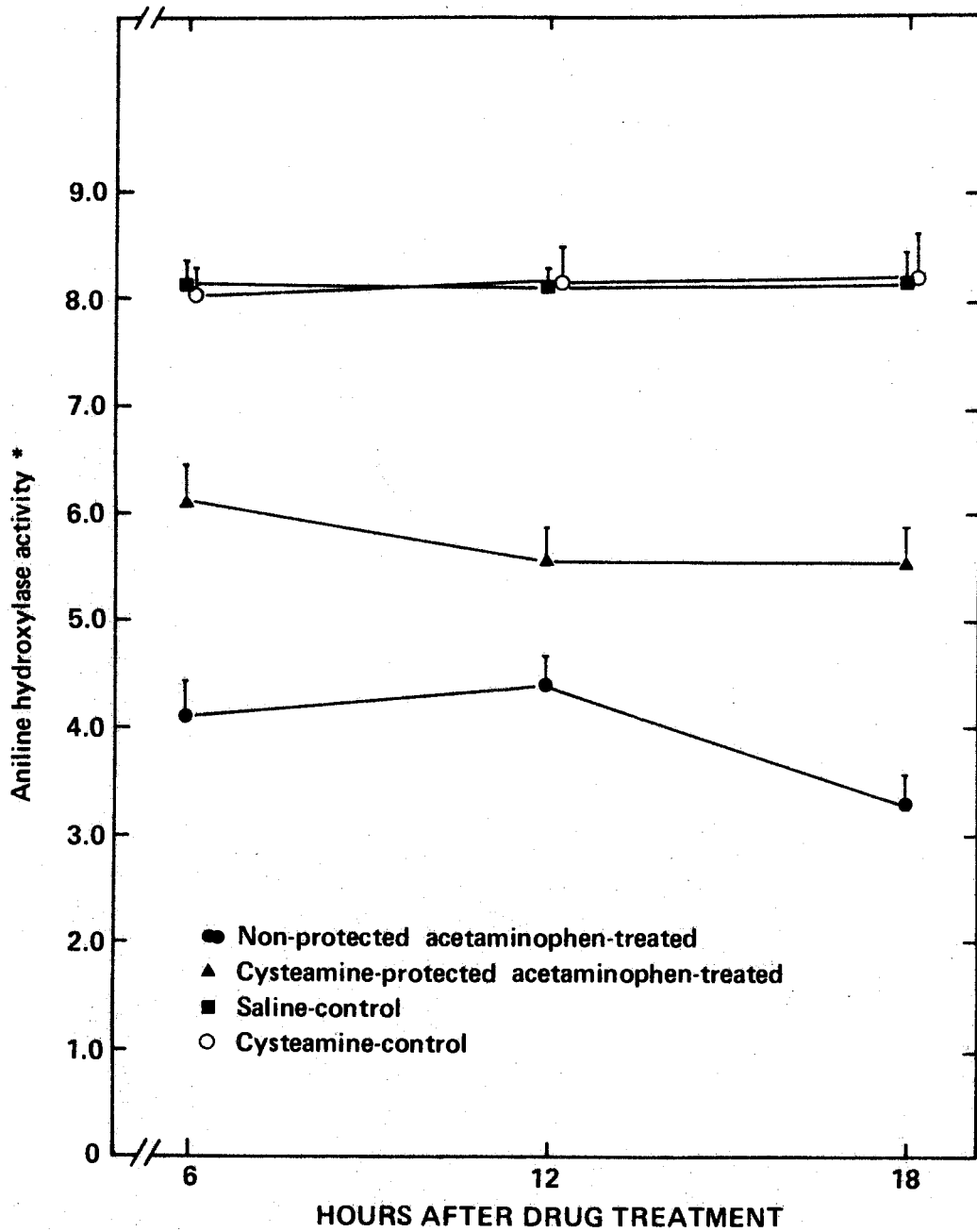
Figure 6: Aniline hydroxylase activity at various time intervals after drug treatments. Aniline hydroxylase activity* is expressed as mg p—amino-phenol formed per g of microsomal protein in 10 min.

TABLE VI   66

EFFECT OF VARIOUS DRUG TREATMENTS ON CYTOCHROME P-450 LEVEL

Time after treatment (Hr.)	Treatment	No. of animals in each group	Cytochrome P-450 level[1]	Percent change from control
6	Saline-control	5	1.62+0.12	
	AAMP[2]	4	1.78+0.10	9
	AAMP+CYS[3]	4	1.68+0.17	3
	CYS[4]	4	1.70+0.18	4
12	Saline-control	4	1.58+0.20	
	AAMP[2]	4	1.78+0.07	12
	AAMP+CYS[3]	4	1.53+0.15	3
	CYS[4]	4	1.68+0.11	6
18	Saline-control	4	1.68+0.20	
	AAMP[2]	4	1.77+0.14	5
	AAMP+CYS[3]	4	1.45+0.21	13
	CYS[4]	4	1.60+0.25	4

[1] Cytochrome P-450 level is expressed as μmoles of cytochrome P-450 per g of microsomal protein. The value represents the mean $\pm$ S.E. in each group of animals.

[2] AAMP; Acetaminophen. Acetaminophen was administered at 600 mg/kg i.p.

[3] AAMP+CYS: Acetaminophen+cysteamine. Acetaminophen was administered at 600 mg/kg i.p., followed 1 hr. later by cysteamine treatment (200 mg/kg i.p.).

[4] CYS: Cysteamine. Cysteamine was administered at 200 mg/kg i.p.

* Significantly different from the saline-control and CYS groups ($p < 0.01$) by Scheffe's multiple range test.

TABLE VIIa

EFFECT OF CYSTEAMINE TREATMENT ON CHANGES IN MICROSOMAL FUNCTION DUE
TO ACETAMINOPHEN POISONING

Time after treatment (Hr.)	Treatment	Lipid Peroxidation in vivo[3]	Lipid Peroxide formed in vitro[4]	Glucose 6-phosphatase activity[5]
6	AAMP[1]	0.89+0.03	0.52+0.07	1.81+0.22
	AAMP+CYS[2]	0.35+0.04*	0.48+0.06	2.47+0.13
12	AAMP[1]	0.91+0.09	1.17+0.07	1.57+0.17
	AAMP+CYS[2]	0.64+0.09*	0.60+0.05*	2.34+0.08
18	AAMP[1]	1.71+0.09	1.83+0.03	0.77+0.11
	AAMP+CYS[2]	0.63+0.07*	0.59+0.05*	2.22+0.13*

1 AAMP: Acetaminophen. Acetaminophen was administered at 600 mg/kg i.p.

2 AAMP+CYS: acetaminophen+cysteamine. Acetaminophen was administered at 600 mg/kg i.p.,
   followed 1 hr. later by cysteamine (200 mg/kg i.p.).

3 Lipid peroxidation in vivo is expressed as absorbance at 232 nm per mg phospholipid
   dissolved in 1 ml of cyclohexane.

4 Lipid peroxide formed in vitro is expressed as μmol malonaldehyde formed per g of 9000
   x g supernatant protein.

5 Glucose 6-phosphatase activity is expressed as micrograms of inorganic phosphorus
   formed per mg of microsomal protein in 15 min at 37C.

* Significantly different from the corresponding acetaminophen-poisoned group by Scheffe's
   multiple range test (p< 0.01).

TABLE VIIb

EFFECT OF CYSTEAMINE TREATMENT ON CHANGES IN MICROSOMAL FUNCTION DUE TO ACETAMINOPHEN POISONING

Time after treatment (Hr.)	Treatment	NADPH-cytochrome c reductase activity[3]	Aniline hydroxylase activity	Cytochrome P-450 level[5]
6	AAMP[1]	0.34+0.04	6.04+0.35	1.78+0.10
	AAMP+CYS[2]	0.58+0.03*	8.09+0.17	1.68+0.17
12	AAMP[1]	0.16+0.01	5.71+0.30	1.78+0.07
	AAMP+CYS[2]	0.44+0.04*	8.30+0.69*	1.53+0.15
18	AAMP[1]	0.15+0.03	5.71+0.18	1.77+0.14
	AAMP+CYS[2]	0.48+0.05*	8.30+0.69*	1.45+0.21

[1] AAMP: Acetaminophen. Acetaminophen was administered at 600 mg/kg i.p.

[2] AAMP+CYS: Acetaminophen+cysteamine. Acetaminophen was administered at 600 mg/kg i.p., followed 1 hr. later by cysteamine (200 mg/kg i.p.).

[3] NADPH-cytochrome c reductase activity is expressed as Δ absorbance at 550 nm per min mg^{-1} microsomal protein.

[4] Aniline hydroxylase activity is expressed as mg p-amino-phenol formed in 10 min per g of microsomal protein.

[5] Cytochrome P-450 level is expressed as μmol of cytochrome P-450 per g of microsomal protein.

* Significantly different from the corresponding acetaminophen-poisoned group by Scheffe's multiple range test ($p < 0.01$).

DISCUSSION

For the sake of clarity, acetaminophen hepatotoxicity will be discussed in two sub-sections: 1) Pathogenesis of acetaminophen-induced hepatic necrosis; 2) Protective action of cysteamine.

Pathogenesis of acetaminophen-induced hepatic necrosis

The results of this study clearly demonstrate that enhanced microsomal lipid peroxidation, as reflected by the significant ($P < 0.01$) increase in diene conjugation in vivo (Table I) and malonaldehyde in vitro (Table II), as an essential feature of acetaminophen hepatotoxicity. This may be the biochemical lesion whereby covalent binding of the toxic metabolite with hepatocellular macromolecules causes cellular necrosis and death. The hypothesis of lipoperoxidative changes of the membranes of the endoplasmic reticulum has been invoked by quite a number of investigators as the primary event in liver injury induced by carbon tetrachloride (Recknagel, 1967; Glende et al, 1976). In contrast to the pattern of lipid peroxidative changes in carbon tetrachloride-induced hepatic necrosis (Reynolds et al, 1972), diene conjugation of microsomal lipids increased as a function of the time elapsed after acetaminophen overdose in hamsters. It is important to note that the maximum elevation of serum transaminase enzymes (McLean and May, 1972) and histological evidence of liver necrosis (Mitchell et al, 1973) occurred within 10 to 24 hours of poisoning.

Alterations in oxidative metabolism of microsomal lipids
can cause drastic changes in the structural integrity of the
membranes of the endoplasmic reticulum, as well as the function-
ality of the microsomal electron transport chain, responsible
for the biotransformation of drugs, steroids, fatty acids and
other foreign compounds. It has been well documented that
lipid peroxidation and drug hydroxylation share the same com-
ponents of the NADPH-cytochrome P-450 electron transport chain:
NADPH-cytochrome c reductase (hemoprotein cytochrome P-450 re-
ductase), hemoprotein cytochrome P-450 and a heat-stable factor
identified as phosphatidylcholine (Lu et al, 1970; Strobel et
al, 1970). Although the molecular mechanism of microsomal
lipid peroxidation remains controversial (Fong et al, 1973;
Pederson and Aust, 1975) it is conceivable that compositional
changes, as well as cross-linking and double-bond shifts of the
fatty acid moieties of membrane phospholipids, will severely
affect the viability of cellular function and lead to cellular
necrosis. A free radical-related mechanism is likely to be res-
ponsible for the panorama of pathological consequences sustained
in acetaminophen-induced hepatic necrosis   (Walker et al, 1974)

Alternatively, microsomal lipid peroxidation in vivo may
subserve a facilitatory function in the microsomal oxidation
of acetaminophen to the toxic metabolite, catalysed by cyto-
chrome P-450-dependent mixed function oxidase. Previously, it
has been shown that the degree of hepatic necrosis was directly
proportional to the covalent binding of the reactive interme-

diate with microsomal proteins in vivo (Jollow et al, 1973) and
in vitro (Potter et al, 1973) upon exhaustion of glutathione
stores in the liver. Furthermore, Potter and Mitchell (1973),
on the basis of analogous studies of covalent binding with another
arylamine, 2-acetylamin fluorene , concluded that acetaminophen
was N-oxidized to the arylating metabolite. Hence the data in
this study suggest that lipid peroxidation occurs concomitantly
with N-hydroxylation of acetaminophen.

Circumstantial evidence supports the view that lipid peroxide
formed in vitro may be related to microsomal oxidation of cer-
tain substrates. Kandutsch (1966) found that 7-dehydrocholes-
terol, when incubated aerobically with hepatic microsomes in the
presence of NADH and NADPH, detergent and ferrous ions, was
oxidized to more polar products. The course of the reaction
could be correlated directly with peroxidative process. It is
perhaps relevant to note that the oxidation of 7-dehydrocholes-
terol shares some common features with NADPH-dependent microsomal
lipid peroxidation, in that both systems are activated by, or
dependent upon, ferric ion chelated by EDTA, and sensitive to
inhibition by anti-oxidants such as α-tocopherol and phenelzine
methosulfate. This explains the protection afforded by α-toco-
pherol against acetaminophen-induced hepatic necrosis in rats
(Walker et al, 1974). A severely poisoned patient was apparently
saved from eventual hepatic failure by prior self-administra-
tion of phenelzine methosulfate (Prescott et al, 1974). Recent-
ly, it has been shown unequivocably that linolenic acid hydro-

peroxide was directly responsible for the hydroxylation of androstenedione when the latter was incubated with partially purified cytochrome P-450, NADPH-cytochrome c reductase and NADPH (Hrycay et al, 1975). Accordingly, lipid peroxide may fulfil a specific metabolic role in drug biotransformation by donating one electron directly to the ferric-microsomal enzyme complex, thereby forming the ferryl ion of cytochrome P-450, the "activated oxygen" species of substrate hydroxylation (Hrycay et al, 1975). Hence the hypothesis that facilitatory interdependence of microsomal lipid peroxidation and N-hydroxylation of acetaminophen could be a determinant in acetaminophen-induced hepatic necrosis, is in harmony with the recent developments concerning the metabolic role of lipid peroxide (Kadlubar et al, 1973; Rahintula and O'Brien, 1975).

Arylation of tissue macromolecules by the toxic metabolite of acetaminophen will not occur until the endogenous glutathione pool is sufficiently depleted, since glutathione would normally conjugate with the reactive intermediate to form mercapturic acid for urinary excretion (Jollow et al, 1974; Mitchell et al, 1974). In view of the putative role of cytochrome P-450 the microsomal peroxidase (Hrycay and O'Brien, 1971a and b), coupled with the in vitro loss of glutathione during ADP-Fe-dependent lipid peroxidation (Hogberg et al, 1975), it is highly likely that in vivo lipoperoxidative process causes the depletion of glutathione in acetaminophen poisoning.

If lipid peroxidation is linked to N-hydroxylation of ace-

taminophen, the peroxidative process has to take place at a specific locus within NADPH-cytochrome P-450 electron transport chain. The progressive decrease in NADPH-cytochrome c reductase activity appears to parallel with the in vivo and in vitro lipid peroxidation (Table I, II and IV). Bidlack and Tappel (1972) claimed that NADPH-cytochrome c reductase functions as an activator of lipid peroxidation, possibly by catalysing the reduction of ferric ions to ferrous ions, which subsequently decompose the endogenous lipid hydroperoxides. Shah and Carlson (1975) presented evidence that the conversion of carbon tetrachloride to trichloromethyl free radicals occurred at the NADPH-cytochrome c reductase site. Consequently, the pattern of change in the enzyme activity in acetaminophen poisoning reflects the interaction of free radical-like species with lipophilic sites on the membrane surface. Furthermore, NADPH-cytochrome c reductase has been demonstrated to generate the superoxide anion $.O_2^-$ (Aust et al, (1972), which may transform to hydroxyl free radical (·OH) (Fong et al, 1973), singlet oxygen $^1O_2$ (Pederson and Aust, 1973) and malonaldehyde (Tappel, 1972). It is tempting to speculate that superoxide anion, which has been associated with a variety of inflammatory reactions (Curnette and Babior, 1974; Oyanagiu, 1976), is likewise implicated in the inflammatory and necrotic responses elicited by acetaminophen in the liver. Hence the molecular events responsible for acetaminophen-induced lipid peroxidation remain to be elucidated.

On the other hand, no significant change was observed in

the level of cytochrome P-450 throughout the course of acetamino-
phen poisoning (Table VI). This finding is in accord with
Mitchell's theory (1973) that acetaminophen hepatotoxicity is
mediated by N-hydroxylation catalysed by cytochrome P-450-re-
quiring-mixed function oxidase system. However, Gazzard et al
(1974) found that cytochrome P-450 level was reduced signifi-
cantly after acetaminophen overdosage in phenobarbital-pre-
treated rats. In view of the potentiating effect of phenobarbi-
tal on acetaminophen-induced hepatic necrosis (Mitchell et al,
1973a), the results obtained in Gazzard's study should be inter-
preted with caution. No correlation has been consist ntly
observed between the change in cytochrome P-450 level and histo-
logical evidence of hepatic necrosis (Gazzard et al, 1974)

The in vivo results of phenobarbital-induced lipid peroxi-
dation on cytochrome P-450 content conflict with in vitro find-
ing which indicted that NADPH-dependent microsomal lipid peroxi-
dation resulted in degradation of cytochrome P-450 heme (Schacter
et al, 1972) and concomitant loss of the hemoprotein (Levin et
al, 1973). It is perhaps important to recognize that in vivo
peroxidative process may be modified by a multitude of factors,
such as the composition and level of unsaturated fatty acids,
lipid- and water-soluble anti-oxidants and the involvement of
the ascorbate-dependent nonenzymatic pathway (Hahn et al, 1972).
After all, the existing method for the determination of cyto-
chrome P-450 (Schoene et al, 1972) does not attempt to distin-
guish between two different forms of pharmacologically active

cytochrome P-450 as isolated and purified by Hoeven and Coon (1974). It is not known which form is responsible for the binding of acetaminophen and the concomitant increase in lipid peroxidation.

The microsomal hydroxylation of aniline in vitro was markedly curtailed at various intervals of time (Table V). Racz and McDonald (1976) reported similar loss of aryl hydrocarbon hydroxylase and N-demethylase activities at twelve hours after various hepatotoxic doses of acetaminophen. It appears that the impairment of in vitro drug metabolism can be attributed directly to the peroxidative decomposition of the membranes of the endoplasmic reticulum. In carbon tetrachloride-induced hepatic necrosis, lipid peroxidation has been demonstrated to be an obligatory factor for the rapid loss of glucose 6-phosphatase and aminopyrine demethylase activities in vitro (Glende et al, 1976). Conversely, when the ascorbate-induced lipid peroxidation was inhibited by EDTA, no change was observed in the catalytic function of the oxidative enzymes (Glende, 1972). Conformational changes induced by acetaminophen binding may drastically alter the spatial relationships of the catalytic sites in the immediate vicinity of the hemoprotein cytochrome P-450 to such an extent that aromatic amine hydroxylation is rendered impossible. Alternatively, the oxidative capacity of the microsomal drug-metabolizing system may have been saturated by excessive formation of the toxic metabolite of acetaminophen.

The activity of the lipid-requiring glucose 6-phosphatase was significantly depressed at six, twelve and eighteen hours.

This may be explained by the progressive increase in in vivo lipoperoxidative decomposition of the membranes of the endoplasmic reticulum. In carbon tetrachloride-induced hepatic necrosis, Reynolds et al (1971) found a dramatic fall in the activity of glucose 6-phosphatase as determined histochemically. In vitro studies further indicated that NADPH- or ascorbate-induced microsomal lipid peroxidation resulted in drastic curtailment of the activity of this lipid-sensitive enzyme (Wills, 1971; Bidlack and Tappel, 1972; Glende et al, 1976). This may be due to the hydrolysis of phosphatidyl ethanolamine moiety of membrane phospholipids (Dutters et al, 1967), or stoichiometric compositional alterations in the arachidonic acid at the β position of microsomal phospholipids (Nichaus et al, 1968).

It should be appreciated that the mechanism of functional interdependence of the microsomal drug hydroxylation system and lipid peroxidation is not completely understood. The possibility exists that an alternative NADH-cytochrome b_5-pathway may facilitate the electron transfer and substrat oxidation in conjunction with the NADPH-cytochrome P-450 system (Archakov et al, 1975). This aspect of microsomal function during acetaminophen poisoning has not been examined in detail.

In conclusion, evidence is presented in this study that facilitatory interaction of microsomal lipid peroxidation and acetaminophen oxidation to the toxic metabolite, is the primary event in acetaminophen hepatotoxicity. The biochemical consequences of acetaminophen-induced hepatic necrosis are reflected in the marked inhibition of in vitro drug metabolizing

activity towards aniline, as well as depression of NADPH-cyto-
chrome c reductase and glucose 6-phosphatase activities. Cyto-
chrome P-450 level was maintained relatively constant, an obser-
vation quite consistent with the obligatory role it subserved
in the N-hydroxylation of acetaminophen.

Protective action of cysteamine

It has been concluded from the above discussion that ace-
taminophen hepatotoxicity arises from the facilitatory interde-
pendence of microsomal lipid peroxidation and the oxidation of
the drug. The results of the present study indicate that cys-
teamine may protect against acetaminophen hepatotoxic effects
by virtue of the antioxidant properties of the sulfhydryl group.
This is evident from the attenuation of in vivo and in vitro
lipid peroxidation as compared with the non-protected acetamino-
phen-treated group. Normal NADPH-cytochrome c reductase activity
was retained (Table 4), which indicates that NADPH-cytochrome
c reductase may be the locus within the NADPH-cytochrome P-450
electron transport chain most susceptible to peroxidative at
back by free radicals. Admittedly in vitro and in vivo lipid
peroxidation was not entirely abolished by cysteamine treatment.
It is possible that a higher dose of the antidote may achieve
better results. The data presented here do not exclude the
possibility that some locus in contiguity with the NADPH-cyto-
chrome P-450 electron transport chain, may also be subject to
lipoperoxidative decomposition, but more sophisticated techni-
ques are required to identify the site of activation.

On the other hand, cytochrome P-450 level was similar in both the non-protected and cysteamine-protected groups. Although the N-hydroxylation of acetaminophen depends upon the activity of cytochrome P-450-requiring mixed function oxidase, no obligatory relationship exists between the cytochrome P-450 level and the severity of liver injury. Gazzard et al (1974) found that ∝-tocopherol at a dose of 400 mg/kg i.p. was ineffective in reversing hepatic damage, in the presence of normal cytochrome P-450 level. However, the protective action of cysteamine appears to be related to its ability to eliminate the facilitatory effect of enhanced microsomal lipid peroxidation on the N-oxidation of acetaminophen to the electrophilic metabolite. Perhaps more definitive conclusion may be drawn by correlating the content of cytochrome P-450 with the peroxidase activity of the hemoprotein as a function of the dose of cysteamine used to reverse acetaminophen hepatotoxicity. The enhanced peroxidase activity of cytochrome P-450 has been suggested to result in significant intracellular oxidation of potential hydrogen donors, such as glutathione, ascorbate and ∝-tocopherol (Hrycay and O'Brien, 1971).

It appears that cysteamine treatment brought about significant maintenance of in vitro drug metabolizing activities, as reflected by the activity of aniline hydroxylase with respect to the non-protected acetaminophen-poisoned animals (Table V, Figure 4). This may be explained by the reduced lipid peroxide formed in vivo and in vitro. Glende et al (1976) showed quite

convincingly that ascorbate-induced NADPH-requiring in vitro
lipid peroxidation was linked to the depression of ethylmorphine
demethylase activity, a microsomal enzyme requiring the co-
operative interaction of the entire segment of the NADPH-cyto-
chrome P-450 electron chain. It may be argued that improvement
in in vitro drug metabolism will enhance the in vivo oxidation
of acetaminophen to the toxic metabolite. However, the possibil-
ity cannot be eliminated that cysteamine may inhibit the in vivo
oxidation of acetaminophen to the electrophilic intermediate,
thereby making more binding sites available for in vitro inter-
action with aniline. More detailed studies on the spectral bind-
ing of cytochrome P-450 with a variety of substrates including
acetaminophen, aniline and protective agents, will elucidate
the molecular interactions involved. Structural considerations
lead one to suspect that acetaminophen will bind at the same
Type II-binding site, namely, the hemoprotein cytochrome P-450
associated site, as aniline.

Circumstantial evidence tends to reinforce the view that
cysteamine functions as an inhibitor of microsomal oxidation in
acetaminophen-induced hepatic injury. Harvey and Levitt (1976)
found that cysteamine markedly reduced the amount of gluta-
thione-derived conjugates in the bile and prolonged the plasma
half-lives of acetanilide, in isolated perfused rat liver. Pre-
viously, Jollow et al (1974) demonstrated that the extent of
urinary excretion of mercapturic acid-conjugates of acetamino-
phen reflected directly the hepatotoxic pathway for the aryla-
tion of microsomal proteins by the toxic metabolite. It is per-

haps more than fortuitous that cysteamine inhibits the microsomal 11-β-hydroxylation of steroids by interacting with the 11-β hydroxylase system consisting of adrenodoxin, adrenodoxin reductase and cytochrome P-450 system (Flemming et al, 1973). Since cysteamine and cystamine (with which cysteamine exists in equilibrium in vivo) are rapidly metabolized and cleared by the kidney, it may be suspected that the pharmacological action of cysteamine must be attributed to an active metabolite which may either be freely circulating or bound to plasma proteins. However, it remains to be seen whether the protective action of cysteamine is related to some yet unidentified metabolite or mixed disulfide complex. In model organic reactions, the fifth axial ligand in cytochrome P-450 is a mercaptide anion which has been shown to co-cordinate quite readily with any sulfhydryl functional group (Chang and Dolphin, 1975).

An alternative hypothesis has been proposed that the protective effect of cysteamine against acetaminophen hepatotoxicity may be attributed to replenishment of endogenous glutathione stores in the liver. Mitchell et al (1973b) observed that covalent binding of the toxic metabolite with microsomal proteins occurred when the endogenous glutathione pool was depleted to 70 per cent in mice. The fundamental role of glutathione appears to protect important nucleophilic sites, such as sulfhydryl moieties in membrane proteins, from electrophilic attack by the reactive intermediate (Mitchell et al, 1973b; Jollow et al, 1974). Dietary supplementation with sulphur-containing amino acid methionine afforded significant protection against liver injury,

presumably by stimulating the Υ-glutamyl cycle of glutathione
metabolism (McLean and Day, 1975). However, the exact relation-
ship between the glutathione pool in the liver and the extent
of liver injury is not established. Strubelt et al (1974)
found that cysteamine treatment at a dose of 50 mg/kg i.p.
although effective in reversing liver damage, only caused a
partial repletion of glutathione stores. In addition, cysteine,
the precursor of glutathione, was not very effective in prevent-
ing liver damage, despite the fact that glutathione content was
maintained normal (Mitchell et al, 1973b).

No conclusive evidence is available to refute or confirm
the hypothesis that the role of free-radical scavenger sub-
served by cysteamine or glutathione is specifically related to
the sulfhydryl group. Dimercaprol (Hughes et al, 1976) has been
reported to potentiate the severity of liver damage substained
by severely poisoned patients. Similarly, thioctic acid, the
potential sulfhydryl donor, failed to prevent hepatic necrosis in
acetaminophen-poisoned rats (Gazzard et al, 1976). Perhaps the
limiting factor of the protective action of cysteamine may not
depend so much on the rate of de novo synthesis of glutathione,
but rather on the kinetics of transmembrane movement of gluta-
thione-liposome complex, as suggested by Malnoe et al (1975).
This explains the apparent failure of glutathione itself to
protect against acetaminophen hepatotoxicity (Gazzard et al,
1974) unless administered in massive doses by intravenous route
in experimental animals (Benedetti et al, 1975).

In the final analysis, the possibility exists that cysteamine may conjugate with the toxic metabolite of acetaminophen. Although Andrews (1976) in a personal communication to Harvey and Levitt (1976) failed to identify any cysteamine-acetaminophen conjugate in the bile upon addition of ^{36}S-cysteamine to the isolated perfused rat liver, the response may be dose- and species-dependent. More detailed studies on whole animals using radioactive acetaminophen and cysteamine will help elucidate the protective mechanism of cysteamine.

In conclusion, the results of this study indicate that cysteamine at a dose of 200 mg/kg i.p. confers partial protection against acetaminophen-induced hepatic necrosis by reducing lipid peroxidation in vivo and in vitro, as well as restoring the microsomal function to some extent. Hence it is suggested that cysteamine acts by inhibiting the formation of the toxic metabolite.

PROTECTION BY CYSTEAMINE AGAINST ACETAMINOPHEN-

INDUCED HEAPTIC NECROSIS: AN ELECTRON-

MICROSCOPIC STUDY

INTRODUCTION

Suicidal attempts with acetaminophen overdose in humans have been observed to cause acute liver injury culminating in fulminant centrilobular hepatic necrosis and liver failure (Prescott et al, 1971; Clark et al, 1973). The prognosis of eventual hepatic failure may often be predicted from the extrapolated plasma acetaminophen level at 4 hours after ingestion of the analgesic. A high risk of severe hepatic necrosis is anticipated if the plasma concentration exceeds 250-300 mg per l (Prescott et al, 1976). The histopathological sequence of events leading to cellular necrosis and death has been analyzed in some detail by Dixon and his colleagues (1971; 1975). Dixon, Nunmo and Prescott (1971), in a study of the development of the lesion extending to 28 days after a single toxic dose of acetaminophen (350 mg/kg oral) in rats, found hydropic vacuolation, centrilobular necrosis and macrophage infiltration, accompanied by appreciable degree of regenerative activity. In a subsequent histological and electron-microscopic investigation, Dixon et al (1975) concluded that coagulative necrosis constituted the basic pathological lesion of acetaminophen hepatotoxicity. This has been confirmed by Portman et al (1975) in severely poisoned patients. They found that the fraction of

surviving hepatic parenchyma as expressed by the hepatocyte volume fraction (HVF) in liver biopsies performed within ten days of poisoning correlated quite closely with the prolonged prothrombin time and the elevation of serum enzymes.

Although cysteamine has been reported to be successful in reversing liver damage in severely poisoned patients (Prescott et al, 1976), mice (Strubelt et al, 1974), and rats (Harvey and Levitt, 1976), no fine structural study has been undertaken to determine the possible alterations of the hepatic lesion caused by cysteamine treatment. Consequently, the present electron-microscopic study is an attempt to evaluate the protective action of cysteamine by examining the morphological changes in the hepatocytes of both the cysteamine-protected and non-protected animals at various time intervals after a toxic dose of acetaminophen.

MATERIALS AND METHODS

The different groups of hamsters described in the previous chapter (Chapter III) subject to the various drug treatments were concurrently used for electron-microscopic study, with the exception the cysteamine-control animals. In addition, only one group of acetaminophen-treated unprotected animals was sacrificed at twenty-four hours. The limited supply of animals, unfortunately, would not allow one to include the corresponding twenty-four cysteamine-protected animals. In all cases, the anterior portion of the left lateral lobe of the liver was processed for electron-microscopic observations. The tissues were

cut into tiny sections which were instantaneously immersed in
3 per cent glutaraldehyde solution, buffered with 0.1 M sodium
cacodylate, pH 7.4 (Sabatini et al, 1963; Carlson, 1973). The
specimens were then placed into small vials containing approxi-
mately 1 ml of the buffered fixative medium and left for two
hours at $4^{o}C$; after which time the tissues were thoroughly
washed with sodium cacodylate buffer for three times, followed
by fixation in two per cent osmium tetroxide buffered with 0.1 M
sodium cacodylate for another 2 hours. After dehydration in a
graded series of alcohols and propylene oxide, the tissues were
transferred to the propylene oxide-ethanol mixture (1:1) and em-
bedded in epoxyl resin according to the method of Luft (1961).
After adding resin-propylene oxide mixture (1:1), they were left
overnight to allow evaporation. Subsequently, the samples were
embedded in pure resin mixture and left at $65^{o}C$ for 3 to 6 days.

The specimens were mounted on epoxyl resin blocks and left
in the oven for three days at $65^{o}C$. Thin sections were cut
with a Reichert OM U2 ultramicrotome, stained with two per cent
uranyl acetate for 20 minutes and for 15 minutes in lead cit-
rate (Carlson, 1973). They were finally examined under a Zeiss
EM-9A electron microscope. Many photographs were taken from
each group of animals to ascertain that no bias were introduced
in the selection of the representative electron-microscopic
pictures for close observation.

RESULTS

In the present investigation, the fine structural appearance of hamster hepatocytes from the saline-control group agrees favorably with that described and reviewed by Jones and Fawcett (1966). Descriptive analysis of the control hepatocyte will therefore be focussed primarily on those organelles exhibiting significant alterations consequent of various drug treatments, in order to emphasize the difference between the control and the experimental animals.

Saline-control animals

Since the animals were allowed access to food and water ad libitum, glycogen deposits accumulate in the cytoplasmic matrix (Fig. 7) and appeared as compact rosette pattern often separated by aggregates of variable size. The granular endoplasmic reticulum (GER) is composed of lamellated membranous cisternae well carpeted with ribosomes (Fig. 8). Smooth-surfaced endoplasmic reticulum (AGER) appears as short anastomizing tubules, often continuous with the GER, creating an impression of a loose network. The cytoplasmic matrix around these tubules may be associated with some glycogen granules.

Double-membrane bound mitochondria, of oval or elongated profiles, abound in the hepatocytes of control animals. They possess platelike cristae and an uniformly dense matrix. Pinocytotic vauoles are occasionally seen in different regions of the liver parenchyma. Lipid droplets of the type described by

Fig. 7: Electron micrograph of a portion of the liver tissue
from the saline-control hamster, showing cytoplasmic
glycogen deposits (G). The endothelial lining of the
sinusoidal space (S) exhibits the typical microvillal
(MV) arrangement. The occurrence of vacuoles (V) is not
considered abnormal. AGER:agranular endoplasmic re-
ticulum. N:nucleus. x 5,400
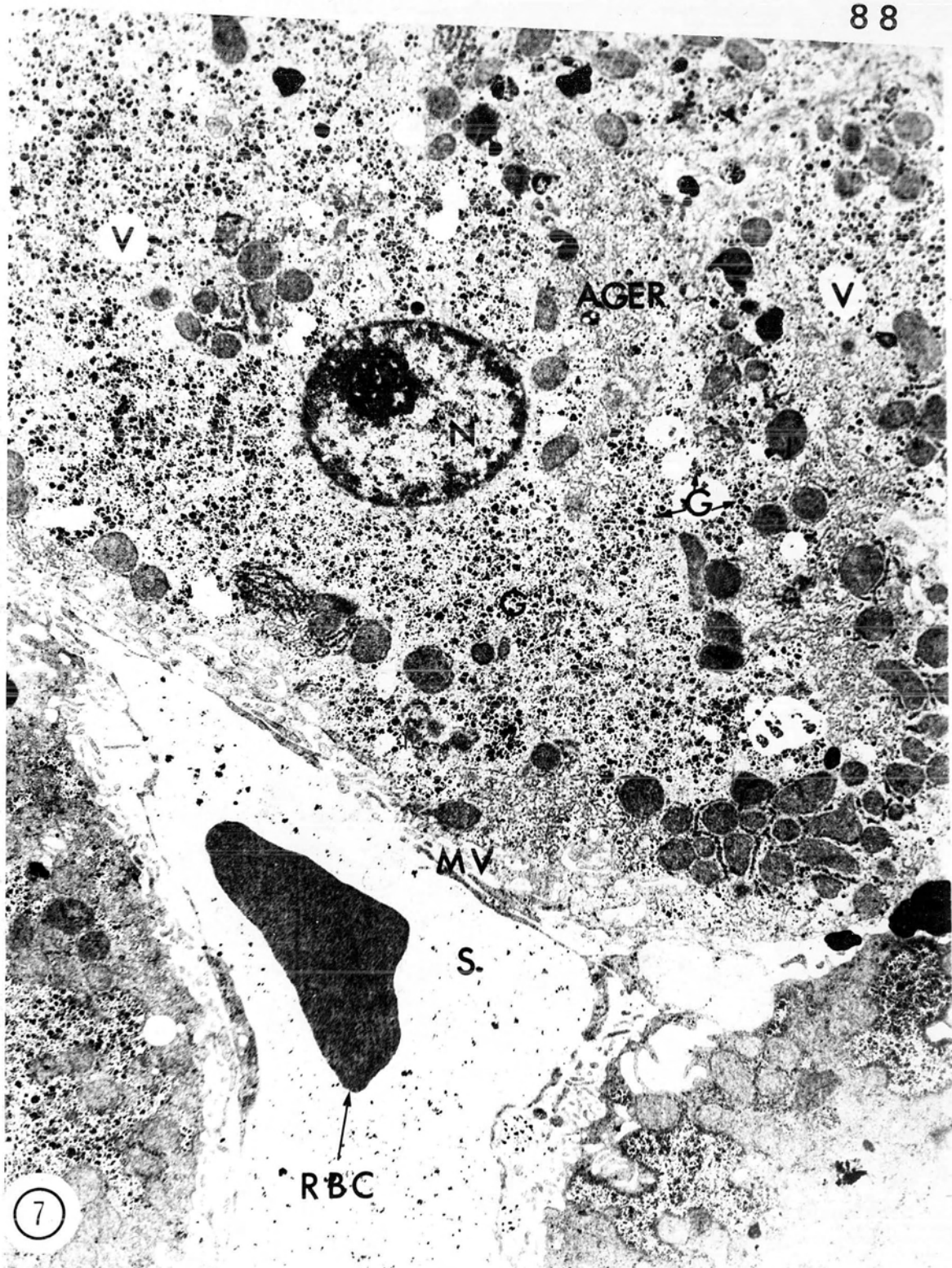
Fig. 8: A bile canaliculus (BCL) separating two hepato-
cytes from a saline-control animal. Note that
rough-surfaced granular endoplasmic reticulum
(GER) is well carpeted with ribosomes, and the
agranular endoplasmic reticulum (AGER) exhibits
the typical loose network pattern. M:mitochon-
dria. G:glycogen granules. PR: ribosomal aggre-
gates.   x 21,900

BCL

GER

M

M

PR

G

G

AGER
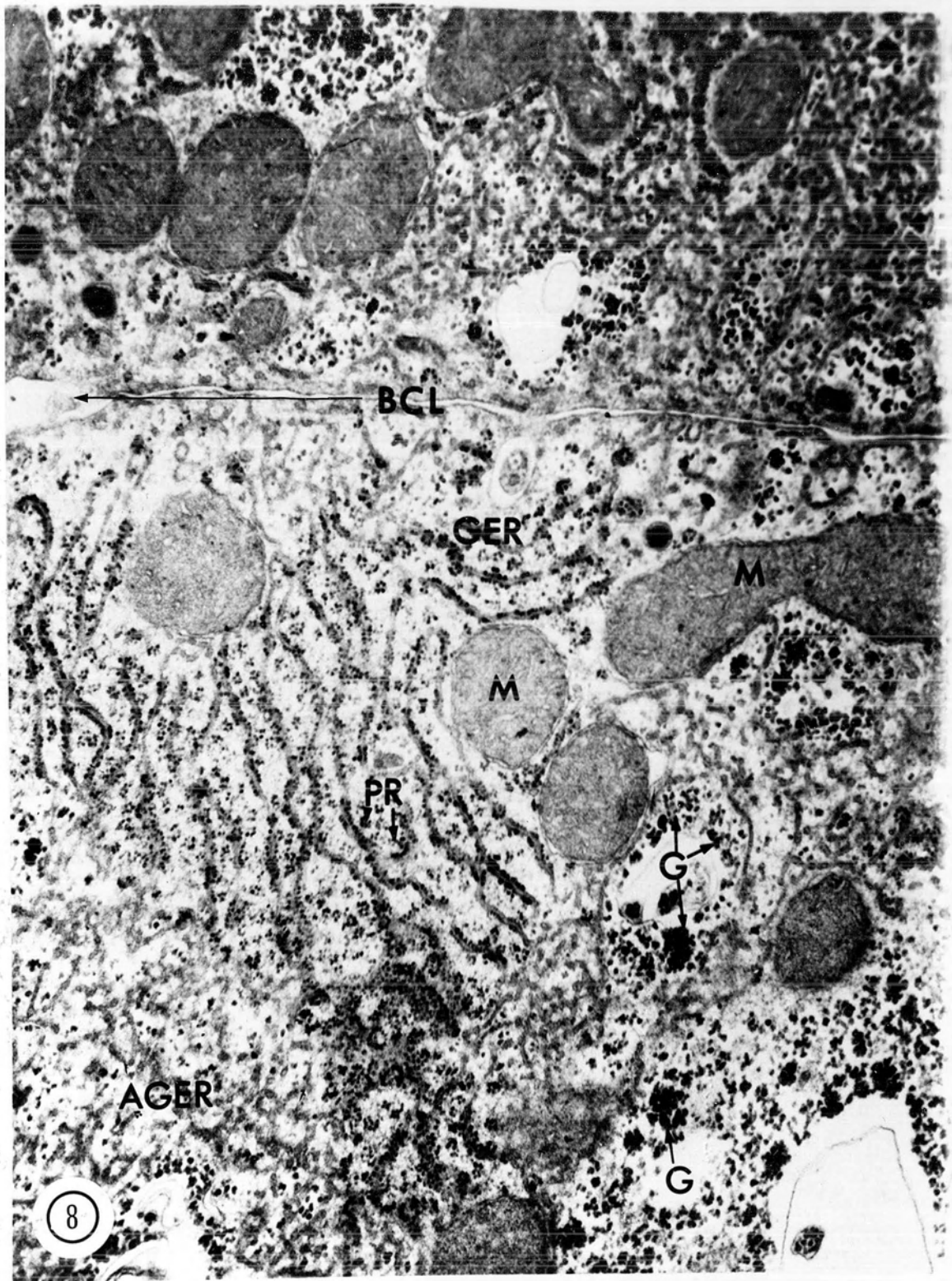
⑧

Nehemiah and Novikoff (1974) as consisting of a distinct peripheral membrane in close apposition to an electron dense layer, are relatively few in the control animals. There are few lipofuscin granules (lysosomes).

The intact endothelial lining of the sinusoids displays the normal irregular array of microvilli and is relatively free of any debris, erythrocytes and neutrophils (Fig. 8). The usual configuration of the bile canaliculus between two contiguous cells is shown in Fig. 8 .

Unprotected acetaminophen-poisoned animals

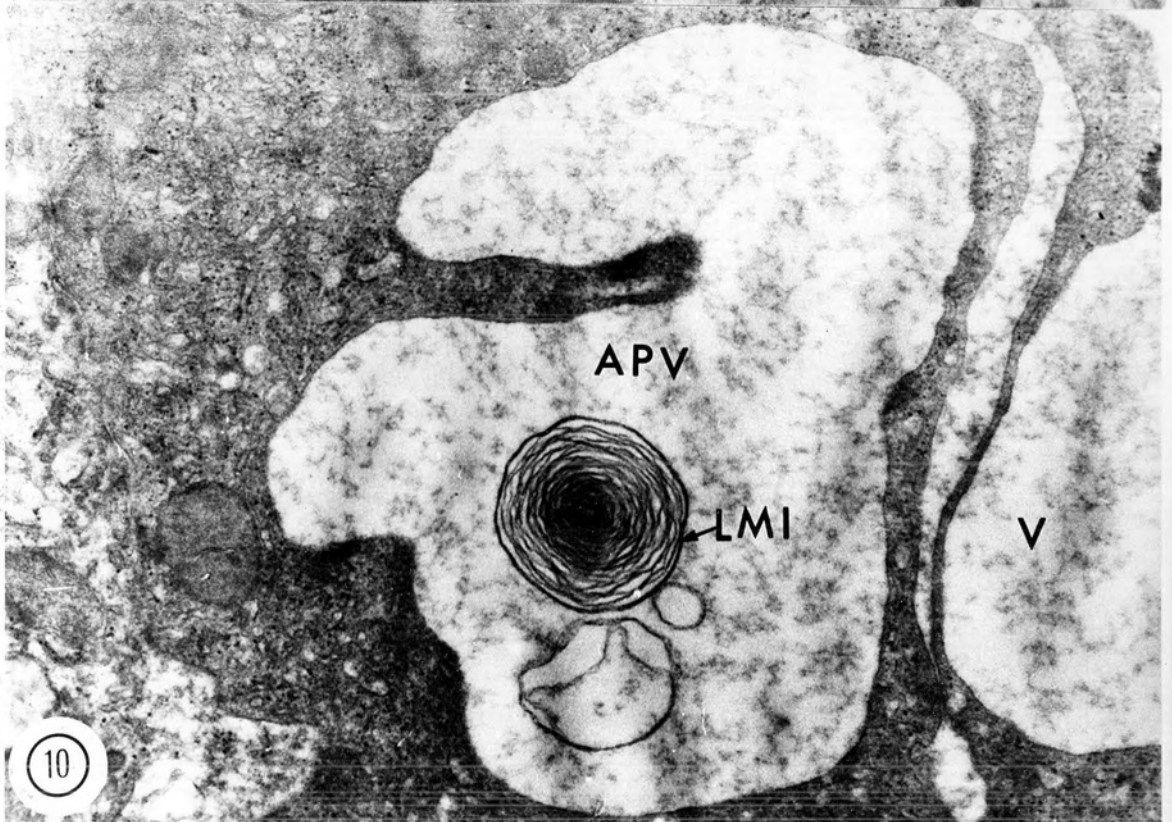### The group sacrificed at six hours after acetaminophen treatment

Examination of the prepared liver sections from the unprotected animals sacrificed at six hours reveals considerable morphological change in the various organelles. Glycogen deposits are nearly depleted from most areas of the cytoplasmic matrix, though occasionally rosettes of glycogen are still evident. The obvious effect of acetaminophen is manifested in the configuration of endoplasmic reticulum. The cisternae of GER become dilated; although the GER is still covered with ribosomes, free ribosomal particles begin to appear in the cytoplasm (Fig. 9). The smooth-surfaced tubules start to aggregate into enlarged masses and assume the shape of a labyrinthine-meshed complex. An aberrant autophagic vacuole consisting of engulfed lamellated membraneous materials embedded in a homogenous electron-dense matrix may sometimes be seen near the bile cana-

Fig. 9: Appreciable dilation of the cisternae of the granu-
lar endoplasmic reticulum (GER) of the liver paren-
chyma from the acetaminophen-poisoned animal six
hours after acetaminophen treatment. Mitochondria
(M) appear normal. FR: free ribosomes. AGER: agranu-
lar endoplasmic reticulum. x 62,700

Fig. 10:Six hours after acetaminophen treatment in the unpro-
tected animal. Lamellated membraneous inclusions(LMI)
are seen within an autophagic vacuole (APV). V: en-
larged vacuole. x 21,900
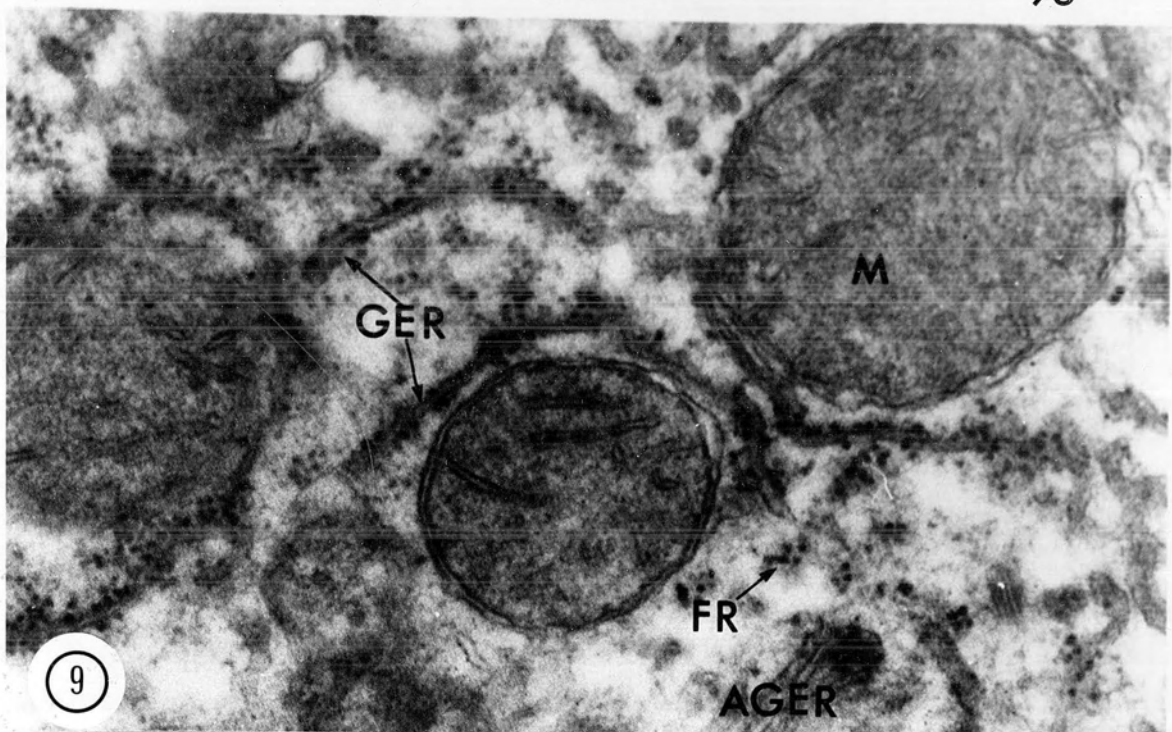
liculus (Fig. 10). Besides, lipid vacuoles containing homogenous electron-dense substance delimited from the cytoplasm by a single membrane, occur in small numbers in most of the hepatocytes. On the other hand, no major change is observed in the configuration of the mitochondria. Lysosomes can sometimes be seen in the cytoplasm, but their frequency does not suggest the presence of active cytoplasmic degradation.

The group sacrificed at twelve hours after acetaminophen treatment

By twelve hours, the degenerative changes induced by acetaminophen have extended to most organelles. The evolution of the necrotic process is evident in the hepatocytes from all animals in this group. Hyperplasia of the agranular endoplasmic reticulum is seen in the condensation of the tubular aggregates and appears as a compact interwoven network enclosed by electron-dense deposits; the normal cytoplasmic matrix is no longer recognizable, but characterized by random distribution of free monomeric ribosomal particles (Fig. 11). The cisternae of the GER have undergone extensive swelling into larger vesicles intermingled with some remnants of the tubular elements of AGER, former membrane-segments can be seen between the swollen sacs of GER. The GER is almost devoid of any membrane-bound ribosomes. The bile canaliculus has become dilated and disorganized.

Fatty infiltration of the liver parenchyma is evident from the abundance of lipid globules of various size and shapes. They display an electron-dense centre surrounded by a halo of

Fig. 11: Hyperplasia of smooth-surfaced agranular endoplasmic reticulum (AGER) twelve hours after acetaminophen treatment in the non-protected animal. Vesiculated free ribosomes (FR) can be found dispersed in the cytoplasmic matrix. M:mitochondria, x 21,900

Fig. 12: Accumulation of lipid vacuoles (LD) most likely to be derived from lysosomes (L) twelve hours after acetaminophen treatment in non-protected animal. LA: membrane-bound lipid aggregates. V:vacuoles. x 21,900

11

12
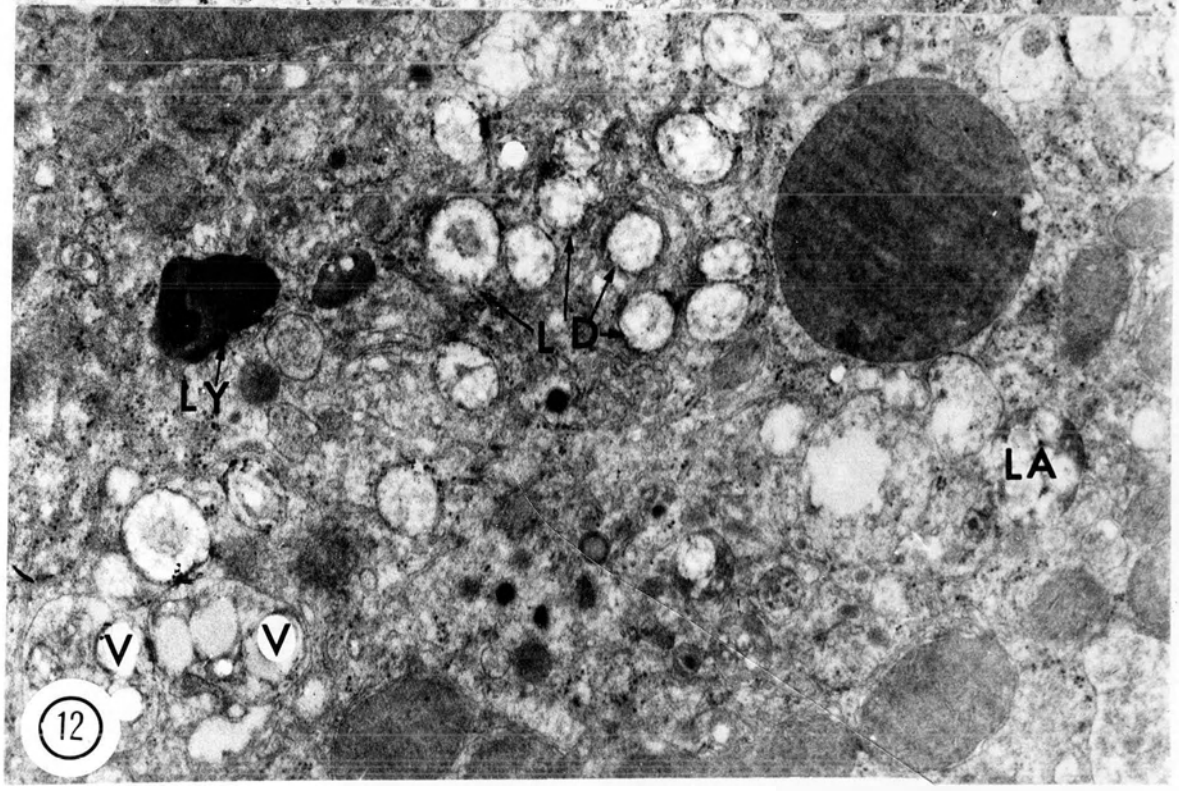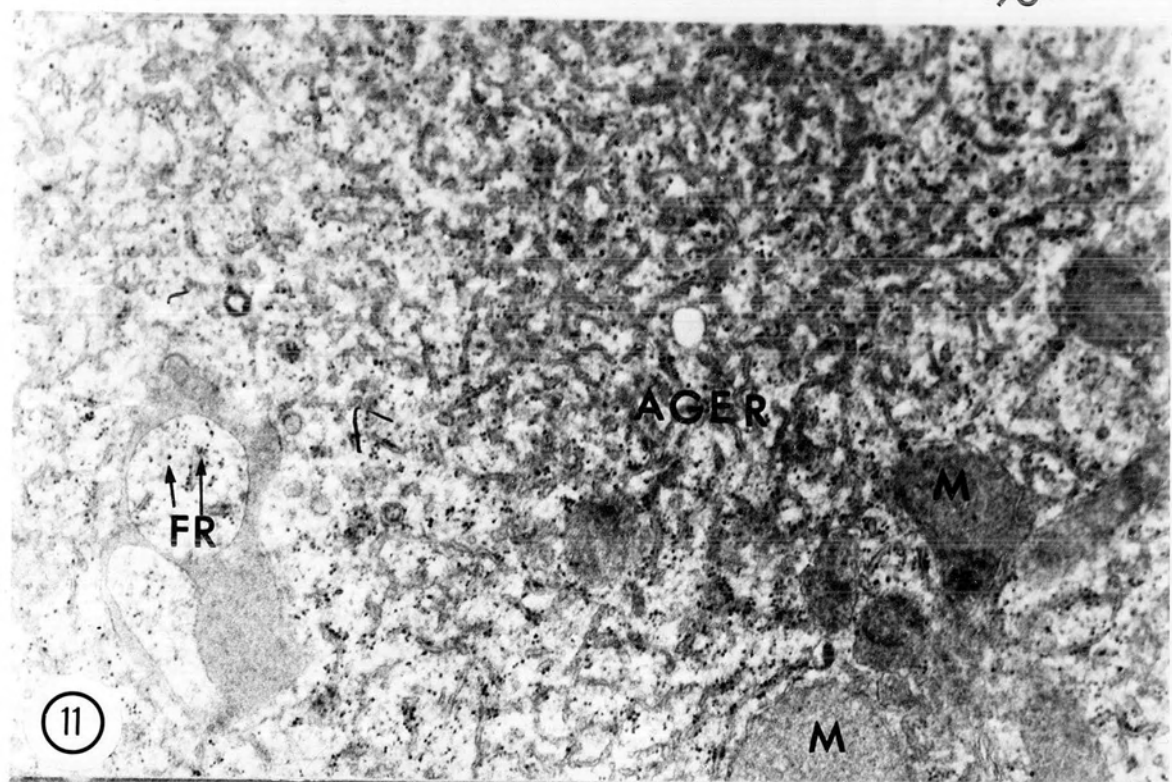
clear homogenous material, which is sometimes associated with one or two crescent cytoplasmic projections (Fig. 12   ). Some lipid droplets become aggregated within membrane-bound sacs, reminiscent of the unique lipolysosomes in hamsters (Nehemiah and Novikoff, 1974). Focal accumulation of primary lysosomes is a constant feature in the twelve-hour group. However, it is noteworthy that a multicentric myeloid body becomes fused with an autophagic vacuole containing remnants of endoplasmic reticulum. (Fig. 13   ). This focal process of cytoplasmic membraneous degradation and autophagic vacuole formation may represent a degenerative response to the hepatotoxin.

Sinusoid congestion seems to be a characteristic feature of the necrotic hepatocytes at twelve hours. In addition to erythrocytes and polymorphic neutrophils, the sinusoid space is crowded with small lipid vacuoles, cellular debris and membrane fragments (Fig. 14   ). The endothelial linings appears to have lost their structural integrity. Some parasinosoidal hepatocytes have engulfed erythrocytes (erythrophagacytosis), and, in addition, large irregularly-shaped vacuoles containing electron-dense granular materials often border the necrotic zones (Fig. 14   ). Even blood platelets appear in the sinusoidal space.

Mitochondria become swollen and the cristae are degenerating into whorled membranes. The peroxisomes, on the other hand, retain their normal configuration.

Fig. 13: Fusion of myeloid bodies (MB) with an autophagic
          vacuole (APV) seen at twelve hours after acetamino-
          phen treatment in the non-protected animal. The
          mitochondria (M) appear swollen with the cristae
          being distorted. L:lysosomes. LD: lipid droplets.
          x 21,900
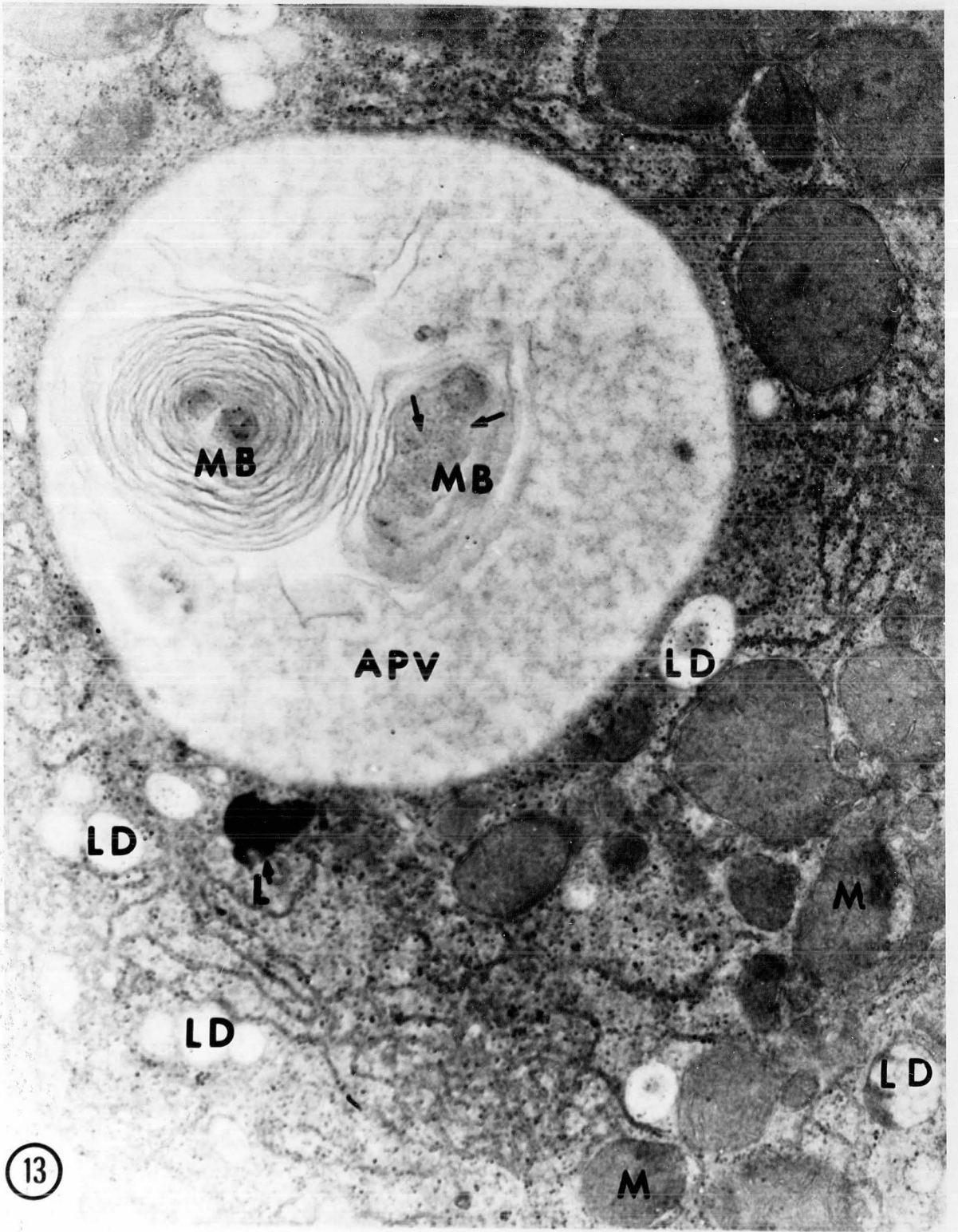
Fig. 14: Sinusoid (S) congestion and marked vacuolation (V)
twelve hours after acetaminophen treatment in the
non-protected animal. Note the labyrinthine mesh
of smooth-surfaced membranes (AGER) is no longer de-
limited by the plasma membrane. N:polymorphonuclear-
leukocytes. LD:lipid droplets. x 5,400
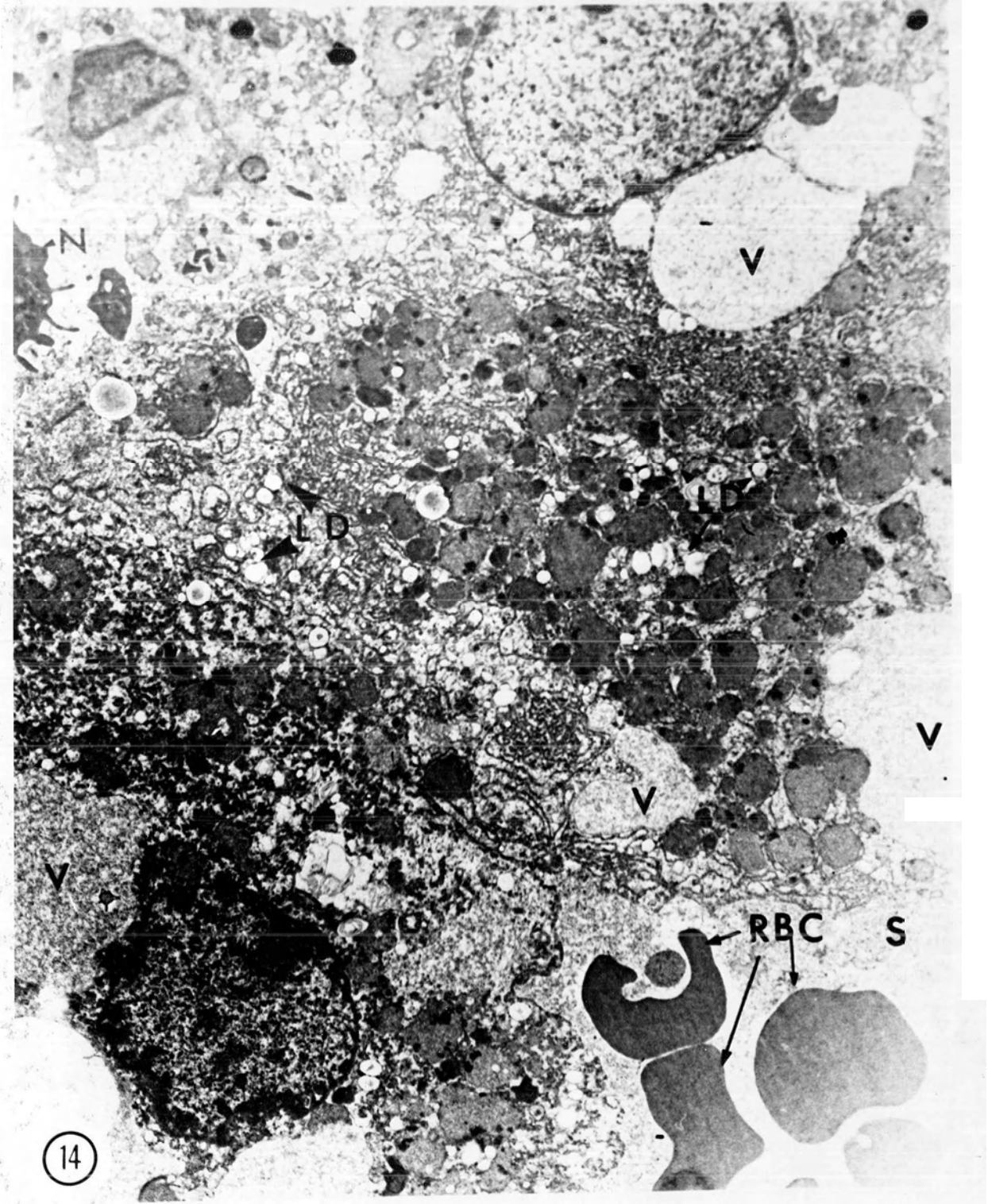
The group sacrificed at eighteen hours after acetaminophen treatment

At eighteen hours, the necrotic process has advanced to such an extent as to suggest irreversible cellular injury. Most of the degenerative changes seen in the animals sacrificed at twelve hours become accentuated. The plasma membranes of most hepatocytes have undergone virtual collapse and denaturation. Although remnants of GER and AGER can still be recognized, vesiculated endoplasmic reticulum organize into myeloid figures bound by a well-delineated unit membrane. Indeed, there exists a tendency for increased formation of multicentric myeloid bodies in response to the hepatotoxin (Fig. 15 & 17).Lipid accumulation is exaggerated in many hepatocytes, as reflected by the occurrence of increased numbers of lipolysosomes (Fig. 16). The mitochondria become mostly enlarged and dense and the arrangement of the cristae is severely distorted. Most nuclei become pyknotic and karyohexic at this advanced stage of necrosis (Fig. 17).

All hepatocytes exhibit various degrees of coagulative necrosis, sinusoidal congestion is very pronounced at this time, and erythrophagocytosis becomes accentuated (Fig.16).

The group sacrificed at twenty-four hours after acetaminophen treatment

At twenty-four hours, coagulative necrosis has progressed to embrace the activity of parasinusoidal Kupffer's cells for phagocytizing membraneous fragments and remnant vesicles (Fig.

Fig. 15: Eighteen hours after acetaminophen treatment in the non-protected animal showing accumulation of lipo-lysosomes (LPL). Parasinusoidal microvilli (MV) become disorganized. x 5,400

Fig. 16: Heterogenous dense body (HDB) enclosing several mye-loid bodies (MB) at eighteen hours after acetamino-phen treatment in the non-protected animal. LD: lipid vacuoles. x 21,900
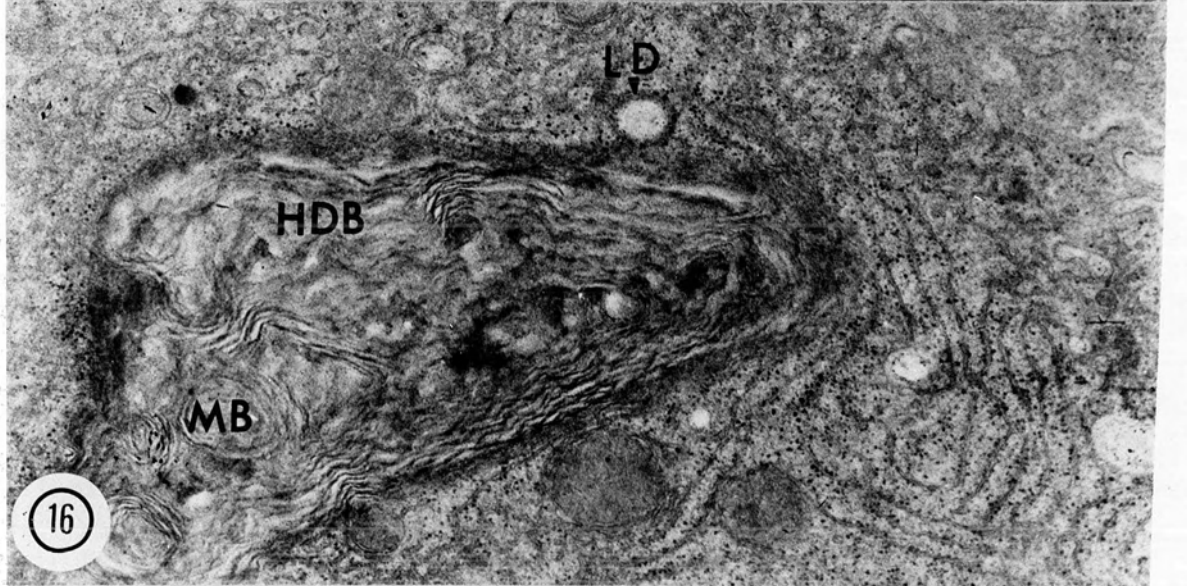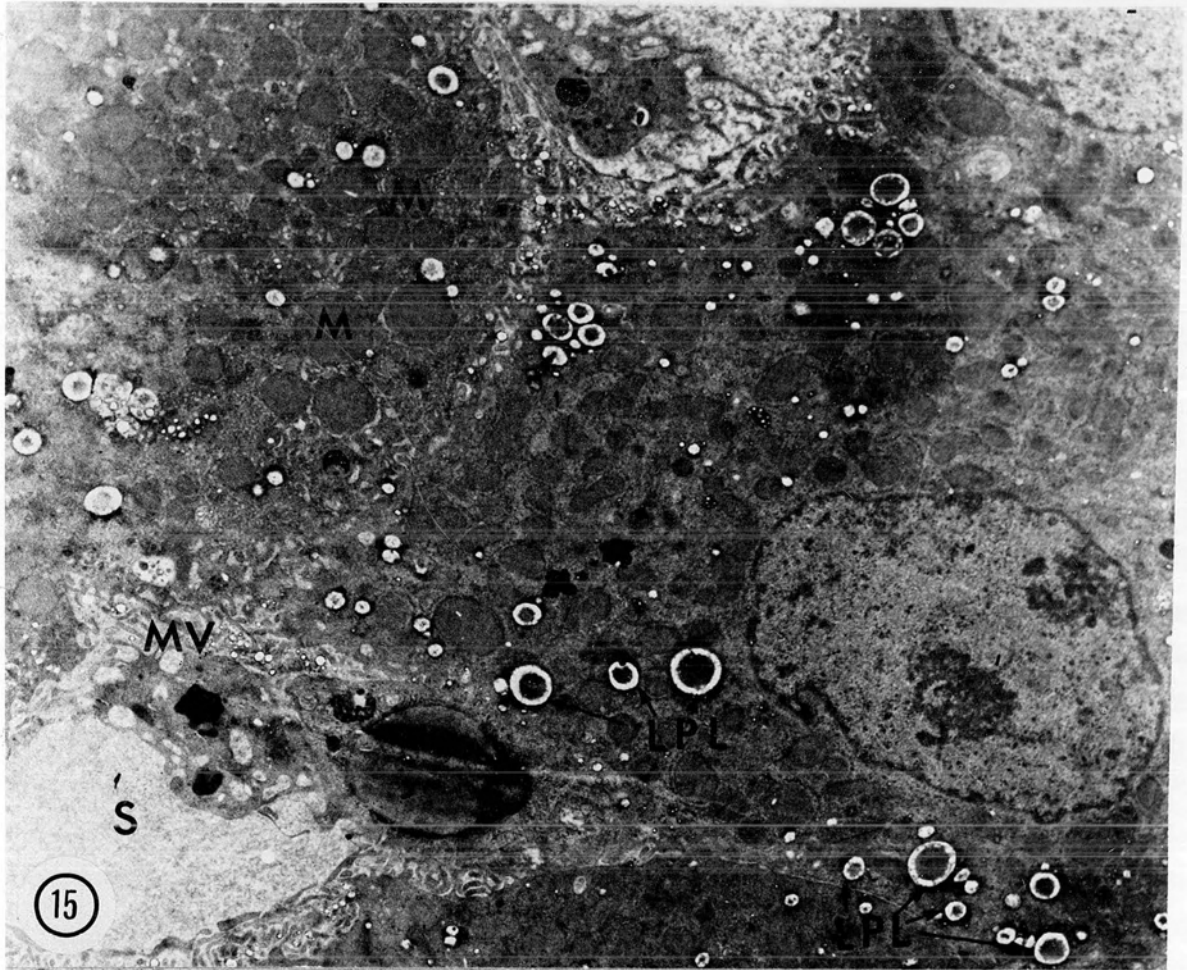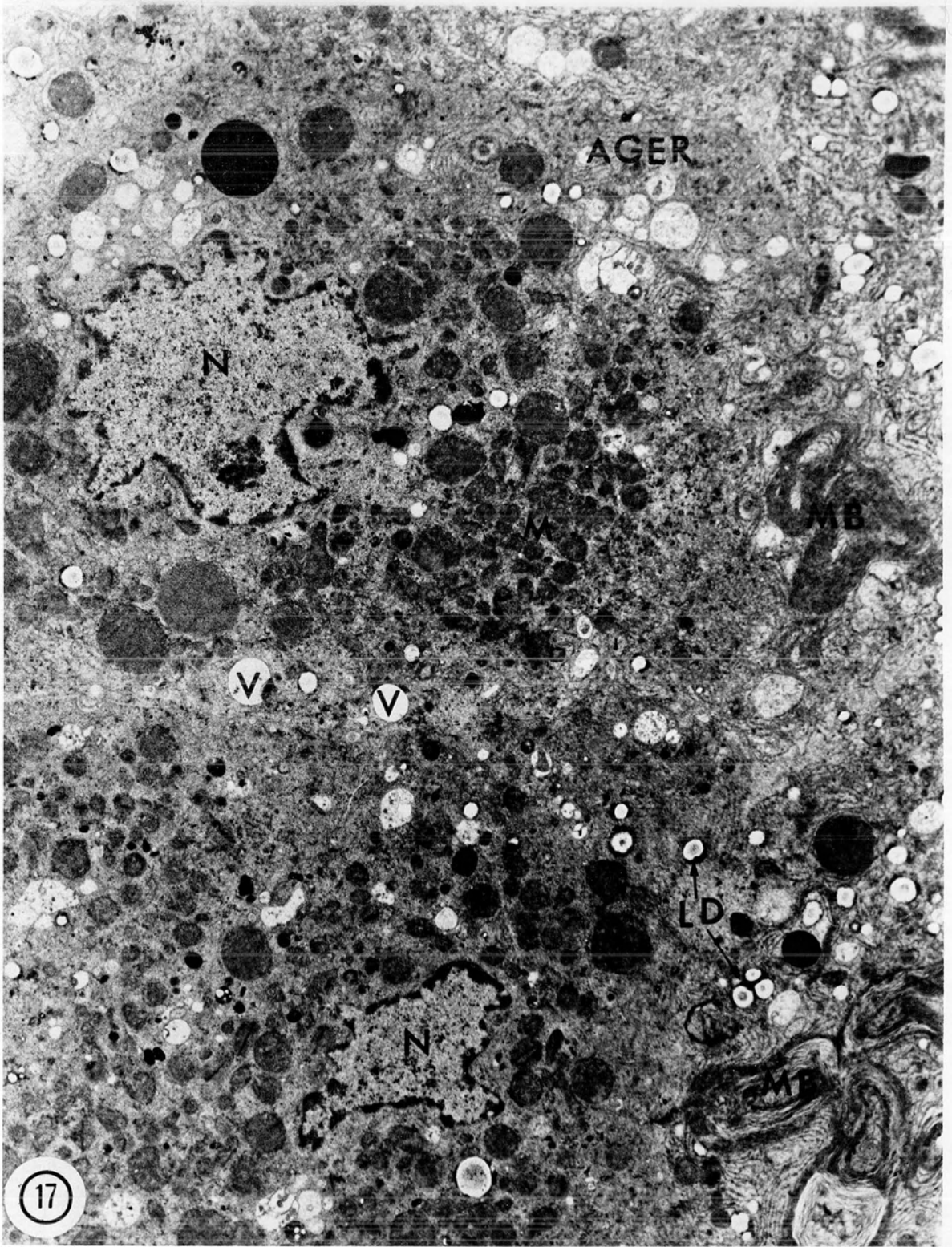
Fig. 17: The appearance of pyknotic nuclei (N) and concentric
and reticular myeloid bodies (MB) at eighteen hours
after acetaminophen treatment in the non-protected
animal. AGER:agranular endoplasmic reticulum.
M:mitochondria. LD: lipid droplets. x 5,400

AGER

N

M

MB

V V

LD

N

MB

M

17

18). Very often, there is extensive accumulation of large
aggregates of myeloid figures and lipid globules (Fig. 19).
Vacuolation consisting of electron-dense granular-like materials,
representing the fine structural counterpart of 'hydropic vacuo-
lation' encountered in light microscopy (Dixon et al, 1975), is
characteristic of necrotic parasinusoidal areas (Fig. 20).
It is perhaps noteworthy that most deaths from acetaminophen
overdose in hamsters occur within eighteen to twenty-four hours.
This observation is consistent with that reported by McLean and
Day (1974).

Cysteamine-protected acetaminophen-poisoned animals

The group sacrificed at six hours after acetaminophen treatment

The cytopathological sequence of events at six hours are
not much different from those observed in the non-protected
animals. While some cisternae of GER are arranged as parallel
strands studded with ribosomes, dilated cisternae can be seen in
some hepatocytes (Fig. 21). Glycogen depletion is clearly
evident; the mitochondria become slightly distended. Large
clear or electron-dense vacuoles and lipid droplets are occa-
sionally observed, but not in abundance. Likewise, a few
aggregates of smooth-surfaced membraneous bodies appear in the
cytoplasmic matrix.

The group sacrificed at twelve hours after acetaminophen treat-ment

Significant morphological difference is observed between

Fig. 18: Accentuated sinusoidal congestion at twenty-four
hours after acetaminophen treatment in the non-
protected animal. Note the phagocytosis of ery-
throcytes (RBC) by Kupffer's cell (K)& the nucle-
us of hepatocyte (N). The inclusion of polymor-
phonuclear leukocyte (PMN) within an enlarged
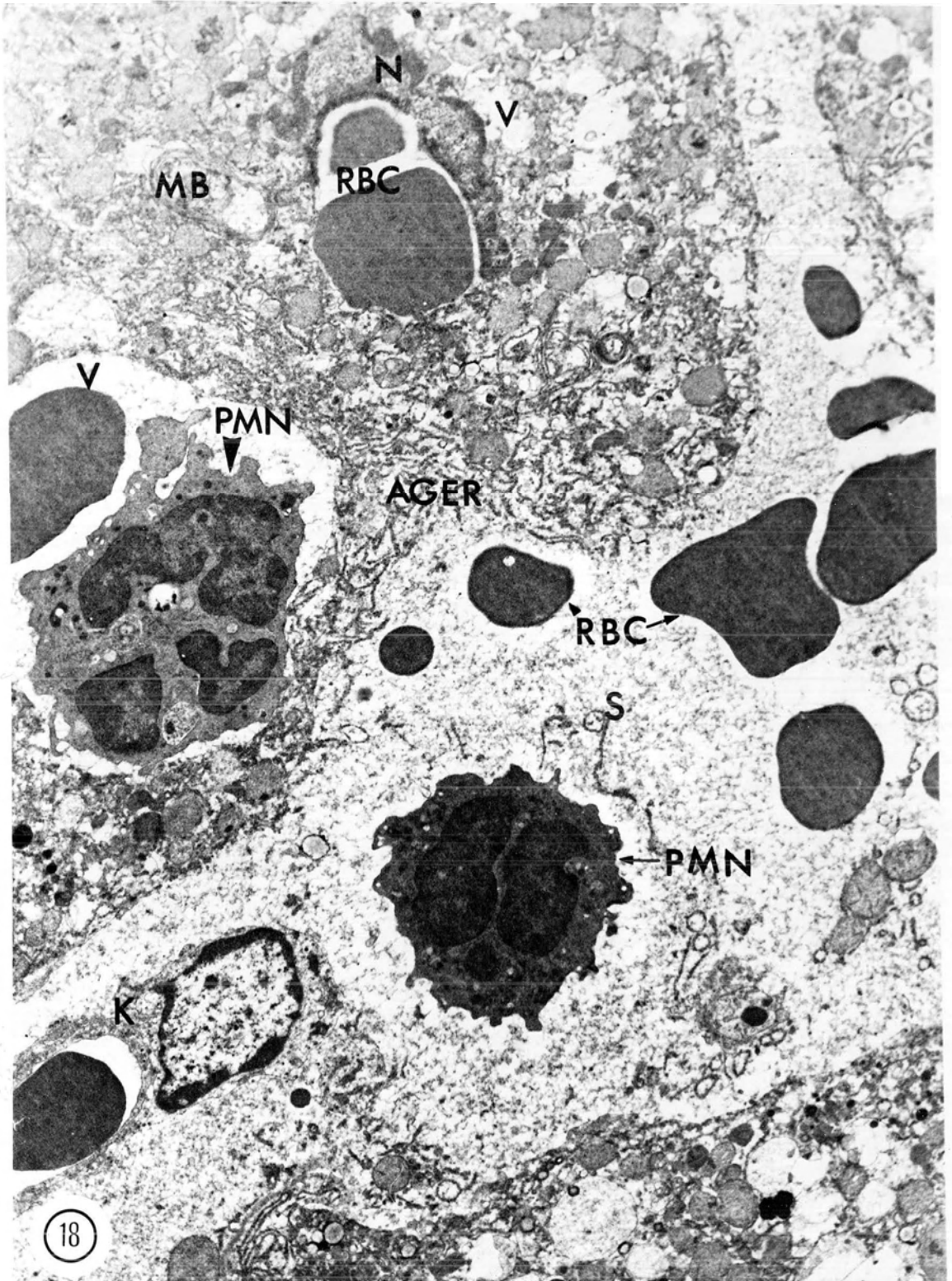vacuole (V) is seen. AGER:agranular endoplasmic
reticulum. x 5,400

Fig. 19: Accumulation of myeloid bodies (MB) twenty-four hours
after acetaminophen treatment in the non-protected
animal. Note the occurrence of heterogenous mutli-
vesicular bodies (arrowhead) and vesiculated materials
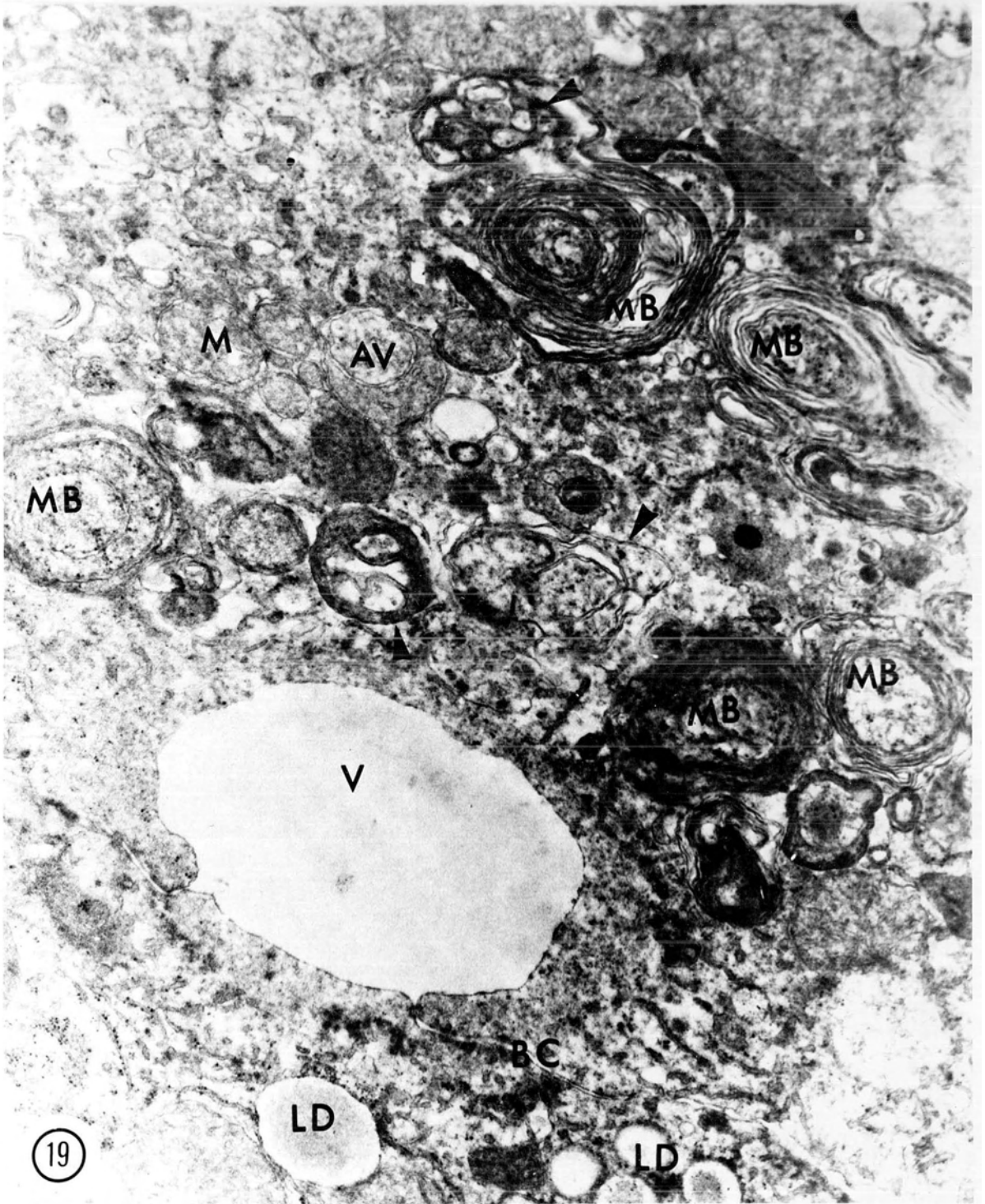within the myeloid bodies (MB). x 21,900

Fig. 20: Virtual obliteration of the sinusoidal space (S)
twenty-four hours after acetaminophen treatment
in the non-protected animal. Erythrocytes (RBC)
and vacuoles containing electron-dense materials
(V) can be seen in the sinusoid. KC: Kupffer's
cell. LD:lipid droplets. x 21,900
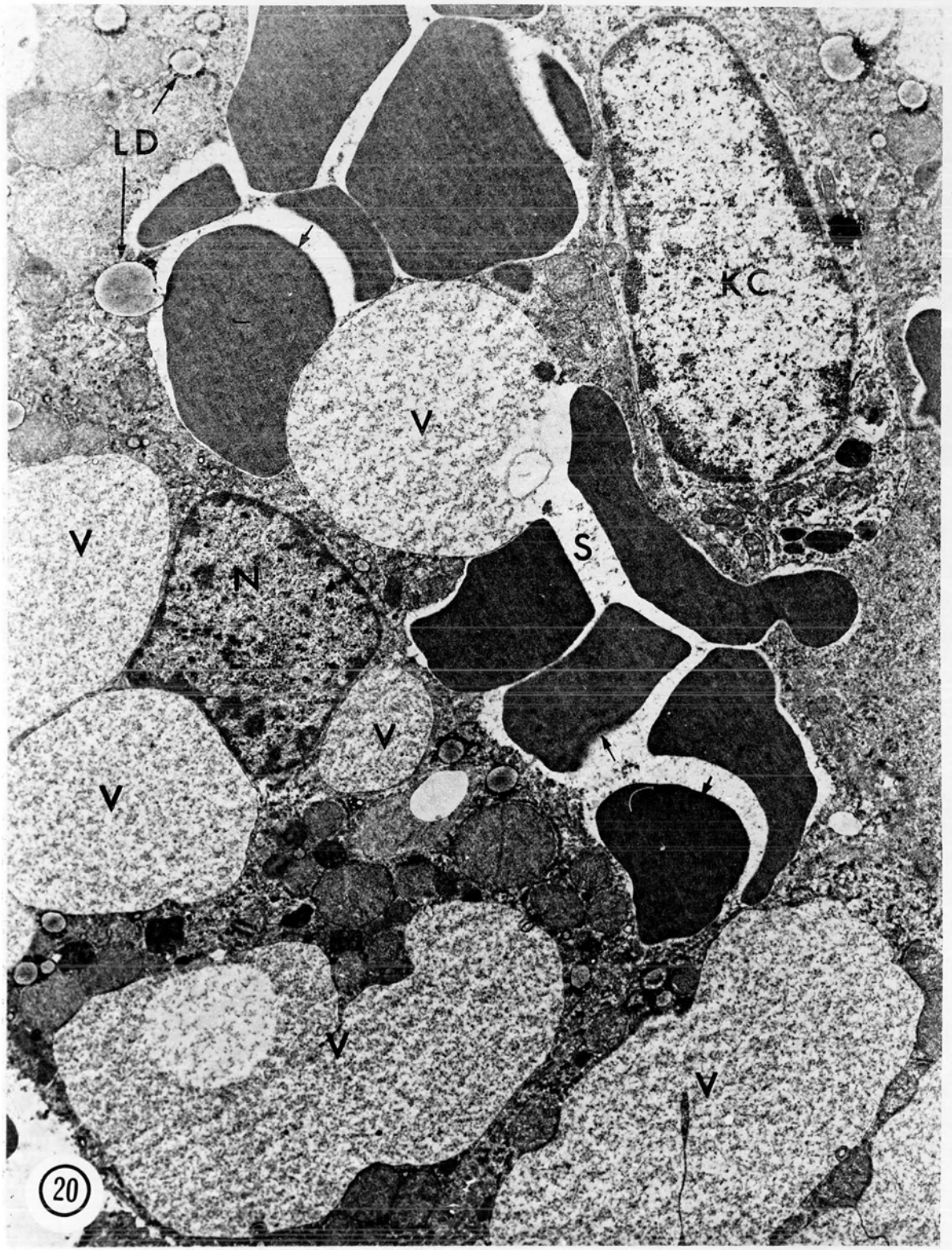
Fig. 21:   The normal configuration of the granular endoplasmic
           reticulum (GER) with ribosomes attached in the cystea-
           mine-protected animal six hours after acetaminophen
           treatment. Note the loose network of the agranular
           endoplasmic reticulum (AGER). x 62,700

Fig. 22:   Polysomal reaggregation (PR) twelve hours after acet-
           aminophen in the cysteamine-protected animal. AGER:
           agranular endoplasmic reticulum. x 62,700
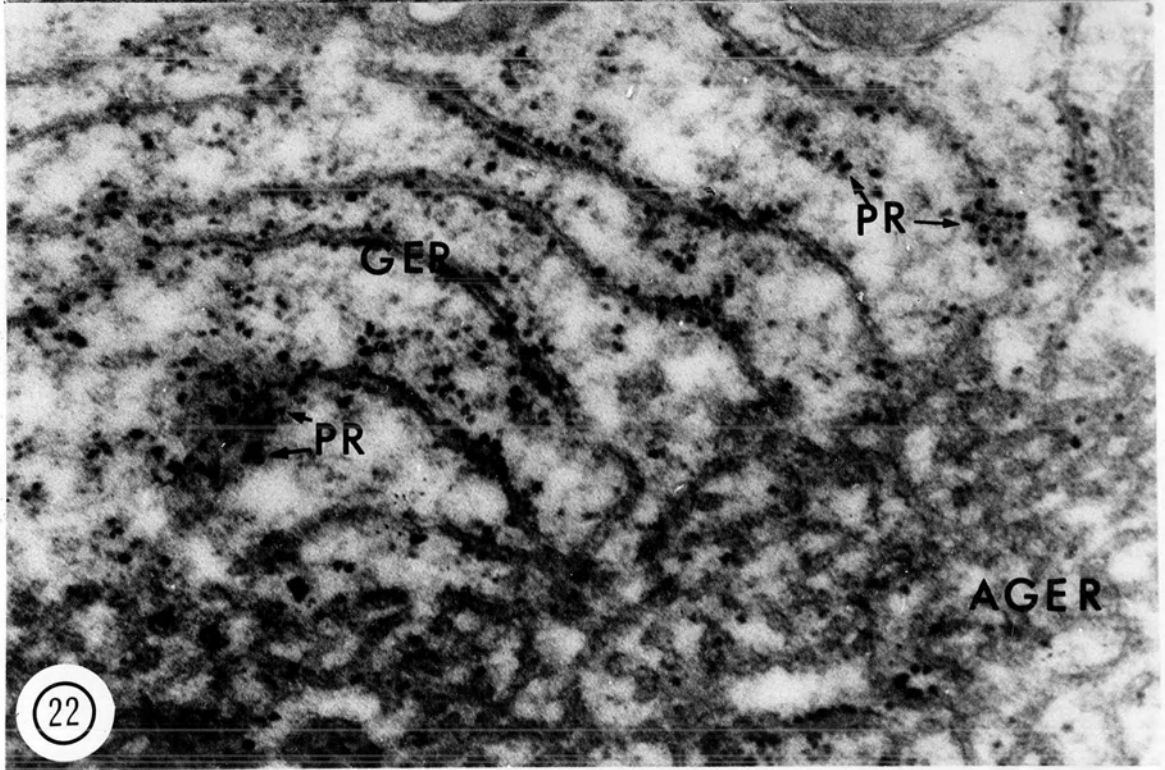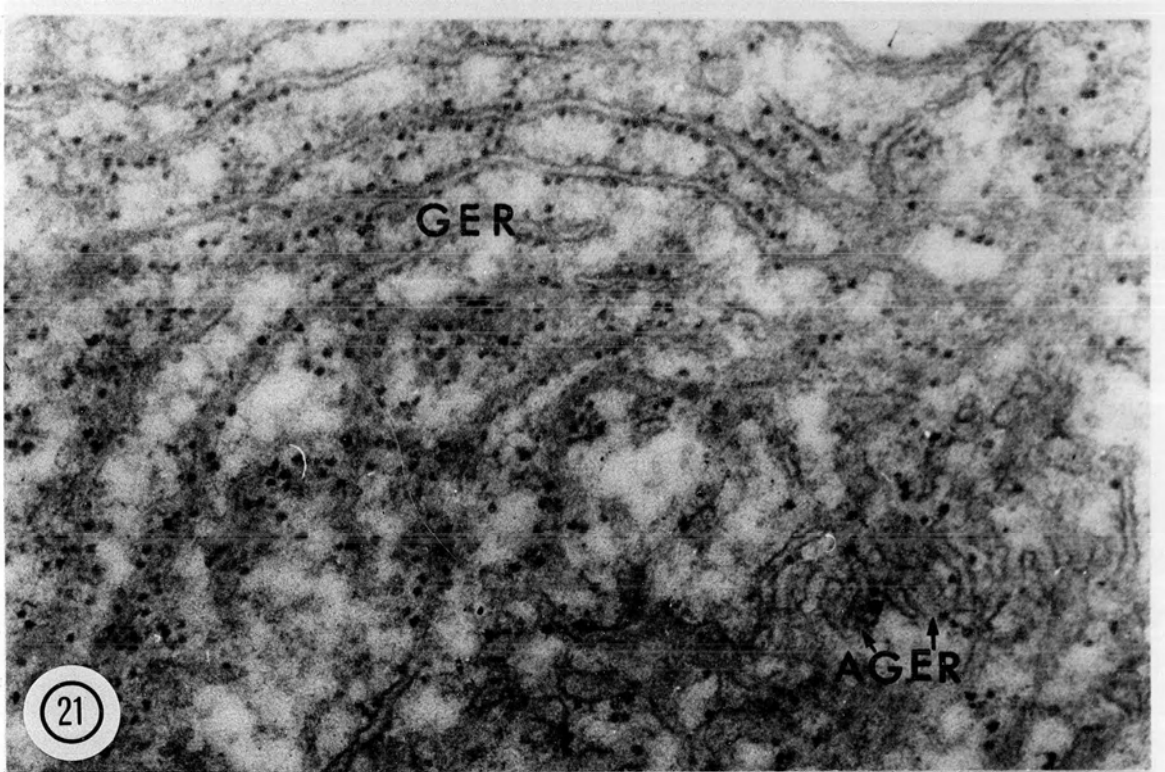
the protected and non-protected animals at twelve hours. The
GER displays the normal tubular arrangement with polysomal
aggregates attached to the strands. On the other hand, free
polysomes organized into geometric conglomeration distribute
themselves in an orderly fashion in the cytoplasmic matrix (Fig.
22). No condensation of the AGER occur, although the AGER
still undergo a certain degree of hyperplasia. Fatty infil-
tration of the necrotic hepatocyte, unlike the unprotected hepa-
tocyte, appears to be limited in nature, although small numbers
of membrane-enclosed lipid aggregates still occur in the cyto-
plasm (Fig. 23). Very few vacuoles, the ultrastructural counter-
part of 'hydropic vacuolation' encountered in light microscopy
(Dixon et al, 1975), can be seen (Fig. 24). In sharp con-
trast to the nonprotected animals, the hepatocytes in the cystea-
mine-protected animals are no longer characterized by extensive
accumulation of lamellated dense bodies. Sinusoid congestion
becomes less critical. Although glycogen depletion still per-
sists, abnormalities observed in mitochondria, nuclei and bile
canaliculus become relatively less in numbers.

The group sacrificed at eighteen hours after acetaminophen treatment

The hepatocytes from the cysteamine-protected animals ex-
hibit signs of partial recovery in that glycogen deposits re-
appear in the cytoplasm and the cisternae of GER tend to become
flattened (Fig. 25). Vesiculation of endoplasmic reticulum
does not occur. Polysomes reassemble themselves in a linear

Fig. 23: Limited accumulation of lipid aggregates (LDA)
twelve hours after acetaminophen treatment in
the cysteamine-protected animal. M:mitochondria.
x 21,900
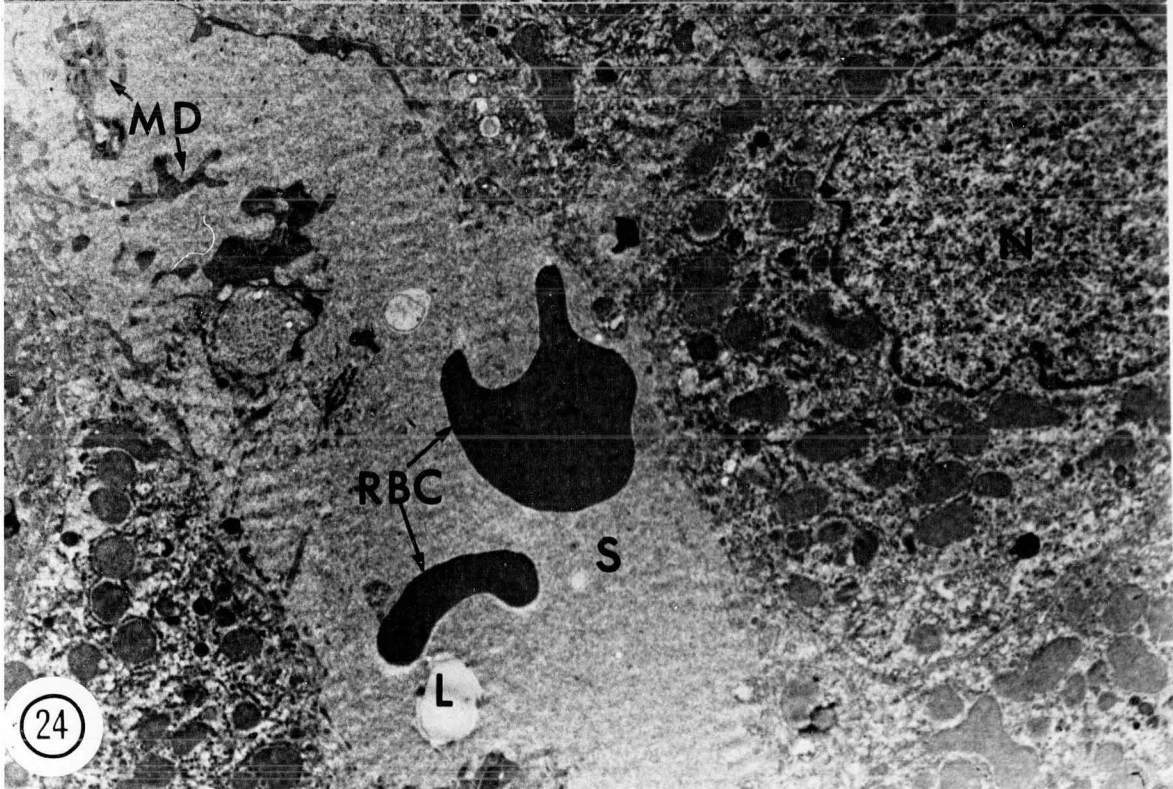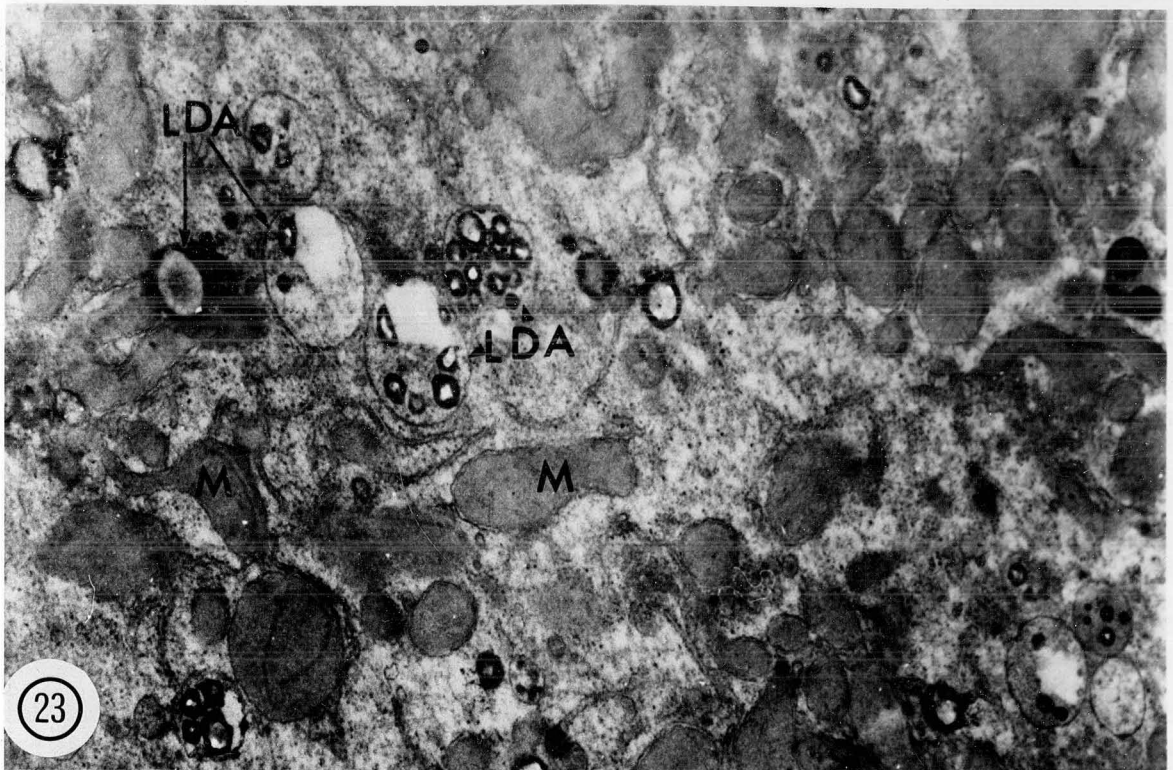
Fig. 24: Absence of parasinusoidal vacuoles twelve hours
after acetaminophen treatment in the cysteamine-
protected animal. The sinusoidal space (S) is
relatively free of any membraneous debris(MD).
L:lipid vacuoles. N:nucleus. RBC:erythrocytes.
x 5,400

Fig. 25:   Repletion of glycogen deposits (G) eighteen
hours after acetaminophen treatment in the
cysteamine-protected animal. The configuration
of the microvillal lining of the sinusoidal
space (S) appears normal. AGER:agranular
endoplasmic reticulum. V: vacuoles.
MV:microvilli. x 5,400

Fig. 26:   Ribosomal aggregates (PRA) seen at eighteen
hours after acetaminophen treatment in the
cysteamine-protected animal. Some lipid va-
cuoles (LD) still persist. M:mitochondria.
x 62,700

25

26
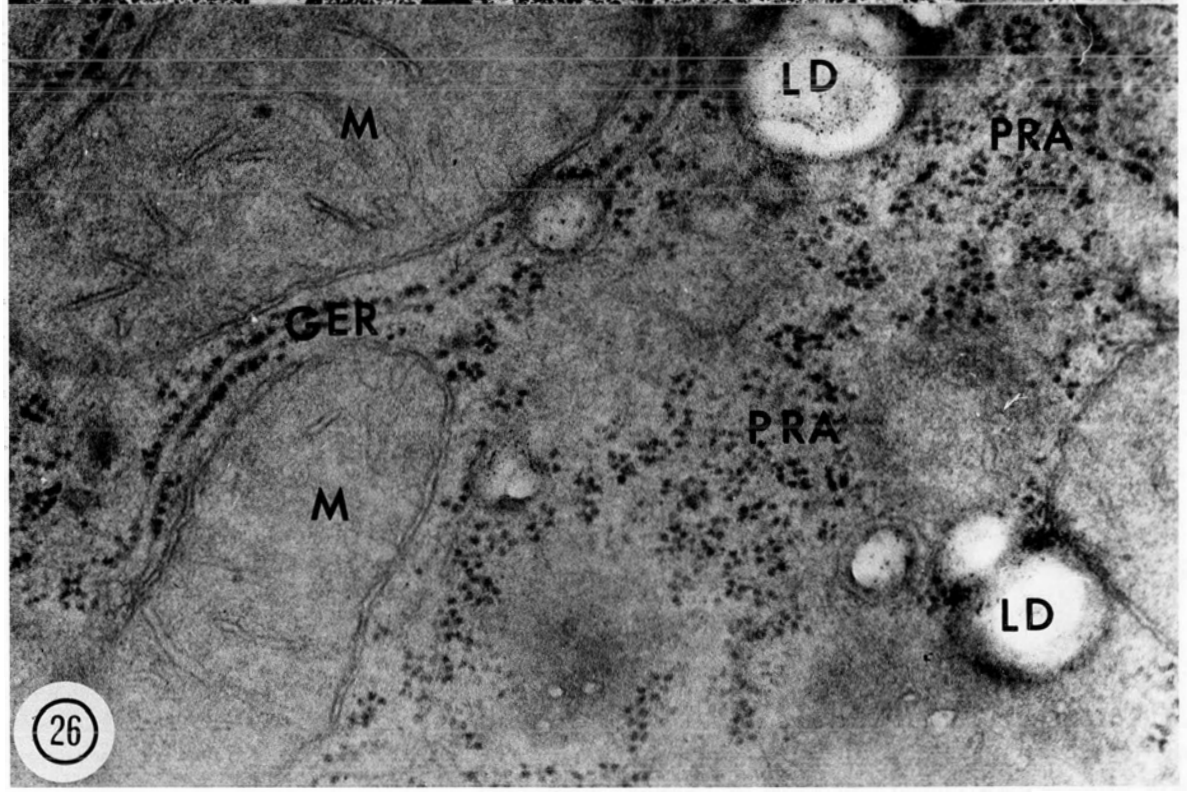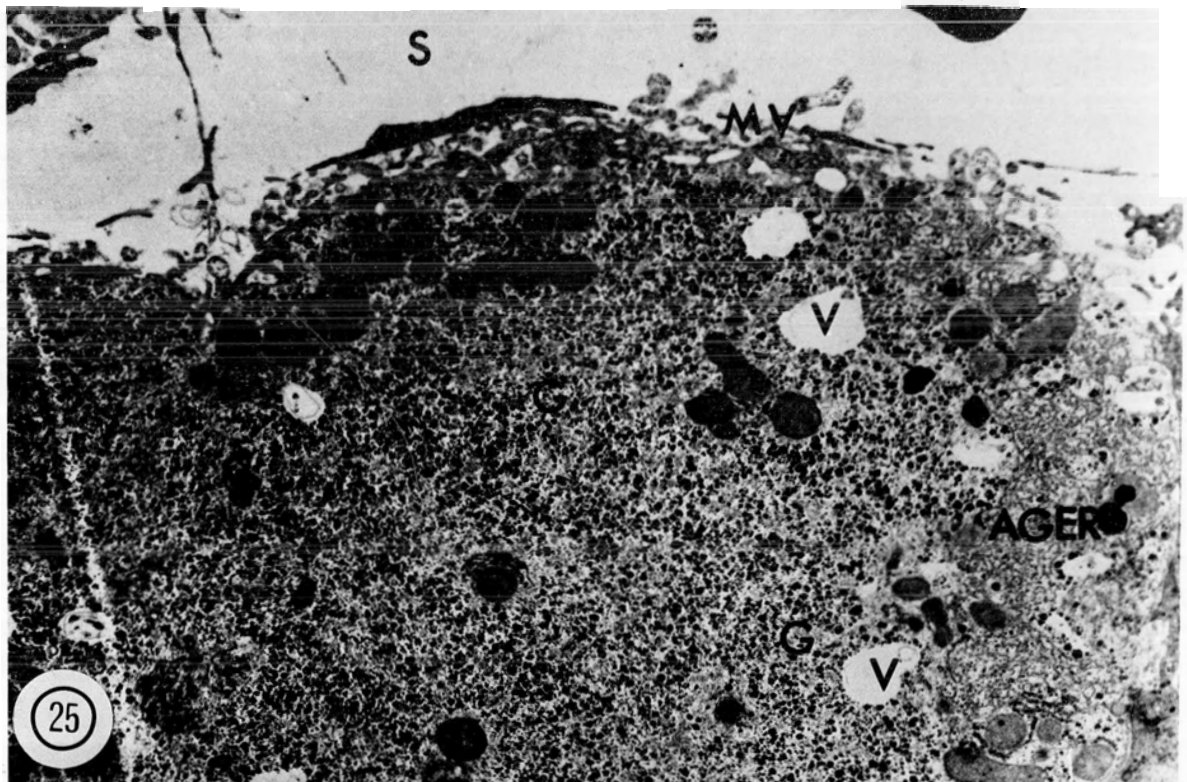
manner around the strands of granular endoplasmic reticulum and some well-organized ribosomal aggregates are visible in the cytoplasmic matrix (Fig. 26   ).   Small numbers of lipid vacuoles occur in some hepatocytes, but the morphological evidence for extensive lipid accumulation at this stage is absent.   The characteristic myeloid bodies as seen in nonprotected animals are entirely absent in the cysteamine-protected group eighteen hours after acetaminophen overdosage.   The microvillal arrangement of the endothelial lining of sinusoids returns to normal.   No degenerative changes are observed in the mitochondria, nucleus and canaliculus.   Pinocytotic vacuoles appear in some hepatocytes, but this cannot be considered to be a pathological finding (Fig. 25   ).

DISCUSSION

The results of this electron microscopic investigation of acetaminophen hepototoxicity shows that glycogen depletion, extensive fatty infiltration, ribosomal disaggregation, hyperplasia of the smooth endoplasmic reticulum and mitochondrial abnormalities and large vacuolations constitute the primary determinants of acetaminophen-induced hepatic necrosis.   In addition, accumulation of myeloid dense bodies appears to be associated with advanced stages of necrosis.   The sequential events leading to cellular death are consistent among the unprotected acetaminophen-intoxicated animals.   This witnesses a significant departure from the findings of Dixon et al (1975) who observed

that lipid accumulation and spherical cytoplasmic inclusion bodies did not feature the pathogenic mechanisms in severely poisoned rats. It is well recognized that rats are relatively resistant to hepatotoxic agents (McLean and Day, 1975) and consequently exhibit considerable variation in the ultrastructural changes of cellular organelles. After all, the panorama of pathological consequences substained may be dose-dependent. In the present study, a high toxic dose of acetaminophen (600 mg/ kg i.p.) was used, as contrasted with the oral dose of 300 mg/kg employed in Dixon's study (1975).

Although several studies have been conducted to investigate the protective mechanism of cysteamine (Strubelt et al, 1974; Mitchell et al, 1973b; Mitchell et al, 1974; Strubelt et al, 1974; Harvey and Levitt, 1976), no electron-microscopic data have been published. The present study is the first endeavour to evaluate cysteamine protection at the fine structural level. The results show that cysteamine treatment is responsible for the attenuation of lipid infiltration, ribosomal disaggregation, vacuolation, as well as curtailment of hyperplasia of the smooth endoplasmic reticulum. The relative absence of these histopathological features of the liver may be explained by the antioxidant properties of cysteamine, since reduced lipid peroxidation formation and enhancement in microsomal enzyme activities have been found in the previous study (Chapter III).

The early dilation of the cisternae of the rough endoplasmic reticulum is unlikely to be specific for acetaminophen,

since the same structural abnormality may be produced by various hepatotoxic agents including carbon tetrachloride (Leduc, 1973) and galactosamine (Scharnbeck et al, 1972). The course of pathological events after six hours in the nonprotected animals appear to resemble those observed in carbon tetrachloride poisoning (Reynolds et al, 1971). Extensive collapse of the cisternae of the rough endoplasmic reticulum occurs along with proliferation of labyrinthine aggregates derived from smooth-surfaced membranes. The finding that the smooth endoplasmic reticulum in cysteamine-protected animals did not condense to a tight network, strongly indicates that this phase of cellular function is related directly to the action of cysteamine. The fine structural integrity of the endoplasmic reticulum, as reflected by the attachment of ribosomes to the cisternae of rough endoplasmic reticulum, implicates that the protein-synthesizing machinery must be conserved.

Severe derangement of the endoplasmic reticulum, characterized by vesiculation and hyperplasia of smooth-surfaced membranes, is a constant feature of acetaminophen poisoning. This tends to substantiate the biochemical finding reported by Mitchell et al (1973b), Racz and McDonnald (1976) that loss of microsomal protein correlated directly with the loss of microsomal function.

Polysomal disaggregation is apparently prevented by cysteamine treatment, as evidenced from the preponderance of aggregates of ribosomes, at twelve and eighteen hours after acetaminophen treatment in cysteamine-protected animals. Although no

data are available on the covalent binding of the toxic metabolite of acetaminophen to nucleic acids and the associated polysomal profile, it is reasonable to assume that the arylating reactive intermediate may prevent the binding of ribosomes to mRNA. Polysomal disaggregation has been suggested as the predominant feature in carbon tetrachloride-induced liver injury (Shah and Carlson, 1975). In view of the protection offered by the antioxidant, N,N'-diphenyl-p-phenylenediamine against polysomal disaggregation in carbon tetrachloride poisoning (Hartman, et al, 1968), it is tempting to speculate that cysteamine may also inhibit the transition of polysomes to monosomes. Autoradiography involving the incorporation of radioactive amino acids into microsomal proteins might be successfully utilized to furnish conclusive evidence for this hypothesis.

The morphologic findings further confirm the role of lipid peroxidation in the pathogenesis of acetaminophen-induced fatty liver. The extensive infiltration of hepatic parenchyma with lipid vacuoles at twelve, eighteen and twenty-four hours in unprotected animals as indicated in this study is consistent with the elevation of hepatic triglyceride levels reported by Buttar et al (1976) in poisoned rats. However, no conclusive evidence can be adduced to support the contention that hyperplasia of the smooth endoplasmic reticulum is associated with the development of drug-induced fatty liver.

Disturbance in lipid metabolism may involve more than mere lipid peroxidative changes. Upon close examination, the lipid

vacuoles in the unprotected animals bear resemblances to the lipolysosomes first described by Nehemiah and Novikoff (1974) in hamsters, in that they are both enclosed by a peripheral membrane in close apposition to a homogeneous electron-dense layer. These lipolysosomes, which have been shown to contain acid phosphatase activities (Nehemiah and Novikoff, 1973), have been implicated in the degradation of intracellular lipids. The occurrence of relatively large numbers of lipolysosome-like structures during the evolution of hepatic lesion may represent a kind of adaptative change of the haptocytes in response to excessive lipid accumulation. Since it is very likely that lipase will be inactivated by the hepatotoxin, the formation of these specialized lipid vacuoles constitutes the alternative pathway for the mobilization of excess lipids from the liver. Indeed, this mechanism seems to explain the occurrence of lipolysosomes in hepatocytes from patients with Wilson's disease (Hayashi and Saernlieb, 1975). Quantitative estimation of the frequencies of lipolysosomes in a spectrum of diseases associated with fatty liver, either genetically endowed or drug-induced, will help elucidate the function of these organelles. Biochemical analysis of the composition and content of lipids will be indispensable.

Recent studies indicate that drug-induced myeloid body formation constitutes the tissue reaction to chemically-induced pathogenic stimuli (Hruban et al, 1972; Yates et al, 1968). Hypocholesterolemic drugs such as triparanol (Hruban et al,

1965) and antibiotics such as clindamycin (Gray et al, 1971) have

been shown to induce myeloid body formation in liver cells.

Morphologically, unicentric and multicentric myeloid bodies are

composed of concentric layers of smooth membranes enveloped by

denser single unit membranes; both types of lamellated dense

bodies may be found in the unprotected animals within twelve to

twenty-four hours after acetaminophen overdosage. The absence of

myeloid bodies in the cysteamine-protected animals further illus-

trates their relevance to the necrotic process. Although the

origin of these myeloid bodies remains controversial (Allison

1968; Dietert and Scallen, 1969), it is generally accepted that

they may arise by sequestration of various membranous cytoplas-

mic organelles which resist degradation by lysosomal enzymes

(Hruban et al, 1972). In view of the hyperplasia of the smooth

endoplasmic reticulum and the eventual denaturation of smooth-

surfaced membranes in acetaminophen poisoning, it is highly

likely that the membranous materials are derived from the AGER

although the possibility of fusion with autophagic vacuoles can-

not be eliminated. Since Mitchell et al (1973a) demonstrated

that most of radioactive acetaminophen was concentrated in the

endoplasmic reticulum, the toxic metabolite of acetaminophen

may become bound to the membranous inclusions of the myeloid

bodies. Triparanol has been found to bind with the sequestered

organelles within the lamellar bodies (Yates et al, 1967).

Further studies on the biochemical composition and morphologic

aspects of these bodies are required to confirm this hypothesis.

Nevertheless, the appearance of myeloid bodies in the later

stages of acetaminophen-induced hepatic necrosis suggests that they may lead to cellular death.

On the other hand, the appearance of these lamellated concentric bodies containing osmiophilic phospholipids in the human hepatocytes reflects a clinical condition termed 'phospholipid fatty liver' (Oda et al, 1970). The characteristic accumulation of abnormal phospholipids, mainly acidic phospholipids (Yamamoto et al, 1971), induced by drug treatments (Iglesia et al, 1974), suggests that phospholipid metabolism has been perturbed by the drug itself or some toxic metabolite formed by the microsomal drug-metabolizing enzymes. It should be recognized that the functional integrity of the microsomal NADPH-cytochrome P-450 electron transport chain depends upon the presence of phospholipids for the oxidative metabolism of steroids, fatty acids and foreign compounds (Strobel et al, 1970). Hence the hepatic drug-induced secondary lipidosis probably arises from the binding of acetaminophen with the membrane phospholipid with the result that the intrahepatic distribution of phospholipids is altered. It is anticipated that compositional alterations in phospholipids as manifested in the enhanced conjugate diene during the course of acetaminophen poisoning in unprotected animals, will undoubtedly contribute towards the occurrence of these pleomorphic concentric bodies seen in this study. It will be premature to establish a causal role of abnormal accumulation of phospholipids .. However, derangement of phospholipid metabolism may somehow facilitate the evolution of hepatic

necrosis and ultimate death.

The advanced stage of acetaminophen introxication is also marked by extensive vacuolation in the vicinity of the necrotic areas. Upon close examination, the vacuoles contain electron-dense granular-like materials, presumably derived from the vesiculation of the endoplasmic reticulum. In carbon tetrachloride hepatotoxicity, 'hydropic vacuolation' has been found to be associated with the marked dilation of the cisternae of the endoplasmic reticulum (Stenger, 1970). The pathological feature reflects continuous intracellular fluid accumulation resulting from alterations in membrane permeability characteristics. However, in view of the augmented lipid peroxidation observed in this study, it is highly likely that changes in physico-chemical properties of membrane phospholipids will impose drastic perturbation in the fluidity and lipid-protein interaction at the plasma membrane level, as has been shown in model membrane studies (Chapman, 1969). This witnesses the derangement of the regulatory mechanism governing the fluxes of ions and fluid across the membrane barrier, and explains the extensive vacuolation and fluid accumulation seen in unprotected animals.

It is noteworthy that in cysteamine-protected hepatocytes, no significant vacuolation occurs at twelve and eighteen hours after acetaminophen overdosage. Since cysteamine treatment has been shown to reduce appreciably the lipid peroxidation in vivo and in vitro (Chapter III), maintenance of the structural in-

tegrity of the membrane will prevent any abnormal accumulation of fluid around the necrotic areas. This further substantiates the view that lipid peroxidation is the determinant of acetaminophen hepatotoxicity.

It should be emphasized that the present study does not exclude the possibility that coagulative necrosis may be the predominant factor responsible for lethal cellular injury, as suggested by Dixon and his colleagues (1975). Sinusoidal congestion and erythrophagocytosis have been observed within twelve to twenty-four hours after acetaminophen intoxication in unprotected animals. The anoxia resulting from reduction in hepatic blood flow explains the appearance of large clear or moderately electron-dense vacuoles bordering the sinusoids. Indeed, Gazzard et al (1974) found that administration of vasodilating agents such as propanolol reduced the wedged-shaped infarct areas of hepatic parenchyma in acetaminophen-poisoned rats. However, Gazzard et al (1975) later found that disturbances in coagualtion in severely poisoned patients were transient and that fresh frozen plasma therapy did not seem to reduce the morbidity or mortality in treated patients. The vascular effects may, after all, be secondary and non-specific.

The pathogenic mechanisms for acetaminophen-induced hepatic necrosis probably reflects the fine interplay of polysomal disaggregation, vacuolation, derangement of phospholipid and neutral lipid metabolism, as well as the loss of functional integrity of the microsomal oxidative enzymes. The morphological features of acetaminophen hepatotoxicity revealed upon

electron microscopy further lend credence to the biochemical findings reported earlier in Chapter III, supporting the hypothesis that the facilitatory interaction of lipoperoxidation and microsomal oxidation is responsible for centrilobular necrosis. Moreover, the ultrastructural observation that cysteamine protection significantly attenuates lipid infiltration and vacuolation and partially maintained the polysomal profile, correlates with the biochemical finding regarding improvement of microsomal function and diminution in diene conjugation (Chapter III).

CHAPTER V

CONCLUDING REMARKS

The biochemical and electron-microscopic results suggest that the facilitatory interaction of microsomal lipid peroxidation and oxidation of acetaminophen to the toxic metabolite, plays an essential role in hepatotoxicity. Cysteamine treatment prevented the biochemical and morphological correlates of hepatic necrosis: derangement of microsomal enzymes, lipid accumulation and polysomal disintegration.

The results of this investigation have incidentally drawn attention to certain interesting aspects which merit further consideration. The postulate that N-hydroxylation and lipid peroxidation mediate acetaminophen hepatotoxicity, demands the lipoperoxidative process to be correlated with extent of oxidation reaction in vivo and in vitro. However, no serious attempts have hitherto been made to synthesize N-hydroxyl-acetaminophen and correlate the evolution of the hepatic lesion with kinetics of oxidation. Furthermore, it is not known whether irreversible binding of the toxic metabolite of acetaminophen with microsomal lipids precedes, or occurs concurrently with, the peroxidative process and the associated liver injury. In this respect, whole animal studies involving the administration of radioactive acetaminophen can elucidate the nature and degree of binding in a dose-related manner.

The appearance of myeloid figures at the advanced stage of necrosis implicates that abnormal phospholipid metabolism may

contribute towards cellular death. This warrants further studies on the changes in the content and composition of phospholipids.

Although the data in this study indicates that cysteamine acts by inhibiting microsomal oxidation, the possibility exists that it may conjugate with the toxic metabolite. Although Andrews (1976) failed to detect any cysteamine-acetaminophen conjugate in the bile in isolated perfused liver of the rats, the evidence is far from conclusive. Definitive conclusions may only be drawn from whole animal studies involving the administration of radioactive acetaminophen and cysteamine. However, neither the metabolic disposition of cysteamine nor the "reactive species" of cysteamine responsible for detoxification is known for certain. It is therefore all the more surprising that cysteamine exerts its protective effects as late as eighteen hours after acetaminophen overdose.

In humans, Prescott et al (1976) found that cysteamine was effective in severely poisoned patients if administered within ten hours. The results of this study further support Prescott's clinical experience. No serious complications were encountered in cysteamine-protected experimental animals. Very recent studies indicated that octylimidazole may be more effective than cysteamine and methionine in interfering with microsomal oxidation (Hughes and Levitt, 1976). More detailed studies are required to elucidate the protective mechanism of octylimidazole.

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