RIBONUCLEOTIDE REDUCTASE IN DIVIDING CELLS: PURIFICATION AND INHIBITION STUDIES WITH 4-HYDROXYNONENAL

A thesis submitted for the degree of Doctor of Philosophy

by

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ABSTRACT

1). The effect of temperature, P_{450} inhibitors (pyrazole and imidazole), sulphydryl reagents (iodoacetamide and N-ethyl maleimide) and glutathione on the activation of CCl_4 in rat liver microsomes was studied. Spin trapping of $CCl_3\cdot$, covalent binding of CCl_4 to protein and CCl_4-dependent MDA formation were used as indices of CCl_4 metabolism. Formation of PBN-$CCl_3\cdot$ adduct, $^{14}CCl_4$ covalent binding to protein and CCl_4-dependent MDA production were dependent on temperature range from 15-40°C. The transition temperature was at 26.7-27.5°C when the activation was measured by formation of PBN-$CCl_3\cdot$ adduct and specific $^{14}CCl_4$ covalent binding. The transition temperature was found to be 34.3°C when CCl_4-dependent MDA production was taken as the index of the activation of CCl_4. Pyrazole, imidazole and iodoacetamide inhibited CCl_4-dependent MDA formation only at high concentrations (10-20 mM), whereas glutathione showed a strong inhibitory effect on CCl_4-stimulated lipid peroxidation. MDA formation was nearly 100% inhibited by 1 mM GSH. GSH also delayed the onset of lipid peroxidation. N-ethyl maleimide (NEM) exerted biphasic effects on CCl_4-dependent MDA formation. The lower concentration of NEM (0.5 mM-1 mM) reduced the MDA production, while the higher concentration of NEM (5-10 mM) enhanced the MDA formation.

2). Ribonucleotide reductase was partially purified from juvenile normal rat liver. The enzyme was purified 30 fold after DEAE-cellulose chromatography. The CDP reductase activity in tissues with different growth states or rates was compared. The enzyme activity was developed well in juvenile rat liver, regenerating liver and hepatoma (cells), while the enzyme activity was undetectable in adult rat liver and sham-operated rat liver. The enzyme activity in Yoshida cells was 3-fold of the activity in Morris 5123tc tumours. Dithiothreitol (DTT) activated the activity of CDP reductase from 48h and 60h regenerating liver, but DTT did not activate the enzyme activity of juvenile normal rat liver. The possible mechanism of the activation of enzyme activity by DTT was discussed and a mechanism of regulation of the ribonucleotide reductase activity in regenerating liver was suggested.

3). The effect of the lipid peroxidation product 4-hydroxynonenal (HNE) on CDP reductase from juvenile normal rat liver was investigated. HNE inhibited the CDP reductase activity. The inhibition was dependent on the concentration of HNE and the incubation time. The enzyme activity was reduced 50% by 0.1 mM HNE. The inhibitory effect of HNE was irreversible. DTT protected the enzyme against HNE suggesting that HNE inhibited the activity of ribonucleotide reductase from rat liver through the mechanism of blockage of functional SH groups in the enzyme protein.

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1. INTRODUCTION

Carbon tetrachloride (CCl_4) is a classic hepatotoxin that has been shown to be activated by enzymic processes to a reactive intermediate. Lipid peroxidation is involved in some of the damaging actions of CCl_4. Lipid peroxidation is a free radical mediated process and during the process of lipid peroxidation, a variety of products are produced. Among these products, 4-hydroxyalkenals have a very important biological activity. They have effects on DNA synthesis and cell proliferation. Ribonucleotide reductase is a key enzyme for DNA synthesis: it catalyzes the conversion of ribonucleotides to deoxyribonucleotides which are DNA precursors. It is well established that the activity of the enzyme correlates closely with tissue and cell growth. This introduction is divided into three sections, the activation of CCl_4 and CCl_4-induced lipid peroxidation, the biological activity of 4-hydroxyalkenals and ribonucleotide reductase.

1. 1 PART I ACTIVATION OF CARBON TETRACHLORIDE AND LIPID PEROXIDATION

Carbon tetrachloride (CCl_4) is a very toxic substance that can cause liver injury in many species with a predictable dose-dependence. It is readily available in pure form and its toxic effects have been studied for many years. The concepts emerged from such studies are helpful for understanding of biochemical mechanisms of other hepatotoxins (Slater, 1984; Slater et al., 1985; Cheeseman et al., 1985).

1. 1. 1 Activation of CCl_4

1. 1. 1. 1 Activation of CCl_4

Although the damaging actions of CCl_4 on the liver were recognised in 1930s, it only was in the 1960s that a fundamental theory was established that CCl_4 has to undergo metabolic activation in order to exert its full range of hepatotoxic effects. The mechanism depended on the metabolism of CCl_4 in the hepatic endoplasmic reticulum to a free radical product capable of initiating lipid peroxidation (Slater, 1966; Slater et al., 1985; Cheeseman, et al., 1985).

CCl_4 is activated through interaction with the NADPH-cytochrome P_{450} electron transport chain, which is located in the endoplasmic reticulum in many types of cell and involves the flavoprotein NADPH-P_{450} reductase and cytochrome P_{450} together in a phospholipid environment (Slater, 1984). The activation of CCl_4 proceeds reductively. The first intermediate of the activation of CCl_4 is believed to be a trichloromethyl radical ($CCl_3\cdot$) (Slater, 1966,1982;

Slater et al., 1985).

$$CCl_4 + e^- \text{——} CCl_4^{-\cdot} \text{——} CCl_3\cdot + Cl^-$$

In principle, the electron donor is NADPH and the electrons are transferred via cytochrome P_{450} reductase to cytochrome P_{450} (see Fig. 1.1.1).

The free radical of $CCl_3\cdot$ is very reactive and can undergo several secondary reactions (see Fig. 1.1.2). It reacts very quickly with oxygen to yield the trichloromethyl peroxyl radical ($CCl_3O_2\cdot$) which is even more reactive than $CCl_3\cdot$ and is believed to be the species that initiates lipid peroxidation (Slater et al., 1985). The $CCl_3\cdot$ radical is very efficient in forming stable covalent bonds with suitable molecules; proteins and lipids are frequent targets (Slater, 1982; Slater et al., 1985).

$CCl_3\cdot$ cannot be measured directly but can be detected using ESR spectroscopy with the spin trap phenylbutyl nitrone (PBN) (Albano et al., 1982; Slater et al., 1985). PBN can react with reactive free radicals, like $CCl_3\cdot$ to form a relatively stable spin-adduct which is detectable by ESR (see Fig.1.1.3). The spin-trapping of $CCl_3\cdot$ by PBN, along with the covalent binding of radiolabelled CCl_4 to microsomal macromolecules and CCl_4-induced lipid peroxidation are useful methods to measure CCl_4 activation indirectly (Cheeseman et al., 1985).

The site of activation of CCl_4 is probably at cytochrome P_{450}. As reviewed by Cheeseman et al. (1985), the conclusion of the role of cytochrome P_{450} in CCl_4 action was drawn from the evidence outlined below: 1) after depletion of cytochrome P_{450} by $CoCl_2$,

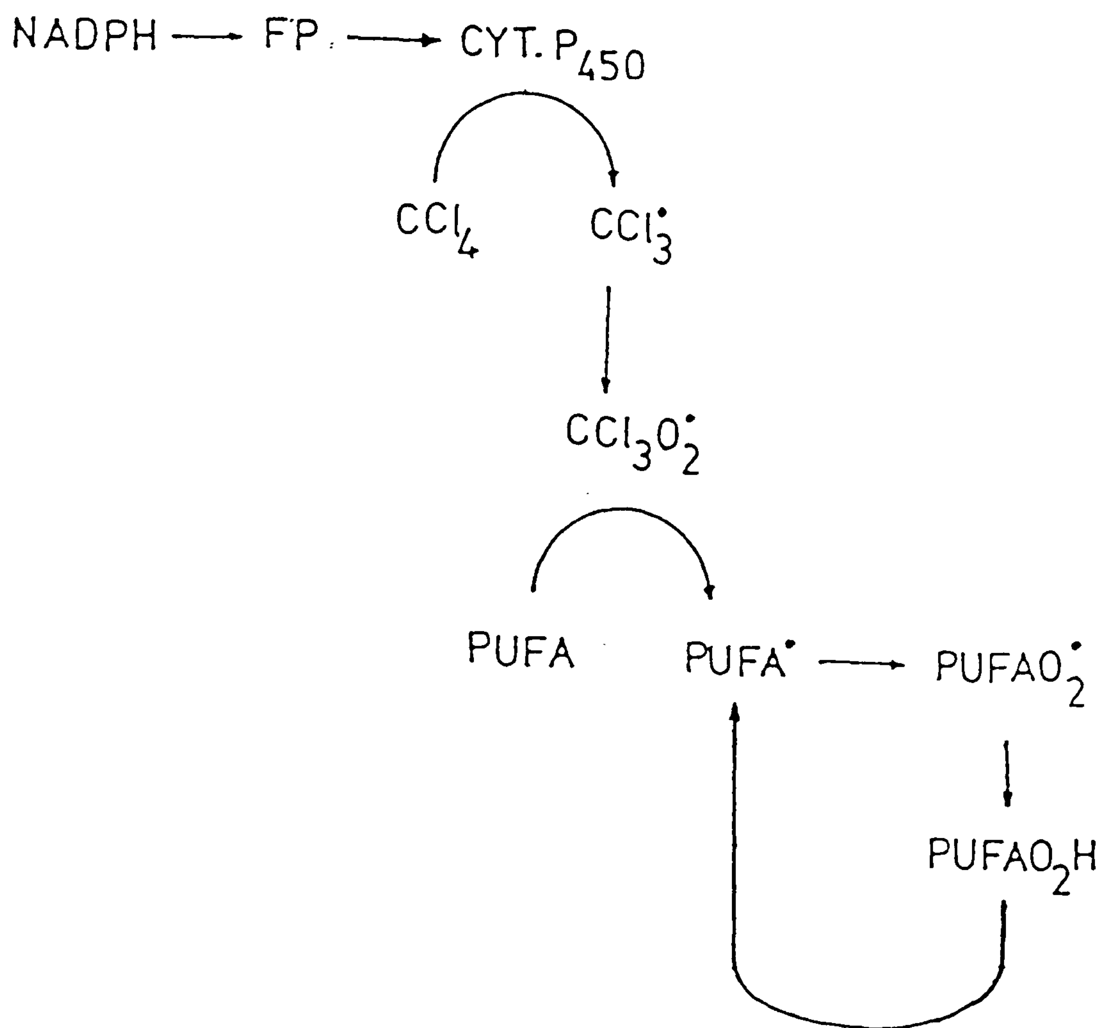


Fig. 1.1.1 Activation of CCl_4 through the NADPH-P_{450} electron transport chain

FP: flavoprotein (NADPH-P_{450} reductase)
(Modified from Slater, 1982)

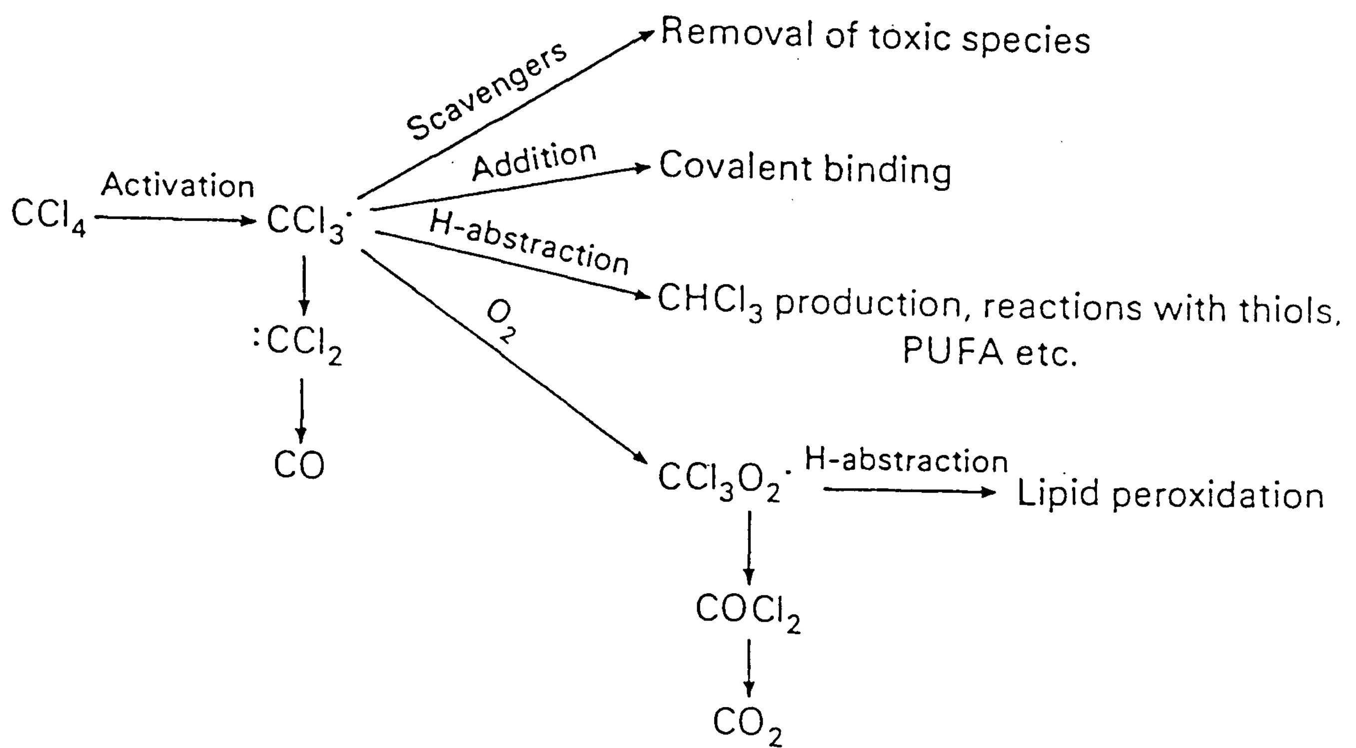


Fig. 1.1.2 Activation and reactions of CCl_4

(Adapted from Slater, 1989)

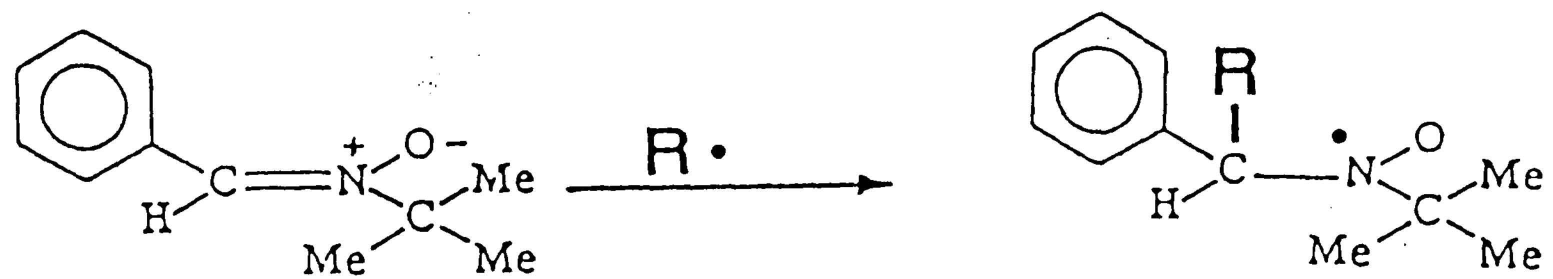


Fig. 1.1.3 PBN - spin adduct formation

(Adapted from Cheeseman et al., 1985)

allylisopropylacetamide and cobalt protoporphyrin *in vivo*, the metabolism of CCl_4 *in vitro* is decreased; 2) the reconstituted systems containing the components of mixed-function oxidase (MFO) system do not metabolise CCl_4 unless the hemoprotein is included; 3) CCl_4 activation is stimulated by the induction of cytochrome P_{450} by drugs such as phenobarbitone, ethanol, benzene, or imidazole; 4) the covalent binding of CCl_4 to microsomal protein is largely to cytochrome P_{450} molecules. Using purified enzymes, NADPH-cytochrome P_{450} reductase and cytochrome P_{450}, Wolf et al. (1980) demonstrated that the enzyme responsible for the metabolic activation of CCl_4 is cytochrome P_{450} rather than NADPH-cytochrome P_{450} reductase. Cytochrome P_{450} can catalyse the reduction of CCl_4, but NADPH-cytochrome P_{450} reducatse alone cannot reduce CCl_4.

However, the studies with classical inhibitor of the MFO system have yielded equivocal results concerning the precise locus of CCl_4 activation along the microsomal electron transport chain. The MFO inhibitors, p-chloromercuribenzoate (pCMB), SKF 525A, CO, menadione and metyrapone have little effect on CCl_4 activation. This may be because CCl_4 is not a typical substrate of P_{450} and its metabolism depends on a specific P_{450} isoenzyme that can be induced by several drugs (Cheeseman et al., 1985). For example, both phenobarbital (PB) and 3-methylcholanthrene (MC) can induce P_{450}, but metabolism of CCl_4 is enhanced only by PB-induction, MC-induced microsomes are equivalent to microsomes from untreated animals (Frank et al., 1982). The work of Noguchi et al. (1982) also demonstrates that the metabolism of CCl_4 to $CCl_3\cdot$ radical is not a general property of the multiple forms of cytochrome P_{450} in microsomes of phenobartital-induced rats. It was limited to a particular form, the 52,000 dalton P_{450} cytochrome which is the first

polypeptide to disappear in liver microsomes treated with CCl_4. Johansson and Ingelman-Sundberg (1985) indicated that the ethanol-inducible form of liver microsomal cytochrome P_{450} ($P_{450\ IIEI}$) was responsible for CCl_4-induced lipid peroxidation. $P_{450\ IIEI}$ can be induced by ethanol in rabbits liver and imidazole or pyrazole in rat liver. In reconstituted membrane system this type of P_{450} was a 100-fold more effective catalyst of CCl_4 metabolism than either of the cytochrome $P_{450\ LM2}$ or $P_{450\ LM4}$. Lauriault et al. (1992) have the same conclusion that ethanol inducible $P_{450\ IIEI}$ is a major catalyst of the microsomal metabolism of CCl_4. Antibody to $P_{450\ IIEI}$ markedly inhibits the metabolism of CCl_4.

There are other indications that the activation of CCl_4 is closely associated with P_{450}. Administration of CCl_4 into the peritoneum produces necrosis mainly in the centrilobular zone of the liver (Slater et al., 1985). Very recent work of Azri et al. (1992) confirms this observation. Using precision-cut slices of liver as the experimental material, they further examined the damage to rat liver by CCl_4. They observed that significant leakage of glucose-6-phosphate dehydrogenase and ß-glucuronidase from centrilobular hepatocytes occurred 9h following CCl_4 administration. The content of the enzymes that are native to periportal (lactate dehydrogenase and sorbitol dehydrogenase) was unchanged in the same slices over the duration of the experiment. A major contribution to this lobular location of injury is the distribution of cytochrome P_{450}. It is known that cytochrome P_{450} is more concentrated in the centrilobular regions of rat liver than in the periportal regions (Gooding et al., 1978; Slater et al., 1985).

Covalent binding of $^{14}CCl_4$, adduct formation with the spin trap PBN and CCl_4-stimulated lipid peroxidation *in vitro* are generally

correlated with the tissue distribution of P_{450} (Slater et al., 1985). 4- and 14-day-old rats are not as capable as adults in their ability to metabolize CCl_4 measured by binding of $^{14}CCl_4$ to microsomal protein and lipid (Cagen and Klaassen, 1980). The concentration of cytochrome P_{450} in new born rat liver microsomes is 2-3 times lower than in adult rat liver (Benedetto, 1981). With microsome preparations from kidney, lung, brain. testis and adrenal, no significant activity for the CCl_4-stimulated peroxidation or of $^{14}CCl_4$ covalent binding was found (Benedetto et al., 1981; Kornbrust and Mavis, 1980). The concentration of P_{450} in microsomal suspensions from these tissues is very low in comparison with liver (Benedetto et al., 1981).

The above discussion is particularly concerned with activation of CCl_4 in microsomes. More recently, it has been observed that CCl_4 can be activated to $CCl_3\cdot$ in rat liver mitochondria (Tomasi et al., 1987). It is found that incubation of liver mitochondria in a medium containing succinic acid, ADP, PBN and in the presence of CCl_4 resulted in the formation of a PBN-spin adduct and lipid peroxidation measured by conjugated diene production. Inhibitors of microsomal monooxygenase at high concentrations, SKF 525A (1 mM), metyrapone (0.2 mM) and p-CMB (0.1 mM) did not affect prodution of $CCl_3\cdot$. However, inhibitors of the respiratory chain such as antimycin A and KCN decreased free-radical formation. Tomasi et al. suggested that the mitochondrial electron-transport chain was responsible for the activation of CCl_4 and cytochrome a_3 was involved in the reduction process, probably by a mechanism similar to that of cytochrome P_{450}-mediated activation.

1. 1. 1. 2 Factors influencing the activation of CCl_4

Many factors, such as the activity of the drug-metabolizing

enzymes, temperature, oxygen concentration and hormones can affect the activation of CCl_4 and therefore influence the liver injury induced by CCl_4.

As early as 1966, McLean and McLean showed that the hepatotoxic action of CCl_4 was increased by prior induction of the drug-metabolizing enzyme system. Since cytochrome P_{450} is the site of activation of CCl_4, any effect which modifies P_{450} activity will influence the activation of CCl_4. Cytochrome P_{450} is a collective term for a large family of hemoproteins associated with the metabolism of drugs (Nebert and Negishi, 1982). P_{450} may function as a monooxygenase, a dioxygenase, an oxidase and as a peroxidase (Bast and Haenen, 1990).

The acute lesion (fatty degeneration and necrosis) can be modified by various pretreatments that affect the activity of the NADPH-cytochrome P_{450} chain (Slater, 1984). Induction of microsomal cytochrome P_{450} by drugs such as phenobarbital, ethanol, isopropanol, benzene and imidazole enhances the activation of CCl_4 and increases its ability to cause lipid peroxidation in liver microsomes. These drugs can induce isoenzymes of liver microsomal cytochrome P450 (Cheeseman et al., 1985; Noguchi et al., 1982; Ingelman-Sundberg and Jornvall, 1984; Johansson and Ingelman-Sundberg, 1985; Hung, et al., 1983). Ethanol treatment of rats caused a 25-fold induction of the microsomal CCl_4-dependent lipid peroxidation. Imidazole and pyrazole enhanced the CCl_4-induced lipid peroxidation in rabbit liver microsomes by 4-5-fold (Johansson and Ingelman-Sundberg, 1985).

In some cases, inhibition of activity of the cytochrome P_{450} system leads to suppression of the activation of CCl_4. Although "classical" inhibitors of cytochrome P_{450} like SKF 525A, CO and metyrapone have little effect on the activation of CCl_4 at lower

concentration as discussed above, high concentrations of these inhibitors still show the effect of reducing CCl_4-stimulated lipid peroxidation or $^{14}CCl_4$ covalent binding to protein or spin trapping of $CCl_3\cdot$ in rat liver microsomes (Cheeseman et al., 1985). Metyrapone at 4 mM still had no effect on Fe^{2+} stimulated peroxidation, but lipid peroxidation induced by CCl_4 was inhibited by 93% (Kornbrust and Mavis, 1980). For more examples, several other drugs are mentioned here to show inhibition of CCl_4 activation due to decrease in the cytochrome P_{450} activity. pCMB (200 nmol/mg protein) produced a 90% loss of P_{450} activities and caused a similar reduction in the extent of peroxidation induced by CCl_4, although it had little or no effect on the cytochrome P_{450} reductase (Kornbrust and Mavis, 1980). Cobaltic protoporphrin IX (CPP) profoundly decreased the level of cytochrome P_{450} in rat liver microsomes; consequently, the associated mixed-function oxidase systems were equally depressed and activation of CCl_4 was much lower in liver microsomes from CPP-treated rats (Cheeseman et al., 1984). Methoxsalen (8-methoxypsoralen) is one of the psoralen derivatives (Labbe et al., 1987). Cytochrome P_{450} content and monooxygenase activities decreased by 60-90% in mice administrated with methoxsalen (250 μmol/Kg). Covalent binding of $^{14}CCl_4$ to protein and CCl_4-induced lipid peroxidation were also inhibited by the same extent. However, lipid peroxidation mediated by reduced endogenous iron was not modified by methoxsalen showing that this drug does not act as a free radical scavenger but prevents CCl_4 activation by decreasing cytochrome P_{450} activity (Labbe et al., 1987). Silymarin is an extract from the wild thistle *Silybum marianum(L.)* Gartneri (Letteron et al., 1990). Silymarin has been shown to protect animals against various hepatotoxins including CCl_4. It inhibits CCl_4-mediated lipid peroxidation in mouse liver *in vivo* evidenced by exhalation of

ethane and in mouse liver microsomes *in vitro* shown by MDA production. Treatment of silymarin also decreases the covalent binding of $^{14}CCl_4$ to microsomal protein *in vivo* and *in vitro*. The inhibition mechanism is apparently due to its inhibition of some monooxygenase activity (Letteron, et al., 1990). Thiol drug diethydithiocarbamate (DEDC) and its two metabolites, disulfiram (DS) and carbon disulfide (CS_2) markedly decreased the activity of aniline hydroxylase, p-nitroanisole-O-demethylase and pentoxyresorufin-O-dealkylase in rat liver microsomes (Lauriault et al., 1992). Hepatocytes isolated from rats treated with DEDC or DS and CS_2 were resistant to CCl_4-induced cytotoxicity and lipid peroxidation.

The concentration of O_2 influences the activation of CCl_4. Kieczka and Remmer (1981) observed that in rat liver microsomes $^{14}CCl_4$ binding to protein and lipid increased continuously with decreasing oxygen concentrations. However, CCl_4-induced MDA formation showed an atypical oxygen dependency with a maximum at about 7% O_2 and a minimum at about 15% O_2. De Groot and Noll (1988 a, b) investigated this subject extensively. They also found that the CCl_4-mediated lipid peroxidation was strongly dependent on the oxygen partial pressure (pO_2). In NADPH-reduced microsomes it was maximal at pO_2 around 5 mmHg and markedly decreased at pO_2 below and above this value. In hepatocytes it was maximal at pO_2 of 7 mmHg. They proposed that this optimum curve resulted from the fact that the formation of $CCl_3\cdot$ radicals at the haem moiety of cytochrome P_{450} is inhibited by O_2 while the formation of $CCl_3OO\cdot$ and the propagation stage of lipid peroxidation require the participation of O_2. The reductive activation of CCl_4 by cytochrome P_{450} takes place at the same site O_2 normally becomes activated, so the activation of CCl_4 is inhibited by O_2.

Changes in body temperature may affect the activation of CCl_4 and protect liver injury induced by CCl_4 (Slater, 1984). Temperature reduction can be made a great protection against CCl_4-mediated liver injury. Marzi et al. (1980) reported that a chlorpromazine (CPZ) induced reduction in body temperature of 5°C prevented CCl_4 induced hepatic necrosis at 24h. They suggested that CPZ delayed the onset of the process but did not prevent its final intensity since CPZ did not inhibit the covalent binding of $^{14}CCl_4$ to cellular constituents or CCl_4-induced lipid peroxidation. Reddrop et al. (1983) observed that promethazine inhibited CCl_4-stimulated lipid peroxidation *in vivo* when added in very low concentrations. The synergistic effects of promethazine and CCl_4 in reducing the body temperature of rats may be one of particular significance to the protective action of promethazine. Administration of promethazine gives a long-lasting 2-3°C reduction in core temperature and this can be expected to lower the rate of metabolism of CCl_4. Villarruel et al. (1990) made a similar observation. They found that thioridazine (TDZ) could partially prevent necrogenic effects in rat liver after administration of CCl_4 at 24h. The protective effect of this drug was partly due to its reduction of the body temperature in CCl_4-treated animals during the initial 24h. Lower body temperature decreases the rate of CCl_4 metabolism.

Cytochrome P_{450} can be phosphorylated in a cAMP-dependent fashion (Mkrtchian et al., 1990). It is found that cAMP dependent phosphorylaytion increases the NADPH dependent production of malondialdehyde. The cytochrome P_{450} inhibitor cyanide abolishes this activity. This observation implies a mechanism of increase in lipid peroxidation in response to hormonal signals (Mkrtchian, et al., 1990).

1. 1. 2. CCl_4-stimulated lipid peroxidation and its pathological role

One of the most important consequences of the activation of CCl_4 is initiation of lipid peroxidation as a damaging reaction of potentially great significance in cytotoxicity. Lipid peroxidation is described as an oxygen-dependent, autoxidative degradation of fats, particularly polyunsaturated fatty acids (PUFA). It is a chain reaction initiated and propagated by free radicals (Pryor, 1976). It comprises three stages: initiation, propagation and termination. The initiation stage occurs when a radical species abstracts one hydrogen from a PUFA to form a lipid radical. This lipid radical will then spontaneously react with oxygen to generate a lipid peroxyl radical. The second stage is a chain reaction leading to propagation. Finally, termination occurs when two radicals react together producing lipid peroxides or when scavengers such as vitamin E act by interrupting this chain reaction. These peroxides can reinitiate the radical chain reactions if they breakdown to further radical species in the presence of a transition metal. They can also undergo rearrangements and cleavages generating different compounds. The whole process can be outlined as shown in Fig. 1.1.4 taking the polyunsaturated fatty acid linolenic acid (18:3 (n-3)) as an example and is fully described for CCl_4-induced lipid peroxidation below.

First CCl_4 is activated by the hepatic NADPH-cytochrome P_{450} system to $CCl_3\cdot$. Second $CCl_3\cdot$ radical reacts quickly with oxygen to yield the trichloromethyl peroxyl radical $CCl_3OO\cdot$. Because $CCl_3OO\cdot$ reacts much faster with a PUFA (such as arachidonate) than does $CCl_3\cdot$ it has been suggested that lipid peroxidation is preferentially initiated in the endoplasmic reticulum by $CCl_3OO\cdot$ rather than $CCl_3\cdot$. Third,

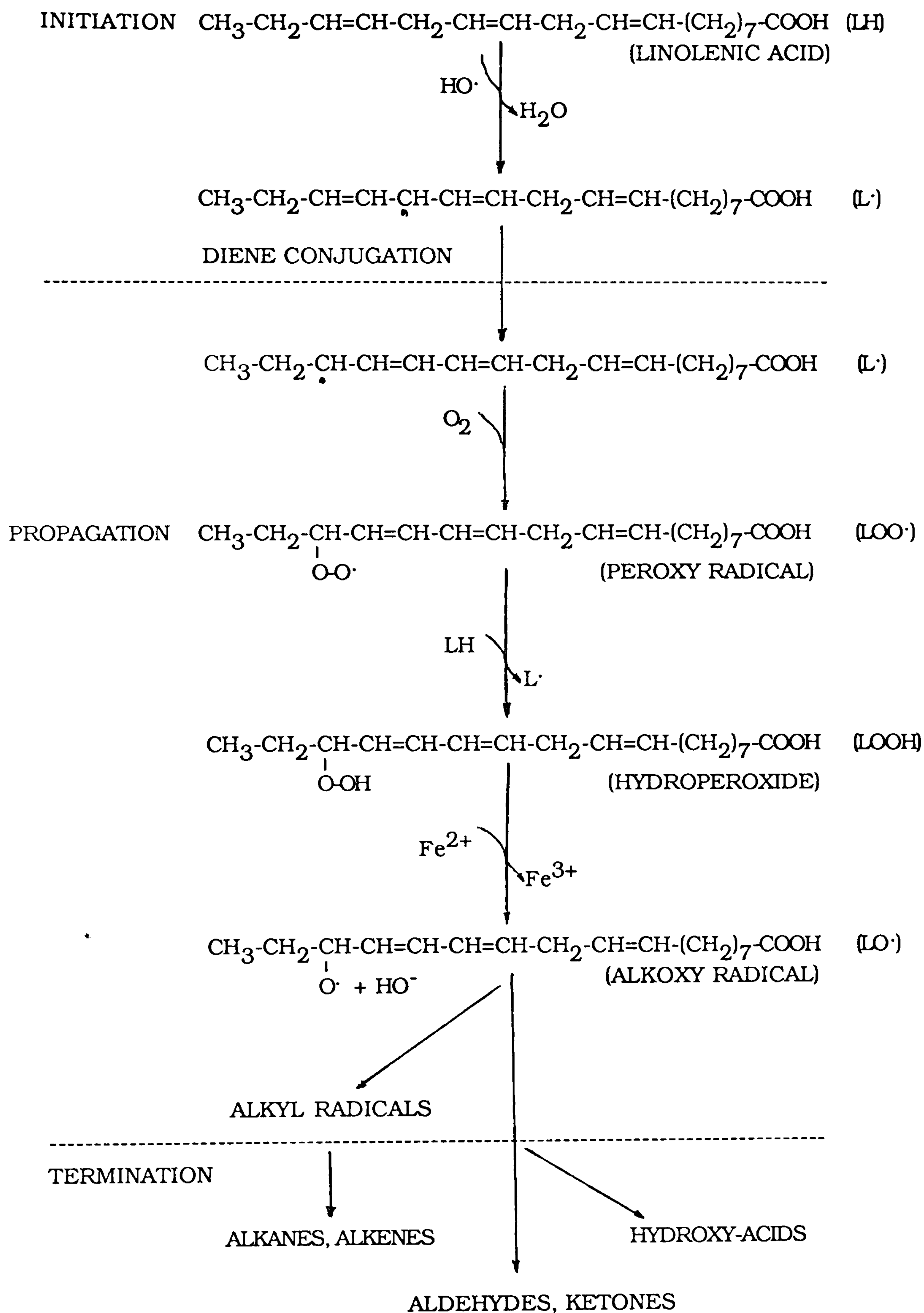


Fig. 1.1.4 Scheme representing the peroxidation of linolenic acid (18:3 (n-3)) induced by hydroxyl radicals

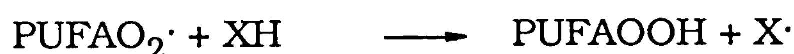
(Adapted from Vaca et al., 1988)

$CCl_3OO\cdot$ reacts with PUFA in the endoplasmic reticulum and other hepatocellular membranes by abstraction of a hydrogen atom from PUFA to initiate the formation of lipid radicals (PUFA·). Lipid radicals interact with molecular oxygen to form the lipid peroxyl radicals

$$PUFA\cdot + O_2 \longrightarrow PUFAO_2\cdot$$

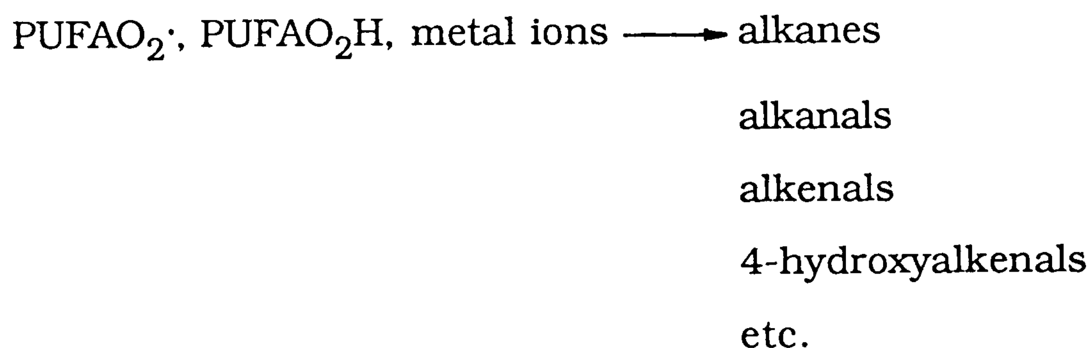
This peroxyl-radical can oxidise its neighbouring molecules by hydrogen abstraction either from another molecule of PUFA or from some other hydrogen donor (XH) to form hydroperoxides and new radicals

$$PUFAO_2\cdot + PUFA'H \longrightarrow PUFAOOH + PUFA'\cdot$$



$$PUFAO_2\cdot + XH \longrightarrow PUFAOOH + X\cdot$$

In this way a chain reaction involving PUFA· is propagated. In the presence of transition metal ions, the hydroperoxides and lipid peroxyl radicals breakdown to form a complex variety of products



$PUFAO_2\cdot$, $PUFAO_2H$, metal ions ⟶ alkanes
alkanals
alkenals
4-hydroxyalkenals
etc.

(Slater, 1984; Slater and Cheeseman, 1987).

It has been proved that CCl_4 induces lipid peroxidation in the livers of various species including rat, hamster, guinea-pig, chicken and mouse (Cheeseman, 1982 b; Lee et al., 1980). Liver microsomes of rabbit are resistant to lipid peroxidation *in vitro.* However, CCl_4 administration to rabbit *in vivo* promoted lipid peroxidation in their liver microsomes *in vitro* (Bernacchi et al., 1983). Rat liver microsomes

and hepatocytes have been used as the useful model of studying toxic effect of CCl_4 (Slater and Sawyer, 1971 a, b, c; Poli et al., 1987; Slater and Cheeseman, 1987).

Lipid peroxidation is involved in many biochemical and biological events such as the aging process, the various diseases and the toxic effects of certain xenobiotics (Slater, 1984; 1987; 1988). It is generally accepted that lipid peroxidation may be important in some types of tissue injury.

Studies with liver microsomes and hepatocytes have clearly demonstrated that lipid peroxidation has a crucial role in the toxic effect of CCl_4 on the liver (Slater et al., 1985; Poli et al., 1987). Lipid peroxidation is strictly related to several CCl_4-induced damaging actions, namely reduction of cytochrome P_{450} level (Glende et al., 1976), alteration of the activity of enzymes including glucose-6-phosphatase (Poli and Gravela, 1982), protein kinase C (Poli et al., 1988 b; Pronzato et al., 1990), Glycerol-3-phosphate acyltransferase (Thomas and Poznansky, 1990), effect on several subcellular organelles and induction of liver necrosis.

Glende et al. (1976) measured cytochrome P_{450} level and aminopyrine demethylase and glucose-6-phosphatase activities in suspensions of microsomes recovered after metabolism of $^{14}CCl_4$ under anaerobic conditions. Massive covalent binding occured in the absence of oxygen. The level of cytochrome P_{450} and the activities of aminopyrine demethylase and glucose-6-phosphatase were virtually unaffected by covalent binding in the anaerobic system, but they were markedly depressed by lipid peroxidation in an aerobic system.

Poli and Gravela (1982) and Poli et al. (1989 a) reported that glucose-6-phosphatase was inactivated by the treatment of hepatocytes with CCl_4. There was a direct relationship between the degree of lipid

peroxidation and the degree of the enzyme inactivation. Promethazine completely prevented the decrease in this enzyme activity and strongly inhibits CCl_4-induced lipid peroxidation in liver microsomes and liver cells. Promethazine is known to have protective activity in relation to CCl_4-induced liver necrosis. The main effect of promethazine on CCl_4-mediated damage in microsomal and isolated hepatocytes is by scavenging $CCl_3OO\cdot$ free radicals that would initiate lipid peroxidation. It has no inhibitory effect on covalent binding of CCl_4 (Cheeseman, 1982 a ; Poli et al., 1989 b). Therefore, the inactivation of glucose-6-phosphatase was not result of covalent binding of CCl_4 to the enzyme protein but the result of lipid peroxidation (Poli and Gravela, 1982; Poli et al., 1989 b). De Groot et al. (1985, 1986) have the same conclusion that $CCl_3\cdot$ radical does not damage glucose-6-phosphatase directly. They found that NADPH/CCl_4 induced lipid peroxidation in microsomes and decrease of glucose-6-phosphatase activity only occurred in the presence of O_2; under anaerobic conditions no lipid peroxidation and no alteration in enzyme activity occurred. Furthermore, Benedetti et al. (1980) could demonstrate that 4-hydroxynonenal, a product of lipid peroxidation, was capable of inactivating glucose-6-phophatase possibly by reacting with essential thiol groups.

Protein kinase C (PKC) is an important transmembrane signal transduction system of many short- and long-term cellular responses including cell proliferation and transformation. Poli et al. (1988 b) and Pronzato et al. (1990) observed that hepatocyte PKC activity was inhibited by CCl_4. Preincubation of hepatocytes with Trolox protected both cytosolic and microsomal PKC activity. Trolox completely inhibited CCl_4-induced lipid peroxidation but did not affect the covalent binding of CCl_4 to hepatocyte proteins. It is indicated that

CCl_4-induced lipid peroxidation inhibited PKC. Moreover, since the degree of inhibition of both PKC activity in microsomes and cytosol was the same, it is suggested that the effect of CCl_4 on PKC could be mediated by a factor able to diffuse from the site of activation of CCl_4 in the membranes of the endoplasmic reticulum to the cytosol. It was shown that products of lipid peroxidation, 4-hydroxyhexenal, 4-hydroxyoctenal and 4-hydroxynonenal inhibit the PKC activity.

Glycerol-3-phosphate acyltransferase (GPAT) is a enzyme of biosynthesis of glycerolipids. The results of Thomas and Poznansky (1990) showed that CCl_4 inactivated GPAT in microsomes in the presence of NADPH. At the same time lipid peroxidation occurred. Addition of promethazine substantially recovered the lost of GPAT activity indicating that lipid peroxidation plays a role in inactivation of microsomal GPAT. Adding 1 mM DTT at the end of incubation protected the enzyme activity, therefore the sensitive SH groups might be damaged during lipid peroxidation, probably by products of lipid peroxidation.

Damage to protein synthesis was involved in CCl_4 toxicity to rat liver slices and related to CCl_4-induced lipid peroxidation (Fraga et al., 1989). CCl_4-induced lipid peroxidation and inhibition of protein synthesis occurred concurrently.

CCl_4-mediated lipid peroxidation has been shown to be involved, at least partly, in the several examples of CCl_4-induced subcellular organelles damage. Examples are liver mitochondrial swelling and uncoupling of oxidative phosphorylation; inhibition of the uptake of Ca^{2+} by liver microsomes, severe impairment of plasma membrane structure resulting in the leakage of intracellular enzymes; interference with the lipoprotein assembly and maturation in the formative side of liver Golgi apparatus (Poli et al., 1987; 1990).

The ultimate consequence of inactivation of enzymes and alteration of functions of subcellular organelles induced by CCl_4-mediated lipid peroxidation might be cell death. At present, the prevailing opinion is that lipid peroxidation plays an essential role in causing acute liver necrosis induced by CCl_4. With isolated hepatocytes lipid peroxidation is shown long before the appearance of signs of cell damage such as the loss of Trypan Blue staining and leakage of cytoplasmic enzymes (Slater, 1985). Both lipid peroxidation and cell injury are prevented by the addition of antioxidants. For example, low concentrations of CCl_4 (0.1-0.15 mM) stimulate early lipid peroxidation. The initial cell death evidenced by the leakage of lactate dehydrogenase or transaminase happens much later. Antioxidants such as promethazine and vitamin E block the lipid peroxidation and prevent the hepatocyte damage (Smith et al., 1983; Poli et al., 1988 a; Mourelle et al., 1989).

The mechanism of injury induced by lipid peroxidation is attributed to three aspects.

First, primary reaction due to the interaction of reactive free radicals produced during lipid peroxidation with the surrounding system. It has been shown that reactive free radicals are able to produce chemical modifications and damage to proteins, lipids, and nucleotides (Slater, 1984). The consequence of these modifications is wide spread disturbances of cellular metabolism.

Second, the toxic and pharmacological effect of products of lipid peroxidation. This mechanism is more important. Highly reactive free radicals are easily trapped in their microenvironment by virtue of their high chemical reactivity. However, the products of lipid peroxidation, like lipid hydroperoxides, aldehydes and 4-hydroxyalkenals can diffuse considerable distances. These products have toxic and pharmacological

activities, therefore they can spread disturbances throughout the cell and even into the extracellular domain (Slater, 1984; Willis, 1980). This mechanism is probably particular important for CCl_4 toxicity. It has been shown by Kornbrust and Mavis (1980) that microsomal lipid peroxidation induced by CCl_4 is highly focal and limited, involving only about 10% of the available membrane fatty acids. The reason for this is a loss of the capacity of microsomes to activate CCl_4, attributed to inhibition of cytochrome P_{450} by lipid peroxidation. Amplification of the toxic consequences of lipid peroxidation is achieved by the products moving from sites of lipid peroxidation on the endoplasmic reticulum to other parts of the cell. Evidence of this is given by Poli et al. (1988 b) and Pronzato (1990). As already mentioned they observed that PKC activity in microsomes and cytosolic was inhibited in proportion to CCl_4-induced lipid peroxidation. They suggested that products of lipid peroxidation which are able to diffuse from the site of activation of CCl_4 to the cytosol were responsible for the inactivation of PKC.

Third, disorganization of the membrane results from lipid peroxidation. Peroxidation of membrane lipids leads to a number of changes in membrane structure and composition, such as decreases in the unsaturation/saturation ratio in membrane phospholipids, changes in the chain-length percentage distribution of fatty acids, and covalent crosslinks between adjacent lipid and protein molecules which are accompanied by increases in the molecular order of the membrane and decreases in its fluidity. Various membrane functions such as selective permeability, enzyme activity, and ion transport are impaired by lipid peroxidation (Slater, 1978; Pryor, 1980). De Groot et al. (1985, 1986) found that lipid peroxidation induced by iron or CCl_4 could lead to the loss of microsomal latency estimated by measuring the activity of

nucleoside diphosphatase and this only occurred when lipid peroxidation had reached a certain extent. Beyond this threshold lipid peroxidation led to severe disintegration of the microsomal membrane resulting in a loss of its selective permeability. The loss of latency evoked by lipid peroxidation was comparable to the loss of latency induced by detergent. They implied that inactivation of glucose-6-phosphatase activity may relate to the loss of integrity of the microsomal membrane apart from the effect of products of lipid peroxidation. Glucose-6-phosphatase is a integral microsomal enzyme: the enzyme activity largely depends on an intact lipid bilayer.

1. 2. PART II BIOLOGICAL EFFECT OF 4-HYDROXYALKENALS

Lipid peroxidation is involved in a range of pathological and biological events such as aging, certain diseases, tumour initiation and promotion and the cytotoxicity of certain xenobiotics (Slater, 1984, 1987). A complex variety of products are generated during the process of lipid peroxidation. Some of them have important biological activities. Among them are the 4-hydroxy-2,3-trans-alkenals series. This is represented by 4-hydroxypentenal (HPE), 4-hydroxyoctenal (HOE), 4-hydroxyhexenal (HHE) and 4-hydroxynonenal (HNE) (Esterbauer, 1991). HNE represents 95% of the aldehyde production (Benedetti et al., 1980). The structure of these 4-hydroxyalkenals is shown in Fig. 1.2.1. This class of products, especially HNE, has received particular attention because of their multiple biological activities.

1. 2. 1 Chemical reactivity

A striking chemical feature of 4-hydroxyalkenals is the fact that at neutral pH they react avidly with SH groups in both proteins and low molecular weight thiols such as glutathione, cysteine and coenzyme A. The reaction product initially formed is a saturated aldehyde with the glutathione residue bound by a thio-ether linkage at carbon atom 3. This primary aldehyde then becomes a very stable cyclic hemiacetal which is the main (95%) end product in aqueous solution (see Fig.1.2.2) (Esterbauer, 1991; Benedetti and Comporti, 1987). The reaction rates of HNE with glutathione are:

forward reaction (HNE + GSH $\longrightarrow$ adduct) $1.09\ M^{-1}\ s^{-1}$

reverse reaction (adduct $\longrightarrow$ HNE + GSH) $9.6 \times 10^{-7}\ M^{-1}\ s^{-1}$

equilibrium constant $8.85 \times 10^{-7}\ M^{-1}$

(Esterbauer, 1981). If isolated proteins and enzymes containing SH

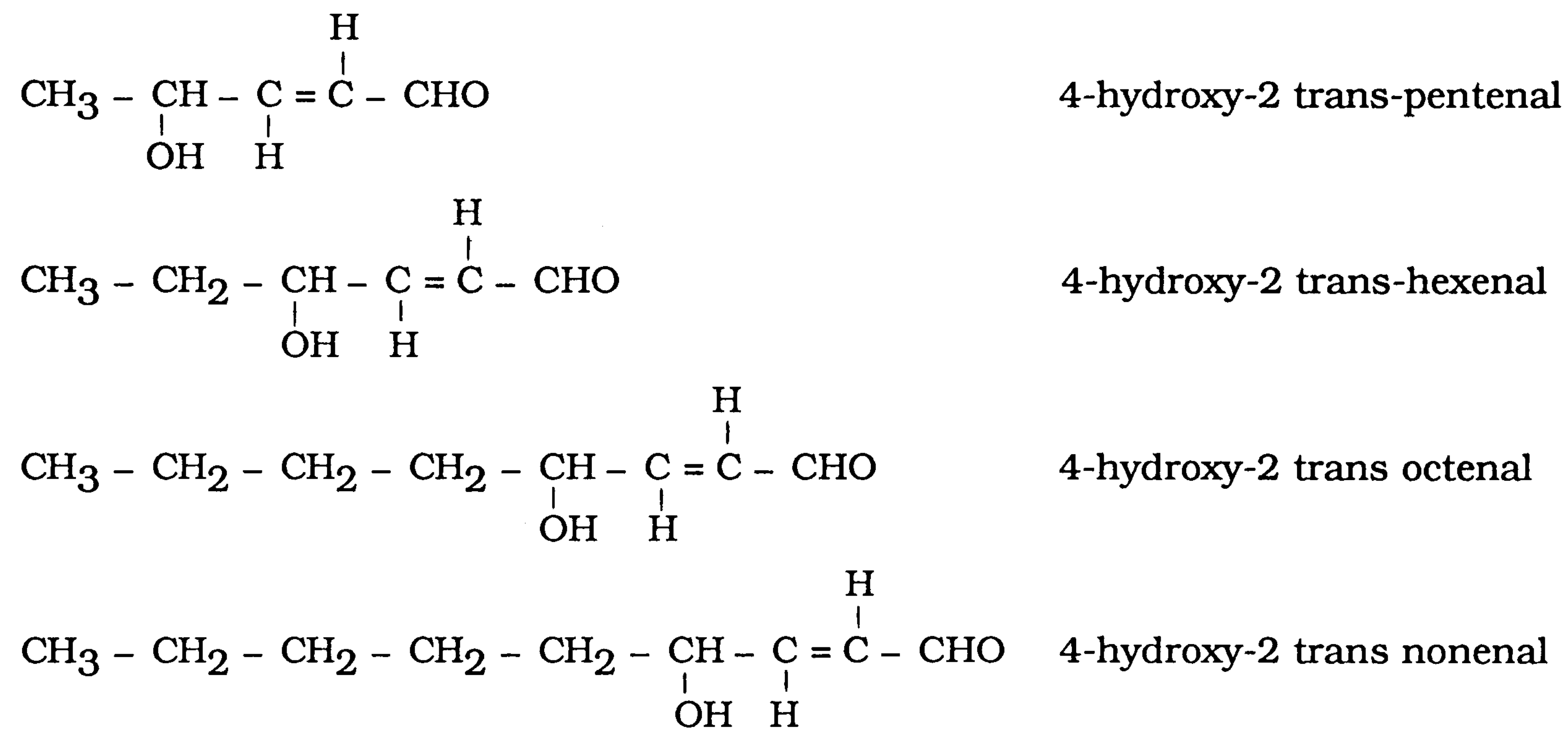


Fig. 1.2.1 Structure of 4-hydroxyalkenals

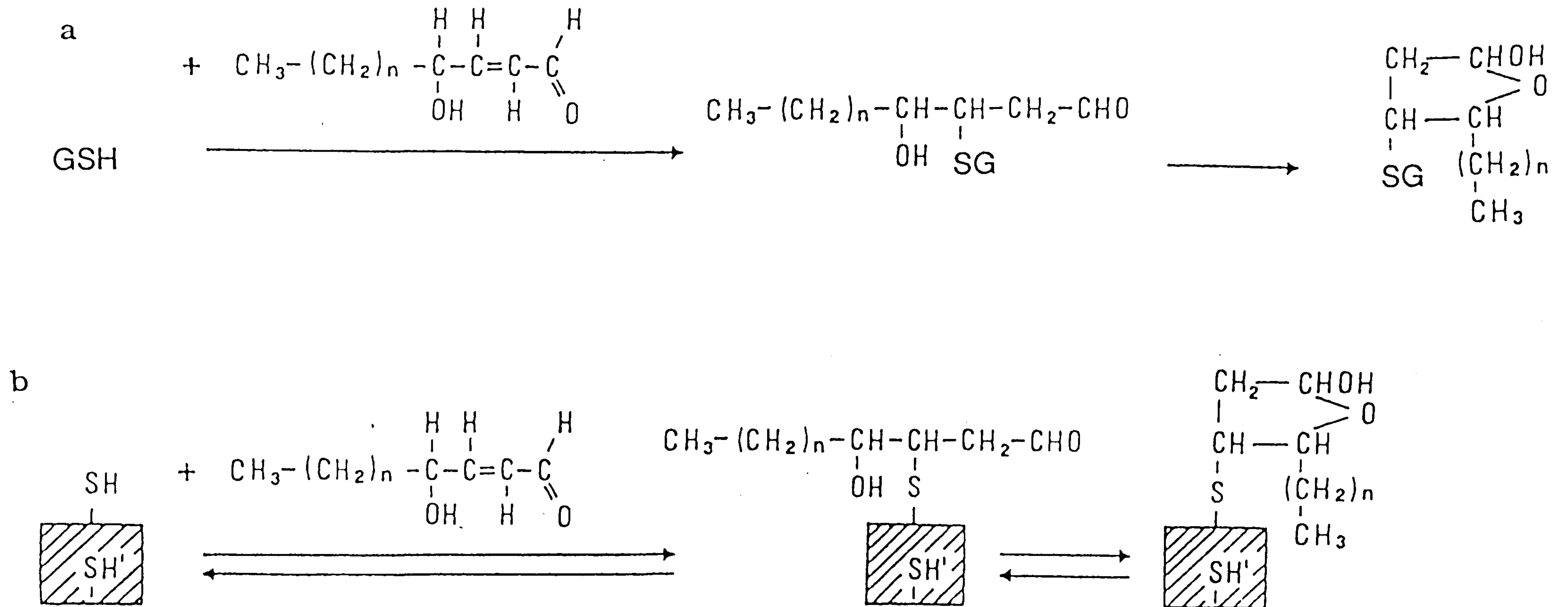


Fig. 1.2.2 Reaction of 4 hydroxyalkenals with SH group
(a) with GSH; (b) with protein containing SH groups
(Adapted from Benedetti and Comporti, 1987)

groups are incubated with 4-hydroxyalkenals, the number of aldehyde molecules bound to the protein equals the number of SH groups lost during the incubation (Benedetti and Comporti, 1987). Apart from cysteine, 4-hydroxyalkenals can react with other amino acid residues, like lysine, histidine, serine and tyrosine (Esterbauer et al., 1991; Uchida and Stadtman, 1992). Under certain conditions (slightly alkaline pH, of 8.8, high concentration of the reactants 10 mM) 4-hydroxyalkenals can react with nearly all amino acids.

1. 2. 2. Cytotoxicity

Unlike reactive free radicals, 4-hydroxyalkenals have a relatively long life-time and can diffuse appreciable distances and thereby extend the cellular damage far from the site of initial toxin activation or even escape from the cell (Slater, 1987; Benedetti, et al., 1981). Therefore, such products generated *in situ* should not merely be considered as remnants or end-products of lipid peroxidation processes but rather as a kind of "second toxic messenger" which mediate and/or potentiate the damage initiated by primary free radicals (Slater, 1987).

1. 2. 2. 1. Inhibitory effects on enzyme activity

A wide range of enzymes on the Table 1.2.1 have been reported to be inhibited by HNE.

The high reactivity of HNE with glutathione implies that HNE formed in biological systems, such as peroxidising microsomes will preferentially attack molecules with SH groups. All inhibitory effects of 4-hydroxyalkenals on enzymes investigated so far can be explained by the inactivation of functional SH groups (Benedetti and Comporti, 1987, Esterbauer. et al., 1988). The fact that most actions are inhibited by the previous addition of thiols in the incubation medium, whereas

Table 1.2.1. Inhibition of enzyme activity by HNE

Enzyme	Source	HNE conctn.- range (μM)	Reference
Adenylate cyclase	rat liver plasma membranes	13.4 - 26.8	Dianzani et al., 1982 Paradisi et al., 1985
S-adenosylmethionine decarboxylase	Yoshida ascites hepatoma cells	250 - 1000	Dianzani et al., 1982
Glucose-6-phosphatase	rat liver microsomes hepatocytes	~ 450 ~ 250	Benedetti et al., 1980
Glyceraldehyde-P-dehydrogenase	Ehrlich ascites tumour cells	150 - 250	Esterbauer et al., 1991
α- and β- DNA polymerases	Yoshida ascites hepatoma cells	210 - 460	Wawra et al., 1986
5'-nucleotidase	rat liver plasma membrane	1300 - 4600	Dianzani et al., 1982, 1989 Paradisi et al., 1985
O^6-methylguanine DNA methyltransferase	human bronchial fibroblasts	~100	Eckl and Esterbauer, 1989
Protein kinase C	rat hepatocytes	~100	Poli et al., 1988b Pronzato et al., 1990

amino acids are generally inactive, points in favour of this explanation (Schauenstein, 1982). HNE discriminates between different types of thiols. When acting *in vitro* on Ehrlich ascites tumour cells at concentrations exceeding 3 mM, the entire subcellular content of nonprotein thiols (which mainly consists of glutathione) is consumed by reaction with the hydroxyalkenals, whereas only 50% of the soluble cell protein thiols react (Schauenstein, 1982). This can explain the mechanism whereby low molecular weight thiols protect protein-thiols against HNE. Further evidence for the explanation that the inhibitory effect of HNE on enzyme activity is due to blockage of enzyme SH groups is that HNE binds to microsomal protein *in vivo* and *in vitro* and the binding mechanism is very likely to involve reaction with SH groups in the protein (Benedetti, et al., 1982). Furthermore, the binding capacity of aldehyde to the protein is responsible for the inactivating capacity of the aldehyde (Benedetti et al., 1984). The lower binding capacity found for 4,5-dihydroxydecenal as compared to HNE correlated with its lower inactivating ability on microsomal glucose-6-phosphatase. Recently polyclonal and monoclonal antibodies which specifically recognize HNE-modified proteins have been established. Immunocytochemical staining with such antibodies give a direct evidence that HNE-modified proteins are in fact formed in animals *in vivo* (Esterbauer et al., 1991).

Other enzymes, not listed in Table 1.2.1 such as hexokinase and lactate dehydrogenase are also inhibited by 4-hydroxyalkenals (Esterbauer et al., 1988). Mitochondral NAD-dependent aldehyde dehydrogenase and glucose-6-phosphate dehydrogenase might be inhibited by 4-hydroxyalkenals too. Kanazawa and Ashida (1991) reported that the two latter enzymes were inhibited by low-molecular weight component of products of linoleic acid peroxidation; the major

components in low-MW were aldehydes.

HNE had a biphasic, dose- and time-dependent effect on adenylate cyclase in rat liver plasma membranes. Very low doses strongly stimulated the enzyme activity after 2 and 5 min of incubation, whereas a progressive, time- and dose-dependent inhibition was observed afterwards. HNE (0.1 μM) elicited the highest enhancement without inhibition whereas 26.8 μM was immediately inhibitory (Paradisi et al., 1985; Dianzani et al., 1990).

1. 2. 2. 2. Inhibitory effect on DNA, RNA and protein synthesis

HNE inhibits protein synthesis, as observed in liver cells (250 μM), Ehrlich ascites tumour cells (140 μM), rabbit reticulocyte lysate (480 μM) (Esterbauer et al., 1991), cells from guinea pig epidermis (guinea pig keratocytes) (100-200 μM) (White and Rees, 1984), human diploid fibroblasts (1-100 μM) (Michiels and Remade, 1991) and bovine aortic endothelial cells (30 μM) (Radu and Moldovan, 1991). It was reported that [^{14}C]-valine incorporation into isolated hepatocytes was inhibited by 30% at 100 μM and by 70% at 250 μM HNE added in the incubation medium (Dianzani, 1982). The inhibitory activity of HNE was about 10 times more effective than that of HPE. Treatment of guinea pig keratocytes in culture with HNE, incorporation of ^{3}H-leucine into protein was inhibited by 50-60% at 100 μM HNE (White and Rees, 1984). At 30 μM, HNE produces a gradual decrease in protein synthesis in bovine aortic endothelial cells during short (2-4h) incubations, followed by return to the control level at 6-8h (Radu and Moldovan, 1991). The protein synthesis was measured by ^{14}C-leucine incorporation. It is suggested that the inhibition of protein synthesis in the reticulocyte lysate of rabbit is primarily due to an impairment of the protein synthetic machinery, probably by an interaction of the

carbonyl compounds with sulphydryl groups essential for protein synthesis (Benedetti, 1981). In support of this explanation, White and Rees (1984) observed that cysteine protects protein synthesis in guinea pig keratocytes against HNE. Addition of 400 μM cysteine to the culture medium in the presence of 200 μM of HNE gave complete protection whereas if the cultures were pretreated with cysteine followed by washing, no protection against the subsequent addition of HNE was obtained.

One of the important effects of HNE is its inhibitory effect on nucleic acid synthesis. It is found that HNE stops or reduces DNA synthesis in Ehrlich ascites tumour cells (150-250 μM) (Schauenstein, 1982; Hauptlorenz, et al., 1985), guinea pig keratocytes (100 μM) (White and Rees, 1984), Yoshida ascites hepatoma cells (40 μM) (Wawra et al., 1986), malaria parasites (50 μM) (Clark et al., 1987), human diploid fibroblasts (0.1 μM) (Michiels and Remacle, 1991), human lymphocytes (100-300 μM) (Tessitore et al., 1987), and K562 and HL-60 human leukemic cells (1 μM) (Barrera et al., 1991). The inhibition of DNA synthesis was shown by the reduced incorporation of labelled thymidine or hypoxanthine into DNA. In cells from guinea pig epidermis (guinea pig keratocytes) inhibition of RNR synthesis (measured by ^{3}H-uridine incorporation) was more sensitive to HNE treatment than protein synthesis whereas DNA synthesis was the most sensitive to HNE (White and Rees, 1984). In Ehrlich ascites tumour cells, the reduction of labelled thymidine incorporation actually results from the inhibition of *de novo* DNA synthesis but not from an inhibition of the thymidine uptake. This was demonstrated by DNA pulse label experiments and ^{86}Rb uptake experiments (Hauptlorenz, et al., 1985).

It is suggested that HNE affects DNA synthesis due to inhibition

of some enzyme activity essential for DNA synthesis, for example DNA polymerases, and again the blockage of the SH groups by HNE plays an important role (Hauptlorenz, et al., 1985). This assumption is supported by the fact that low concentrations of HNE selectively attack protein SH groups contained in the nucleus. Whilst 3 mM HNE discriminates between non-protein and protein thiols as mentioned above, less than 0.2 mM HNE can discriminate between cytoplasmic and nuclear protein thiols: the latter are more sensitive to hydroxyalkenals than those of the cytoplasm. For example, after incubation of Ehrlich ascites tumour cells with HNE (0.2 mM) for 30 min, cytoplasmic protein thiols decrease only 1.7% whilst nuclear soluble proteins thiols decrease 27% (Schauenstein, 1982). When EATC are incubated with 200 μM HNE, DNA synthesis is inhibited up to 90% after 30 min incubation, but respiration of the cell remains practically unaffected and glycolysis is inhibited only by some 15%. Respiration and glycolysis are controlled by SH enzymes. This finding is in accordance with the above observation (Schauenstein, 1982).

There is another possibility of inhibition of DNA synthesis by HNE. The inhibition may result from block of some signalling events that are necessary for progression through the G1 phase since HNE can alter the cell cycle as described below (Barrera, et al., 1991 a).

1. 2. 2. 3. Altering cell cycle and mitosis

4-hydroxyalkenals are able to disturb cell cycling.

Dianzani (1982) reported that HPE influences the cell cycle of ascites AH-130 Yoshida hepatoma cells *in vivo* during the exponential phase of growth. At a dose of 25 mg/kg and 50 mg/kg, HPE significantly increase the mean DNA synthetic period (S-phase) and decreases the number of cells participating in the cell cycle, especially

at the lower dose. Moreover, HPE decreased the peak of the mitotic wave, suggesting an impairment of the S-phase.

Poot et al., (1988) studied the disturbance of cell proliferation by HNE in close detail. Using amnitotic fluid fibroblast-like cells and human diploid skin-derived cells they found that HNE (10-20 μM) caused an accumulation of cells in the G1 and G2 phase. This is due to the fact that HNE provokes a retardation of the transit of the cells from G0 to G1 and a rapid rate of exit from G0/G1 and S phase but diminished rate of exit from G2. Additionally, HNE elicited a permanent arrest in the G2 phase of the first cell cycle and in the G1 phase of the second cell cycle even if added to quiescent cell culture. These results indicate that HNE might cause proliferating cells to enter a quiescent state (Poot et al., 1988).

4-hydroxyalkenals are able to affect microtubules. Microtubules, together with microfilaments and intermediate filaments, are the major cytoskeleton components of mammalian cells. They play a role in the secretory processes, the distribution of cell organelles and the movement of chromosomes during mitosis (Olivero et al., 1990). It is observed that HNE affects the cellular distribution of microtubules (Olivero et al., 1990). At a low concentration (10 μM) HNE slightly affects microtubules in 3T3 fibroblasts, which became smaller than their normal counterparts and the microtubule network appeared to be formed of short fibres. After treatment with 100 μM HNE cytoplasmic microtubules disappeared. This effect of HNE may result from preventing tubulin polymerization or by provoking microtubule depolymerization. Tubulin is a main functional protein of microtubules. It was found that incubation of purified tubulin with HNE caused a reduction, both in the rate and the extent of polymerization. The inhibition showed a direct relationship between the amounts of HNE

and the degree of inhibition. The maximum inhibitory effect (78%) was observed at 1mM of HNE (Olivero et al., 1990). Similar results were obtained by Miglietta et al. (1991). They observed that 0.5 mM HNE inhibited polymerisation of microtubular protein in calf brain *in vitro*. The mechanism of the inhibition may be the interaction of HNE with SH groups of tubulin since the inhibition was prevented by addition of cysteine. SH groups are fundamental for the polymerization of tubulin (Dianzani, 1982; Olivero et al., 1990; Miglietta et al., 1991).

1. 2. 2. 4. Damage to DNA and DNA function

It has been demonstrated that free radicals induce DNA damage in liver systems. So far there is no direct evidence that 4-hydroxyalkenals damage DNA or influence DNA function, however, recently there is increasing information that suggests lipid peroxidation is an important event in free radical induced DNA damage and products of lipid peroxidation may play an important role (Vaca, 1988; Esterbauer, 1990). Several examples are given below to show the relationship between lipid peroxidation and damage of DNA and DNA function.

A high dietary level of polyunsaturated fats in the absence of vitamin E resulted in decreased hepatic DNA template activity. It is presumed that peroxidative damage to the DNA occurred since DNA template activity of rats fed less polyunsaturated fats and vitamin E was normal (Summerfiedd and Tappel, 1984).

DNA isolated from liver slices after incubation with 1 mM t-butyl hydroperoxide (t-BOOH), 1 mM $BrCCl_3$ or 50 μM ferrous iron for 2h was damaged. The enhancement of ethidium bromide fluorescence by DNA from treated slices was decreased. Meanwhile, the TBARS released into the incubation medium from liver slices was increased.

DNA damage and TBARS release were both time dependent. In this experimental system $BrCCl_3$ did not induce significant DNA damage and was the weakest TBARS inducer. Butylated hydroxytoluene (BHT) at 1 mM inhibited both DNA damage and TBARS production. These results indicate that lipid peroxidation and DNA damage in liver slices were concurrent (Fraga and Tappel, 1988).

When mitochondria of rat liver were incubated in an *in vitro* lipid peroxidation system (NADPH-ferrous chloride) marked lipid peroxidation occurred. DNA isolated from these mitochondria had a completely different Agarose gel electrophoresis pattern. The lipid peroxidation and DNA damage are not protected by scavengers of superoxide anion, hydrogen peroxide and hydroxyl radicals. However, alpha-tocopherol protected against both lipid peroxidation and mitochondrial DNA damage (Hruszkewycz, 1988).

Transforming activity of plasmid pBR 322 DNA, as assayed by transforming *E. coli* CSR 603 was inactivated by incubation of DNA with the reaction mixture for NADPH-dependent lipid peroxidation of liposomes. The inactivation was dependent on the extent of the lipid peroxidation as determined by measuring the production of TBARS. The DNA was broken by the incubation. ·OH scavengers exhibited only a weak effect on the formation of DNA strand breaks, but did not inhibit lipid peroxidation. The chloroform extracts from peroxiding liposomes caused inactivation of the transforming activity of plasmid DNA, but did not induce DNA strand breaks. These results indicate that the factor which inactivates the transforming activity is not a free radical since it could be extracted into chloroform, whereas the factor producing DNA breaks might be free radical intermediate since it was not extractable with chloroform (Akasaka, 1986).

As illustrated in Fig. 1.2.3 damage to DNA may be produced in

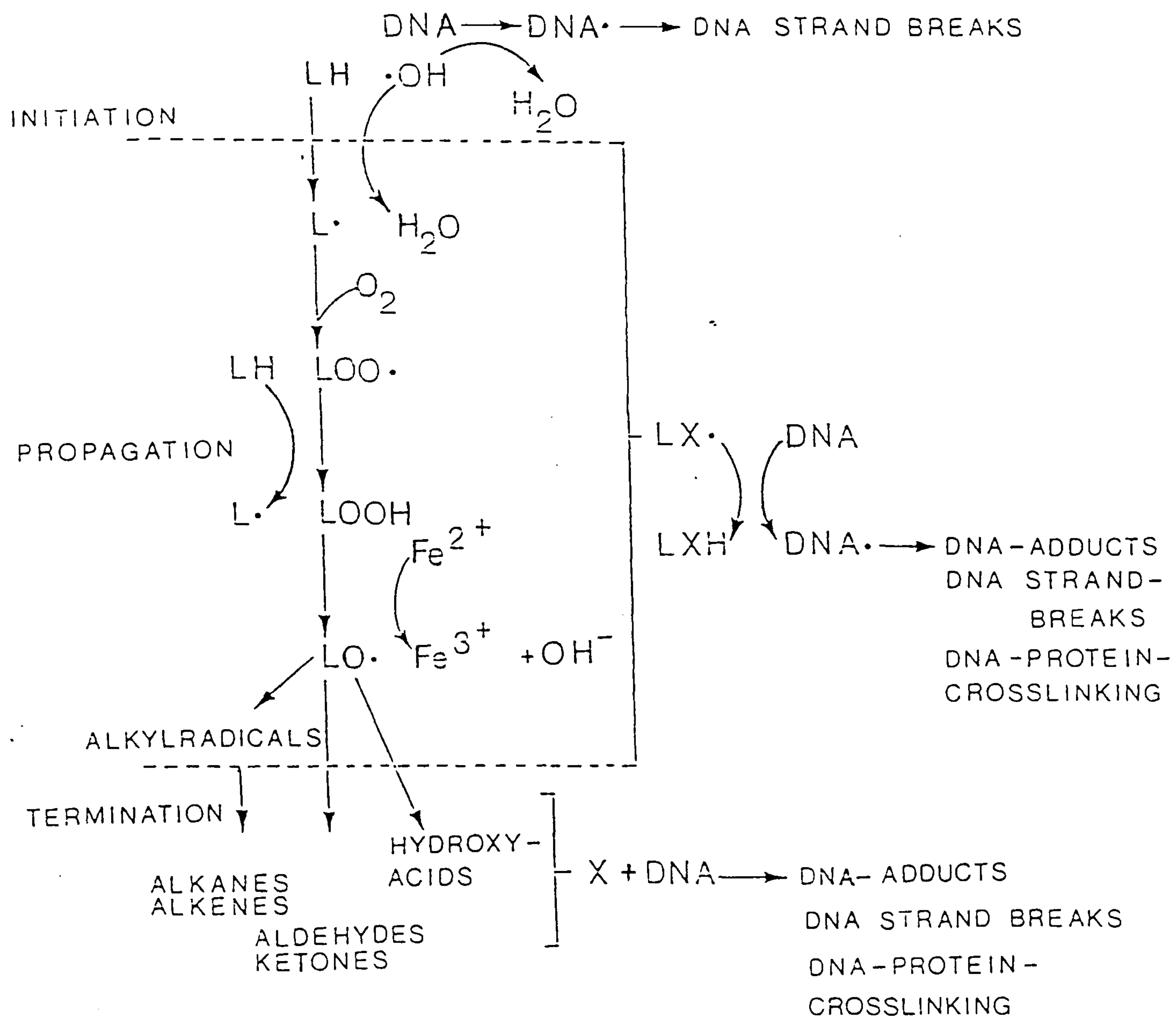


Fig. 1.2.3 Possible pathways for the interaction between lipid peroxidation products and DNA

(Adapted from Vaca et al., 1988)

the various steps of lipid peroxidation. The third mechanism would be represented by the reaction of DNA with the variety of non-radical products of the lipid peroxidation process. These reactions lead to modification of nucleobases and to severe structural deterioration of DNA (Vaca et al., 1988). However, the interaction of products of lipid peroxidation with DNA has been disputed in the literature. According to Akaska (1986) in a system consisting of plasmid pBR 322 DNA and [U-^{14}C]-arachidonic acid-containing liposomes, no binding of peroxidation products of the radiolabelled fatty acid to DNA was observed. On the other hand, Vaca and Harms-Ringdahl (1989 a, b) reported that the products of lipid peroxidation do interact with chromatin inside the cell nucleus. Using rat liver nuclei labelled with ^{3}H-arachidonic acid which is a main substrate for lipid peroxidation, Vaca and Harms-Ringdahl (1989 a, b) found that the product of lipid peroxidation induced by ascorbate-Fe^{2+} and NADPH-ADP-Fe^{2+} interacted with DNA and nuclear proteins. Inhibition of lipid peroxidation by the iron chelator Desferrioxamine B prevented the association of peroxidation products to nuclear macromolecules.

One of the lipid peroxidation products, malondialdehyde can interact with DNA. Its damage to DNA and genotoxicity have been extensively studied *in vivo* and *in vitro* (Vaca et al., 1988; Esterbauer et al., 1991). It is possible that 4-hydroxyalkenals also react directly with DNA but to my knowledge no experimental evidence has been shown. There is only indirect evidence that suggest this class of product of lipid peroxidation may associate with DNA. The formation of fluorescent products from the interaction of peroxidizing lipid with DNA has been characterized in a series of studies (Vaca et al., 1988). A fluorescence spectrum was observed when DNA was reacted with peroxidizing arachidonic acid. This spectrum was different from that

obtained from the reaction between MDA and DNA. The results indicated that reactive carbonyls other than MDA, formed during the peroxidation of arachidonic acid, may also react with DNA. A similar experiment was performed by Frankel et al. (1987). They examined the fluorescence formation between DNA and a series of aldehydes, carbonyl compounds and other products obtained by thermal and catalytic (in presence of ferric chloride and ascorbic acid) decomposition of pure methyl linolenate hydroperoxides. They found that the aldehydes gave the most reactive intermediates with DNA. In addition, it is observed that HNE can react with deoxyguanosine *in vitro* at pH 7.4 to form cyclic adducts (Winter et al., 1986) indicating that HNE has the ability of alkylating DNA (Witz 1989). Recently, it is observed that a potential activated metabolite of HNE, 2,3-epoxy-4-hydroxynonenal (EH) modifies DNA and RNA. Endogenous HNE may be metabolised to form EH *in vivo* (Sodum and Chung, 1991). Reaction of EH with RNA and single-strand DNA yielded different proportions of adenosine and guanosine adducts. In contrast to the single stranded nucleic acids, reaction with native DNA produced only deoxyguanosine adducts. In double-stranded DNA, yields of adduct were considerably lower than in single-stranded DNA or RNA (Sodum and Chung, 1991).

1. 2. 2. 5. Lethal effects on cells

Apart from the cytoxicity of 4-hydroxyalkenals discussed above, these compounds also alter respiration, Ca^{2+} uptake, phosphate transport, cytochrome P_{450} and levels of cellular glutathione and onset of lipid peroxidation (Esterbauer, 1988; Esterbauer et al., 1991). These toxic effects can induce cell injury, suppression of cell growth and cell death.

Cadenas et al. (1983) reported that HNE at 0.6-2.4 mM

enhanced oxygen-induced low-level chemiluminescence and ethane and n-pentane formation by hepatocytes. HNE at 2 mM enhanced these toxic indices over 7-fold. HNE also caused a rapid loss of cellular glutathione in the form of a glutathione conjugate with the alkenal when it was added to a suspension to hepatocytes. White and Rees (1984) found that HNE at 0.5-1.5 mM initiated lipid peroxidation in rat liver microsomes.

Morphological changes were also observed in the cells injured by HNE. Normal cultured hepatocytes have a polyhedral shape and 1-2 prominent nuclei, and all cells are well attached to the surface of the dishes (Eckl and Esterbauer, 1989; Esterbauer et al., 1990). After treatment of cultures with 100 μM HNE for 3h, a large number of spherical cells with an intense granular structure and pycnotic nuclei appeared. These cells also appeared to have lost contact with the surface. The cells did not recover from this damage and 24h later all the cells were dead. With higher concentrations (0.3-0.5 mM) of HNE it is observed that guinea pig keratocytes (cells from guinea pig epidermis) rounded up and became detached within 1h of treatment (White and Rees, 1984).

The inhibition of cell growth is shown by decreases in cell number and viability. HNE at 1 μM decreased human diploid fibroblasts viability by 20% compared with control. At 100 μM of HNE, the cell viability was totally lost (Michiel and Remacle, 1991). K562 cells growth was strongly inhibited by 100 μM HNE. With treatment of cells with HNE for 1h, the cell number did not increase during 6 days culture period and was only 25% of control. The viability of treated-cell culture lost 30% (Barrera et al., 1991 b).

High concentrations of HNE can eventually cause the death of the cell. Ehrlich ascites tumour cells were killed by 3 mM HNE

(Schauenstein, 1982).

Table 1.2.2 shows the concentration of HNE and time of treatment acutely toxic for mammalian cells. Most of the HNE effects described as inhibitory and toxic to cells are at relatively high concentrations, i.e. in the range of 0.1-10 mM. It seems most unlikely that this high concentration of hydroxyalkenals exists in biological systems, except perhaps in cell membranes (see below). The levels of HNE that can be expected in normal tissues are in the range of about 0.1-3 μM (Esterbauer et al., 1989), and under conditions of oxidative stress the HNE concentration can increase to 5-10 μM. Normal rat liver for example contains 0.5-2.5 μM nmol HNE/g wet tissue, and under vitamin E deficiency this figure may increase to 5.4 nmol/g wet tissue (Esterbauer et al., 1990). The intracellular steady state concentration of HNE in CCl_4-intoxicated hepatocytes was found to be about 7 μM (Poli et al., 1985).

The main reason for the need of relatively high concentrations to get an inhibition of the function *in vitro* is probably the fact that biological fluids contain a large amounts of masking substances preventing the contact of HNE with the enzyme targets. It is shown by the fact that when the aldehydes are assayed on purified enzymes, their active concentration decreases consistently (Dianzani, et al., 1990).

Another reason may be the fact that aldehydes are quickly catabolized by cells and cell particles. Liver cell suspensions can tolerate exposure to a constant concentration (10 μM) of toxic aldehydes (Poli et al., 1989 a). Incubation of isolated rat liver cells with HNE resulted in a rapid loss of the aldehyde from the incubation mixture as measured by h.p.l.c. Within 1 min 92% of 0.1 mM and 50% of 1.0 mM HNE were consumed by 2 x 10^6 cells/ml. The rate of HNE

Table 1.2.2 Cytotoxicity of HNE towards different cell types

Cell Type	LD_{50}, Time
isolated rat hepatocytes	1000 μM, 1h
culturated rat hepatocytes	100 μM, 20 h
intraperitoneal Ehrlich ascites tumor cells	500 μM, 2 h
culturated Ehrlich ascites tumor cells	100 μM, 6 h
Chinese hamster ovary cells	100 μM, 1.5 h
umbilical vein endothelial cells	25 μM, 3 h
human skin fibroblasts	> 40 μM, 1 week
glutathione synthetase deficient skin fibroblasts	20 μM, 1 week
fibroblasts in logarithmic growth	18 μM, 20 h
fibroblasts growth arrested	5 μM, 20 h

(Adapted from Esterbauer et al., 1991)

loss increased linearly with the cell number over the range 5 x 10^4-5 x 10^5 cells/ml (Esterbauer et al., 1985).

On the other hand, the concentration of HNE may be very high in cell membranes. It is observed that aldehydes derived from lipid peroxidation are unequally distributed between the lipid and aqueous phases. Therefore, it is possible that liposoluble compounds such as HNE might reach concentrations in the membrane environment higher than those detected in whole cells. It is assumed that the membrane concentration of HNE is in the range of 4.5-100 μM; this is enough to inhibit several enzyme functions and it has been proposed that HNE might attack critical target proteins within the lipid bilayer (Poli, et al., 1987).

1. 2. 3. Genotoxicity

The genotoxic activities of 4-hydroxyalkenals were investigated in Chinese hamster cells (Brambilla et al., 1988). Sister-chromatid exchange increased after treatment with HPE, HHE, HOE, while HNE and 4-hydroxyundecenal had a weak, although statistically significant, effect. The concentration of 4-hydroxyalkenals was 20-60 μM. DNA fragmentation increased after exposure to the five 4-hydroxyalkenals tested. The genotoxicity of HNE was also observed in rat hepatocytes (Eckl and Esterbauer, 1989; Esterbauer et al., 1990). After incubation with 0.1-10 μM HNE for 3h, the percentage of cells with micronuclei, the frequency of chromosomal aberration and number of sister-chromatid exchanges were significantly increased in primary cultures of rat hepatocytes. HNE at 10 μM or below is not cytotoxic towards the culture hepatocytes. Therefore the observed genotoxic effects are indeed induced by the aldehyde itself and not by some unknown factor produced by severely damaged or dying cells.

Mutagenicity of HNE was observed in V79 Chinese hamster lung cells at the hypoxanthine-guanine phosphoribosyltransferase locus. It was found that at the concentrations ranging from 10-45 μM, HNE induced a dose-dependent increase in the number of mutations to the 6-thioguanine resistance (Vaca, 1988; Eckl and Esterbauer, 1989).

Clastogenic factors (CF) are chromosome-damaging materials in plasma of irradiated persons and patients with so-called spontaneous chromosomal instability. CF have a molecular weight lower than 10,000 Da. Exposure of human lymphocytes or Chinese hamster cells to superoxide-generating systems can produce CF. CF are produced or released by cells. In a recent report by Emerit et al. (1991) it was shown that when cultures of human lymphocytes exposed to hypoxanthine and xanthine oxidase ($O_2^{\cdot-}$-generating system) CF were induced. HNE was formed in 50% of CF induction samples, while it was absent in all control samples. After addition of as low as 0.1 μM HNE in the culture, clastogenic activity was observed. So it was suggested that HNE is a clastogenic component of CF.

In direct contrast to reports of its mutagenicity, HNE has shown its ability to inhibit the mitogenic effect of another mitogen (Kiss et al., 1992). In fibroblasts the mitogenic effects of sphingosine involves a rapid rise in the cellular content of phosphatidic acid (PtdOH). It is observed that in fibroblasts (NIH 3T3 cells), HNE selectively inhibits sphingosine-stimulated phospholipid hydrolysis resulting in the inhibition of PtdOH formation (Kiss et al., 1992).

1. 2. 4. Effect on cell proliferation

HNE and other 4-hydroxyalkenals possess the capacity of blocking cell proliferation at concentrations where lethal effects are low or absent. Schauenstein (1982) and Hammer et al. (1988) reported

that HNE inhibited Ehrlich ascites tumour cell (EATC) proliferation. After incubation of EATC *in vitro* with 0.16 mM HNE for 120 min, the cell viability did not differ essentially from control cells, but when treated cells were reimplanted into healthy mice, considerable inhibition of tumour growth occurred: 90% of animals remained absolutely tumour-free after 10 days, and 10% of the animals showed considerable inhibition of tumour growth. No animal showed unchanged tumour growth. Hauptlorenz et al. (1985) also observed that during exposure of cultured EATC to concentration of 10-20 μM HNE, the proliferation was strongly inhibited. These concentrations of HNE do not affect cell viability and membrane integrity as measured by the Trypan Blue exclusion method and rubidium uptake. Barrera et al. (1991b) found that repeated treatment of the HL-60 human promyelocytic cell line with HNE (1 μM) caused a strong inhibition of cell growth without affecting cell viability. Moreover, HNE treatment induced differentiation of HL-60 cells. HL-60 cells can be induced to differentiate terminally into either monocyte/macrophage-like cells or granulocytes by a variety of chemical agents. Like a common inducer of HL-60 cell differentiation, DMSO, HNE induced chemiluminescence production, phagocytosing capabilities and enzymatic changes.

There are several possibilities that may explain the inhibition of cell proliferation induced by HNE:

(1) Inhibition of the DNA polymerase system and DNA synthesis (Schauenstein, 1982; Hauptlorenz, et al., 1985). DNA polymerase α is an SH-containing enzyme, which can be blocked by sulphydryl reactive agents including α, β-unsaturated aldehydes, such as HNE. However, isolated DNA polymerase α is rather resistant to HNE (ID_{50} = 21 μM), it is therefore unlikely that inhibition of cell proliferation and overall synthesis of DNA results from inhibition of the DNA polymerase system

(Esterbauer et al., 1991).

(2) Disturbance of the cell cycle (Poot et al., 1988; Miglietta, et al., 1991) and alteration of the microtubular system (Olivero et al., 1992). As mentioned above, HNE arrests cells in G2 and G1 phase, delays onset of proliferation due to a retardation of the transit of the cells from G0 to G1 and prolongs the G1 phase. This severe disturbance of the cell cycle indicates that HNE-treated cells show a diminished DNA synthesis and a diminished rate of proliferation.

(3) Interference of the expression of certain genes (Dianzani et al., 1990).

The property of HNE interfering with the gene expression was displayed at very low concentrations. HNE specifically blocks c-myc oncogene expression by a human erythroleukemic cell strain (K 562) cultivated *in vitro.* This effect occurs at concentrations of 0.1-1 μM and is accompanied by the increase of the expression of the gamma-globin gene (Dianzani, et al., 1990). The c-myc protein is involved in the regulation of cellular multiplication.

Low concentration of HNE has also been suggested to reduce ornithine decarboxylase (ODC) gene transcription (Barrera et al., 1991a). ODC is the first regulatory enzyme in the biosynthesis of polyamines. It plays a key role in proliferation both of normal and neoplastic cells and its induction seems to be a universal mechanism of growth stimulation by hormones, drugs and tumour promoters. It has been reported that the inhibition of ODC activity is associated with a block in cell cycle progression at the G1 phase. Barrera et al. found that HNE at 1-10 μM inhibits the induction of ODC activity in leukaemic cells by foetal serum. Since HNE did not affect the half-life of ODC and the same HNE concentration did not inhibit ODC activity when added to cytosol, they suggested that HNE affects ODC synthesis

and may act by reducing ODC mRNA transcription.

The mechanism involved in the regulation cell differentiation still remains relatively obscure (Barrera, 1991 b). It seems that HNE-induced HL-60 cell differentiation is related to regulation of gene expression. HNE inhibits c-myc gene expression in K562 cell. The down regulation of this gene is a common feature of several differentiation processes (Barrera, 1991 b). Induction of HL-60 cell differentiation by DMSO enhanced synthesis of heat shock proteins (Barrera, 1991a). This effect has also been observed after treatment of hepatoma cells with HNE (Cajone and Bernelli-Zazzera, 1989). It is reported that treatment of HeLa cells with HNE has the same effect as heat shock in causing the appearance of a protein that binds to the sequence of DNA specific for the induction of heat-shock gene expression (Cajone et al., 1989).

However, in contrast to the inhibitory effect on proliferation of cells, HNE exhibits some stimulatory effects on gene expression and cell proliferation.

Ascorbic acid has shown to stimulate collagen synthesis via stimulation of procollagen gene transcription. Chojkier et al., (1989) reported that lipid peroxidation and aldehydes induced by ascorbic acid and iron accumulated in culture cells may be necessary for the stimulation of Type I collagen synthesis and collagen $\alpha(1)$ gene transcription in cultured AF_2 human fibroblasts. They observed that coincubation of cells with ascorbic acid and α-tocopherol blocked the production of TBARS, malonaldehyde-protein adducts and 4-HNE-protein adducts as well as ascorbic acid stimulated-collagen production indicating that products of lipid peroxidation mediated the ascorbic acid-induced stimulation of collagen gene expression. They suggested that protein adducts formed with aldehydes derived from peroxidation

such as HNE may play a role in the increased transcription of the collagen gene.

Adenylate cyclase and phosphatidylinositol-4,5-bisphosphate (PIP_2)-phospholipase C (PL-C) are effecter enzymes coupled, through regulatory G proteins, to specific membrane receptors for hormones and growth factors. Their reaction products (cAMP for adenylate cyclase, diacylglycerol (DG) and inositol triphosphate (IP3) for PL-C) play a prominent role in the control of cell division and proliferation. HNE at very low concentrations stimulate both of these two enzymes (at higher concentration, HNE inhibits adenylate cyclase, see Table 1.2.1), 0.1-1 μM for adenylate cyclase in Fisher rats liver plasma membrane, 0.01-1 μM for PL-C in rats liver (Dianzani, et al., 1990, Rossi, et al., 1990) and 0.01 μM-1μM for PL-C in plasma membranes isolated from rat neutrophils (Rossi et al., 1991).

Moreover, HNE (0.001-1 μM) reduces junctional communication (JC) among bovine aortic endothelial cells in culture. JC is reduced progressively with increasing concentration of HNE. The decrease in JC does not seem related to general, unspecific inhibition of protein synthesis. At concentrations between 0.001-1 μM, there is no reduction in protein synthesis for up to 8h treatment with HNE. Intercellular communicating junctions are considered to be important for cellular homeostasis, regulation of cell growth and differentiation. Loss of JC, may help transformed cells to escape from growth control. Reduction of JC is considered to be a general mechanism, among others, for promotion of uncontrolled cellular proliferation (Radu and Moldovan,1991).

Clearly, there are contradictory reports concerning the effect of HNE on cell proliferation and this topic needs further investigation to resolve this contradiction.

From the discussion above, it can be seen that 4-hydroxyalkenals, particularly HNE, have two different effects depending on the concentration. At 10-100 μM or above HNE cause acute and unspecific cytotoxic effects, such as inhibition of DNA-, RNA- and protein synthesis and leading to cell death. At 0.1-1 μM or below, HNE exhibits physiological functions, modulation of cell proliferation/differentiation by affecting associated enzyme activity and gene expression. HNE is a product of lipid peroxidation and under conditions of oxidative stress the concentration of HNE in tissues can increase to 5-10 μM (Esterbauer et al., 1989). The membrane concentration of HNE may reach 100 μM (Poli et al., 1987). At this concentration HNE will show its toxic effects. In fact some cytoxicities of HNE, such as inhibition of glucose-6-phosphatase activity, reduction of P_{450} content and alteration of Ca^{2+} uptake are observed under the condition of lipid peroxidation induced by chemicals, for example CCl_4. Lipid peroxidation also exists in normal tissues at very low level. The physiological concentration of HNE ranged from 0.1-3 μM (Esterbauer, 1990; Esterbauer et al., 1989). At this concentration level, HNE plays a role in the control of cellular proliferation/differentiation. It has been suggested that lipid peroxidation has a very important role for control of cell proliferation (Cheeseman et al., 1986; Slater 1988). The fact that HNE has a regulatory function on cell proliferation supports this hypothesis.

1. 3 PART III RIBONUCLEOTIDE REDUCTASE

1. 3. 1 Ribonucleotide reductase of *Escherichia coli* and mammals

Ribonucleotide reductase (ribonucleoside diphosphate reductase, EC 1.17.4.1.) and ribonucleoside triphosphate reductase (EC 1.17.4.2.) catalyse the conversion of all ribonucleotides into their corresponding deoxyribonucleotides. Since the pool of deoxyribonucleoside triphosphates is relatively small compared to the rate of DNA synthesis, ribonucleotide reductase is considered to play a critical role for DNA synthesis, maintaining an adequate and balanced supply for the direct precursors of DNA (Thelander and Reichard, 1979; Lammer and Follmann, 1983; Cory, 1989).

Based upon substrate specificity and cofactor requirements, the reductase can be classified into three types. The first type reduces ribonucleoside diphosphates and is best represented by the enzyme from *Escherichia coli* (*E. coli*). This type of enzyme is also found in mammals, higher plants and in some fungi (Thelander and Reichard 1979; Wright et al., 1989; Lammers and Follmann 1983). The second type of enzyme reduces ribonucleoside triphosphates, requires 5´-deoxyadenosylcobalamin as a factor which is represented by the enzyme from *Lactobacillus leichmanni* (Carell and Seeger, 1980). The third type is characteristic of *Bacillus megaterium* and *Rhizobium melilate.* This enzyme requires deoxyadenosylcobalamin as the co-factor but reduces the ribonucleoside diphosphates (Thelander and Reichard, 1979, Carell and Seeger, 1980). There is another type of enzyme in which manganese is involved in ribonucleotide reduction (Lammer and Follmann, 1983).

1.3.1.1. *Escherichia coli* enzyme

The well characterized ribonucleotide reductase from *E. coli* is composed of two non-identical proteins B1 and B2, both required for activity (see Fig. 1.3.1). Protein B2 (Mr=78,000) contains two identical polypeptide chains (ββ), that do not dissociate except under denaturing conditions. Each chain contains a non-haem iron centre and an oxidized tyrosine free radical which is essential for the enzymatic reaction (Reichard and Ehrenberg, 1983; Atkin, et al., 1973). This radical is derived from a tyrosyl residue of the polypeptide chain and is stabilised by the iron centre. The radical can be recognized by a sharp peak at 410nm in the optical spectrum and a characteristic electron paramagetic resonance (EPR) signal (Thelander and Reichard, 1979; Sjoberg and Reichard, 1977). Protein B1 (Mr=160,000) is a dimer of polypeptides (αα´) which are identical in size but appear to differ in their N-terminal amino acid sequence. B1 contains two apparently identical binding sites for substrates (CDP, UDP, GDP and ADP) and four sites for allosteric effectors. Of four allosteric effector sites, two sites ('h' sites) bind dATP with high affinity and, in addition, bind ATP, dGTP, and dTTP and determine substrate specificity of the enzyme. The remaining two sites ('l' sites) bind dATP with low affinity and, in addition, only bind ATP and govern the overall activity of the enzyme (Thelander and Reichard, 1979; Reichard and Ehrenberg, 1983). The active enzyme contains B1 and B2 in a 1:1 stoichiometry. The catalytic site of the holoenzyme is at the interface of the two proteins comprised of residues from both subunits with B1 contributing redox-active sulphydryl groups that donate the electrons necessary for the reduction and B2 contributing the tyrosine free radical and the non haem iron (Thelander and Larsson, 1976;

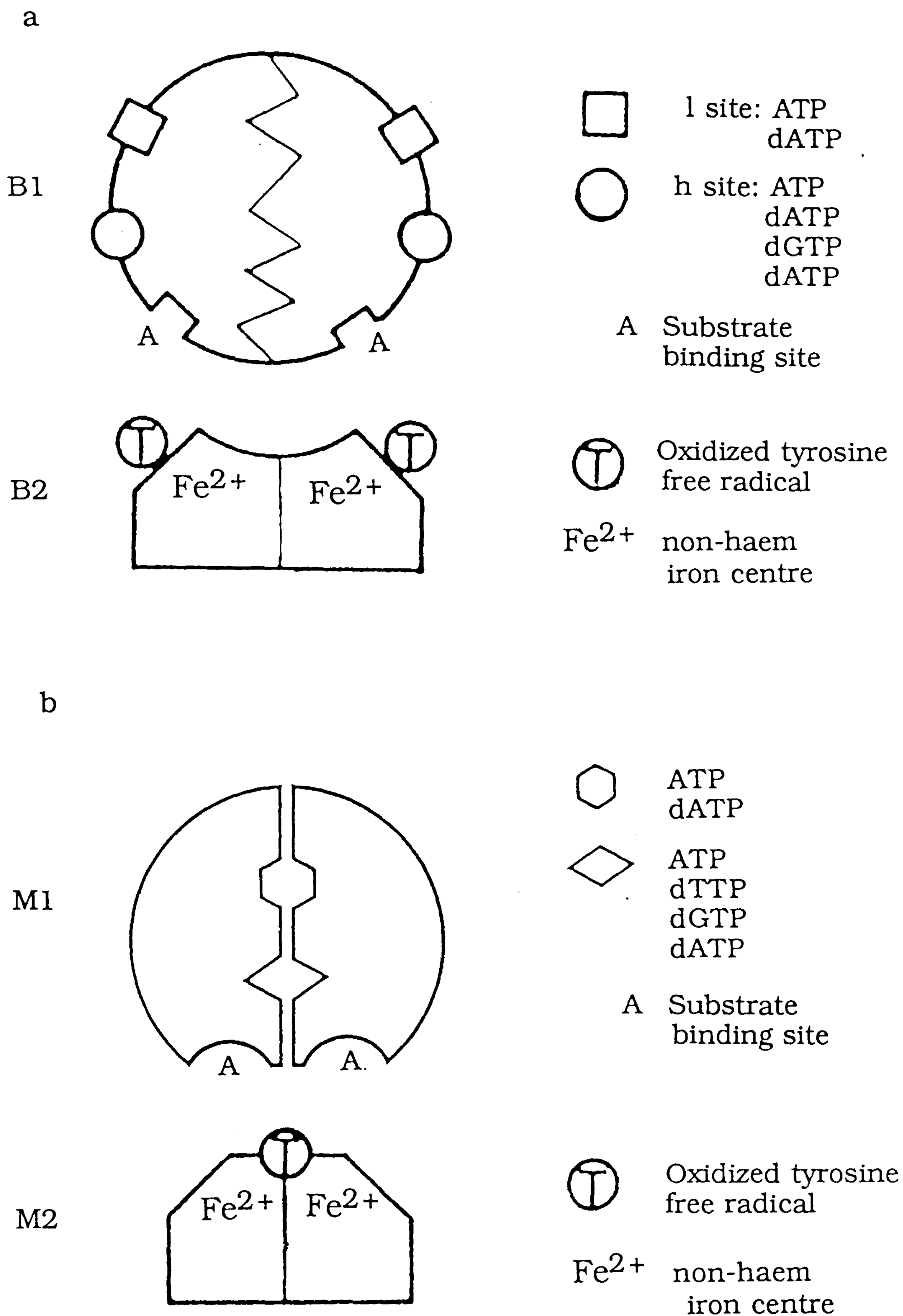


Fig. 1.3.1 Model for *E. coli* and mammalian ribonucleotide reductase
(a) *E. coli* enzyme; (b) mammalian enzyme
(Modified from Wright, 1989 and Whitfield and Youdale, 1989)

Thelander and Reichard, 1979; Lin et al., 1987). The cross-linking data support a model in which both of the β-subunits interact closely with only one of the two α subunits (Mathews et al., 1987). C-terminal ends of the B2 protein are involved in the interaction with the B1 protein (Climent et al., 1991).

1.3.1.2. The mammalian enzyme

Mammalian ribonucleotide reductase appears to resemble the *E. coli* enzyme with respect to its substrate, iron and free radical involvement. Studies on the enzyme from Ehrlich ascites hepatoma tumour of mice (Cory et al., 1978), rat Novikoff hepatoma (Moore, 1977), human lymphoblast cell line (Molt-4F) (Chang and Cheng, 1979), rabbit bone marrow (Hopper, 1972), calf thymus (Thelander et al., 1980; Engstrom, et al., 1979), mouse fibroblast 3T6 cell (Larsen et al., 1982), and rat regenerating liver (Youdale and MacMances, 1979; Youdale rt al., 1982) have revealed that several properties of the mammalian enzyme are similar to those of the enzyme obtained from *E. coli*.

In mammalian cells, the enzyme consists of two non-identical subunits called M1 and M2. These two components have functions equivalent to the B1 and B2 proteins of the *E. coli* reductase (Cory et al., 1978, 1979; Thelander and Reichard, 1979; Thelander et al., 1985; Nutter and Cheng, 1989) (see Fig. 1.3.1). Protein M1 is a dimer and it contains two kinds of effector binding sites, one kind specific for ATP or dATP (activity site) and the other kind capable of binding ATP, dATP, dTTP and dGTP (specificity site), but only one catalytic site which forms, or becomes functional when M1 subunits couple with M2 subunits. There is no indication of the presence of high- and low-

affinity binding sites for dATP (Thelander et al., 1980). The M2 protein is a dimer too and it contains two non-haem iron centres and a tyrosyl free radical essential for activity (Thelander et al., 1985; Ochiai et al., 1990). Both iron and radical centre seem to be the same as in the bacterial protein (Thelander et al., 1985; Akerblom et al., 1981; Graslund et al., 1982). Molecular weights of the intact enzyme and separated components of some mammalian reductases were summarised by Nutter and Cheng (1989) (see Table 1.3.1).

1.3.1.3. Mechanism of ribonucleotide reductase reaction

In *E. coli* and mammalian cells the reduction of the four ribonucleotides occur at the diphosphate level, (NDP to dNDP) and involves the replacement of a hydroxyl group by a hydrogen. It appears likely that the enzyme reaction involves a direct replacement of the hydroxyl group at carbon 2′ of NDP by a hydrogen (Thelander and Reichard, 1979; Lammer and Follmann, 1983). In addition to the ribonucleotide reductase, two small proteins, thioredoxin and thioredoxin reductase, which is a flavoprotein, also participate in the reduction process as hydrogen carriers. The electrons are originally from NADPH (Thelander and Larsson. 1976; Thelander and Reichard, 1979). The scheme for the ribonucleotide diphosphate reduction in *E. coli* is shown in Fig. 1.3.2. Reduced thioredoxins do not directly donate H to a substrate but first reduce an enzyme disulphide bridge. During enzymic catalysis, the B1 subunit alternates between two forms, the active dithiol form and the inactive disulphide form, which is continuously regenerated by thioredoxin (Sjoberg and Reichard, 1977; Lammer and Follmann, 1983). There are at least 4 redox-active cysteine thiols in the reductase B1. The role of thiol residues in the reductase catalysis is much more complex: two pairs of thiols are

Table 1.3.1 Molecular weights of mammalian ribonucleotide reductase and their components

Source	Holoenzyme	Component	
		Effector-binding (M1)	Non-haem iron (M2)
Rat Novikoff ascites	200,000 - 250,000 *	180,000 * 90,000 #	
Regenerating rat liver	280,000 †	45,000 # , † (CDP reduction)	120,000 † 75,000 + 47,000 #
Human lymphoblast	210,000 ⊕	100,000 ⊕	100,000 ⊕
Calf thymus		84,000 #	55,000 #
		98,000 ⊕	110,000 * , ⊕
		84,000 #	58,000 #
Mouse Ehrlich tumour	340,00 § (CDP reduction) 254,000 § (ADP reduction)	127,000 § 95,000 §	81,000 §
Mouse lymphosarcoma		89,000 #	

Methodological criteria; * gel filtration, # SDS-polyacrylamide gel electrophoresis, ⊕ sedimentation velocity, § sedimentation equilibrium, † molecular exclusion high performance liquid chromatography.
(Adapted from Nutter and Cheng, 1989)

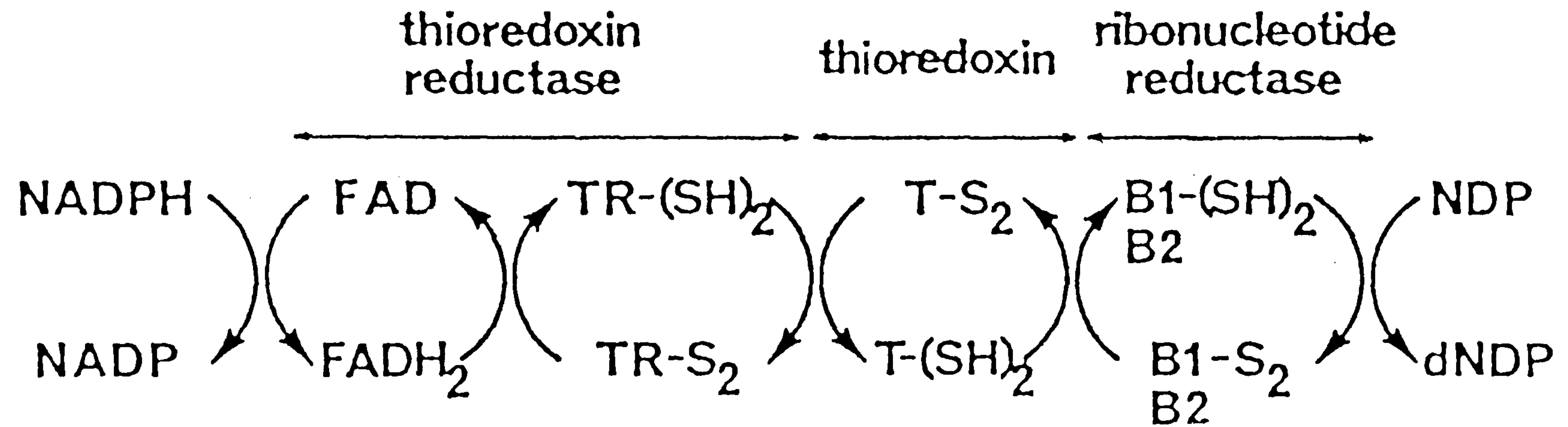


Fig. 1.3.2 Reduction of ribonucleotide diphosphate to deoxyribonucleotide diphosphate in *E coli.*

(Adapted from Thelander and Reichard, 1979)

involved in catalysis, one being responsible for substrate reduction and the other for shuttling reducing equivalents from the external reducing system into the active site (Ashley and Stubbe, 1989). The role of the B2 free radical in the substrate reduction is not very clear, but it directly participates in the catalytic process together with the oxidation-reduction active dithiols of B1 (Thelander and Larsson, 1976; Sjoberg and Reichard, 1977; Ashley and Stubbe, 1989).

A single *E. coli* reductase reduces all four ribonucleotides (Thelander and Reichard, 1979; Cory, 1989). The mammalian enzymes, on the other hand, seem to be more complicated. Youdale et al (1982) proposed that there are four different L1 (or M1) subunits each specific for the reduction of a particular ribonucleotide (ADP, UDP, CDP and GDP) attached to one L2 (or M2) subunit in rat liver. A similar conclusion suggesting that there may be more than one kind of M1 subunit in rat liver comes from Whitfield (1989) who observed that the M1 C-terminal anti-serum inhibits CDP-reductase activity almost completely in extracts from 24h regenerating liver, but this anti-serum does not affect ADP reductase activity. Carter and Cory (1989) found that elevated CDP and ADP reductase activity in hydroxyurea-resistant cells (hydroxyurea is a specific inhibitor of the reductase, see below) are not coordinately increased indicating that the two enzymes are not identical. Based on the observations of the enzyme from Ehrlich hepatoma tumour cells, mouse L cell lines, and CHO cell lines, Cory and Fleischer (1982 a) suggested that the intact mammalian reductase is made of a non-haem iron component (M2) that is common to all substrates and an effector-binding component (M1) which is specific for each substrate. There are conflicting conclusions. Chang and Cheng (1979) reported ADP- and CDP- reductase activity may exist in the same enzyme of human lymphoblast cells (Molt-4F) but have different

active sites. With the enzyme from calf thymus, Ericksson (1979) claimed that only one enzyme catalyses the CDP, UDP, GDP, and ADP reduction. However, their data are not sufficient for the conclusion that the four substrate are all reduced by the same enzyme (Cory and Fleischer, 1982 a).

1.3.1.4. Location of the enzyme

Although it was once postulated that the ribonucleotides were reduced in the nucleus, using immunohistochemical and subcellular fractionation techniques it has been demonstrated that the enzyme is exclusively localised in the cytoplasm and the activity is present in the cytoplasm (Elford et al., 1972; Chang and Cheng, 1979; Hansson et al., 1986; Kucera and Paulus, 1986).

1.3.2 Relationship between ribonucleotide reductase activity and the growth of tissues and cells

The levels of deoxyribonucleoside triphosphates in cells are very low. It is found that the dNTP pools are sufficient to support DNA synthesis for only 1-5 min (Elford, 1974). Therefore, the reductive conversion of ribonucleotides to deoxyribonucleotides is believed to be a crucial and rate-controlling step in the pathway leading to the biosynthesis of DNA and the enzyme that catalyses the reduction, ribonucleotide reductase is considered to play a key role in cell division and its activity closely correlates with the tissue growth (Elford, 1970,1972; Takeda and Weber, 1981).

1.3.2.1 Activity in proliferating tissue

Based on research into the relationship between reductase

activity and growth rate of hepatomas, Elford et al. (1970; 1972) found an excellent correlation between tumour growth rate, which is defined as the average time required for the development of a viable transplantable tumour, and the activity of the reductase (see Fig. 1.3.3). Differences of 200-fold in enzyme specific activity exist between the very fast- and the slow-growing hepatomas. The experiments of Takeda and Weber (1981) support this finding. They showed that the reductase activity is markedly increased in hepatomas compared to the normal liver and the rise in activity correlates positively with the growth rate of the tumours.

A strong correlation between the enzyme activity and tissue proliferation is also found in the profile of the enzyme activity during neonatal development in several rat organs. Rat foetal liver extract has a very high level of enzyme activity. At the time of birth, there is a sharp decrease in activity. One week after birth there is only a very small amount of activity remaining, 1/20 of the newborn liver activity and only 1/200 of the 18-19-day foetal liver extract activity. No activity can be detected in adult rat liver. In developing rat spleen, the maximum activity occurs at a time when the onset of white cell production is initiated, at six days after birth. In the developing rat thymus, the enzyme is very low at the time of birth and then rapidly increases. The maximum peak of activity occurs at about the time that the thymus is able to colonise the lymphoid system and synthesize a new pattern of immunoglobulins (Elford, 1972). The activity of the enzyme is also markedly increased in regenerating liver (King and Lancker, 1969; Elford, 1974; Cory and Fleischer, 1982 b; Takeda and Weber, 1981). The main events preceding cellular division and cellular multiplication after partial hepatectomy are DNA synthesis, which in the rat starts at 18h post operation, and mitosis which starts 6h later

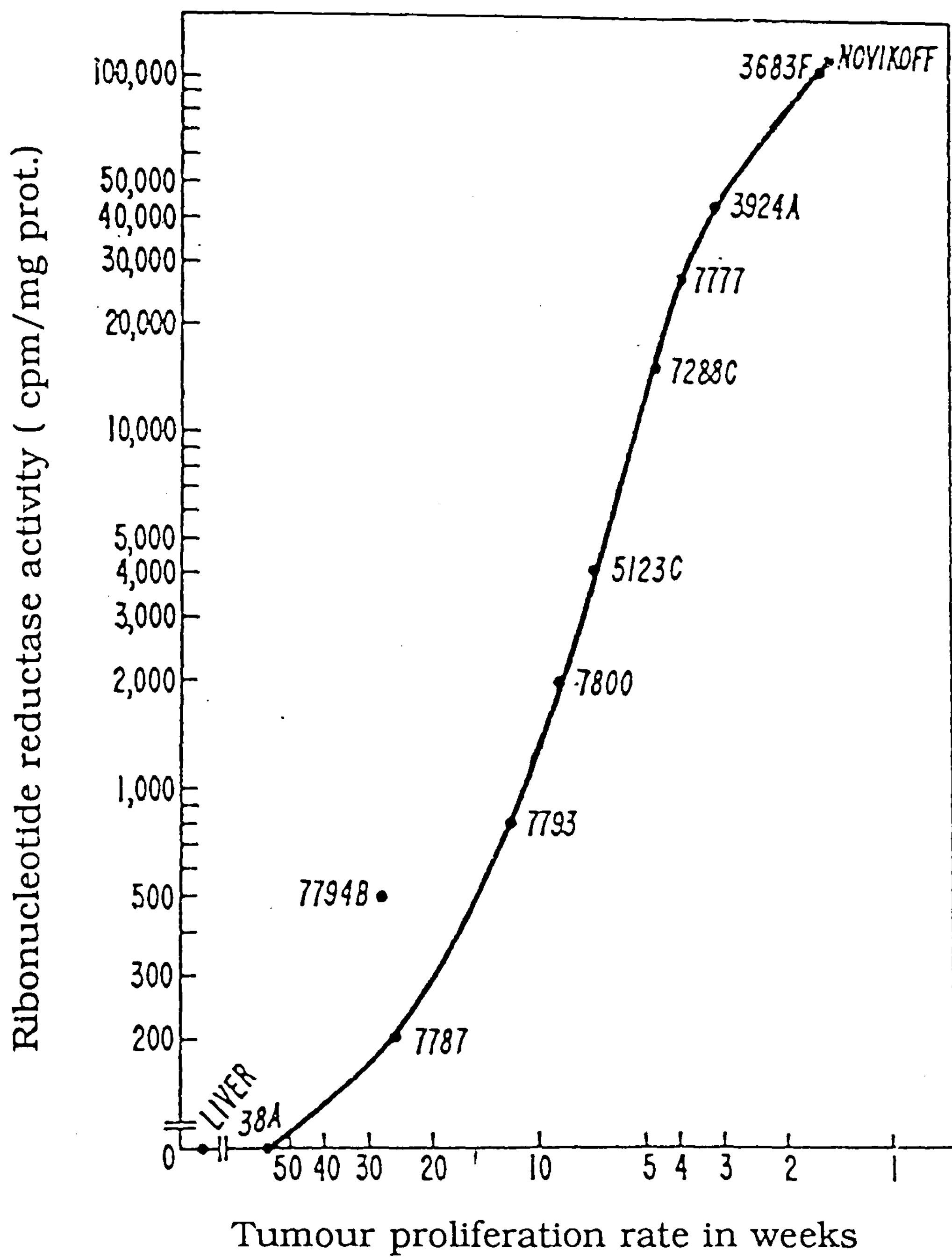


Fig. 1.3.3 Relationship between tumour growth rate and ribonucleotide reductase activity

(Adapted from Elford, 1972)

(King and Lancker, 1969). According to Elford (1974) there is no detectable activity in normal rat liver, or in 5h, 12h, and 18h regenerating rat liver. However, appreciable activity is seen 24h after hepatectomy and is further increased after 36h.

Further evidence for the close involvement of reductase in replication is provided by the organ distribution of this enzyme activity. Among 14 different tissues of the rat the activity is highest in the organs of active cell renewal: namely, in the thymus, bone marrow, spleen, mucosa of small intestine, and colon mucosa. In other organs activity is only found in trace amounts in the brain and adipose tissue (Takeda and Weber, 1981).

1.3.2.2. Activity during cell cycle

Many investigations have uncovered a critical association between the mammalian reductase activity and cell growth. There is a strict relationship between the measured reductase levels and the fraction of the cell population that is replicating DNA (Turner et al., 1968). The activity of the enzyme has been shown to vary in cells at different phases of the cell cycle. The enzyme activity is low in resting cells during G0-G1 and increases dramatically just prior to and during periods of DNA synthesis, S phase, and then returns to a low basal level near the end of S phase (Eriksson and Martin, 1981; Cheranne and Caldini, 1986; Rubin and Cory, 1986).

The mammalian enzyme is composed of two proteins M1 and M2. The proteins are associated but can be separated into two inactive components which constitute an enzymatically active complex when recombined. In contrast to the enzyme in *E. coli*, the two subunits of the mammalian reductase are not coordinately increased and decreased during the cell cycle (Lammers and Follmann, 1983; Nutter

and Cheng, 1989). Although it is in agreement that the two subunits of mammalian enzyme are not coordinately expressed, there are two opposite conclusions concerning which subunit is the controlling or limiting component for enzyme activity in G1 when holoenzyme is low.

Cory and Fleischer (1982 b) reported that in Ehrlich ascites tumour cells, the decrease in CDP reductase activity which accompanies decreased proliferation *in vivo*, is correlated with the decrease in the effector-binding component (M1) since the addition of this component to cell-free extracts stimulated CDP reductase activity, and the addition of non-haem iron content component (M2) had no effect. Rubin and Cory (1986) found that in L1210 murine leukemia G1 phase cells, addition of exogenous effector-binding subunit markedly increased the level of CDP reductase activity indicating an excess of the non-haem iron subunit and a limiting amount of the effector-binding subunit. Whitfield et al (1989) demonstrated that in regenerating rat liver cells functional M2 subunits start accumulating rapidly around 2h before the onset of DNA replication. Only when the M2 subunits reach a certain level do M1 subunits appear and combine with waiting M2 subunits to form the active reductase holoenzyme needed for DNA replication. Thus, from these results it is suggested that the M1 subunit controls the level of reductase holoenzyme.

By contrast, another group of experiments show that M1 is in excess and the enzyme is controlled during the cell cycle by the level of M2 protein. The increased overall enzyme activity in cells from the S phase of the cell cycle is shown to be due to an increased M2 protein activity, and the M1 protein is practically constant in G1 and S phase cells. When mouse S49 lymphoma cells are arrested by Bt_2cAMP (N^6,O^2-dibutyryl 3'-5'-monophosphate) in the G1 phase, addition of M1 protein gave no stimulation of the reductase activity from treated

cells, but the addition of M2 protein resulted in a stimulation of the activity indicating that the decreased enzyme activity in Bt_2 cAMP-treated, G1-arrested cells is mainly due to the limiting M2 protein activity. When a population of dividing cells was subfractionated as follows:

(i) 90% cells in G1

(ii) 31% cells in G1 and 68% cells in S/G2

(iii) 95% cells in S/G2

the specific activity of M1 protein was found to be similar in all 3 fractions and close to that of an unfractionated population, however, the specific activity of protein M2 is very similar in fraction (ii) and (iii), but in the fraction (i) it is decreased to approximately one-third (Eriksson and Martin, 1981). In hydroxyurea-resistant, M2-over-producing mouse mammary tumour TA3 cells, the concentration of the M2-specific tyrosyl radical increases when cells pass from the G0/G1 phase to the S phase of the cell cycle. Using two independent methods to obtain a synchronized cell population, centrifugal elutriation and isoleucine starvation, it was found that both S and G2 cells contain high levels of protein M2, whereas very little is found in G0/G1 cells (Erissson et al., 1984). In bovine kidney MDBK cells, no correlation was observed between the protein level of M1 which was determined by the use of a two-site monoclonal antibody-enzyme immunoassay and the number of cells in the S phase. Instead, M1 protein is constant through the cell cycle. However, M2 protein increases in S phase in parallel with an increase in reductase holoenzyme activity. Addition of an excess of pure M1 gave no stimulation of the enzyme activity. Addition of purified M2 protein resulted in a pronounced stimulation of enzyme activity (Engstrom, et al., 1985). These studies suggest that, at least in these cultured cell lines, the M2 protein is limiting for

ribonucleotide reductase activity during the cell cycle (Wright, 1989). Other evidence that supports the view that M2 controls the enzyme activity come from Strandart et al (1985, 1986) who showed that unfertilised clam and sea urchin maintain a pool of functional M1 subunits and a pool of untranslated M2 mRNA which translate only after fertilisation.

Mann et al. (1988) defined the kinetic feature of M1 expression related to transitions into, within, and out of the cell cycle by directly studying its expression during a variety of cell kinetic events. They observed that the M1 subunit is continually expressed by rapidly-cycling cultured cells throughout the cell cycle. The level in G1 is even slightly higher than in later parts of the cell cycle, whereas quiescent (G0) cells have virtually no M1. Their data further reinforce the view that M1 protein levels do not limit activity during G1. These results indicate that when proliferation ceases, M1 falls in these 'out of cycle' cells. This is in agreement with the result that protein M1 is not present in all cells, it is only present in proliferating cells of rat tissues (Engstrom et al., 1985). A similar observation was made by Whitfield et al., (1989). They observed that cells in intact rat liver make functional M1 protein only when replicating DNA. When quiescent, or after finishing DNA replication the liver produces a fragment of the functional M1 protein. This fragment in the quiescent, intact liver is not functional.

However, the precise relationship between the changes in the M1 and M2 level during the cell cycle has not yet been determined (Sato and Cory, 1986).

1. 3. 2. 3 The relationship between other enzymes associated with DNA metabolism and cell proliferation

Apart from ribonucleotide reductase, other enzymes are involved in pyrimidine and purine metabolism, such as thymidylate synthetase, thymidine kinase, uridine kinase, and uridine phosphoribosyltransferase etc. However, ribonucleotide reductase plays a pivotal role in the biochemical programme of proliferation. Elford et al. (1970) indicated that although thymidine kinase is markedly elevated in the hepatomas and that the increases are related to growth rate, discrepancies were observed and this enzyme is a salvage pathway enzyme and not a *de novo* synthetic one. The activity of thymidylate synthetase appears elevated in hepatoma tumours, but the degree of correlation between this enzyme activity and growth rate of the tumour is quite limited, especially in the rapidly growing tumour (Elford et al., 1970). The largest measurable difference in thymidine kinase and thymidylate synthetase activities between the fast-growing, poorly differentiated tumours and slow-growing, well-differentiated tumours, are less compared with the difference in ribonucleotide reductase activity, only about 20-fold in the case of thymidylate synthetase and 50-fold in the case of thymidine kinase (Elford, 1972). Ribonucleotide reductase activity in fast-growing tumours is 200-fold higher than the enzyme activity in slow-growing tumours. Takeda and Weber (1981) observed that when cells are freshly plated and allowed to grow for a 96h period, ribonucleotide reductase is the first enzyme activity to rise at 6h among the enzymes mentioned above. The only other parameters measured that rise as early as 6h after plating are the incorporation of cytidine and deoxycytidine into macromolecules. These results indicate that the correlation of the ribonucleotide reductase activity with DNA synthesis and tissue growth rate is much

closer than the other enzymes associated with DNA synthesis.

Additionally, the physiological reducing complex, thioredoxin/ thioredoxin reductase is also shown not to be the rate-limiting factor accounting for the large differences in the reductase activity observed between the various hepatomas (Elford, 1972).

1. 3. 3. Regulation of ribonucleotide reductase activity

Ribonucleotide reductase from all sources so far examined exhibits complex control by different mechanisms of regulation from holoenzyme activity level to the molecular level.

1. 3. 3.1 Product feed back regulation

The enzyme activity has been shown in both mammalian and bacterial systems to be regulated in a very strict allosteric fashion by nucleoside 5'-triphosphates and nucleoside 5'-diphosphates that can serve as positive and negative effectors (Thelander et al., 1980; Eriksson et al., 1981; Cory, 1973; Cory et al., 1985 b; Hards and Wright, 1984). There are two genetically independent regulatory domains on the protein M1 molecule, one responsible for regulating substrate specificity of the enzyme and the other for regulating its overall activity (Erikson et al., 1981) (see Table 1.3.2).

The most widely cited regulatory model for the mammalian reductase is that described by Thelander and Reichard (1979) and Thelander et al. (1980). According to this model, the regulation of enzyme activity is proposed to occur as follows. The holoenzyme is activated when ATP causes the formation of an M1 dimer containing two kinds of effector sites and one kind of catalytic site. When ATP occupies both of the effector sites, the M1 catalytic site switches to its

Table 1.3.2 Regulation of E. coli and mammalian ribonucleotide reductase by ribonucleoside triphosphate

Activity site		Specificity site	
Binds	Effects	Binds	Substrate reduced
ATP	activation	ATP }	CDP
dATP	inhibition	dATP }	UDP
		dTTP	GDP
		dGTP	ADP

(Adapted from Eriksson et al., 1981)

CDP/UDP reductase mode. However, when the increasing amount of dTTP formed from dUDP eventually displaces ATP from one of the M1 effector sites, the catalytic site switches from its CDP/UDP reductase mode to its GDP reductase mode. The increasing amount of dGTP displaces dTTP from the effector site and stops GDP reduction by switching the catalytic site to its ADP reductase mode. Finally, the cycle or pulse ends when dATP displaces ATP from one of the effector sites and switches off the enzyme. However, this model was constructed using data obtained with cell-free extracts, and there are indications that *in vivo* regulation of the enzyme may not entirely agree with this model. Using an intact cell assay system, Hards and Wright (1984) modified the model. They suggested that GTP, or perhaps dCTP, should be included in the model for the enzyme regulation (for activation of CDP and ADP reduction). Since it is indicated that mammalian reductase consists of one of four distinct M1 subunits plus one M2, in the new modified model, it is assumed that all four possible M1 subunits would possess a similar regulatory site to which dATP binds to inhibit enzyme activity. Each subunit should also possess a nucleoside specific site but, unlike the *in vitro* model, it is proposed that each M1 subunit can bind only those nucleotides which activate the particular substrate it binds. Cory et al., (1985 b) demonstrated that nucleoside 5′-diphosphates and the direct products of the enzyme reaction, deoxynucleoside 5-diphosphates can also serve as positive and negative effectors respectively. dNDPs act as non-competitive inhibitors with respect to the substrate.

The question arises whether the difference in reductase activity between the various tissue and cell phases are the consequence of an alteration in the enzyme from an inactive to active state as mediated by allosteric agents, or whether they represent an increase in enzyme

synthesis. Takeda and Weber (1981) suggested that it is unlikely that the enzyme activity in rapidly proliferating tissues is controlled by modulation of the enzyme activity by feedback inhibitors. In most mammalian systems the inhibition of purified enzyme activity achieved by dNTPs was not verified in the actual cellular concentration of these metabolites. In normal, regenerating and newborn rat liver and in slowly and rapidly growing hepatomas, there is a growth-rate-related increase in the levels of dATP, dGTP, and dTTP. The concentrations required to inhibit 50% of the activity of CDP reductase from regenerating liver and rapidly growing hepatoma are much higher than those normally found in tissues. Thus, Takeda and Weber proposed that the activity of reductase in the liver and in hepatoma is controlled chiefly by the amount of the reductase present in the cell. Elford et al., (1970) believed that the gross changes in enzyme activity observed in a series of hepatoma tumours represent different rates of enzyme synthesis and degradation in response to the cell requirement for DNA synthesis rather than response to an alteration in the state of the enzyme from an inactive to an active state. The allosteric control probably exerts a fine control on the reaction by assuring that a balanced supply of deoxyribonucleotides is synthesized and preventing an excess supply of free deoxyribonucleotides since excess deoxyribonucleoside triphosphates are cytotoxic to cells (Cory, 1989). However, Albert and Gudas (1985) claimed that the increased reductase activity seen in mouse T-lymphoma S49 S phase cells compared with G1 arrested cells is not due to a large induction of the synthesis of either the M1 or M2 subunit. Allosteric control of the enzyme accounts for some changes in dNTP pools, or enzyme activity during the cell cycle, especially in G1 phase.

1. 3. 3. 2 Regulation through free radical activation

The enzyme activity of protein M2 depends upon the presence of the tyrosyl radical. The regeneration of the radical is an intrinsic property of M2 protein provided that iron, dithiothreitol and oxygen are present (Thelander et al., 1983;1985). The thymus enzyme might not permanently contain a free radical species but instead forms such a species only during the reaction (Engstrom et al., 1979). These facts imply that cells may have a mechanism for controlling reductase activity by generating the free radical of M2. Two experiments support this proposal. Using a cell line (mouse fibroblast 3T6) resistant to hydroxyurea, Akerblon et al. (1981) and Graslund et al. (1982) observed a 20-fold increase in M2 tyrosine free radical content compared to that seen in non-resistant cells, but only about a 3-fold increase in the amount of M2 protein indicating that part of the increase of enzyme activity is due to the modulation of the free radical. Probast et al. (1989) observed that the radical concentration in cultured Ehrlich ascites cells decreased under hypoxia and increased again upon re-aeration. At the same time, CDP reductase activity changed in the same way as the radical concentration: CDP reduction was greatly diminished and reactivated respectively under hypoxia and re-aeration. However, it is suggested that normally there is no pool of radical-free M2 protein present in cells (Eriksson et al., 1984; McClarty et al., 1987 b). There is still no concrete evidence whether the balance between radical containing and radical-free enzyme reflects an additional regulatory mechanism.

1. 3. 3. 3 Regulation through the changes of the level of enzyme protein

It is suggested that perhaps in the cell the components of the

reductase do not exist as a tightly bound entity because the subunit components of the enzyme are dissociable (Cory and Fleischer, 1979). This may be an important factor in the regulation of this enzyme in the intact cell. This suggestion plus the fact that M1 and M2 can exist independently in the cell cycle indicate that M1 and M2 are able to be regulated differently in the cell. In recent years, some studies concern in particular the molecular regulation of M1 and M2. Usually these studies have used a valid model, hydroxyurea-resistant mutant cells, since these cells have an elevated activity of ribonucleotide reductase and the increased enzyme activity is related to an increase in the enzyme level rather than to marked alterations in activity (Choy et al., 1988; McClarty et al., 1987 a,b; Hurta and Wright, 1990 a, b; Bjorklund et al., 1990). Mutants altered in the reductase activity would be valuable tools for analyzing the complex structural, regulatory, and functional properties of the mammalian enzyme (Wright, 1989). In general, compared with wild-type cells, M1 protein and M2 protein levels are increased in the mutant cells though the increase in the M2 level is much higher than in M1. For example, hydroxyurea-resistant mutant mouse L cells contain approximately 50-fold more M2 than its wild-type counterpart whereas M1 protein is over-produced 2-3-fold in mutant cells compared to wild-type cells (McClarty et al.,1987 a, b). Both protein increases are accompanied by corresponding elevation in the levels of mRNA for both subunits and increased rates of transcription of both genes. Usually the M2 mRNA transcript was dramatically overproduced in the mutant cell while only the most highly resistant lines show increases in M1 mRNA as well (McClarty et al., 1987 a, b; Choy et al., 1988; Hurta and Wright,1990 a,; Albert et al., 1990). The increased level of M2 mRNA is frequently associated with amplification of the M2 gene. In contrast, M1 gene amplification in

hydroxyurea resistant cell lines appears to occur only rarely, and seems to be associated with the most highly drug resistant cells, for example in the cells which are resistant to 15 and 30 mM hydroxyurea (McClarty et al., 1987a; Wright, 1989; Hurta and Wright, 1990 b). Moreover, M1 and M2 protein levels are further elevated when the drug (hydroxyurea) is present, i.e. mutant cells grown in the presence of hydroxyurea contain more M1 and M2 protein than the same cells grown in the absence of the drug. However the levels of M1 and M2 mRNA are essentially unchanged when the mutant cell line is grown under two different conditions, one is presence of HU and the other is absence of HU (McClarty et al., 1987 a; 1988). These results suggest that these kinds of change in M1 and M2 proteins occur by a post-transcriptional mechanism, such as an increase in half-life or through an increase in protein biosynthetic rate (Choy et al., 1988). It is observed that when mutant cells are cultured in the presence of hydroxyurea, the half-lives of both proteins M1 and M2 are increased by approximately 2-fold but the increase in the rate of M1 and M2 biosynthesis occurs slowly. The level of protein M2 rises much more rapidly than protein M1 (McClarty et al., 1988). These observations show that multiple mechanisms are involved in the regulation of the two proteins of the enzyme.

The regulation of M1 and M2 protein levels through the changes of mRNA concentrations seems to be a common mechanism for a increase of enzyme activity in hydroxyurea-resistant mutant cells for achieving resistance to hydroxyurea (McClarty, 1987 a, b). Perhaps this is also an important regulation mechanism which accounts for both the S phase dependent reductase activity expressed as the cell pass through the cell cycle, and the enzyme elevation in rapidly dividing tissues. From the results of measuring the levels of M1 and M2 mRNA

during the cell cycle in centrifugally elutriated cells and cells synchronized by isoleucine or serum starvation, it has been shown that the levels of both transcripts are very low or undetectable in G0/G1 phase, have a pronounced increase as cells progress into S phase, and then decline when cells progress into G2 + M phase (Bjorklund et al., 1990). Many investigations have shown that *de novo* synthesis of the reductase is a major contributor for the increase of the enzyme activity in rapidly proliferating tissue since the protein synthesis inhibitor prevented expected increases in enzyme activity during the neonatal development of spleen and thymus in the rat (Elford , 1972) and when cells approached S phase (Lammers and Follmann, 1983).

1. 3. 4 Inhibition of ribonucleotide reductase activity

1. 3. 4. 1 Chemical inhibitors

The complex structure and molecular mechanism of the reaction of ribonucleotide reductase enzymes make them vulnerable to many kinds of disturbances (Lammers and Follmann, 1983). The enzyme from all sources is easily inhibited by one or more of the following inhibitors.

i) inhibitors which destroy or hinder generation of the radical.

ii) inhibitors which affect the metal ion which has structural or catalytic function.

iii) inhibitors which affect the effector-binding component (M1 or B1) or specially inactivate the catalytically-active dicysteine system.

iv) inhibitors which block the substrate or the effector-nucleotide binding sites and require a functional enzyme.

v) inhibitors which cause disturbance of the native protein

structure which is important for activity.

Several different compounds have been shown to be relatively specific inhibitors of ribonucleotide reductase (see Fig. 1.3.4).

i) Free radical scavengers and inhibitors of free radical formation

(a) Hydroxyurea (HU)

HU is a known antineoplastic drug employed in clinical chemotherapy for solid tumour as well as acute and chronic leukemia and a widely used tool for reversible inhibition of replicative DNA synthesis in cell synchronization *in vitro* and *in vivo* (Lammers and Follmann, 1983; Wright, 1989). It is a highly specific and extensively studied inhibitor of ribonucleotide reductase. It is generally agreed that its mechanism of action is by scavenging and destroying the tyrosine free radical of the M2 or B2 subunit of the reductase. In studies using homogeneous *E. coli* reductase *in vitro*. Atkin et al. (1973) observed that the drug was shown to scavenge and destroy the radical of protein B2 resulting in an inactive enzyme. Meanwhile the iron is not removed nor apparently affected in any way by the treatment with HU. The experiments of Larsen et al. (1982) gave further support that the free radical in protein B2 is the target of HU. Using a model system using the free radical salt, $(SO_3S)_2NO\cdot$, replacing the tyrosine free radical of the reductase, they found that there was a positive correlation between the free radical scavenging ability of HU and its analogues, and the ability to inhibit ribonucleotide reductase activity. In an analogous mode of action such as with the bacterial enzyme, HU also acts as a radical scavenger of the tyrosyl radical of the M2 subunit of mammalian reductase as well (Lammers and Follmann, 1983). After incubation of HU-resistant, M2 overproducing mouse fibroblast 3T6 cells with 50 mM HU for 15 min. The EPR spectrum of free radical

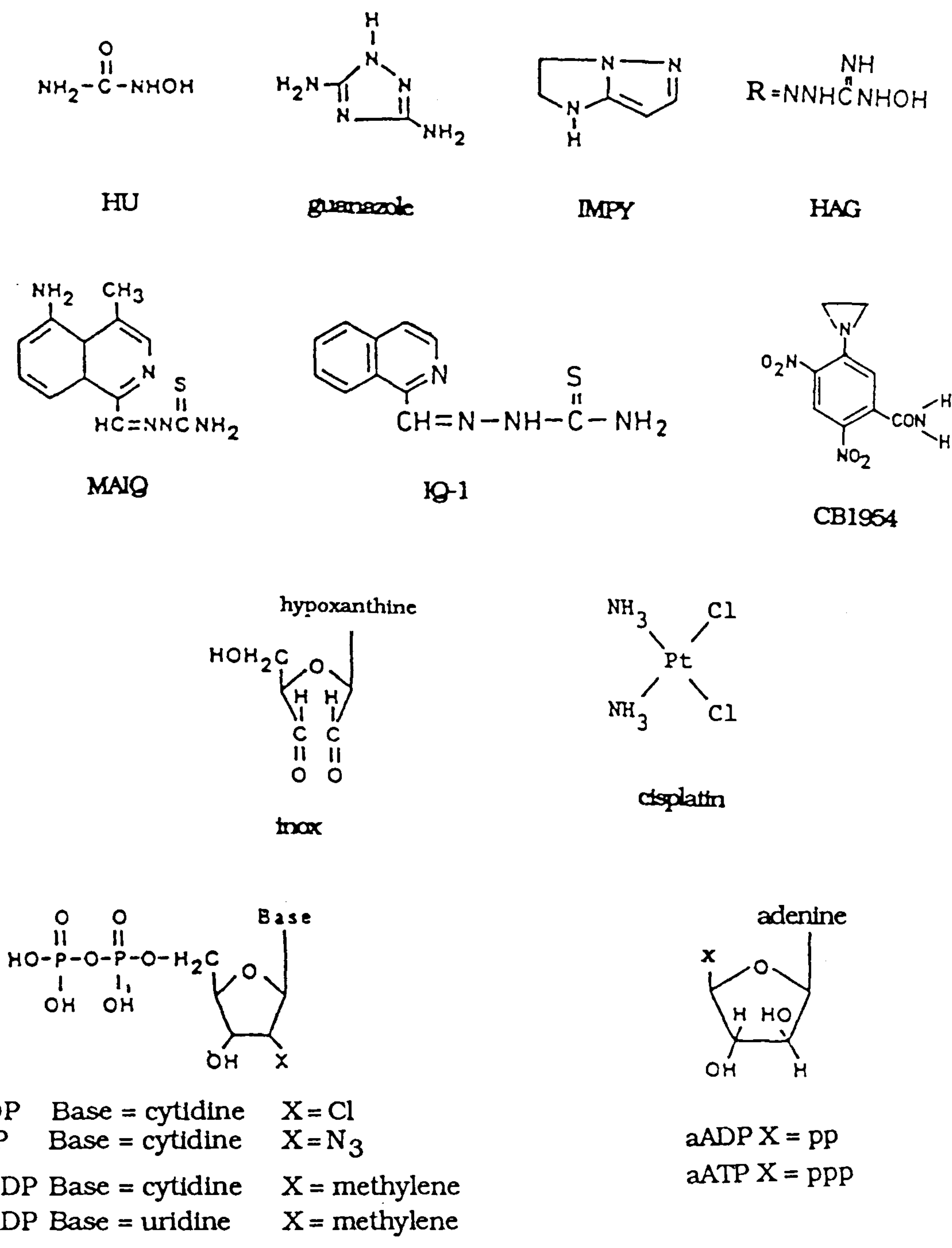


Fig. 1.3.4 Structures of inhibitors of ribonucleotide reductase

disappeared completely (Akerblom et al., 1981). When HU-resistant mutant mouse L cells were grown in the presence of HU the free radical concentration decreases dramatically compared with the same cell line grown in the absence of drug. This is the result of HU destroying the M2 tyrosyl free radical, leading to the loss of the EPR signal (McClarty, 1987a).

The reductases from regenerating liver (King and Lancker, 1969), calf thymus (Engstrom et al., 1979), human cells (Chang and Cheng, 1978; Larsen et al., 1982), mouse cells (Akerblom et al., 1981), *E. coli* (Atkin et al., 1973), bacteriophage T_4 (Yeh and Tessman, 1978; Vant't Riet et al., 1979) are all inhibited by HU. Reduction of CDP, ADP, and GDP by different mammalian enzymes is as sensitive to HU (IC_{50} = 2-4 x10^{-4} M) as is the *E. coli* enzyme (Lammers and Follmann, 1983). HU inhibits the homogeneous calf thymus enzyme in a concentration dependent fashion. The activity is inhibited 90% by 1mM HU (Engstrom, et al., 1979). This degree of inhibition is very similar to that observed with homogeneous *E. coli* reductase (Engstrom et al., 1979). The M2 activity in HU-resistant mouse fibroblast 3T6 cells is increased 30-fold. The reductase isolated from such cells shows the same sensitivity towards HU inhibition as the enzyme from normal cells (Akerblom et al., 1981). However, mouse TA3 cells, selected for resistance to HU, also overproduce the M2 subunit of the reductase and the enzyme from such cells is more resistant to HU than the enzyme from parent cells (Larsen et al., 1982). The mechanism of HU inhibition is different for enzymes from different sources. The effect of HU on the thymus enzyme is reversible (Engstrom et al., 1979). After removal of the drug by gel filtration, the enzyme regained its original activity. Like the intact calf thymus reductase, isolated M2 of thymus enzyme was affected reversibly by treatment with HU

(Thelander et al., 1980). The same, reversible inactivation of the reductase occurred during preincubation of T_4 enzyme or its isolated subunits with HU (Bergland and Sjoberg 1979). However this reversible effect is in strong contrast to what happens with the enzymes from Ehrlich ascites tumour cells and *E. coli*. HU inhibits *E. coli* enzyme in an irreversible fashion (Atkin et al., 1973; Thelander and Reichard, 1979). For the partially-purified reductase from Ehrlich ascites tumour cells, HU is an irreversible inhibitor (Cory et al., 1978; Cory and Fleischer, 1979).

The reductase of phage T_4 and *E. coli* is a single enzyme controlling the reduction of four common nucleotides: CDP, ADP UDP and GDP. The effect of HU on each reduction activity of T_4-infected cells has been investigated. Yeh and Tessman (1978) found that HU inhibited reduction of the four common substrates in T_4, but HU had considerably smaller effect on the reduction of UDP and GDP than on the reduction of CDP and ADP. Contrary to this, Bergland and Sjoberg (1979) found that HU was a potent inhibitor of the reduction of all four ribonucleotide substrates in phage T_4; the differences between the four substrates were negligible. The same investigations were performed on the enzymes from *E. coli* and human cells. Bergland and Sjoberg reported (1979) that there is no difference in the effect of HU on reduction of GDP and CDP in *E. coli*. Chang and Cheng (1978) reported that CDP reductase activity from human cells (Molt-4F and KB cells) was more sensitive to inhibition by HU than was ADP reductase activity.

HU inhibits DNA synthesis in eukaryotic cells and also in prokaryotes. This effect is mediated via inhibition of ribonucleotide reductase leading to decreased dNTP pools (Skog et al., 1987; Yeh and Tessman, 1978). A consequence of the depletion of the necessary DNA precursors is inhibition of cellular growth (Platz and Sjoberg, 1984). In

most mammalian cells, HU concentration of about $2x10^{-3}$ M prevents DNA replication, cell proliferation, and *in vitro* ribonucleotide reduction (Lammers and Follmann, 1983). HU at 200 μM will inhibit the growth of L1210 cells in culture (Sato et al., 1982). In the presence of 60 μM of HU, the growth rate of mouse S49 T-lymphoma and human CEM T-lymphoblastoid cells was diminished by about 25% (Skog et al., 1987). Therefore, HU has been widely used in chemotherapy to inhibit the growth of neoplastic cells, especially in the treatment of leukemia (Yeh and Tessman, 1978).

(b) Other inhibitors

2,3-dihydro-1*H*-pyrazolo-(2,3-*a*)-imidazole (IMPY) is a relatively potent and selective inhibitor of DNA synthesis without affecting RNA or protein synthesis. It inhibits the reductase from HEP-2 cells and L1210 cells (Cory and Fleischer, 1980; Goddard et al., 1987). The results from Cory and Fleischer indicate that the site of the IMPY target is on M2. The ADP reductase activity was inhibited to a greater extent than was CDP reductase activity by IMPY and the inhibition is irreversible. IMPY is also shown to be a inhibitor of HSV-1 reductase, but the effect is reversible (Spector and Jones, 1985).

Guanazole inhibits the mammalian enzyme by a mechanism similar to HU, acting as a radical scavenger and destroying the tyrosine free radical of protein M2 (Larsen et al., 1982). The inhibitory effect of guanazole on the mammalian enzyme (human epidermoid carcinoma cells) is 10-fold more effective than on the HSV-1 enzyme (Spector and Jones, 1985).

Thiosemicarbazone derivatives comprise the most active inhibitors of the mammalian enzyme (Lammers and Dollmann, 1983). Formylisoquinoline thiosemicurbazone (MAIQ) and 1-formylisoqui-

nolinethiose thiosemicarbazone (IQ-1) are more potent inhibitors than HU on human cell reductase activity and cell growth (Chang and Cheng, 1978). MAIQ is at least 100 times more active than HU (Carter and Cory, 1989). In the presence of oxygen both of them can destroy the tyrosine free radical of the M2 subunit of purified calf thymus reductase (Thelander and Graslund, 1983). This is in consistent with the observation using Ehrlich tumour enzyme that only the addition of exogenous M2 subunits to the MAIQ-treated intact enzyme and the enzyme from the MAIQ-treated cells can restore enzyme activity (Cory and Fleischer, 1979). Although MAIQ and IQ-1 act directly on the M2 subunit, they may have different mechanisms of inhibition of the reductase compared with HU (Carter and Cory, 1989). The iron chelates of these two drugs are the active form of the inhibitor rather than the drugs themselves (Thelander and Graslund, 1983; Moore and Startorelli, 1989).

The aminoguanidine (HAG) derivatives are inhibitors of CDP reductase activity of L1210 cells (Cory et al., 1985 a; Matsumoto et al., 1990; Weckbecker et al., 1988). These derivatives, which have a core structure resembling HU, are supposed to act through the same mechanism as HU (Matsumoto, et al., 1990). However, the reduction activity in extracts from drug-treated L1210 cells was not restored by the addition of either exogenous non-haem iron or effector-binding subunits (Cory et al., 1985 a).

EDTA acts as an inhibitor of the enzyme only at high concentrations (Lammers and Follmann, 1983), and this effect is due to the inhibition of the enzyme regeneration. Once the radical-iron centre is formed, the enzyme seems to be stable to EDTA (Thelander, et al., 1983).

ii) Inhibitors affecting iron centre

(1-aziridinyl)-2,4-dinitrobenzamide (CB1954) inhibits the enzyme from Walker carcinoma in rats. At the same concentration of CB 1954, the degree of inhibition of the enzyme was proportional to the enzyme concentration in the assay. This suggests that the cytosolic protein contains a fixed number of enzyme sites that are titrated out by CB1954. The inhibition is irreversible and from structure-activity studies it is likely that the amide group becomes bound to the enzyme, possibly by chelation of iron ion (Tisdale and Habberfield, 1980).

iii) Inhibitors of component M1 or B1

Oxidized inosine (inox) is one of the periodate-oxidized dialdehyde derivatives. It inhibits Ehrlich tumour CDP reductase. The inhibitory effect of inox on CDP reductase is stronger than on ADP reductase activity and the inhibitory mechanism of inox is probably irreversible (Cory et al., 1976). Cory and Fleischer (1979) reported that addition of exogenous M1 can restore the enzyme activity which is decreased by inox, and suggested that inox inactivates the M1 subunit.

Cisplatin is one of the most widely used anticancer drugs. Cisplatin inhibits the CDP reductase from *E. coli*. Under anaerobic conditions, using the dithiol-reduced form of the enzyme it was found that ribonucleotide reductase is extremely sensitive to cisplatin. Inhibition was essentially instantaneous and irreversible. Inhibition occurs specifically by reaction of cisplatin with the dithiol at the active site and other dithiol pairs in the B1 subunit (Smith and Douglas, 1989).

The intact enzyme from Ehrlich tumour cells loses the activity by treatment with the sulfhydryl agent, pCMB and NEM . The effector-binding subunit is much more reactive with these agents (Sato and

Cory, 1986).

iv) Inhibitors requiring functional enzyme

Unlike the inhibitors discussed above, 2'-chloronucleoside 5'-diphosphates cytidine (CclDP) and 2'-azidonucleoside cytidine (CzDP) comprise another class of ribonucleotide reductase inhibitor that requires a functional enzyme (both subunits) to generate the inhibitor. Thelander and Larsson (1976) have extensively studied CclDP and CzDP effect on *E. coli* enzyme. CclDP inactivates the B1 subunit. The inactivation of B1 showed an absolute requirement for active B2 and is prevented by normal substrate CDP. The interaction of CclDP with the enzyme is controlled by allosteric effectors in the same way as the binding of CDP. So the interaction occurs at the substrate binding site of the enzyme. CclDP irreversibly modifies the active dithiols in B1. CzDP inactivates protein B2, the activity of protein B1 was not decreased by CzDP. In the absence of functional B1, no inactivation of B2 occurs. The inactivation is due to the selective destruction of the free radical of B2. CDP protects protein B2 against CzDP-inactivation.

In inactivating the reductase both CclDP and CzDP behave like irreversible enzyme inhibitors. Since the enzyme had to be in an active conformation in order to be inactivated by CclDP and CzDP, it is suggested that a reaction product of CclDP or CzDP, but not the compounds themselves inactivated B1 or B2, i.e. CclDP and CzDP are suicide inhibitors (Thelander and Larsson,1976). CzDP also decreased the calf thymus ribonucleotide reductase, but it acts as a reversible inhibitor competitive with the normal substrate, CDP (Engstrom et al., 1979). Similarly the inactivation of CDP reductase of *E. coli* by 2'-methylene-5'-diphosphate cytidine (CmdDP) and 2'-methylene uridine(UmdDP) appears to be irreversible. CDP reductase is capable of

catalyzing the interconversion of both compounds to a reactive species capable of enzyme inactivation (Baker et al., 1991).

The reductase isolated from rat Novikoff tumour is inhibited by arabinonucleotides. aADP acts as a competitive inhibitor with respect to substrate indicating it interacts with the substrate sites. The enzyme from *E. coli* also interacts with arabinonucleoside triphosphates at the triphosphate-specific effector sites : aATP is a potent inhibitor, but aCTP stimulates substrate reduction (Ludwig and Follmann, 1979).

v) Inhibitors causing disturbance of the protein structure

The nonapeptide YAGAVVNDL, which in sequence is identical to the C-terminus of HSV component 2, can inhibit HSV ribonucleotide reductase *in vitro,* and the inhibition is accompanied by a dose-dependent dissociation of the enzyme subunits. These data imply that the nonapeptide inhibits enzyme activity by competing with component 2 for a critical binding site and effectively prevents the formation of the active holoenzyme (Pierrette et al., 1987; McClements et al., 1988). The peptide corresponding to the C-terminal region of the B2 subunit inhibits the enzymatic activity and binds to the B1 subunit indicating that the peptide inhibits *E. coli* enzyme by binding to the heterodimer interface in competition with the B2 subunit (Bushweller and Barttelt, 1991; Climent et al., 1991). This type of inhibition is also found to occur in the mammalian enzyme. The N^{α}-acetyl derivative of FTLDADF which represents the C-terminal heptapeptide of component 2 of the mouse enzyme is a potent inhibitor of calf thymus ribonucleotide reductase (Yang, et al., 1990).

1. 3. 4. 2 Natural inhibitors

There are also some common natural inhibitors such as negative

allosteric effectors dATP, dTTP, and to a lesser extent, dGTP (Lammers and Follmann, 1983), RNA (Cory, 1973), proteases (Sato and Cory, 1986), some phenolic metabolites isolated from mushroom (Fitzgerald et al., 1984) and gossypol from cotton seed oil (McClarty et al., 1985).

RNA markedly inhibits ribonucleotide reductase from mouse Ehrlich ascites tumour cells, at concentrations which could be of physiological significance, (I_{50} = 5 μM). The inhibition appeared to be dependent on the base composition of the RNA (Cory, 1973). The enzyme is inhibited by tRNA and rRNA from Ehrlich ascites cells, by yeast tRNA and by poly (C). Poly (I), poly (G) and poly (U) are only slightly inhibitory. Poly (A) does not inhibit the enzyme activity.

The activity of CDP reductase from Ehrlich tumours was rapidly lost from the intact enzyme treated with trypsin. This is due to the great sensitivity of the non-haem iron subunit to trypsin. When the trypsin-treated enzyme was supplemented with exogenous non-haem iron subunit, considerable reductase activity was restored. On the other hand, the effector subunit was more sensitive to chymotrypsin than was the non-haem iron subunit, and the loss of reductase activity in the intact enzyme was due to the specific loss of the effector-binding subunit (Sato and Cory, 1986).

γ-L-Glutaminyl-4-hydroxybenzene is a substance that has been implicated in the induction of sporulation in the common mushroom. It inhibits the enzyme from L1210 leukemia cells reversibly and this inhibition is responsible for inhibition of DNA synthesis in the L1210 cell line (Fitzgerald et al., 1984).

Gossypol is in relatively common use in several countries as a male contraceptive and may be useful as an antitumour agent. Gossypol is a potent inhibitor of the reductase activity of mouse L cell on the

reduction of CDP and ADP (McClarty et al., 1985).

1. 3. 4. 3 Physical effectors

Some physical effectors also decrease the enzyme activity. The M1 subunit of the Ehrlich tumour enzyme loses its activity rapidly at 55°C while M2 is remarkably stable (Sato and Cory, 1986). The whole-body γ-irradiation of mice with a dose of 6 Gy and the whole-body X-irradiation of 18h post-partially-hepatectomised rats causes a decrease in enzyme activity (Avakyan, 1987; King and Lancker, 1969).

Ribonucleotide reductase plays a critical role in DNA synthesis. 4-hydroxynonenal inhibits DNA synthesis and affects cell proliferation. It has been found that the level of lipid peroxidation is very low in dividing tissues and cells (Slater et. al., 1990). Therefore, this thesis relates studies into the activity of ribonucleotide reductase in regenerating liver; livers taken from rats at different stages of development and cells from hypatomas were also studied. The inhibitory effect of the lipid peroxidation product, HNE on ribonucleotide reductase was also investigated. This thesis also details investigation into the activation of CCl_4 as an agent capable of inducing lipid peroxidation.

2. MATERIALS AND METHODS

2. 1 MATERAL

Reagents were purchased from the following suppliers:

BDH Chemicals Ltd., Poole, England: Thiobarbituric acid (TBA), sucrose, magnesium chloride, ethanol, ether, chloroform, trichloroacetic acid (TCA), toluene, potassium chloride, sodium carbonate, cupric sulphate, acetic acid, potassium hydroxide, ammonium sulphate, perchloric acid, metaphosphoric acid, ethylenediaminetetra acetic acid disodium (EDTA-Na_2), sodium chloride, sodium hydroxide, hydrochloric acid, disodium hydrogen orthophosphate, potassium dihydrogen orthophosphate, potassium tartrate, Folin & Ciocalteu's phenol reagent, tri-sodium citrate (Na_3-citrate), sodium dodecyl sulphate (SDS), glycerol, 2-ß-mercaptoethanol.

Sigma Chemical Company Ltd: Bovine serum albumin, Trizma base(Tris), glucose-6-phosphate, acetamide, glutathione (GSH), dimethyl sulphoxide (DMSO), imidazole, pyrazole, N-ethyl maleimide (NEM),iodoacetamide, dithiothreitol (DTT), ATP, 2'-deoxycytidine 5'-monophosphate (dCMP), 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB), phosphate-buffered saline (PBS), acrylamide, N,N'-methylene-bis acrylamide, N,N,N',N'-tetramethylethylene-diamine (Temed), Bromophenol blue, glycine, Brilliant Blue G-colloidal, MES (2-[N-morpholino]ethanesulfonic acid), high molecular weight standard protein mixture, N-α-p-tosyl-l-lysine chloromethyl ketone (TLCK), Trypsin inhibitor (type II-S : Soybean), DEAE-cellulose (fine mesh), Dowex 50W-(+H) (200-400 mesh), Sephadex-G25, CM-sephadex C-25 (40-120 μ).

Pharmacia: ATP-Agarose (AGATP^{TN} Type 3).

Calbiochem: Flake polyethylene-glycol (Aquacide III).

NEN Research Products: Proteosol, Econofluor.

Packard, Canberra Company: Opti-fluor.

Bethesda Research Laboratories, Inc: Ammonium persulphate.

Boehringer Mannheim GmbH: NADP (sodium salt), glucose-6-phosphate dehydrogenase.

Amersham International Plc.: ^{14}C-CCl_4, (5-^3H)cytidine 5'-diphosphate, ammonium salt (^3H-CDP) (18.4 ci/m mol).

4-hydroxy-trans 2,3-nonenal (HNE) was kindly provided by Professor H. Esterbauer, Institute fur Biochemie, University of Graz, Austria.

Male Wistar rats from Harlan Olac Ltd. were used in all experiments. The animals had access to food and water *ab libitum*.

The animals for regenerating liver were housed for a minimum of three weeks under a strict lighting regimen of an inverse 12 hour light:12 hour dark cycle with food only available for 8h in the dark period. All partial hepatectomy operations were performed under light anaesthesia by the procedure of Higgins and Anderson (1931) and always at 7:00 pm. In each case the left and right lobes, equivalent to approx. 2/3 of the original weight of the liver were removed. For each operation 4-10 rats (250-320g body weight) were used. When ribonucleotide reductase was to be prepared from the regenerating and sham livers the animals were sacrificed at 12 hourly intervals following the operation. In the sham group only the minor lobes were taken.

Yoshida ascites hepatoma cells were maintained by transplantation every 5 days into the abdomen of male Wistar rats. Cells were passaged by removing 1 ml of ascitic fluid from the host rat

and injecting the cell suspension into the next carrier animal. The cells were harvested at the end of the passage period. The cell suspension was centrifuged at 800 x g for 5 min. and the supernatant was discarded. The cell pellet was washed 3 times with phosphate-buffered saline (PBS). The viability of cells was always ≥ 90%.

Morris Hepatoma 5123tc solid tumours were obtained from S. M. Clutton, Department of Biochemistry, Brunel University. Tumours were transplanted in male Buffalo rats (bred in Brunel University) every 21 days by injecting 0.5 g of minced tumour material into the thigh-muscle of rats.

Cultured Yoshida ascite hepatoma cells were obtained from Dr. Marie-Luce Carrie, Department of Biochemistry, Brunel University. Cells were cultured in MEM medium at 37°C. The culture medium was changed every 1-2 days.

2. 2 METHODS

Preparation of microsomes from rat liver

The rats used for making microsomes were males of the Wistar strain, 7 weeks-old and 200-220g body weight. Rats were starved overnight prior to the preparation of microsomes. Livers were removed immediately after the rats were killed by cervical dislocation, weighed and put into ice-cold 0.25M sucrose solution. After washing with 0.25M sucrose, livers were minced using scissors and rinsed further with 0.25M sucrose to remove blood. The minced livers were homogenized in 0.25M sucrose (1:4-5, w/v) using a loose-fitting Potter-Elvehjem (glass-Teflon) homogeniser. The homogenate was centrifuged twice at 14,000 x g (MSE-18) for 10 min. The pellet was discarded and the supernatant was centrifuged at 165,000 x g (MSE-

65) for 40 min. The pellet was rinsed with 0.1 M Tris/HCl (pH 7.4) and then gently homogenised in the same buffer by hand using a small glass homogeniser. The microsomal pellet was collected by centrifugation at 165,000 x g for 25 min. and stored at -70°C (Slater and Cheeseman, 1987).

Lipid peroxidation initiated by CCl_4/NADPH in rat liver microsomes

In this system the NADPH was generated using glucose-6-phosphate, $NADP^+$ and glucose-6-phosphate dehydrogenase, so the stock solution of lipid peroxidation incubation mixture contained 83.5 mM KCl, 37 mM Tris/HCl (pH 7.4), 5.5 mM glucose-6- phosphate (sodium salt), 0.25 mM $NADP^+$ (sodium salt), 106 mM acetamide and 8 international units of glucose-6-phosphate dehydrogenase. The microsomal pellet was resuspended in ice-cold 0.15M KCl, so that 1 ml of suspension contained microsomes equivalent to 1g wet weight of original liver. The standard 3 ml reaction mixture contained 2.2 ml stock solution, 0.3 ml microsomal suspension, 0.5 ml H_2O or test drug solution under study and 10 µl CCl_4. The reaction was started by adding the microsomal suspension with or without addition of 10 µl of a CCl_4 solution in dimethyl sulphoxide (CCl_4/DMSO, 1:4, v/v) to the tube fitted with a ground glass socket and stopper containing stock solution. The incubation was carried out in a water bath at the required temperature for 15 min. with gentle shaking and in the dark. (Slater and Sawyer, 1971 a; Slater and Cheeseman, 1987).

To stop the reaction, 6 ml of 10% TCA was added to the medium at the end of incubation. After leaving on ice for 15 min. the mixture was centrifuged at 800 x g for 10 min. and 2 ml of supernatant was taken for assay of content of MDA

Malonaldehyde (MDA) estimation

To estimate the MDA content, 2 ml of protein-free sample was mixed with 2 ml of 0.67% thiobarbituric acid and heated at 100°C for 10 min. The absorbance of the solution was read at 535 nm using a Beckman DU-62 spectrophotometer. The MDA content was calculated using the molar extinction coefficient of 1.49 x 10^5 M^{-1} cm^{-1} (Slater and Cheeseman, 1987).

Extraction of $PBN/CCl_3\cdot$ produced during lipid peroxidation induced by CCl_4 in rat liver microsomes

The standard reaction mixture in this experiment was essentially the same as that used for the general lipid peroxidation induced by CCl_4 in rat liver microsomes except 0.5 ml 18 mM PBN was used instead of 0.5 ml H_2O. The final concentration of PBN was 3 mM. The reaction was started by the addition of the microsomal suspension with or without CCl_4/DMSO and took place in the dark at certain temperatures between 20-40°C for 15 min. Ice-cold toluene (1 ml) was mixed well with the reaction mixture at the end of incubation. The upper phase of toluene containing $PBN/CCl_3\cdot$ was separated by centrifugation at 800 x g for 10 min. and immediately frozen in liquid nitrogen for later analysis by ESR spectroscopy.

Covalent binding of ^{14}C-CCl_4 to rat liver microsomal protein

The method was essentially the same as that used for the general CCl_4-induced microsomal lipid peroxidation, but using ^{14}C-labelled CCl_4. The incubation was at certain temperatures for 15 min and kept in the dark. The reaction was terminated with 6 ml ice-cold 10% TCA. Two controls were required. One was for the non-specific covalent

binding in which microsomes were incubated with reaction stock solution without the NADPH generating system and ^{14}C-CCl_4. The other was for CCl_4-stimulated lipid peroxidation in which liver microsomes were incubated in the presence of NADPH but in the absence of CCl_4. After the reaction was terminated, the protein precipitate was collected by centrifugation at 800 x g for 10 min. The pellet was suspended in 5 ml of 5% TCA and the suspension was heated at 100°C in open tubes for about 10 min. and then centrifuged to remove 5% TCA. The protein pellet was suspended in 7.5 ml of the mixture of ethanol/ether/chloroform (2:2:1, v/v/v) and allowed to stand at room temperature for 45 min. with occasional mixing. The protein pellet was collected and washed with acetone and ether in the same way as it was washed with the ethanol/ether/chloroform mixture as described above. The remaining ether was removed by placing the tube in cold water and gradually warming the water until the ether was completely removed. The dried protein pellet was weighed and put into a mini-scintillation vial. H_2O (0.1 ml) and Protosol (0.5 ml) were added to the vial in order to disslove the protein. The mixture was kept at 37°C until the protein was fully dissolved. Econofluor scintillation liquid 4.5 ml was used to check the radioactivity of the liver microsome protein on the liquid scintillation counter (Cheeseman, 1984).

Determination of NADPH production

NADPH was generated from the system of glucose-6-phosphate, $NADP^+$ and glucose-6-phosphate dehydrogenase. NADPH production was checked by recording the changes of absorption at 340nm using a Cary 219 spectrophotometer. A standard incubation medium was prepared which contained 36 ml 0.15M KCl, 24 ml 0.1M Tris/HCl (pH 7.4), 102 mg glucose-6-phosphate, 3.6 ml H_2O and 6µl glucose-6-

phosphate dehydrogenase (5 mg protein/ml, 0.26 units/ml). 3 ml of this stock solution were dispensed into control and test quartz cuvettes. The cuvettes were put in the spectrophotometer which was kept at the appropriate temperature as required in the experiments. Then the baseline was checked to be flat. Afterwards 0.2 ml of KCl:Tris/HCl (pH 7.4) mixture (0.15M:0.1 M = 3:2) and 0.2 ml of 3 mg/ml (in KCl:Tris/HCl mixture) $NADP^+$ were mixed in to the control and test cuvettes, respectively. The cuvettes were left in the chamber of the spectrophotometer for certain times as required in the experiments, then the spectrum from 450-300 nm was scanned at the conditions of range=2.0, scan rate=2.0 nm/sec., period=10 sec. and display=10. The absorption at 340nm was measured.

Isolation of ribonucleotide reductase (RNR'ase): crude extract

The medium for the isolation of RNR'ase contained 0.25M sucrose, 10 mM potassium chloride, 5 mM magnesium chloride and 2 mM dithiothreitol (DTT) (final concentrations) in 0.1 MTris/HCl (pH 7.4). The ratio of tissue to medium was 1:4-5 (w/v) when the enzyme was prepared from livers or solid tumours. If the enzyme was isolated from Yoshida cells, the medium volume was the same as the volume of the pelleted cells after centrifugation at 800 x g for 5 min. The materials (livers, tumours, cells) were homogenized in the medium using a loose-fitting Potter-Elvehjem (glass-Teflon) homogeniser and the slurry was centrifuged at 100,000 x g (MSE-65) for 1h. The precipitate was discarded and the clear supernatant was kept as the crude enzyme extract. All procedures were carried out below 4°C (Youdale and MacManus, 1979). This crude enzyme extract, about 20 mg protein/ml, was usually used for the further partial purification, but if the material was too small, for example cultured Yoshida cells, the

crude extract was used directly for determination of the ribonucleotide reductase activity.

Partial purification of ribonucleotide reductase

A. Precipitation with ammonium sulphate:

Solid ammonium sulphate (0.25 g/ml, corresponding to 45% saturation) was slowly added to crude extract under stirring at 0°C. After 30 min. of additional stirring at 4°C, the suspension was centrifuged at 100,000 x g for 20 min. The pellet was dissolved in a small volume (usually 10% of original volume of crude extract) of 0.1M Tris/HCl (pH 7.4) containing 2 mM DTT and 5 mM $MgCl_2$ (Youdale and MacManus, 1979, Engstrom *et al.*, 1979). The protein solution was desalted by either passing through a Sephadex G-25 column (sample to column 1:15, v/v) equilibrated with the same buffer or dialysing overnight against the same buffer with two buffer changes. The dialysate was centrifuged at 800 x g for 10 min. to remove remaining precipitate. The resulting protein solution was used for routine determination of the enzyme activity and the effect of inhibitor on the activity.

B. Chromatography on DEAE-cellulose:

If the enzyme preparation was used for further purification, the protein precipitate resulting from 45% saturation of ammonium sulphate was dissolved in a small volume (10% of original crude extract) of 20 mM Tris/HCl (pH7.4) containing 2 mM DTT and 5 mM $MgCl_2$ and dialysed overnight against the same buffer with two buffer changes. Usually 10 ml of dialysate (from 20 g of liver of 7-10 rats aged three weeks) was adsorbed to a DEAE-cellulose column (2 x 22 cm) equilibrated with 10 mM potassium phosphate buffer

(pH 7.0) (Engstrom et al., 1979) The protein was eluted in a stepwise manner with

(1) 10 mM potassium phosphate buffer (pH 7.0),

(2) the same buffer containing 0.08 M KCl,

(3) the same buffer containing 0.16 M KCl

(4) the same buffer containing 0.25 M KCl

at a rate of 1 ml/min until the optical density at 280 nm of the eluate was less than 0.100 (Mattaliano et al., 1981). The fractions of eluate containing protein were pooled according to the value of optical density at 280nm. Proteins were concentrated by adding ammonium sulphate to 80% saturation (0.56 g/ ml). The protein precipitate was collected by centrifugation and dissolved in 0.1M Tris/HCl (pH 7.4) containing 2 mM DTT and 5 mM $MgCl_2$. The salt in the protein solution was removed by using a Sephadex-G25 column. The protein content in this enzyme preparation was about 10 mg/ml.

C. Chromatography on ATP-Agarose column

The enzyme preparation after DEAE-cellulose chromatography was further purified by using an ATP-agarose column as described by Spector and Averett (1983). The protein solution (about 1.5ml, 12.5 mg prot/ml) was applied to a 1x12.5 cm ATP-agarose column equilibrated with 0.1M Tris/HCl (pH 7.4) containing 2 mM DTT and 5 mM $MgCl_2$. The column was then washed with the same buffer and the effluent was collected. This protein solution is referred as the ATP-Agarose enzyme preparation. After the absorbance at 280nm of the eluate from the column was less than 0.100, the washing buffer was changed to the buffer containing 0.5M KCl. Then the column was washed with the buffer containing 2 mM ATP and this protein solution was also collected.

D. Chromatography on CM-Sephadex

The enzyme preparation after DEAE-cellulose chromatography (about 2.4 ml of 15.8 mg prot/ml protein solution) was diluted 1:1 with H_2O and the pH was reduced to 5.2 with acetic acid. The resulting precipitate was pelleted by centrifugation at 10,000 x g and the supernatant (5 ml) was added to CM-Sephadex (6 ml) which was equilibrated with a solution of 20 mM MES-HCl (pH 6.0) containing 2 mM DTT and 5 mM $MgCl_2$. The gel was removed and washed by the same buffer till the protein free. The protein solution was collected (CM-Sephadex fraction 1). Then the gel was further washed with the buffer containing 1M NaCl. The resulting solution from the wash with the buffer plus 1M NaCl was dialysed against two changes of 200 ml of water containing protease inhibitor (TLCK, 60 mg/L; soybean trypsin inhibitor , 30 mg/L) and mercaptoethanol (0.1 ml/L) for 15h (CM-Sephadex fraction 2). After drying with flake polyethylene glycol, proteins of both CM-Sephadex fractions were resolved in 0.1 M Tris/HCl containing 2 mM DTT and 5 mM $MgCl_2$ (Youdale et al., 1982).

The whole scheme of purification of RNR'ase from rat liver is shown in Fig. 2.1.

Assay of CDP reductase activity

The CDP reductase activity was assayed with the method of Elford (1974), measuring the reduction of ^{3}H-CDP. The standard incubation solution contained 5 μCi ^{3}H-CDP (specific activity 18.4 Ci/mmol, final concentration in the incubation solution 0.787 μM), 3.3 mM ATP, 1.4 mM $MgCl_2$, 10 mM DTT, 20 mM Tris/HCl (pH 7.4) and enzyme protein 0.5-1 mg in a total volume of 0.345 ml. Incubation was at 30°C for 1h. The reaction was stopped by the addition of 1 ml of 1.25 M perchloric acid and 2.5 μmol of cold (unlabelled) carrier dCMP.

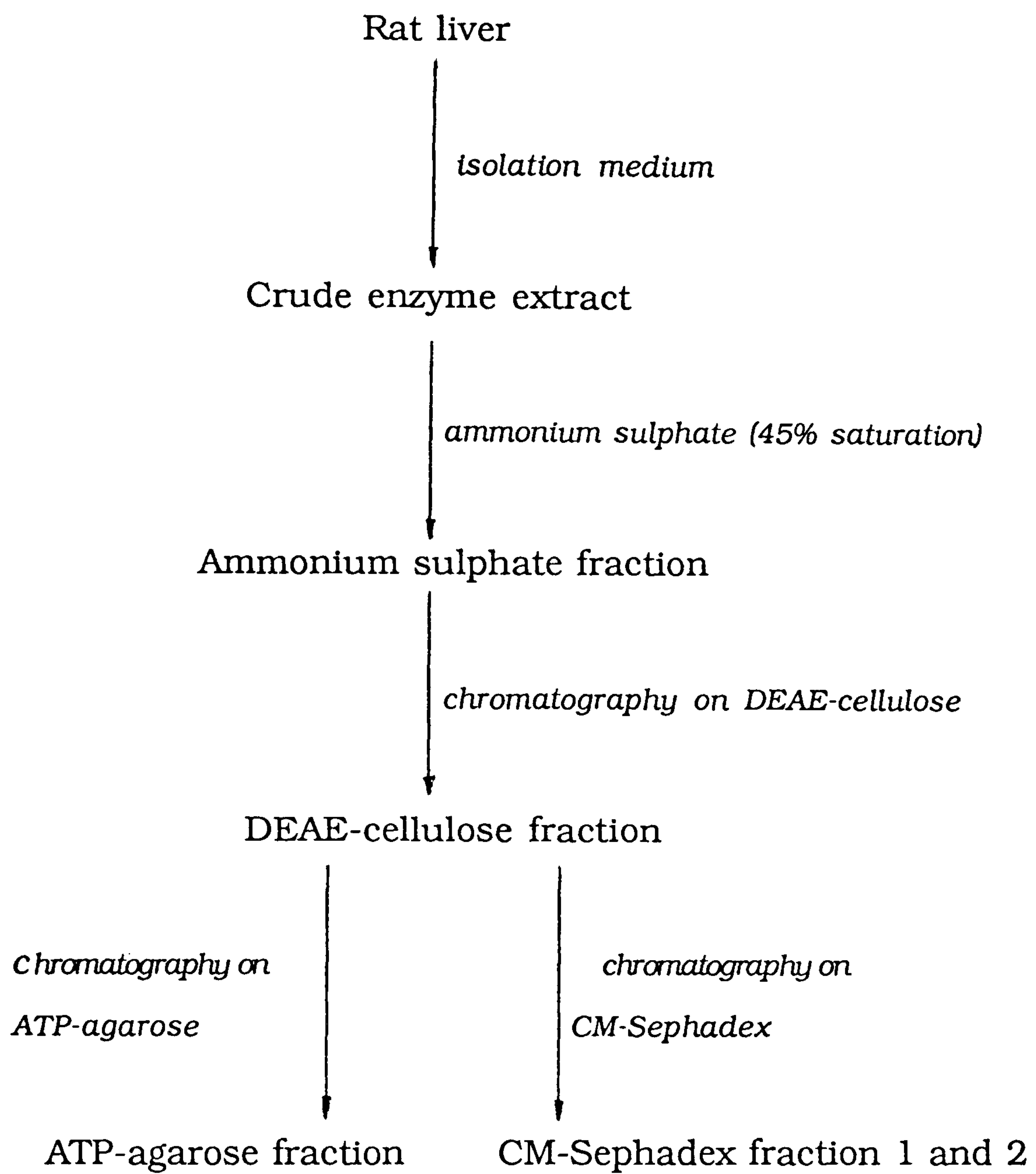


Fig. 2.1 Scheme of purification of ribonucleotide reductase from rat liver

The mixture was left on ice for 10 min and denatured protein was removed by centrifugation. The nucleotide in the deproteinised supernatant was converted to the monophosphate by 20 min. incubation in a boiling water bath. Then the solution was neutralised with 4N KOH and stood on ice for 2-12 hours. After removal of precipitated potassium perchlorate, the solution was transferred to 13.5 x 0.5 cm column of Dowex 50W-(H^+) (200-400 mesh) resin and eluted with 0.2N acetic acid. All CMP was removed in the first 40 ml of eluant and the dCMP was collected in the next 16-20 ml. The radioactivity of the eluent which contained dCMP was read on a liquid scintillation analyser (Tri-carb 2000CA, United Technologies Packard) using aqueous Opti-fluor scintillation liquid. All the radioactivity determinations were corrected for a small amount of radioactivity that was present with a control containing no enzyme. One unit of activity of enzyme was defined as that amount of enzyme that catalyses the conversion of 1000 DPM (about 0.024 p moles) of CDP to dCDP in 1 h at 30°C (Richard et al., 1961; Steeper and Steuart, 1970; Elford, 1974).

Effect of compounds on CDP reductase activity

Unless otherwise stated, the compounds were incubated with the enzyme under the standard reaction conditions. Then the activity of the enzyme was determined as normal.

Regeneration of DEAE-cellulose

DEAE-cellulose was first soaked in water (for new cellulose) or in 0.5N NaOH-0.5N NaCl (for used cellulose) for about 12 hours. After drying by using a Buchner funnel fitted with two filter papers, the cellulose cake was treated as follows:

(1) Resuspending in 1N NaOH for 3 hours with occasionally mixing,

(2) washing with water,

(3) resuspending in 0.5N HCl for 1 hour,

(4) washing with water,

(5) resuspending in 1N NaOH again for 2 hours,

(6) washing with water.

The pH of the suspension should be 7.0 when washing with water. The pH was checked using pH test paper (pH 1-14 and pH 6-8) (Himmelhoch, 1971).

Regeneration of Sephadex-G25

Sephadex-G25 was used to remove excess HNE in the HNE-enzyme mixture and ammonium sulphate salt when the enzyme was partially purified. After HNE or ammonium sulphate had passed though the column, 3 volumes of water was passed down the column. The gel was poured out and washed with water several times. If the gel was not white and translucent, it was soaked overnight in 0.5N NaOH-0.5N NaCl, then washed with water.

Regeneration of Dowex 50W-(H^+):

New or used resin was washed 3 times with 2N NaOH, followed by water, then 3 times with 2N HCl (the last time for 12 hours), finally with water. Each time when washing with water the pH of the resin suspension should be pH 7.0.

SDS gel electrophoresis

Casting separating gel:

7.5% acrylamide, 0.1% SDS, 0.375M Tris/HCl pH 8.8 separating

gel was made by combining the reagents as following: 10 ml 30% acrylamide/bis (29.2% acrylamide and 0.8% N,N'- methylene-bis-acrylamide), 10 ml 1.5M Tris/HCl (pH 8.8), 0.4 ml 10% sodium dodecyl sulphate (SDS), 2.0 ml 1.5% ammonium persulphate and 17.6 ml H_2O. After degassing the mixture under vacuum for 5 min., 20 µl of Temed was added and immediately poured into a glass plate sandwich (16 x 20 cm) to a depth of 13-14 cm. The polymerization mixture was overlayed with about 2 ml of H_2O. This mixture was allowed to polymerize for 1 hour at room temperature, then the water and the unpolymerized gel mixture were removed from the gel surface.

Casting stacking gel:

4.0% acrylamide, 0.1% SDS, 0.125M Tris/HCl pH 6.8 stacking gel was prepared as follows: 1.3 ml 30% acrylamide/bis, 2.5 ml 0.5M Tris/HCl (pH 6.8), 0.1 ml 10% SDS, 0.5 ml 1.5 % ammonium persulphate, 5.65 ml H_2O were mixed. The mixture was degassed under vacuum for 5 min and 7.5 µl Temed was added. The resulting mixture was immediately added to the glass sandwich to a depth of 2.5 cm. A comb was carefully inserted into the mixture. The gel was allowed to polymerize for 45 min. at room temperature. The ammonium persulphate solution for the two gels was freshly prepared (O'Farrell, 1975, Hames and Rickwood, 1990).

Preparation of the sample:

Protein samples were diluted to 2.5 mg protein/ml with sample buffer which contained 4.0 ml H_2O, 1.0 ml 0.5M Tris/HCl (pH 6.8), 0.8 ml glycerol, 1.6 ml 10% SDS, 0.4 ml 2-ß-mercaptoethanol and 0.2 ml 0.05% Bromophenol blue (sodium salt) in a total volume of 8.0 ml. Diluted protein solutions were heated at 95°C for 4 min (O'Farrell, 1975, Hames and Richwood, 1990).

After washing the wells of the stacking gel with water, 20 µl or

10 μl of samples and 10 μl of standard protein mixture were loaded onto the gel. Electrophoresis was carried out at 60 V for stacking gel and 100 V for separating gel in the running buffer of 4.5g Tris, 21.6g glycine and 1.5g SDS in 1.5 L H_2O, cooling with running tap water (20°C). The time of running in stacking and separating gel were about 1.5h and 12h respectively. When the samples had migrated to a level of 1.5 cm above the bottom of the separating gel, the electrophoresis was stopped. The gel was carefully taken off the glass and put into fixing solution (7% acetic acid in 40% methanol v/v). After 1 hour of fixing, the gel was transferred to the staining solution and remained in staining solution for 2 hours. The staining solution was prepared from a mixture of 200 ml Brilliant Blue G-colloidal and 800 ml deionized H_2O. Afterwards 120 ml of this mixture was mixed with 30 ml methanol. The stained gel was destained by washing with 10% acetic acid in 25% methanol for 60 sec and 25% methanol for up 24 hours with several changes of 25% methanol (O'Farrell, 1975, Hames and Rickwood, 1990).

Determination of protein content

Protein content of microsome suspension and enzyme preparation was determined by the method of Lowry (1951) using bovine serum albumin as a standard.

Determination of cell viability

Yoshida hepatoma cells' viability was assessed by using Trypan Blue. 4 volumes of diluted cell suspension was mixed with 1 volume of 0.04% Trypan blue. The numbers of viable and non viable cells were counted using a microscope and haemocytometer.

$$Viability = \frac{\textit{No. of viable cells}}{\textit{No. of viable cells + No. of non viable cells}} \times 100\%$$

Determination of the content of acid soluble thiol

The content of acid soluble thiol groups in enzyme preparation, liver homogenate and DTT solution was determined by the method of Beutler et al (1963). Precipitation solution which was 1.69g glacial m-phosphoric acid, 0.2g EDTA-Na_2 and 30g NaCl in 100 ml H_2O (1 ml) was added to a mixture of 0.2 ml enzyme preparation (about 1.7 mg protein) and 0.2 ml H_2O or drug solution. The resulting mixture was left on ice for 5 min. Following centrifugation at 800 x g for 10 min, 1 ml supernatant was mixed with 4.0 ml 0.3 M Na_2HPO_4 (pH 9.0) and 0.5 ml DTNB-reagent (0.04% 5,5'-dithiobis-(2-nitrobenzoic acid) and 1% Na_3-citrate). The absorption at 412nm of the mixture was read within 5 min on the Beckman spectrophotometer. The concentration of SH (mM) was calculated by comparing to a standard curve produced using GSH as standard. DTT (1 ml) solution was directly mixed with 4.0 ml 0.3M Na_2 HPO_4 and 0.5 ml DTNB-reagent. The absorption at 412nm was read within 5 min.

Determination of the content of protein thiol

Liver homogenate or enzyme preparation 0.2 ml was mixed with 2.0 ml TCA/EDTA solution (1 vol. of 5 mM EDTA plus 1 vol. of 5% TCA). The mixture was allowed stand on ice for 10 min following centrifugation at 800 x g for 10 min. The supernatant was discarded and the protein pellet was washed with TCA/EDTA solution again as above. Afterwards the protein pellet was resuspended in 5 ml or

Tris/EDTA/SDS (0.1 M, pH 7.4/5 mM/ 0.5%). The suspension (1 ml) was mixed with 2 ml Tris/EDTA (0.2 M, pH 8.6/2 mM) and 0.03 ml DTNB (10 mM in ethanol) and left at room temperature in the dark for 15 min. Then the absorption at 412nm and 550nm was readed on a Beckman spectrophotometer. The concentration of thiol was calculated from the difference of absorption at 412nm and 550nm by comparing to a standard curve produced using GSH.

Preparation of aqueous 4-hydroxy trans 2,3-nonenal(HNE)

50 µl of HNE/CH_2Cl_2 solution (about 8-10 mg HNE/ml) was taken and the CH_2Cl_2 was almost completely evaporated at room temperature under N_2. 1 ml H_2O was added and shaken for 1 to 2 min. Traces of CH_2Cl_2 were removed under vacuum and afterwards the solution was filtered through folded filter paper. The concentration of this solution was checked by the absorption at 223nm using a Beckman DU-62 spectrophotometer. The concentration of this aqueous HNE solution was approximately 3.1 mM and stable for one week if kept at 4°C.

Removal of excess HNE from RNR'ase preparation

In some experiments HNE and the RNR'ase preparation were pre-incubated together. In order to remove HNE the mixture of enzyme and HNE was put down a Sephadex G-25 (21 x 1 cm) and the protein eluted with 0.1 M Tris/HCl (pH 7.4) containing 2 mM DTT at 4°C. Fractions were collected and the fraction containing the protein was used for the reductase assays.

3. RESULTS

3.1 CARBON TETRACHLORIDE ACTIVATION IN RAT LIVER MICROSOMES

3. 1. 1 Effect of temperature on the activation of CCl_4 by rat liver microsomes

The effect of temperature on the activation of CCl_4 in microsomes from normal rat liver was studied. Spin trapping of $CCl_3\cdot$ and ESR spectrometry, covalent binding of CCl_4 to protein and MDA formation were used as indices of CCl_4 metabolism.

3. 1. 1. 1 Production of $CCl_3\cdot$

The free radical $CCl_3\cdot$ can be spin-trapped with phenyl-t-butylnitrone (PBN) to form PBN-$CCl_3\cdot$ adduct which is a relatively stable free radical detectable by ESR (Albano, et. al. 1982). The ESR spectrum obtained from incubating liver microsomes with CCl_4 and PBN in a standard lipid peroxidation reaction mixture (see "Methods") at 37°C is shown in Fig.3.1.1a. The spectrum showed a triplet of doublets with g value of 2.006. The effect produced by omitting CCl_4 is shown in Fig. 3.1.1b. The intensity of signals without CCl_4 was much lower than that with CCl_4. These signals may come from the background free radicals. It is possible to quantitate production of $CCl_3\cdot$ by measuring the intensity of the signal due to the PBN-$CCl_3\cdot$ adduct. Fig. 3.1.2 demonstrates the effect of temperature on the formation of PBN-$CCl_3\cdot$ adduct in liver microsomes. The results of the formation of the PBN adduct produced during the incubation of liver microsomes

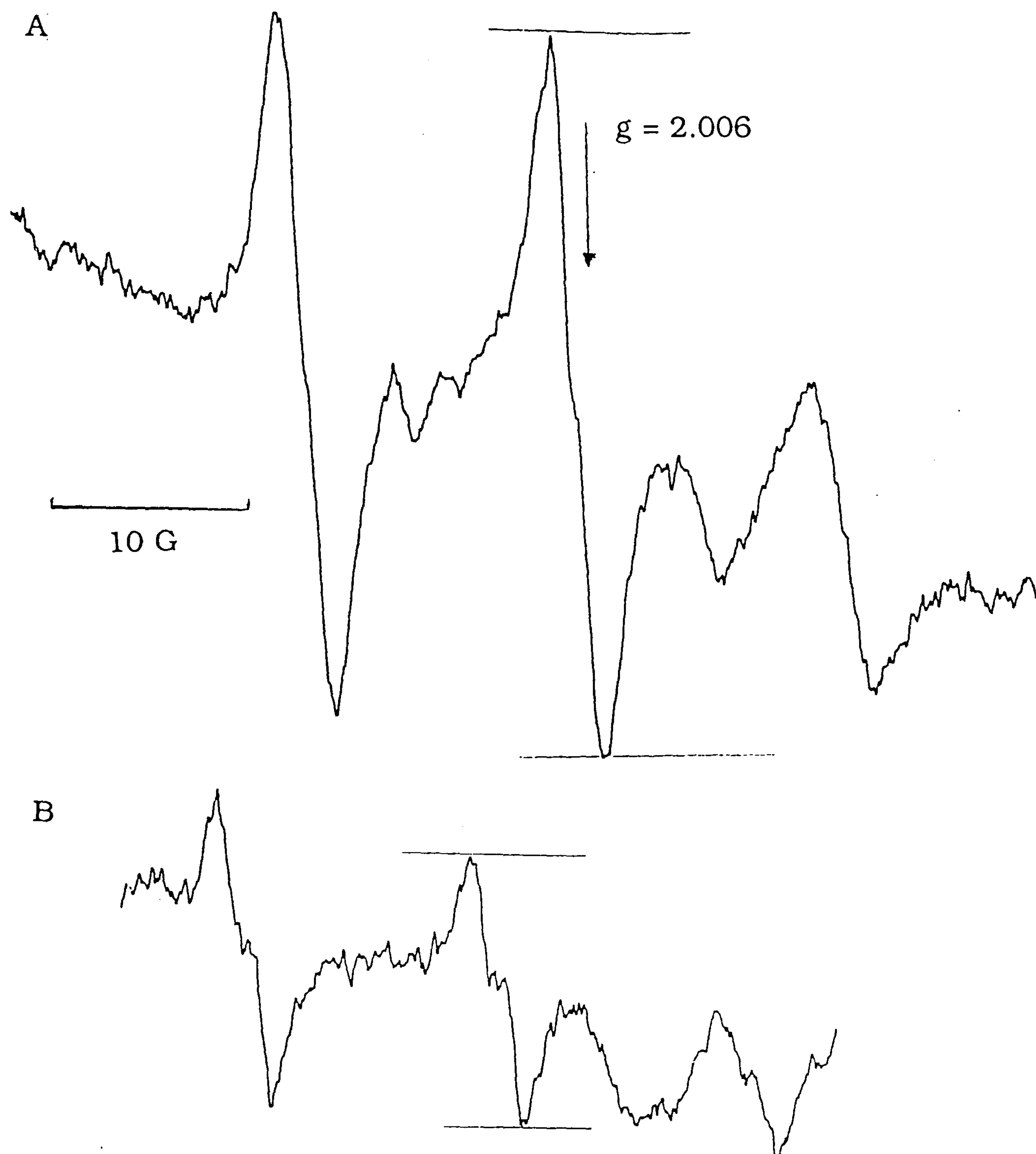


Fig. 3.1.1. ESR spectra of PBN-$CCl_3^{\cdot}$ adduct in rat liver microsomes

This represents a scaled down version of the ESR spectrum of PBN-$CCl_3^{\cdot}$ adduct. Rat liver microsomes were incubated with or without CCl_4 at 37°C for 15 min in the presence of PBN. Spectrometer setting: Temperature=213K, Microwave power =10dB (20.5 mW), Modulation amplitude=2 Gauss, Mod. frequency =100 kHz, Microwave frequency=9.51 GHZ. gain=1.25 x 10^6. A) with CCl_4 , B) without CCl_4.

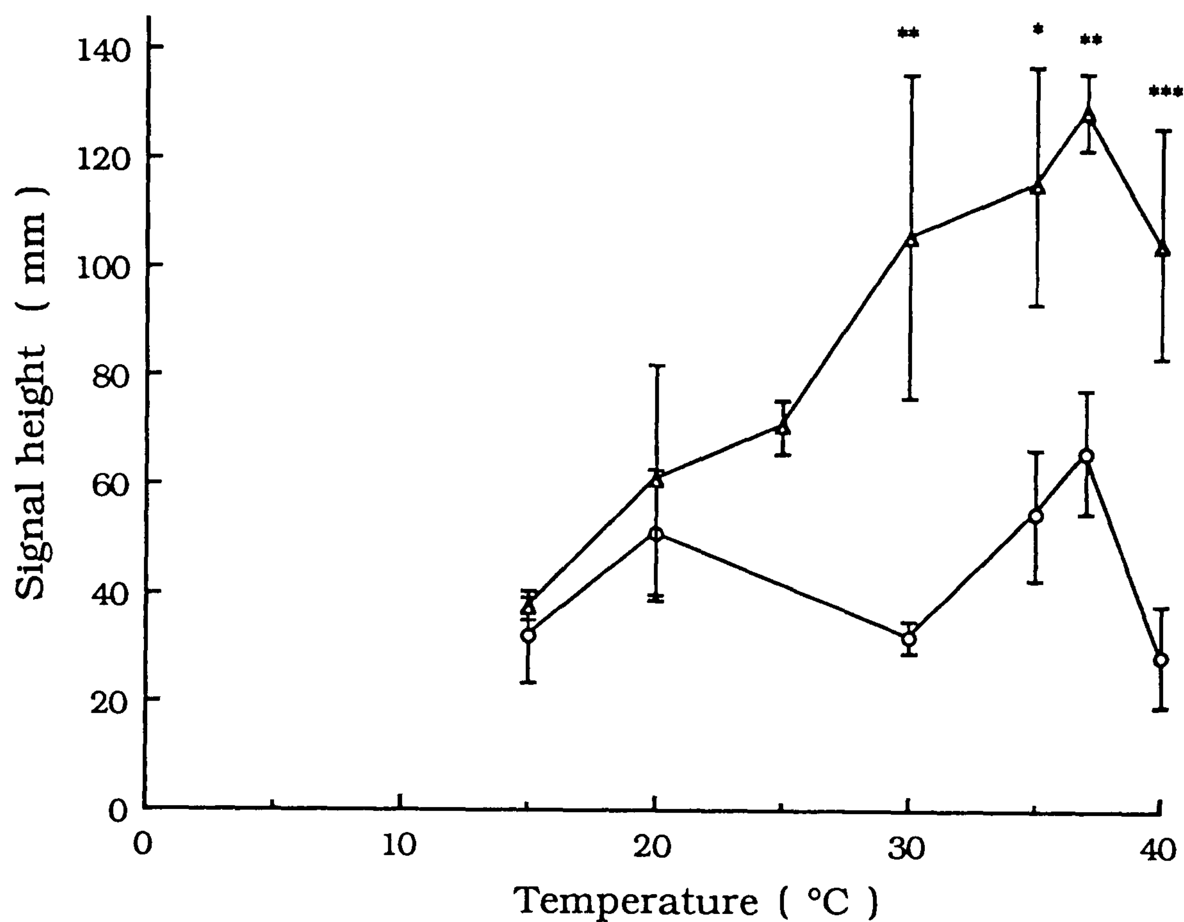


Fig. 3.1.2. Temperature effect on the activation of CCl_4 in rat liver microsomes determined as PBN-CCl_3· adduct formation

Lipid peroxidation was carried out for 15 min. in the dark. Mean values are given ± S.E.M. for four separate experiments. (o) without CCl_4; (Δ) with CCl_4. * $P< 0.05$; ** $P< 0.01$; *** $P < 0.001$ (Student's t-test) for differences between samples with and without CCl_4.

with or without CCl_4 in the presence of 3 mM PBN were expressed in arbitrary units as the height of the middle peak of the PBN adduct ESR triplet signals. It can be seen that the formation of PBN adducts increases as the temperature of incubation was increased up to 37°C. It was shown that only when the temperature was ≥ 30°C, could a significant difference of PBN adduct formation above control levels be stimulated by CCl_4 addition. However at 40°C, the formation of PBN adduct was less than that at 35°C and 37°C (Fig. 3.1.2). This phenomenon was apparent both with and without CCl_4. Although PBN adduct signals were observed in the absence of CCl_4, there was no significant difference in the intensity of signals at different temperatures except at 40°C. Fig. 3.1.3 shows an Arrhenius plot of PBN-$CCl_3\cdot$ adduct formation. It exhibited a discontinuity at 27.5°C. The energies of activation were calculated by linear regression analysis of the slopes of the Arrhenius plots and found to be 6.80 and 1.66 kcal/mol below and above the temperature break, respectively.

3.1.1.2 Covalent binding of CCl_4 to hepatic microsomal protein

Using $^{14}CCl_4$ the amount of covalent binding of ^{14}C-CCl_4 to hepatic microsomal protein can be estimated by measuring the protein-bound radioactivity. Fig. 3.1.4 shows the effect of temperature on covalent binding of CCl_4 to microsomal protein. Throughout the range of temperature used (20°C-40°C), the binding of ^{14}C-CCl_4 to the protein increased gradually in a temperature-dependent fashion. At lower temperatures (20°C and 25°C), the binding of $CCl_3\cdot$ derived from CCl_4 activation was not significantly elevated above non-specific (NADPH-independent) ^{14}C-CCl_4 covalent binding. The Arrhenius plot

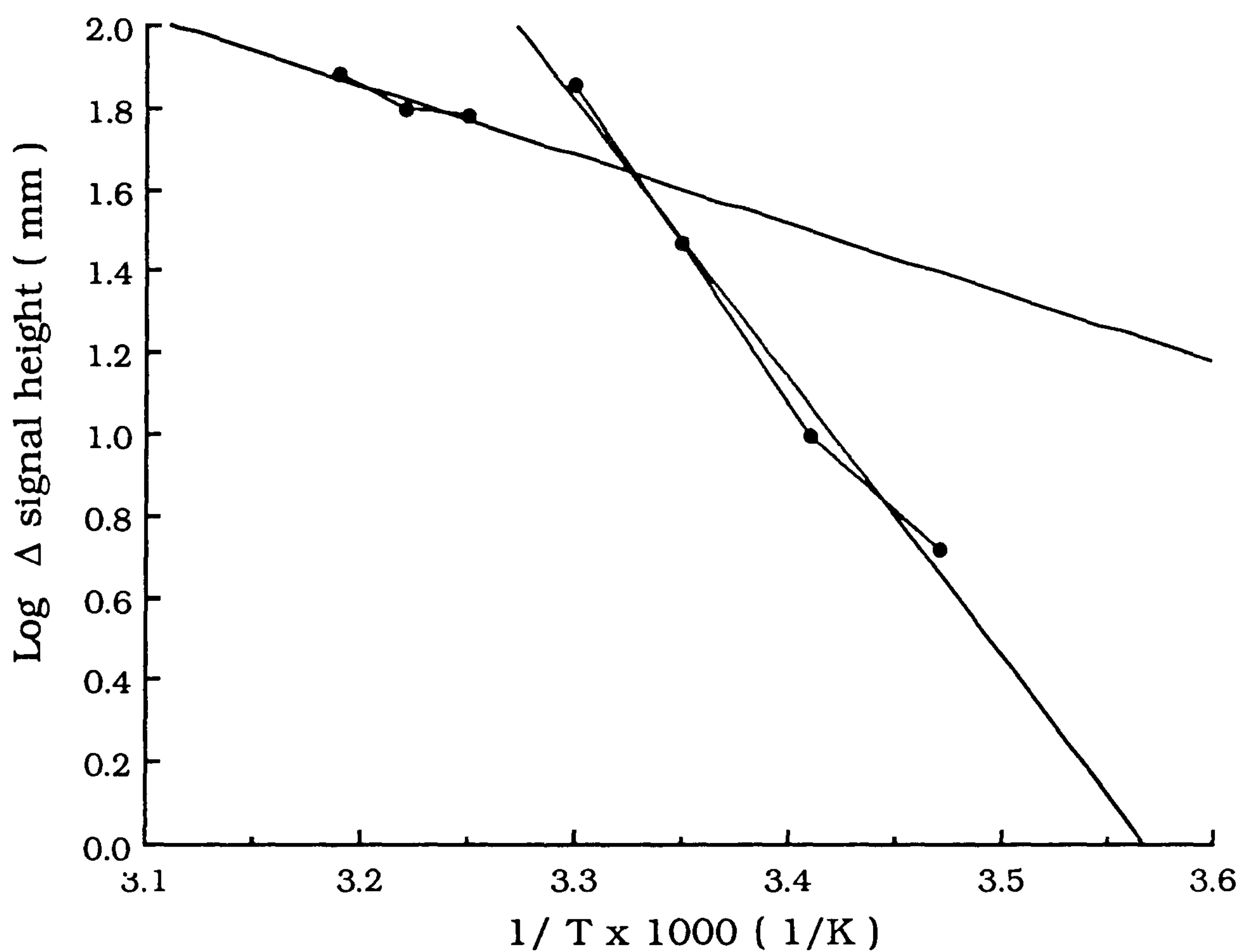


Fig. 3.1.3. Arrhenius plot of PBN-CCl_3· adduct formation

The data were from Fig. 3.1.2. Δ signal height = signal height of samples with CCl_4 - signal height of samples without CCl_4.

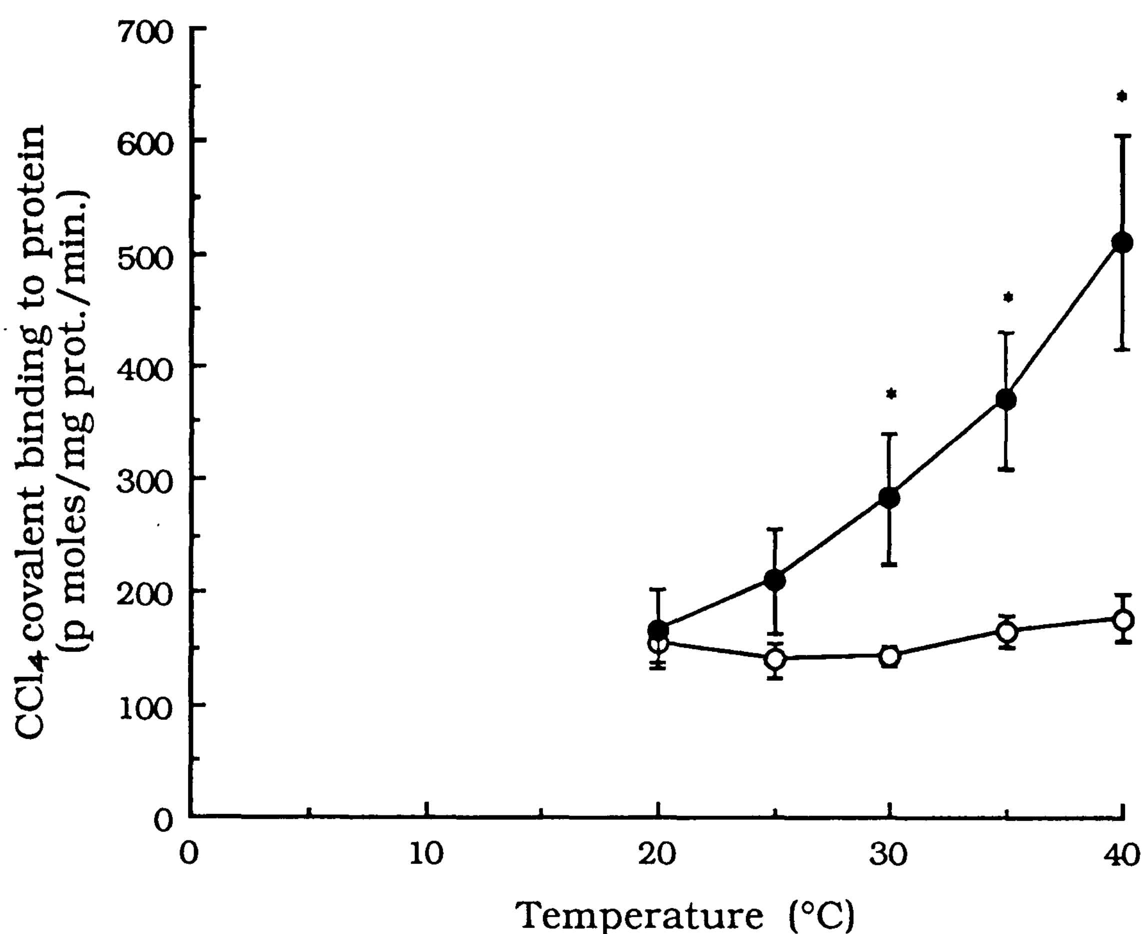


Fig. 3.1.4. Temperature effect on the activation of CCl_4 in rat liver microsomes measured as covalent binding to protein

Lipid peroxidation was carried out for 15 min in the dark. Mean values are given ± S.E.M. for three experiments. (o) non-specific binding(no NADPH in the incubation mixture); (●) specific binding. * $P < 0.05$ (Student's t-test) for differences between specific and non-specific binding.

for $^{14}CCl_4$ covalent binding to microsomal protein was shown in Fig. 3.1.5. It broke at 26.7°C. The activation energies for $^{14}CCl_4$ binding were 11.83 kcal/mol and 3.36 kcal/mol at temperatures below and above 26.7°C.

3. 1. 1. 3 Malondialdehyde production

In microsomal systems, the measurement of MDA by the thiobarbituric acid reaction has been widely used as an index of CCl_4-induced lipid peroxidation and, by implication, of the activation of CCl_4. The production of MDA at different temperatures from 15°C to 40°C in the presence or absence of CCl_4 is illustrated in Fig. 3.1.6. It was demonstrated that when the temperature was higher than 25°C, MDA was produced even without CCl_4, but only when the temperature was higher than 25°C, did CCl_4 show a significant stimulatory effect on MDA production. The degrees of stimulation as compared to the control (without CCl_4) were 17.5%, 56.5%, 123.5% and 145.5% at 25°C, 30°C, 35°C, and 40°C respectively. Fig. 3.1.7 showed Arrhenius plot for CCl_4-stimulated MDA formation. The break in the Arrhenius plot occurred at 34.3°C. The activation energies of CCl_4-stimulated MDA fromation was 2.83 kcal/mol at the temperature higher than 34.3°C, and 9.8 kcal/mol at the temperature lower than 34.3°C.

3. 1. 1. 4 Temperature effect on NADPH generation

NADPH is required for the activation of CCl_4. In the model used here, NADPH was generated from a glucose-6-phosphate dehydrogenase dependent system:

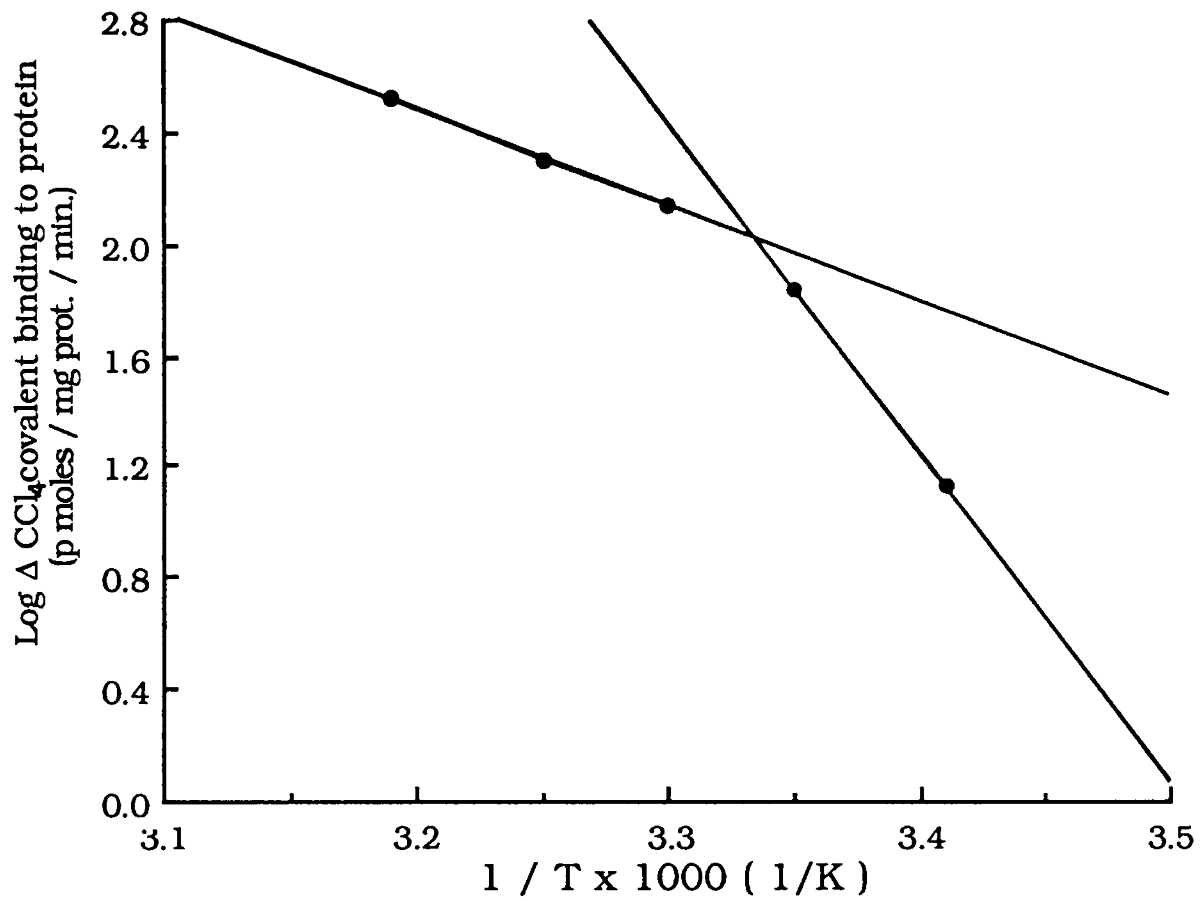


Fig. 3.1.5. Arrhenius plot of the amount of $^{14}CCl_4$ covalent binding to microsomal protein

The data were from Fig. 3.1.4. Δ $^{14}CCl_4$ binding = specific binding - nonspecific binding.

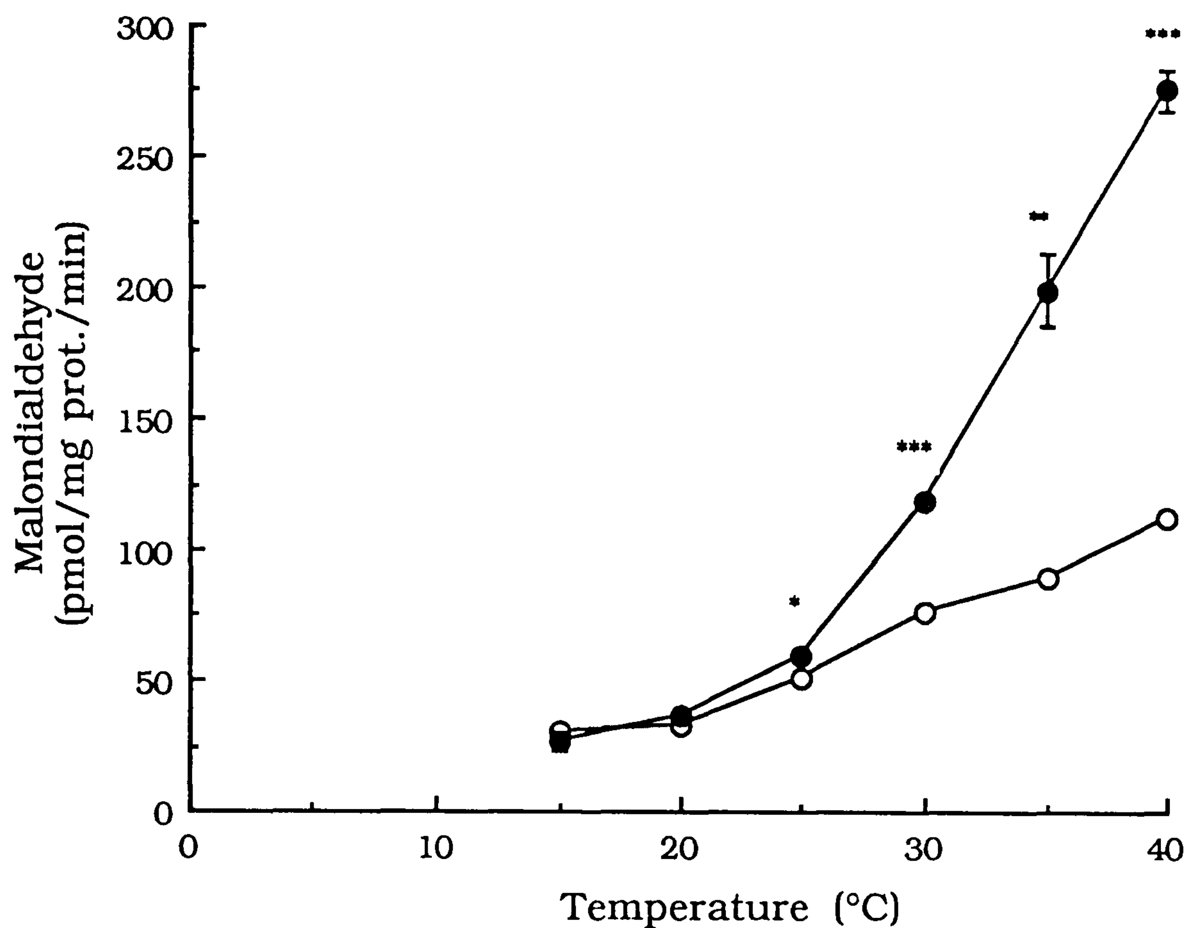


Fig. 3.1.6. Temperature effect on activation of CCl_4 in rat liver microsomes determined as stimulation of MDA production

Lipid peroxidation was carried out for 15 min. in the dark. Mean values are given ± S.D. for two experiments with total 4 replicates. (○) without CCl_4; (●) with CCl_4. * $P< 0.05$; ** $P<0.01$; *** $P< 0.001$ (Student's t-test) for differences between samples with and without CCl_4.

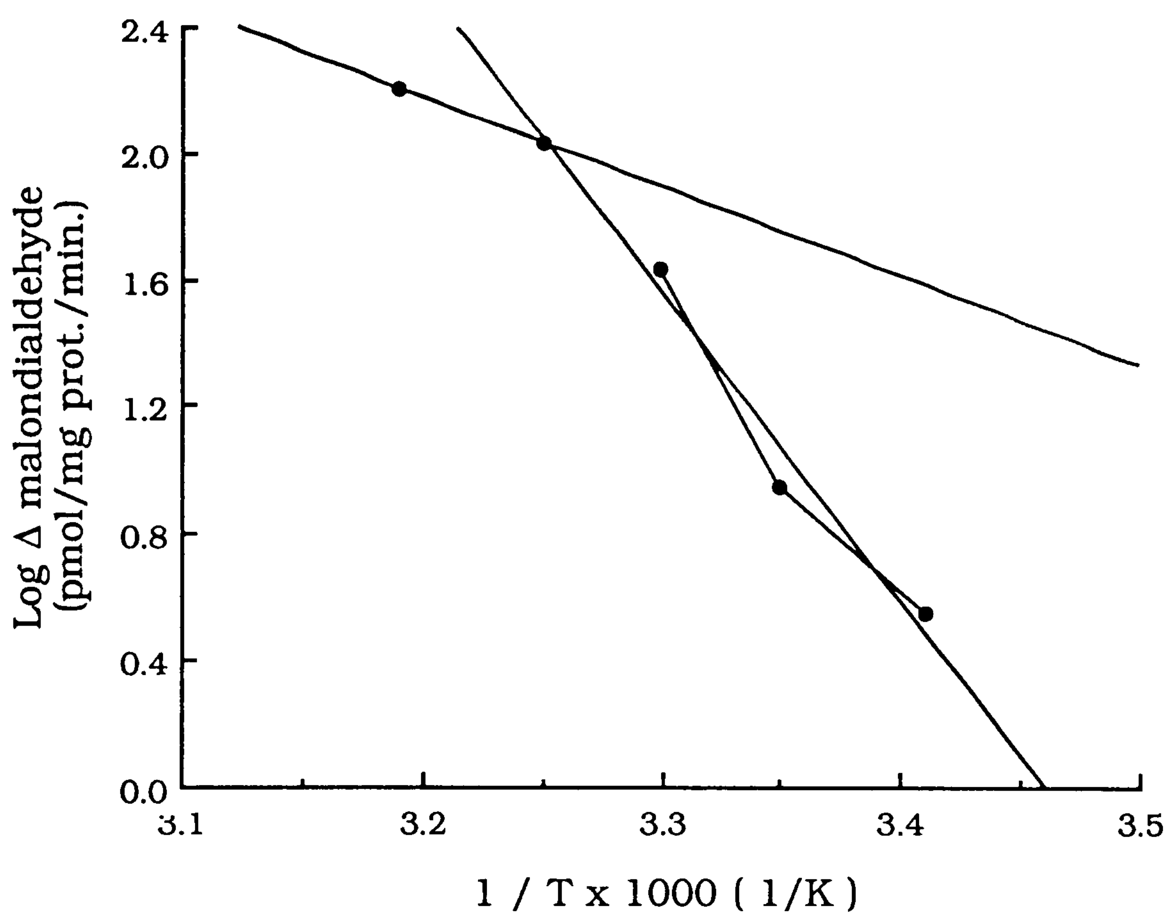
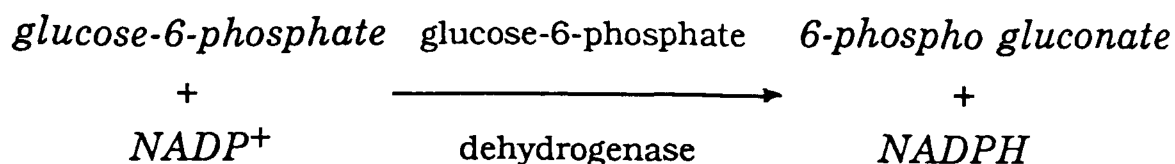


Fig. 3.1.7. Arrhenius plot of CCl_4-stimulated MDA production

The data were from Fig. 3.1.6. Δ MDA = MDA of samples with CCl_4 - MDA of samples without CCl_4.



It is important therefore to check the effect of temperature on NADPH generation. The results are shown in Fig. 3.1.8. The higher the temperature, the faster the generation of NADPH, as expressed by absorbance at 340nm.

3. 1. 2 Effect of selected agents on the activation of CCl_4 by rat liver microsomes

The effect of two inhibitors of the cytochrome P_{450} system, (pyrazole and imidazole), two sulphydryl reagents, (iodoacetamide and N-ethyl maleimide, NEM) and glutathione (GSH) on the activation of CCl_4 in rat liver microsomes was investigated. The activation of CCl_4 was measured by lipid peroxidation as determined by the production of MDA. The degree of inhibition of MDA production by each compound was compared to the control samples. The indicated concentrations of the compounds are the final concentrations used in the incubation mixture.

The data in Fig. 3.1.9-12 and Table 3.1.1 illustrate the effect of these selected agents on the formation of MDA. Pyrazole, iodoacetamide and imidazole are weak inhibitors. Even at the highest concentrations used, 10 mM or 20 mM, they failed to produce 50% of inhibition (Fig. 3.1.9, 3.1.10 and Table 3.1.1). Pyrazole inhibited lipid peroxidation by 30% at 10 mM and iodoacetamide only inhibited lipid peroxidation by 15% at 10 or 20 mM. No increase in inhibition occurred when the concentration of iodoacetamide was increased to

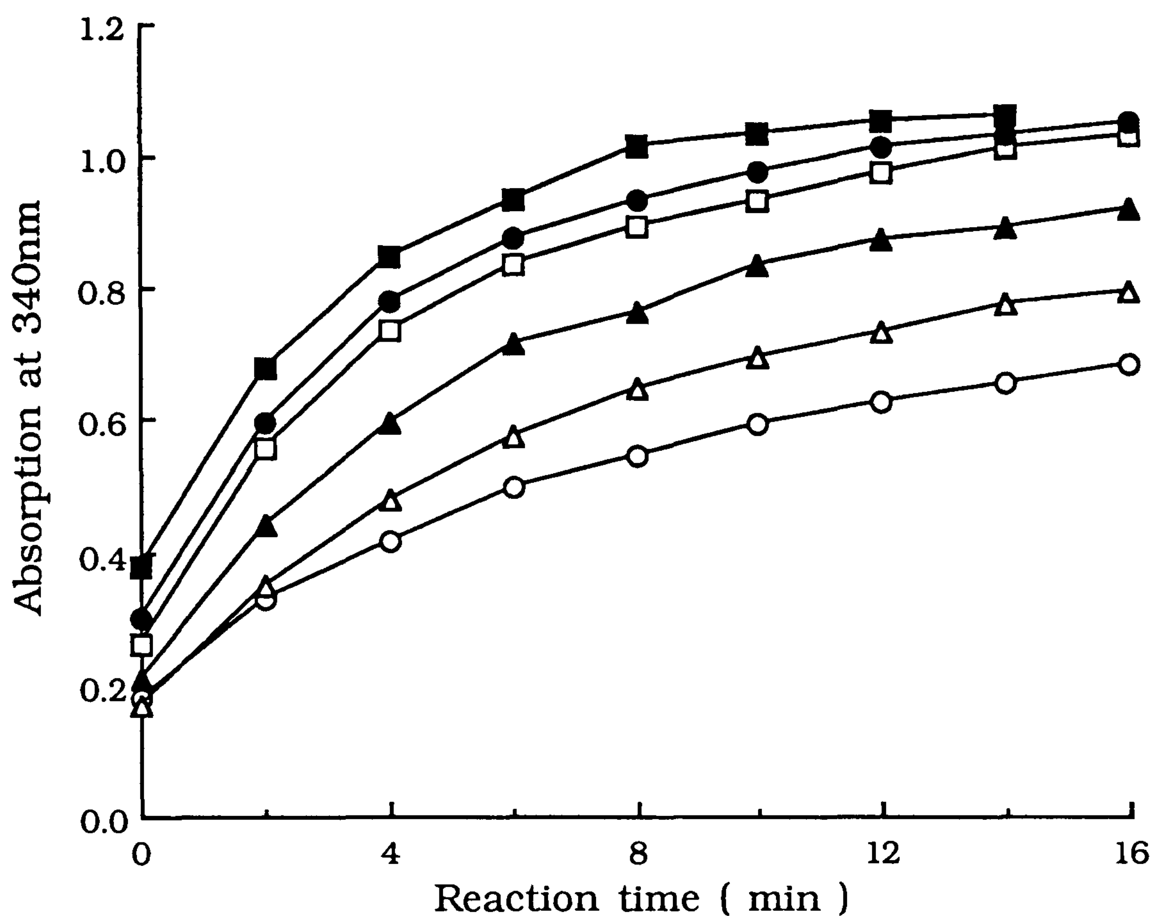


Fig. 3.1.8 NADPH production rate in NADPH generaing system at different temperature

NADPH content, as expressed in absorbance at 340nm, was checked in the NADPH generating system of glucose-6-phosphate, NADPH$^+$ and glucose-6-phosphate dehydrogenase at the indicated time at different temperatures as described in "Materials and Methods." Mean values are given ± S.D. with duplicate. (○) 15°C; (△) 20°C; (▲) 25°C; (□) 30°C; (●) 35°C; (■) 40°C.

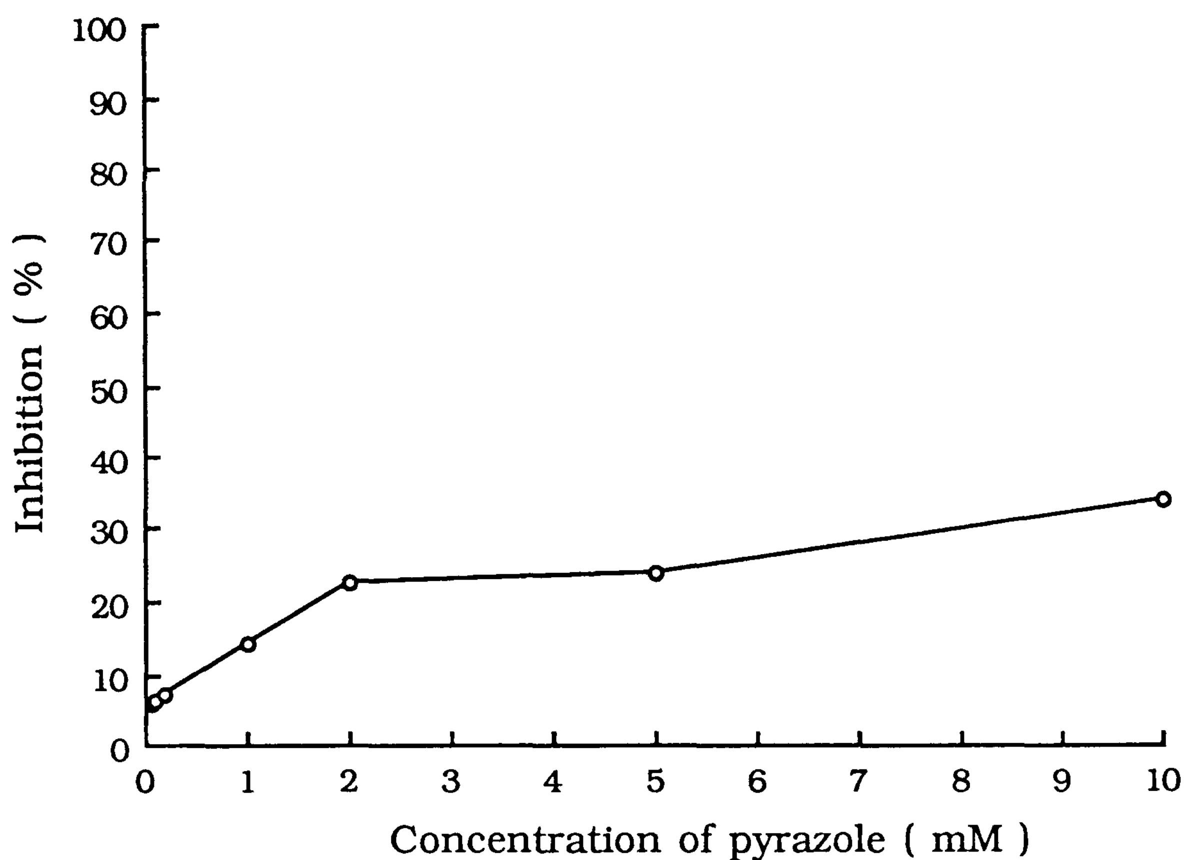


Fig. 3.1.9. Effect of pyrazole on lipid peroxidation stimulated by CCl_4 in rat liver microsomes

Lipid peroxidation was carried out at 37°C for 15 min. in the dark and estimated by measuring MDA production. MDA content in control (without pyrazole) was 257.9 pmol/mg prot./min. Each concentration point represents the mean of 4 determinations.

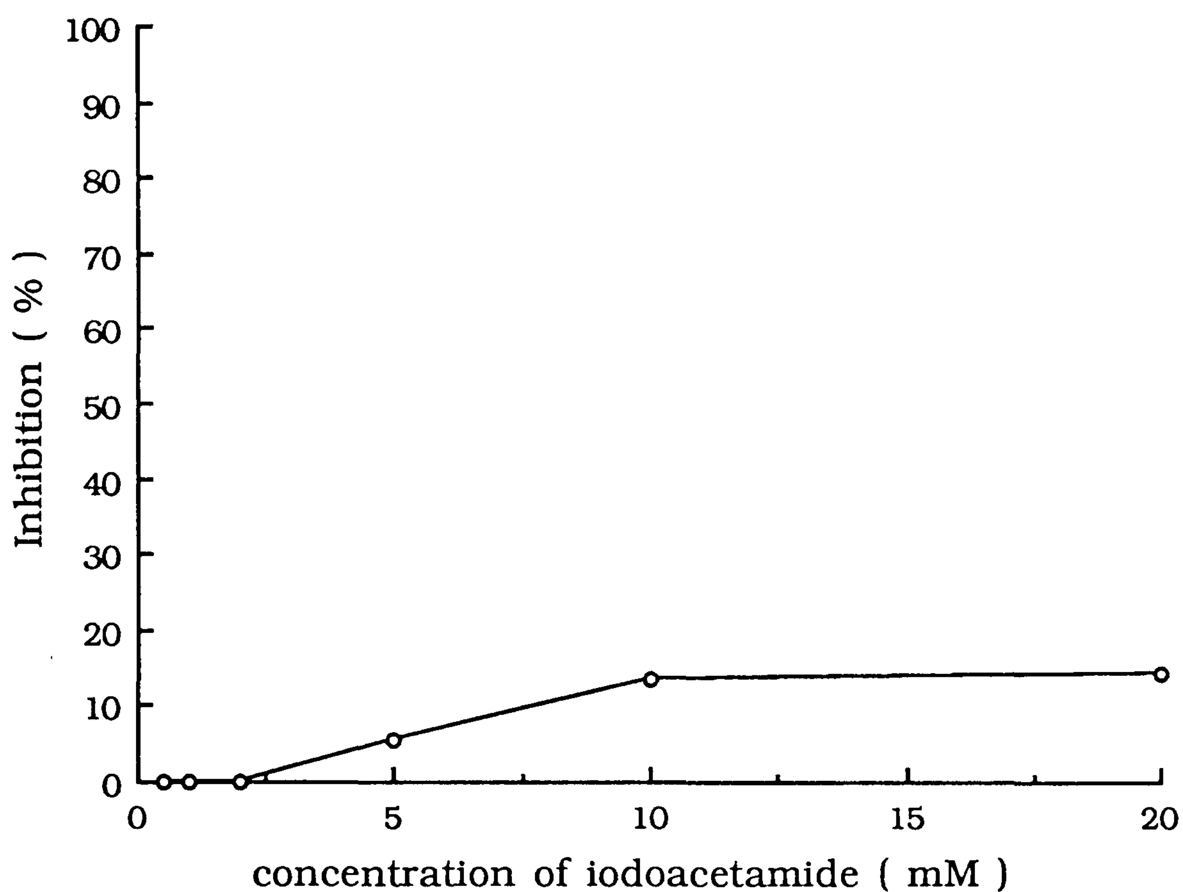


Fig. 3.1.10. Effect of iodoacetamide on lipid peroxidation stimulated by CCl_4 in rat liver microsomes.

Lipid peroxidation was carried out at 37°C for 15 min in the dark and estimated by measuring MDA production. MDA content in control (without iodoacetamide) was 171.6 pmol/mg prot./min. Each concentration point represents the mean of 2 determinations.

Table. 3. 1. 1 Effect of imidazole on lipid peroxidation stimulated by CCl_4 in rat liver microsomes

Concentration of imidazole (mM)	Malonaldehyde (pmol/mg prot./min.)	Inhibition (%)
0	267.8 ± 28.2	
1	259.7 ± 36.0	2.9
5	290.4 ± 25.5	- 8.6
10	273.7 ± 34.6	- 2.7
20	188.0 ± 91.6	29

Lipid peroxidation was carried out at 37° C for 15 min. as described in " Materials and Methods ". Mean values are given ± S.D. for two separate experiments with a total of 4 replicates.

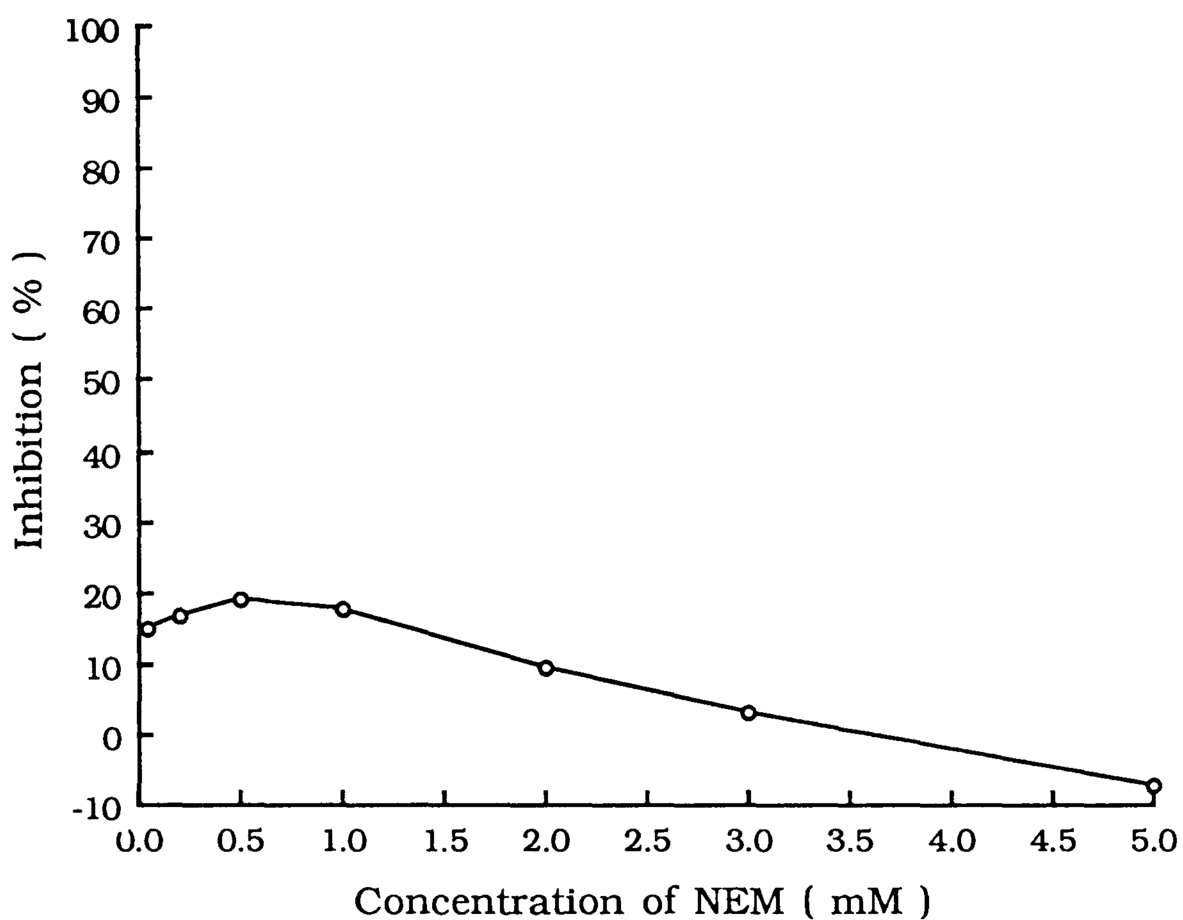


Fig. 3.1.11. Effect of N-ethyl maleimide (NEM) on lipid peroxidation stimulated by CCl_4 in rat liver microsomes

Lipid peroxidation was caried out at 37°C for 15 min. and estimated by measuring MDA production. MDA content in control (without NEM) was 306 pmol/mg prot./min. Each concentration point represents 4 replicates.

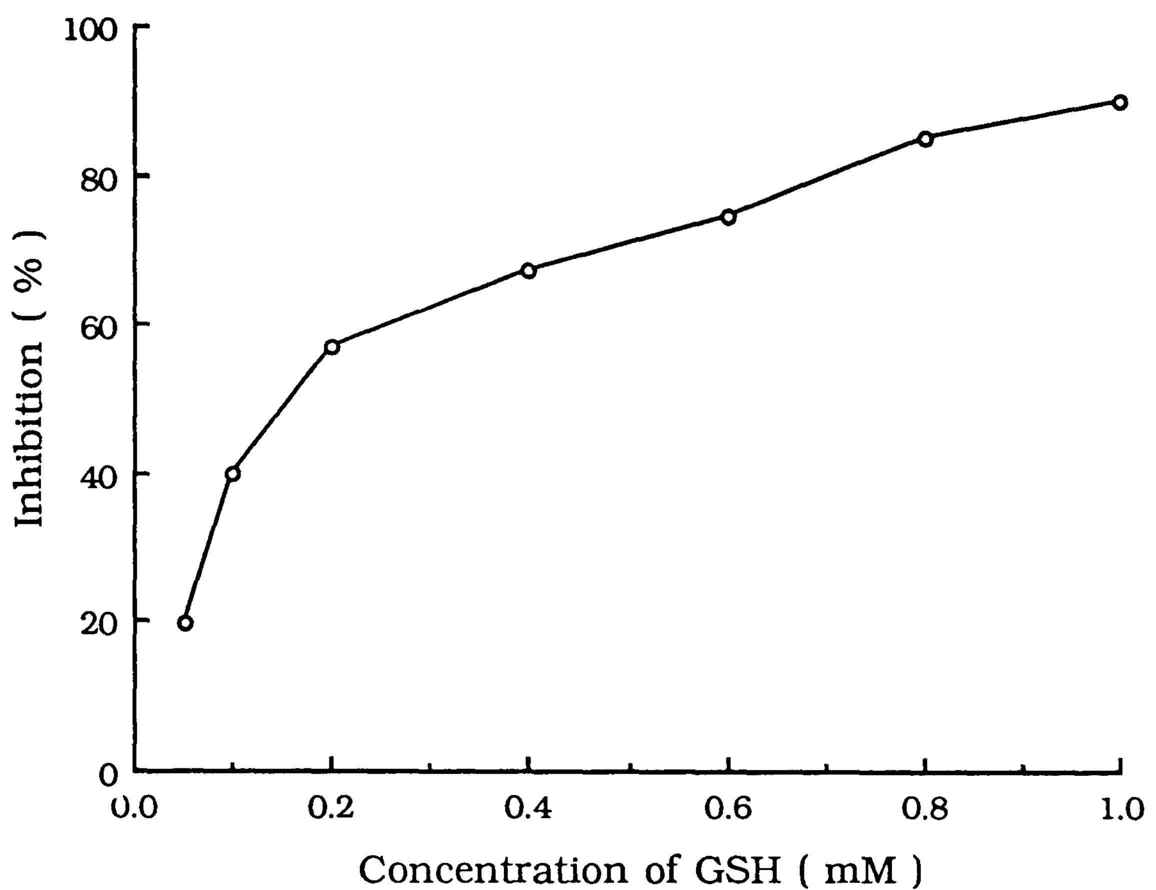


Fig.3.1.12. Effect of reduced glutathione on lipid peroxidation stimulated by CCl_4 in rat liver microsomes

Lipid peroxidation was carried out at 37°C for 15 min and estimated by measuring MDA production. MDA content in the control (without GSH) was 187.7 pmol/mg prot/min. Each concentration point represents the mean of 4 determinations.

more than 10 mM. Low concentrations of imidazole had no inhibitory effect on lipid peroxidation, but increasing the concentration eventually produced 29% inhibition at 20 mM (Table 3.1.1). For NEM, the effect on lipid peroxidation was different. As shown in Fig. 3.1.11, low concentrations of NEM had a slight inhibitory effect on MDA production. The highest inhibition extent was about 20% at 0.5-1.0 mM of NEM. However, when the concentration of NEM was higher than 1.0 mM, the inhibition decreased and at 5 mM NEM had a slight stimulatory effect on MDA production. Fig. 3.1.9 represents the results obtained for the inhibitory effect of GSH on lipid peroxidation. It can be seen that GSH strongly inhibits lipid peroxidation in a concentration-dependent fashion. MDA production was inhibited 50% by a concentration of less than 0.2 mM.

Using GSH as the inhibitor, the time course of the inhibitor effect on lipid peroxidation was studied. An illustration of the effects of different concentrations of GSH on CCl_4-stimulated lipid peroxidation in liver microsomes at different incubation times is given in Fig. 3.1.13. It can be seen that throughout the incubation time employed, 3 min. to 30 min., MDA production was inhibited by 0.08 mM, 0.2 mM, and 0.5 mM GSH. Although CCl_4-dependent lipid peroxidation increased with the incubation time under both conditions of with and without GSH, the extent and rate of increase above control levels was much lower when GSH was present, especially at higher concentrations. For example at 0.5 mM GSH, MDA production no longer increased after 10 min. incubation whilst in the absence of GSH lipid peroxidation continued to increase for at least another 5 min. GSH prolonged the initiation period of lipid peroxidation and this effect was concentration-dependent. In the absence of GSH, a 3 min lag was seen before MDA production increased at a higher rate. The presence of

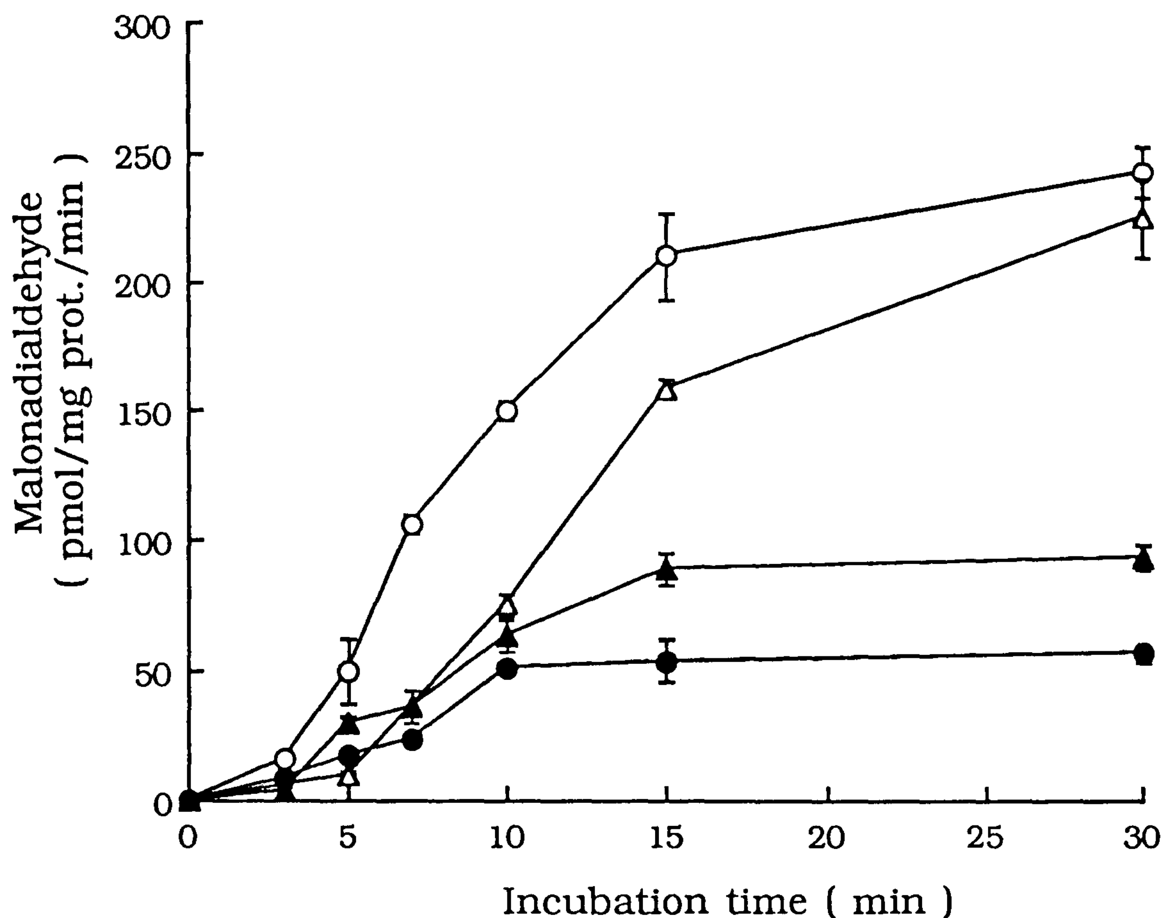


Fig. 3.1.13. Effect of reduced glutathione on the production of malondialdehyde in rat liver microsomes stimulated by CCl_4 at different incubation times

The temperature of lipid peroxidation incubation was 37°C. GSH concentrations represent final concentrations in the incubation mixture. Mean values are given ± S.D. (o) without GSH; (▵) 0.08 mM GSH; (▲) 0.2 mM GSH; (●) 0.5 mM GSH.

0.08 mM and 0.2 mM GSH increased the lag phase to 5 and 7 min respectively. No further delay of onset of lipid peroxidation was found when the highest concentration of GSH (0.5 mM) was present, but the rate of lipid peroxidation was strongly decreased.

3. 2 RIBONUCLEOTIDE REDUCTASE

3. 2. 1 Purification of ribonucleotide reductase

The purpose of this aspect of the investigation was

(i) to prepare a partially purified enzyme for determination of RNR'ase in a variety of tissues and under a variety of conditions, and

(ii) to develop a method of purification for obtaining a pure preparation of the enzyme with the intention of raising an antibody to it.

The rats used for the preparation of ribonucleotide reductase were 3-4 weeks old. The body weight of these rats was generally about 65g although values as low as 50g or as high as 92g were observed. The liver weights were in the range of 2.2-3.5g. One gram of liver usually yielded 41 mg of crude extract protein, 11 mg of 45% ammonium sulphate fractionation protein and 0.76 mg protein of enzyme preparation after chromatography on DEAE-cellulose (Table 3.2.1). A typical purification of the rat liver up to and including chromatography on DEAE-cellulose column is summarized in Table 3.2.2. The apparent recovery of total enzyme activity after ammonium sulphate precipitation step was about 220%. This was an inaccurate estimation probably due to the underestimation of the ribonucleotide reductase activity in the sample of crude extract, because of RNA and nucleoside kinase (NDP kinase) activity (Cory, 1973; Spector and Averett, 1983).

Table 3.2.1 Ribonucleotide reductase yield and activity of different preparations from 3-4 weeks old rat liver

Body Wt.	Liver Wt.	Crude extract			Ammonium sulphate fraction			DEAE-cellulose		
(g)	(g)	mg prot./ml	units/mg prot.	mg prot./g liver	mg prot./ml	units/mg prot.	mg prot./g liver	mg prot./ml	units/mg prot.	mg prot./g liver
65±6 (7)	3.1±0.3 (7)	20.8±0.7 (7)	25.5±2.7 (7)	41.3±10.5 (7)	40.6±2.8 (6)	185.3±26.5 (6)	11.5±3.2 (6)	12.5±2.8 (4)	940±114.2 (4)	0.76±0.3 (4)

Mean values are given ± S.E.M. with the number of animal groups and enzyme preparations shown in parentheses. Each animal group contained 5-10 rats, and each enzyme preparation was obtained from 5-10 livers. The value of inhibition extent of crude extract by 4 mM HU was from two separate experiments. The determination of enzyme activity was performed under standard reaction conditions as described in "Material and Methods". Each assay was carried out in duplicate.

Table. 3. 2. 2 Purification of rat liver ribonucleotide reductase from 36 g of juvenile normal rat liver.

	Volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg prot.)	Recovery (%)	Purification (fold)
Crude extract	38	908.2	29788.4	32.8 ± 0.9	100	
45% ammonium sulphate precipitate	6.0	260.7	66081.4	253.4 ± 5.6	221.7	7.7
DEAE-cellulose	1.5	22.5	23935.5	1063.8 ± 96.1	80.3	32.4

The enzyme activity was determined under standard reaction conditions as described in "Methods". The mean values of specific activity of enzyme are given ± S. D. with each assay was carried out in triplicate.

This will be discussed later. This is also the reason that the recovery after chromatography on DEAE-cellulose was so high, about 80%; sometimes 100% was observed. However when compared with the preparation of ammonium sulphate fractionate extract, the recovery of the enzyme protein after DEAE-cellulose chromatography was reasonable. This was about 36%.

After precipitation with ammonium sulphate, the resulting enzyme preparation was chromatographed using a DEAE-cellulose column. Usually 260 mg protein (6-9 ml) in 20 mM Tris-HCl containing 2 mM DTT and 5 mM $MgCl_2$ was applied to a 2 x 22 cm DEAE-cellulose column equilibrated with 10 mM potassium phosphate buffer (pH 7.0). The protein was eluted from the column in a stepwise manner with 10 mM potassium phosphate containing different concentrations of KCl. Ribonucleotide reductase activity was eluted with 10 mM potassium phosphate buffer containing 0.16 M KCl (Fig. 3.2.1). There was a little activity in the fractions of eluate of the buffer containing 0.25 M KCl. The elution pattern was very reproducible.

The whole procedure, from the homogenising of the liver to a purified enzyme preparation after chromatography on DEAE-cellulose, required two and a half days. The activity of three different preparations of the enzyme were stable for at least one month at -70°C when stored in 0.1M Tris/HCl (pH 7.4) containing 2mM DTT and 5 mM $MgCl_2$.

In Fig. 3.2.2 the results of analysis of the enzyme by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-page) is shown. The rat liver enzyme preparation after chromatography on DEAE-cellulose yielded several major bands on SDS-page and, in addition, some minor components. The results indicate that this kind of enzyme preparation was far from homogeneous.

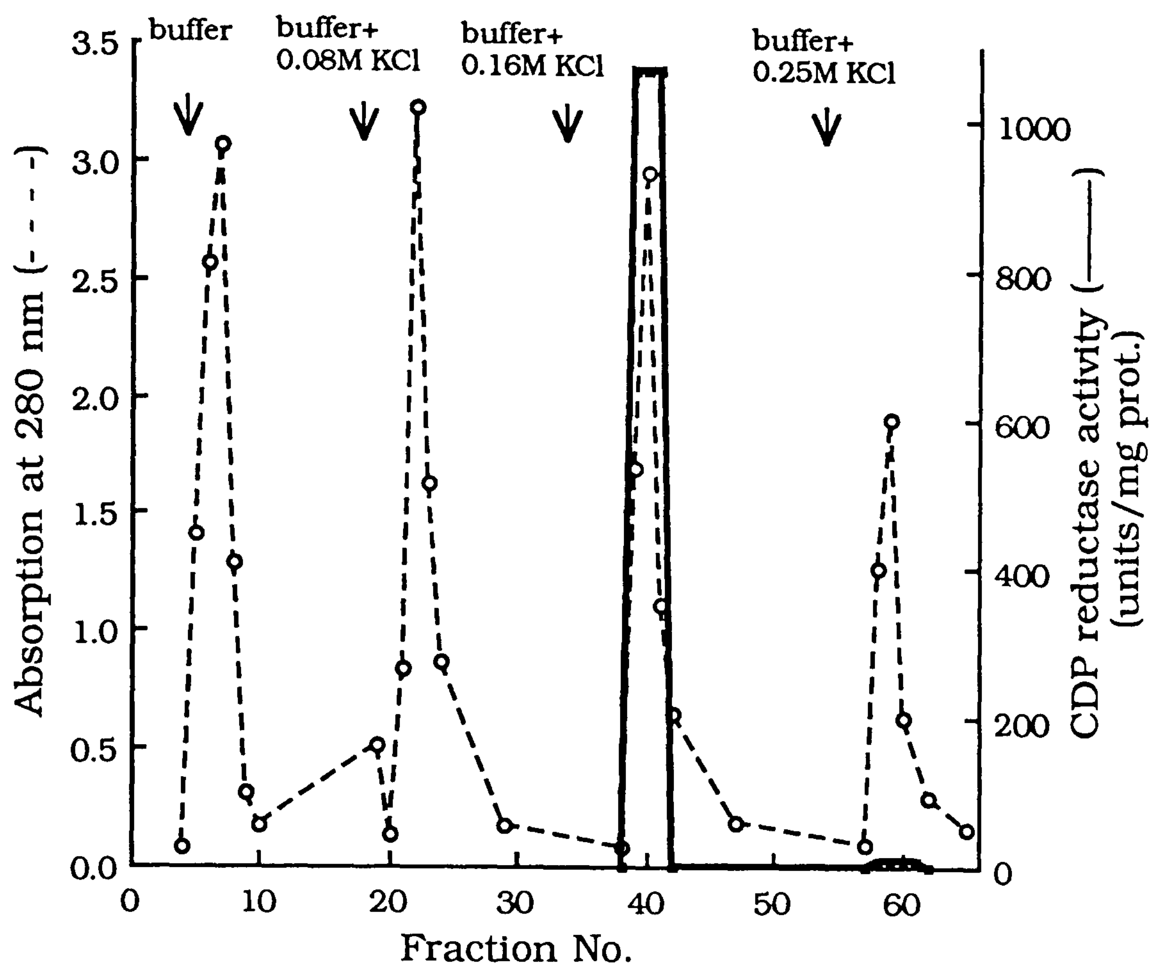


Fig. 3.2.1. DEAE-cellulose chromatography of rat liver ribonucleotide reductase

This represents a standard DEAE-chromatography profile as described in the text. The eluate buffer was 10 mM potassium phosphate. The volume of the eluate of each fraction was 6.0 ml. Fractions containing protein(indicated by the absorption at 280nm) were collected and the CDP reductase activity was determined under standard reaction condition as described in " Materials and Methods." (-o-) A_{280}; (-■-) enzyme activity.

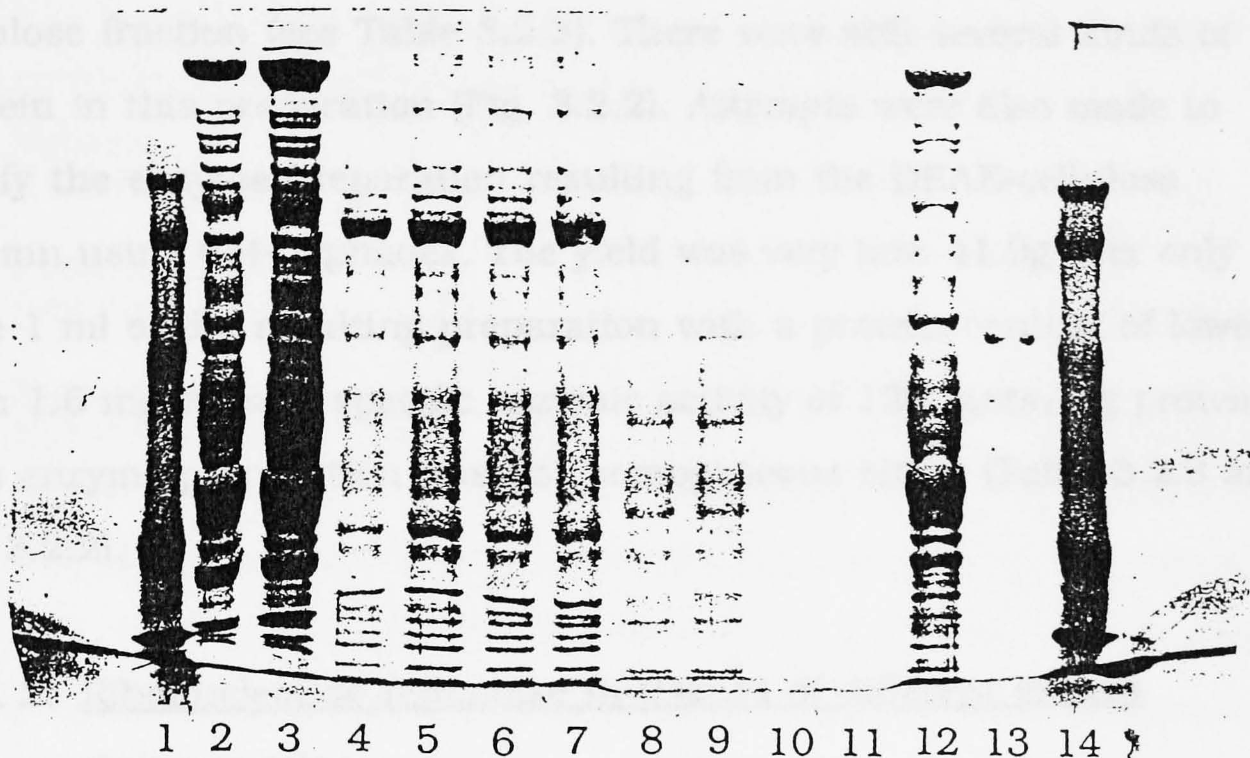


Fig. 3.2.2. SDS-polyacrylamide gel electrophoresis of preparations of ribonucleotide reductase from rat liver

Samples from left to right: DEAE-cellulose eluted with buffer + 0.16M KCl (2 and 3); DEAE-cellulose eluted with buffer + 0.25M KCl(4 to 7); CM-Sephadex fraction 1 (8 and 9); CM-Sephadex fraction 2 (10 and 11); ATP-agarose eluted without ATP (12); and ATP-agarose eluted with 2 mM ATP (13).

Standards (1 and 14) are from the top: ß-Galactosidase (Mr=116,000), Phosphorylase (Mr=97,4400), Bovine serum Albumin (Mr=66,000), Egg Albumin (Mr=45,000), Carbonic anhydrase (Mr=29,000).

Chromatography on an ATP-Agarose affinity column was tried in an attempt to further purify the protein after chromatography on DEAE-cellulose. This procedure was not very successful and not pursued further. The enzymic activity of the resulting enzyme preparation from the ATP-Agarose column eluting with the same buffer lost about 80% compared to the enzyme preparation of the DEAE-cellulose fraction (see Table 3.2.3). There were still several kinds of protein in this preparation (Fig. 3.2.2). Attempts were also made to purify the enzyme preparation resulting from the DEAE-cellulose column using CM-Sephadex. The yield was very low: 41.9g liver only gave 1 ml of the resulting preparation with a protein content of lower than 1.6 mg/ml and specific enzymic activity of 132 units/mg protein. This enzyme preparation was not homogeneous either (Table 3.2.3 and Fig. 3.2.2).

3. 2. 2. Ribonucleotide reductase in tissues of different growth states or rates

Using a partially-purified enzyme preparation, (45% ammonium sulphate fractionation) as the experimental sample, the CDP reductase activity in different tissues of different growth rate was determined and compared.

3. 2. 2. 1 Ribonucleotide reductase activity in juvenile and adult normal rat liver.

Table 3.2.4 gives a comparison of the ribonucleotide reductase activity in juvenile (3-4 week-old) and adult (11-12 week-old) rat liver. It is evident that there was a marked difference between the

Table 3.2.3 Rat hepatic ribonucleotide reductase yield and activity after chromatography on ATP - Agarose and CM-Sephadex

	ATP - Agarose	CM-Sephadex	
		Fraction 1	Fraction 2
Volume (ml)	2	1	1.5
Protein content (mg prot./ml)	2.2	1.6	undetectable
Enzyme activity (units/mg prot.)	233.3, 181.4	132.5	undetectable

The data are from a single purification experiment. Fractions 1 and 2 of CM-Sephadex represent, respectively the enzyme preparations which were obtained by washing the CM-Sephadex with buffer only and with buffer plus 1M NaCl. The protein solutions applied to ATP-Agarose column and CM-Sephadex were the enzyme preparations obtained from DEAE-cellulose chromatography. The specific activity of the enzyme was 1063 units/mg prot. prior to being applied to the ATP-Agarose and 973 units/mg prot. prior to being applied to CM-Sephadex.

Table. 3.2.4 Ribonucleotide reductase activity in juvenile and adult rat liver

	Age	
	3-4 weeks	11-12 weeks
Body Wt. (g)	65 ± 6 (7)	323 ± 14 (5)
Liver Wt. (g)	3.1 ± 0.7 (7)	12.3 ± 1.2 (5)
Enzyme activity (units/mg prot.)	185 ± 26,0 (4)	undetectable

The enzymic activity was determined under standard reaction conditions as described in " Materials and Methods ". Mean values are given ± S.E.M. with the number of experiments shown in parentheses. For the 3-4 week-old group, each experimental sample was from 5-10 rats. For the 11- 12 week-old group, each experimental sample was obtained from an individual rat. All assays of enzyme activity were performed in duplicate.

juvenile and adult rat liver in the enzymic activity. In liver of 3-4 week-old rats, the specific activity was 185 units/mg protein. However, no enzyme activity could be detected in livers of 11-12 week-old rats.

3. 2. 2. 2 Ribonucleotide reductase activity in hepatoma tumours

CDP reduction was detected in Yoshida ascites hepatoma cells and Morris 5123tc solid tumours carried in Wistar rats. From the results of Fig. 3.2.3 it is indicated that an ammonium sulphate fractionation from Morris 5123tc tumours showed only 13% of the CDP reductase activity as compared to a similar fraction from Yoshida cells. The range of enzyme activities found in the Yoshida cells under study was from 1379.5 to 4215.9 units/mg prot. In Morris 5123tc tumours activities ranged from 255.7 to 452.8 units/mg prot.

The viability of the freshly collected Yoshida cells was generally greater than 90%. The weight of Morris 5123tc solid tumours ranged from 2.73 g to 7.02 g. There was no correlation between the CDP reductase activity and the weight of the solid tumour.

3. 2. 2. 3 Ribonucleotide reductase activity in cultured Yoshida hepatoma cells

The Yoshida ascites hepatoma cells were collected from the host rat. After washing with PBS, the cells were cultured in MEM culture medium at 37°C. At the indicated time, the cells were collected and washed with PBS by centrifugation and cell-free extracts prepared as described in "Methods". CDP reductase was measured directly in the crude extract. The results are shown in Fig. 3.2.4. For freshly harvested cells from the host rat, the CDP reductase in crude extract enzyme

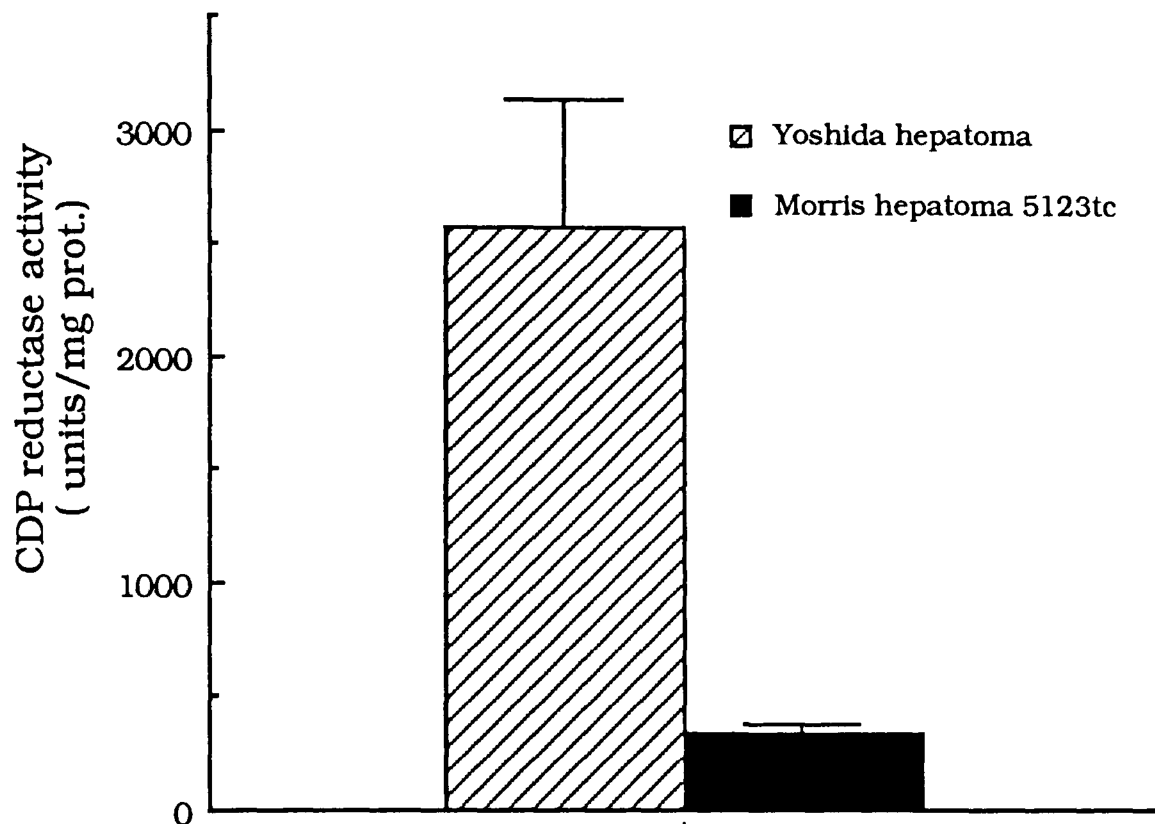


Fig. 3.2.3. Ribonucleotide reductase activity in Yoshida ascites hepatoma cells and Morris hepatoma 5123 tc solid tumours

The CDP reductase activity was determined under standard reaction conditions as described in "Materials and Methods." Mean values are given ± S.E.M. for 5 batches of Yoshida cell suspensions and 5 Morris hepatoma 5123 tc tumours. Each assay was carried out in duplicate.

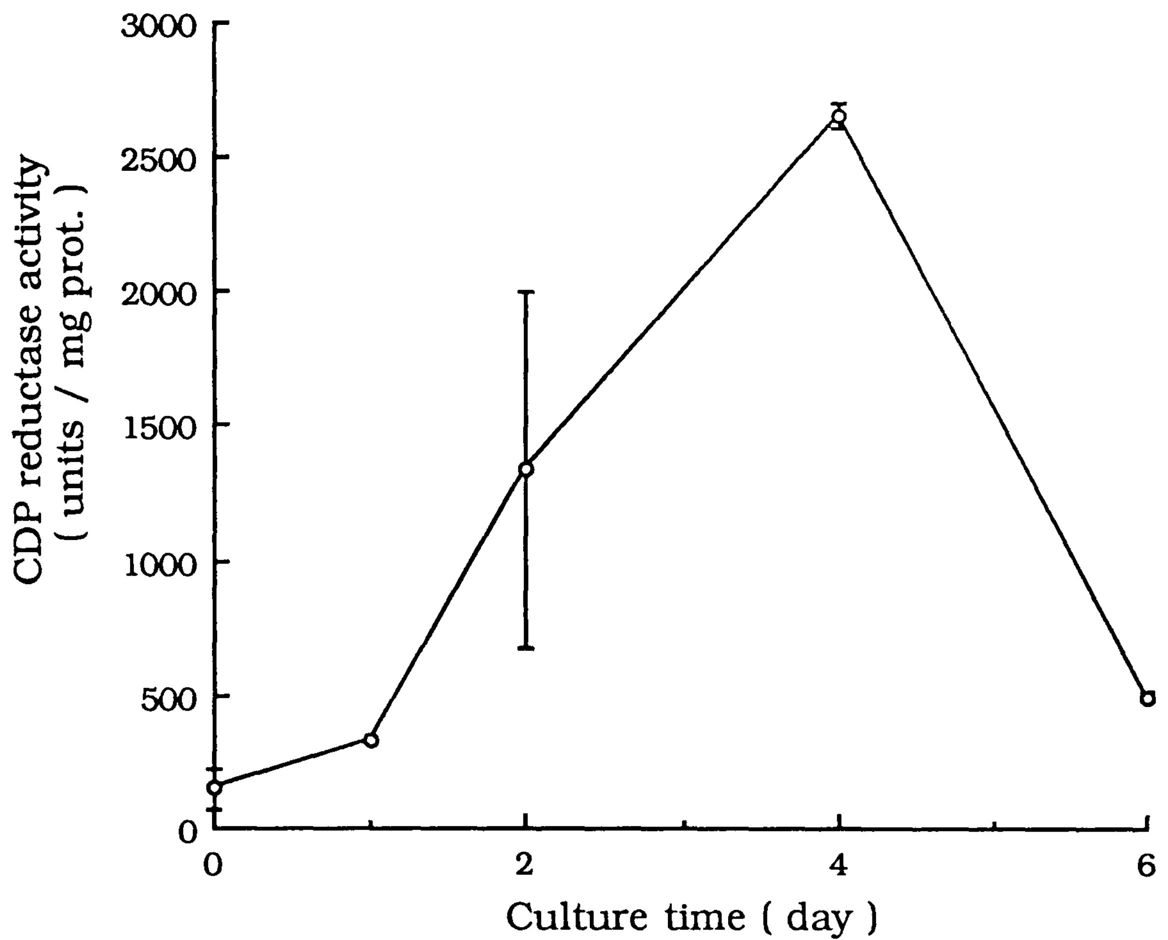


Fig. 3.2.4. Time course of ribonucleotide reductase activity in Yoshida ascites hepatoma culture cells

CDP reductase activity was determined under standard reaction conditions as described in "Materials and Methods". Mean values are given ± S.D.

preparation was about 149 units/mg prot. During 6 days of culture period the activity of reductase activity increased over 4 days. At day two, the enzymic activity was markedly elevated, 9 fold compared to the activity at day 0. At day four, the activity peaked at 2669 units/mg prot., 17 fold above the day 0 value. Afterwards the activity started to decline. On day 6, it had returned to a level only slightly above that found in freshly harvested cells. Since the reductase activity was measured in the crude extract, the specific activity was lower than in the ammonium sulphate fraction sample. The enzymic activity at Day 0 was 149 units/mg prot. in the crude extract, but it was 1358 units/mg prot. in the 45% ammonium sulphate fraction.

3. 2. 2. 4 Ribonucleotide reductase activity in regenerating liver

Following partial hepatectomy, the remaining minor lobes of the liver grow rapidly in order to restore the original mass of the liver. As shown in Table 3.2.5, the net weight of minor lobes of liver from partially hepatectomised rats increased compared with sham operated control livers. The amount of regeneration of liver mass relative to sham operated liver was increased by 69.7% at 36h, 92.7% at 48h, 116% at 60h and 93.6% at 72h post operation. In the sham operated rats the weight of the minor lobes was on average 2.69g during the period of study.

At intervals of 12 hours, the partially hepatectomized and sham operated rats were killed and the minor lobes of liver were sampled. Ammonium sulphate fractions were prepared from the minor lobes for CDP reductase activity determination. Fig. 3.2.5 illustrates the time course of the CDP reductase activity in regenerating liver. The enzyme activity increased in liver tissue following partial hepatectomy. The

Table 3. 2. 5 Weight of the minor liver lobes following either partial hepatectomy or sham operations

Time post operation	36 h	48h	60h	72 h
Weight of minor lobe of liver (g)				
Regenerating liver	4.26 ± 0.33 (4)	5.03 ± 0.54 (2)	5.44 ± 0.72 (5)	6.06 ± 0.51 (4)
Sham-operated liver	2.51 ± 0.35 (2)	2.61 ± 0.37 (2)	2.51 ± 0.19 (2)	3.13 ± 0.54 (4)

Mean values are given ± S.E.M. with the number of animals shown in parentheses.

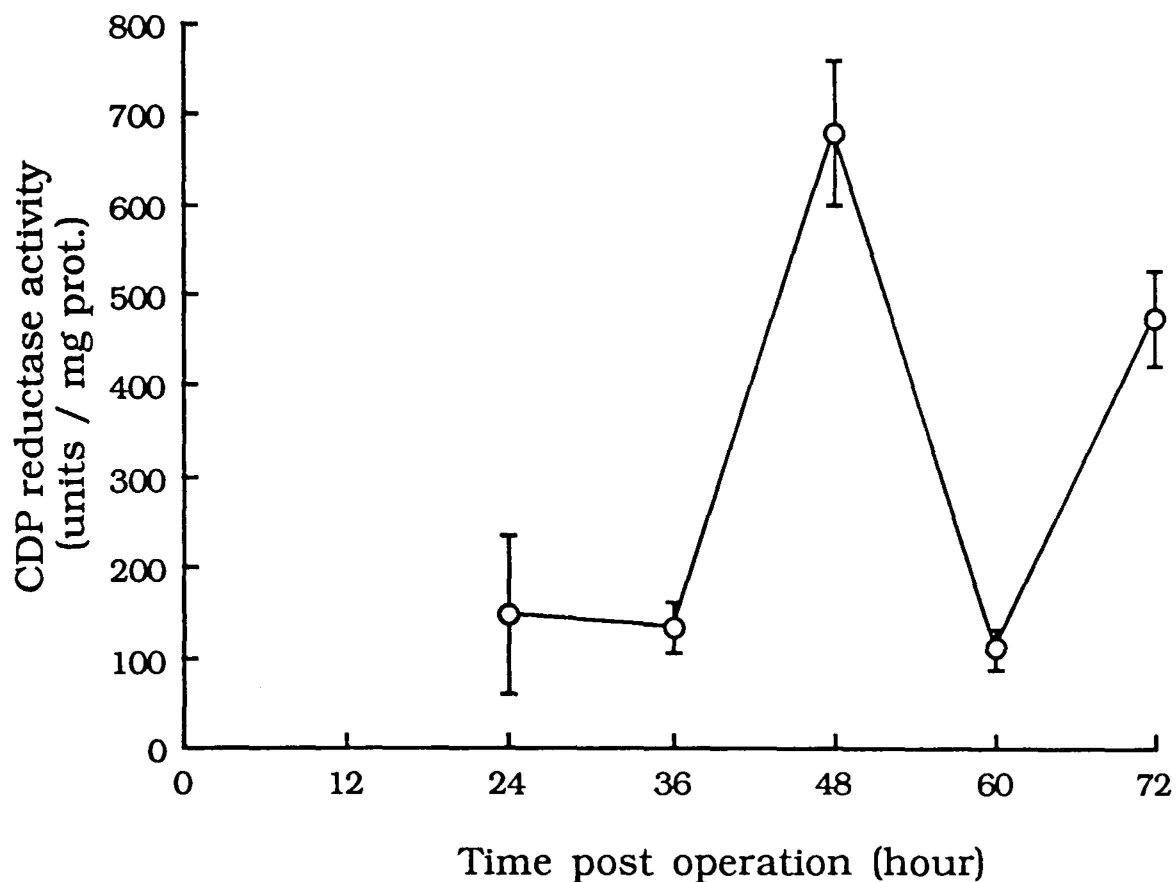


Fig. 3.2.5. Ribonucleotide reductase activity in regenerating rat liver

Rats were subject to partial hepatectomy at '0 hours' and liver was sampled for ribonucleotide reductase activity at the times shown. The CDP reductase activity was determined under standard reaction conditions as described in "Materials and Methods". Mean values are given ± S.E.M. The number of rats was 10 at 24h, 9 at 36h, 11 at 48h, and 4 at 72h post operation. Each assay was carried out in duplicate.

time course appears to exhibit a periodic cycling with two peaks, one at 48h post operation and another at 72h post operation. The maximal reductase activity was observed at 48h post operation which was 4-5 fold higher than the activity at 24h, 36h and 60h post operation. The enzyme activity of regenerating liver at 72h was 2-3 fold higher than that at 24h, 36h and 60h post operation. CDP reductase activities were undetectable in all sham operated livers.

Table 3.2.6 demonstrates the CDP reductase activity of the mixture of enzyme preparations from 48h and 60h regenerating rat liver. The activity of the mixture was 292 units/mg protein. This value was very close to the calculated activity of the enzyme mixture which was 282 units/mg protein. This result indicated that there was only additive effect when these enzymes were mixed together.

3. 2. 3 Elevation and inhibition of the activity of ribonucleotide reductase from juvenile rat liver and regenerating liver

Dithiothreitol (DTT) is necessary for CDP reductase activity as a reducing agent in this assay system. In this study it was found that there were differences in the optimal DTT concentration for exerting the full CDP reduction activity between the enzyme from juvenile normal rat liver and regenerating liver. Fig. 3.2.6 illustrates the dependence of the activity of CDP reductase from juvenile normal rat liver and regenerating rat liver (48h and 60h post operation) on DTT concentration ranging from 1mM to 15 mM. It can be seen that higher concentration of DTT can elevate regenerating liver's CDP reductase activity. The enzyme activity of 48h regenerating liver was enhanced 160% when DTT concentration was increased to 5 mM. The enhancement was not significantly increased when the DTT

Table 3.2.6 CDP reductase activity in the mixture of enzyme from 48h and 60h regenerating rat liver

Enzyme	Activity (units/mg prot./h)	
	calculated	observed
48h		428.8±21.9
60h		136.7±11.2
48h+60h	282.7	292.8±17.8

The activity of CDP reducatse was determined under standard conditions as described in "Material and Methods". The DTT concentration in the reaction solution was 10 mM. The reaction time was 1h. Protein content in the reaction mixture was 1 mg. For the measurement of the CDP reductase activity of mixed enzymes from 48h and 60h regenerating liver, 0.5 mg protein of each enzyme preparation was added to the CDP reduction mixture. Mean values are given ± S.D.

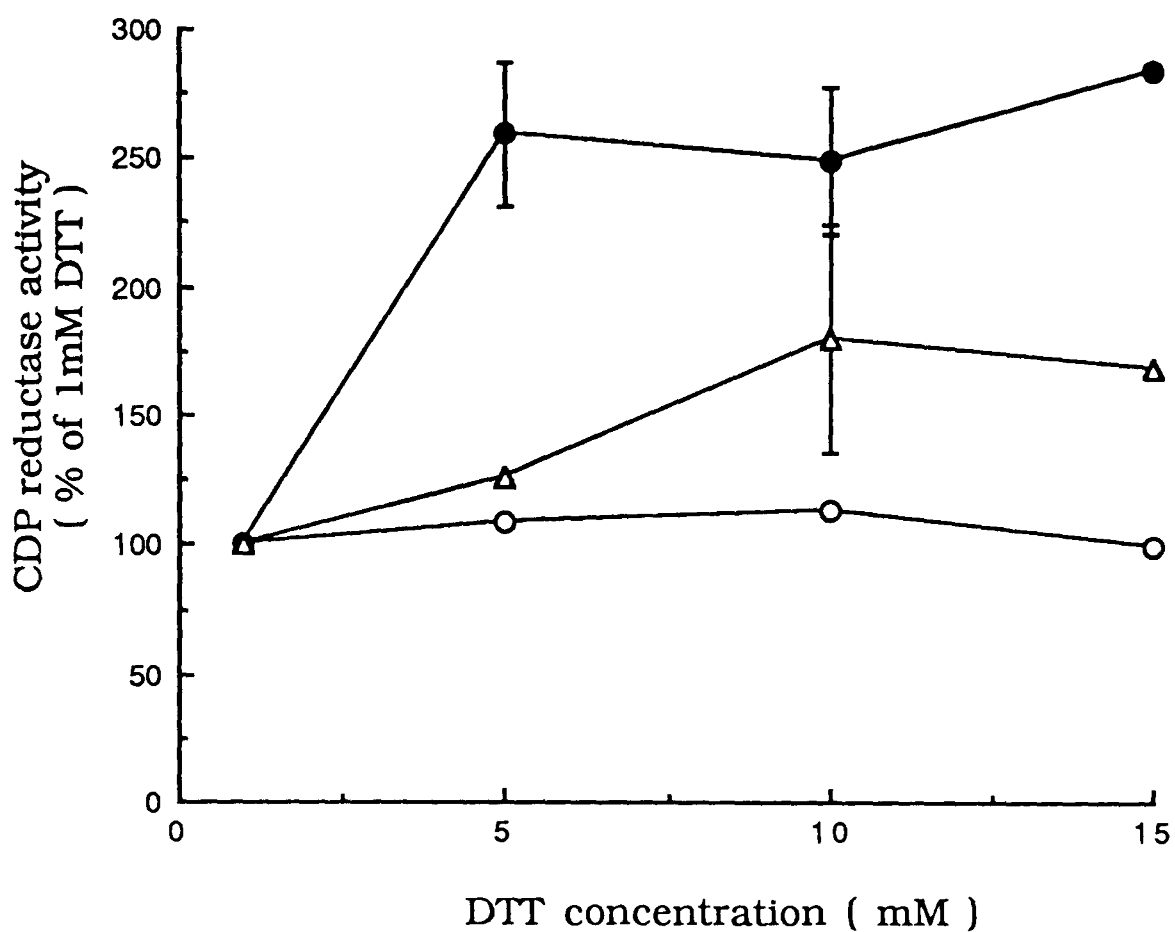


Fig. 3.2.6. Dependence of CDP reductase activity on DTT concentration

The CDP reductase activity of enzyme from juvenile normal rat liver and regenerating rat liver (48h and 60h) was determined at different concentrations of DTT. The enzyme activity at 1 mM DTT was taken as 100%. Mean values are given ± S.E.M. Each assay was carried out in triplicate. (o) juvenile normal rat liver; (●) 48h regenerating rat liver; (Δ) 60h regenerating rat liver.

concentration increased further to 15 mM. For the enzyme from 60h regenerating liver the degree of activity enhancement by DTT was lower than for the enzyme from 48h regenerating liver. Comparing to the activity at 1mM DTT, the activity at 5 mM DTT and 10 mM DTT were increased 27% and 81% respectively. No further enhanced activity was observed when the DTT concentration was increased to 15 mM. In contrast to the enzyme from regenerating liver, the enzyme activity of normal juvenile liver did not respond to changing of the DTT concentration. In the range of 1 mM to 15 mM DTT there was no significant difference in the reduction activity (Fig. 3.2.6).

To investigate if continual higher DTT concentration was necessary for the reductase from regenerating rat liver to exert its full activity, the effect on the CDP reduction by the enzyme from 48h regenerating liver of addition of higher concentration of DTT (10 mM) to the enzyme assay mixture which contained lower DTT (1 mM) at different incubation times was studied (Fig. 3.2.7). At 10 mM DTT the dCDP formation was 2.2 fold of the value at 1 mM DTT during 60 min. incubation time. Addition of 10 mM DTT to the 1 mM DTT-containing enzyme assay mixture 20 min. and 40 min. after the reaction started, the rate of the dCDP production was completely restored indicating that the activity of the enzyme had returned to full level.

The function of DTT as an activator of CDP reductase activity was further studied. First the enzyme protein was preincubated with a higher concentration of DTT (10 mM) and 8 mM $MgCl_2$ at 30°C for a certain time. Then the preincubation mixture was transferred to CDP reduction mixture for regular activity assay in which the DTT concentration was diluted to 1 mM. The results are shown in Fig. 3.2.8-10. After preincubation the activity of CDP reductase from 48h and 60h regenerating liver was enhanced (Fig. 3.2.8 and Fig.3.2.9).

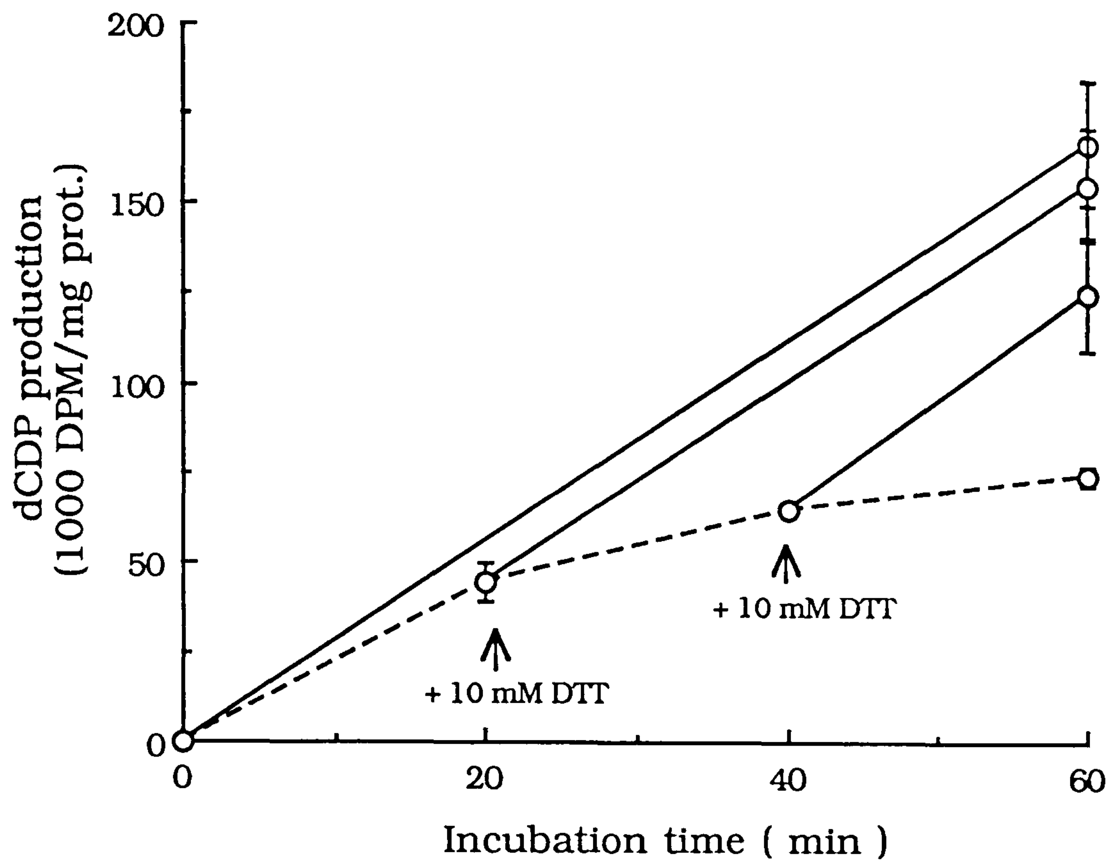


Fig. 3.2.7. Effect of addition of a high concentration of DTT on CDP reductase activity from 48h regenerating rat liver

Enzyme protein from 48h regenerating rat liver was incubated with CDP reduction mixture containing 1 mM DTT at 30°C. At the indicated time point (shown by the arrow in the figure) 3 of the samples were stopped for dCDP determination, 3 of the samples received 5 µl 630 mM DTT solution to make the DTT concentration in the assay 10 mM and incubation was continued to 60 min. The amount of enzyme protein in assay was 0.39 mg. The mean values are given ± S.D. All assay were carried out in triplicate. (-------) 1mM DTT; (———) 10 mM DTT.

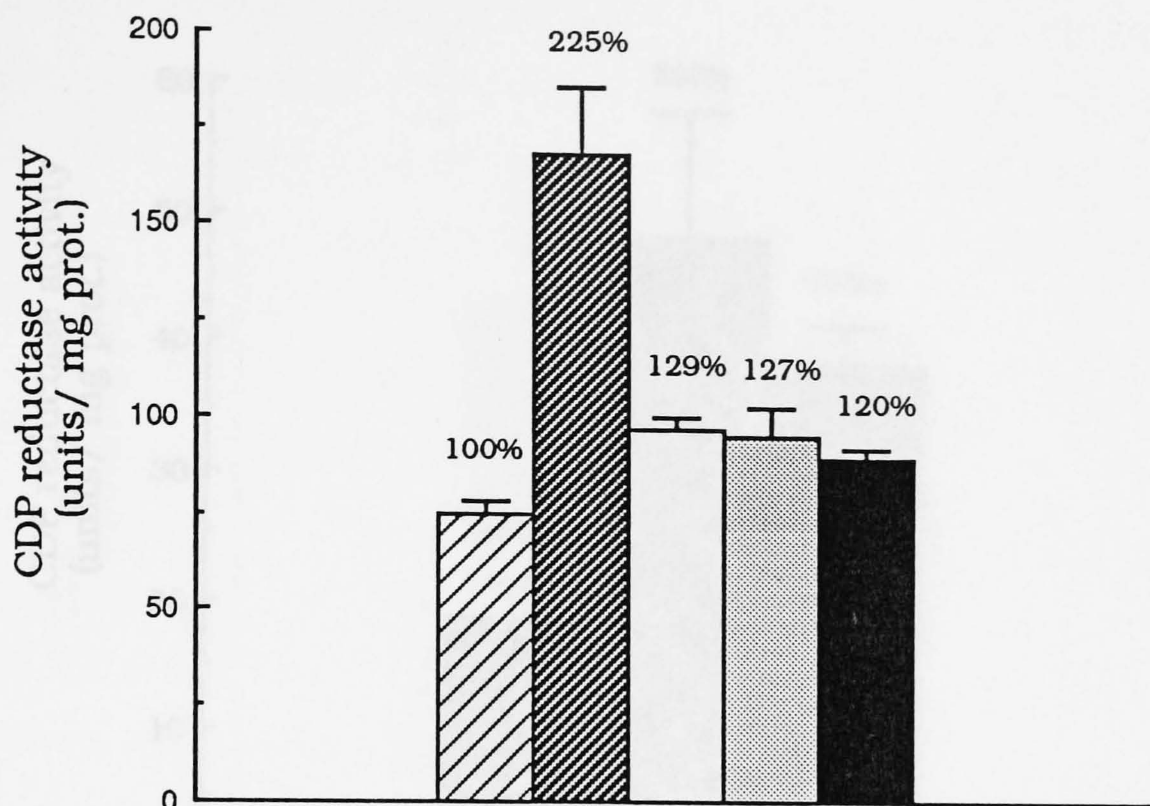


Fig. 3.2.8. Effect of preincubation of enzyme protein from 48h regenerating liver with DTT/Mg^{2+} (10mM/8mM) on CDP reductase activity

The protein (3.7 mg) of enzyme preparation from 48h regenerating liver was preincubated with 10mM DTT and 8 mM $MgCl_2$ in total volume of 330 μl at 30°C for 20, 40 and 60 min. At the time indicated 34.5 μl aliquots of this incubation mixture were added to the CDP reduction mixture (310.5 μl) for the regular activity assay in which the DTT concentration was diluted to 1 mM. The amount of enzyme protein in assay was 0.39 mg. Mean values are given ± S.D. (▨) without preincubation, DTT concentration in assay was 1 mM; (▩) without preincubation, DTT concentration in assay was 10 mM; (□) preincubation for 20min.; (▒) preincubation for 40 min.; (■) preincubation for 60min.

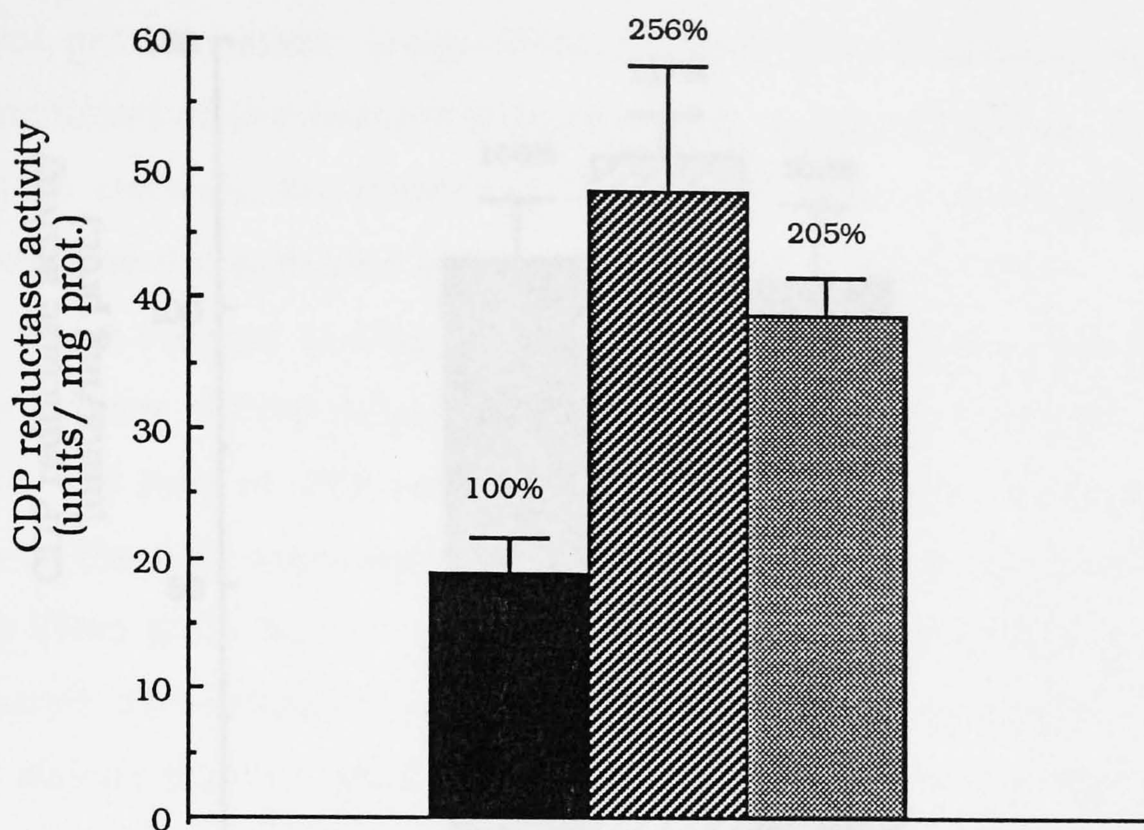


Fig. 3.2.9. Effect of preincubation of enzyme protein from 60h regenerating liver with DTT/Mg^{2+} (10mM/8mM) on CDP reductase activity

The protein (1.8 mg) of enzyme preparation from 60h regenerating liver was preincubated with 10mM DTT and 8 mM $MgCl_2$ in total volume of 110 µl at 30°C for 20 min. Then 34.5 µl aliquots of this incubation mixture were added to the CDP reduction mixture (310.5 µl) for the regular activity assay in which the DTT concentration was diluted to 1 mM. The amount of enzyme protein in assay was 0.58 mg. Mean values are given ± S.D. (■) without preincubation, DTT concentration in assay was 1 mM; (▨) without preincubation, DTT concentration in assay was 10 mM; (▩) preincubation for 20min.

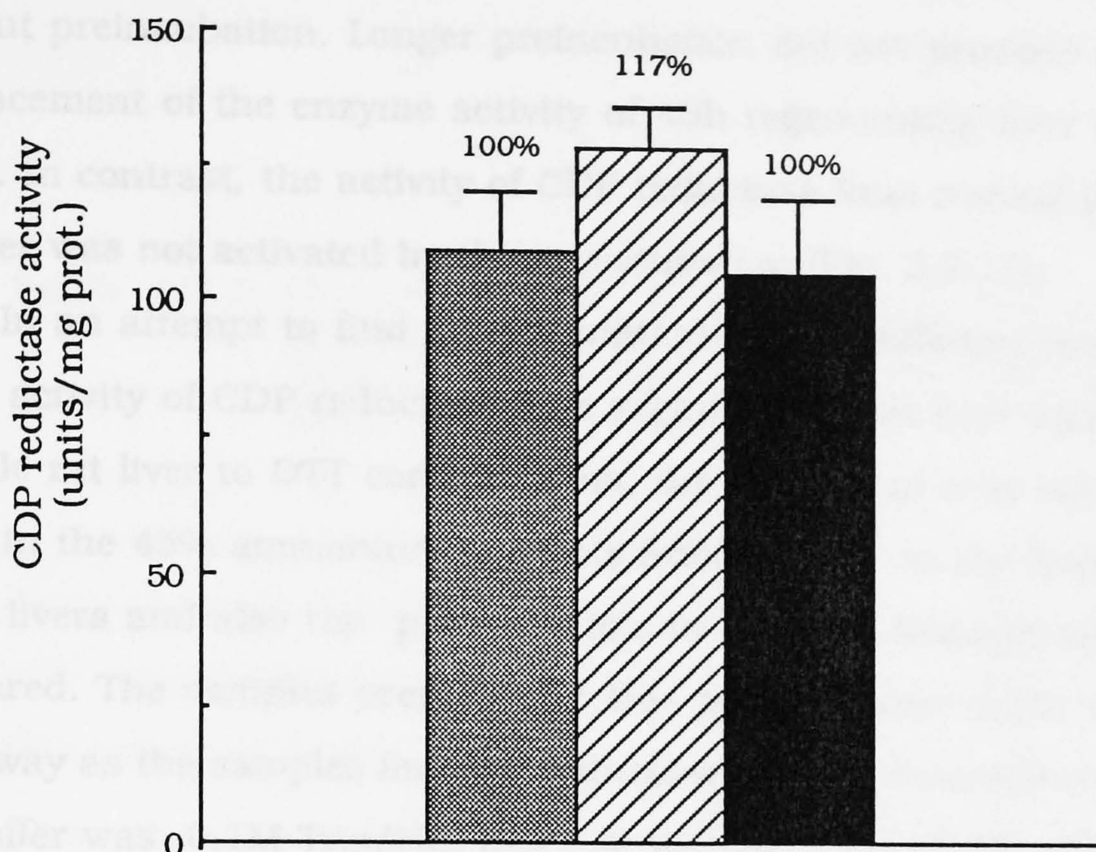


Fig. 3.2.10. Effect of preincubation of enzyme protein from juvenile normal rat liver with DTT/Mg^{2+} (10mM/8mM) on CDP reductase activity

The protein (3.9 mg) of enzyme preparation from juvenile normal rat liver was preincubated with 10mM DTT and 8 mM $MgCl_2$ in total volume of 110 µl at 30°C for 20 min. Then 34.5 µl aliquots of this incubation mixture were added to the CDP reduction mixture (310.5 µl) for the regular activity assay in which the DTT concentration was diluted to 1 mM. The amount of enzyme protein in assay was 1.23 mg. Mean values are given ± S.D. (▩) without preincubation, DTT concentration in assay was 1 mM; (▨) without preincubation, DTT concentration in assay was 10 mM; (■) preincubation for 20min.

The activity was increased about 29% for 48h regenerating liver enzyme (Fig. 3.2.8) and 105% for 60h regenerating liver enzyme (Fig. 3.2.9) after 20 min preincubation compared to the enzyme activity without preincubation. Longer preincubation did not produce more enhancement of the enzyme activity of 48h regenerating liver (Fig. 3.2.8). In contrast, the activity of CDP reductase from normal juvenile rat liver was not activated by the preincubation (Fig. 3.2.10).

In an attempt to find an explanation for the different responses of the activity of CDP reductase from regenerating rat liver and normal juvenile rat liver to DTT concentration, the content of acid soluble thiols in the 45% ammonium sulphate fraction and in the homogenate of the livers and also the protein thiols in the liver homogenates were measured. The samples prepared for this purpose were made in the same way as the samples for CDP reductase activity determination but the buffer was 0.1M Tris/HCl (pH 7.4) without 2 mM DTT and 5 mM $MgCl_2$. The results are shown in Tables 3.2.7, 3.2.8 and Fig. 3.2.11. The content of acid-soluble thiols in the 45% ammonium sulphate fraction of 48h regenerating liver was significantly higher than that from 60h regenerating liver. There were no significant differences in acid soluble thiols content between 48h regenerating liver and juvenile normal rat liver or between juvenile normal rat liver and 60h regenerating liver (Table 3.2.6). Similarly, there were no differences of the content of protein thiols in homogenates of normal juvenile rat liver and regenerating rat livers (Table 3.2.7). However, the content of acid soluble thiols in homogenates from regenerating liver exhibit a periodic cycle. The thiol content exhibited maxima at 24h, 48h, and 72h post operation compared to minima at 36h and 60h post operation (Fig. 3.2.11).

The inhibitory effect of hydroxyurea, a specific inhibitor of

Table 3.2.7 The content of acid soluble thiols in enzyme preparation of 45% ammonium sulphate fraction from juvenile normal rat liver and regenerating rat liver

	Thiols content (nmoles/mg prot.)
Juvenile normal liver	5.1 ± 0.7 (4)
48h regenerating liver	6.9 ± 0.5 (5)
60h regenerating liver	4.2 ± 0.5 (5)

The enzyme preparation was prepared as the same as for CDP reductase activity determination except the buffer was 0.1M Tris/HCl (pH7.4). The content of acid soluble thiols was measured as described in "Methods". Mean values are given±S.E.M with the number of animal shown in parentheses. Thiols content in fraction of 45% ammonium sulphate from 60h regenerating liver was significantly lower ($P < 0.005$) compared to 48h regenerating liver.

Table 3.2.8 Content of protein thiols in the homogenate from juvenile normal rat liver and regenerating rat liver (nmoles/mg protein)

Juvenile normal	Regenerating liver				
liver	24h	36h	48h	60h	72h
94.5 ± 1.3	94.5±3.7	90.5±2.9	90.3±4.6	89.5±3.0	92.8±3.5
(4)	(5)	(5)	(5)	(6)	(5)

Homogenates were made from juvenile normal rat liver and from regenerating liver at various times post operation with 0.1M Tris/HCl (pH 7.4). The content of protein thiols was measured as described in "Methods". Mean values are given±S.E.M with the number of animal shown in parentheses.

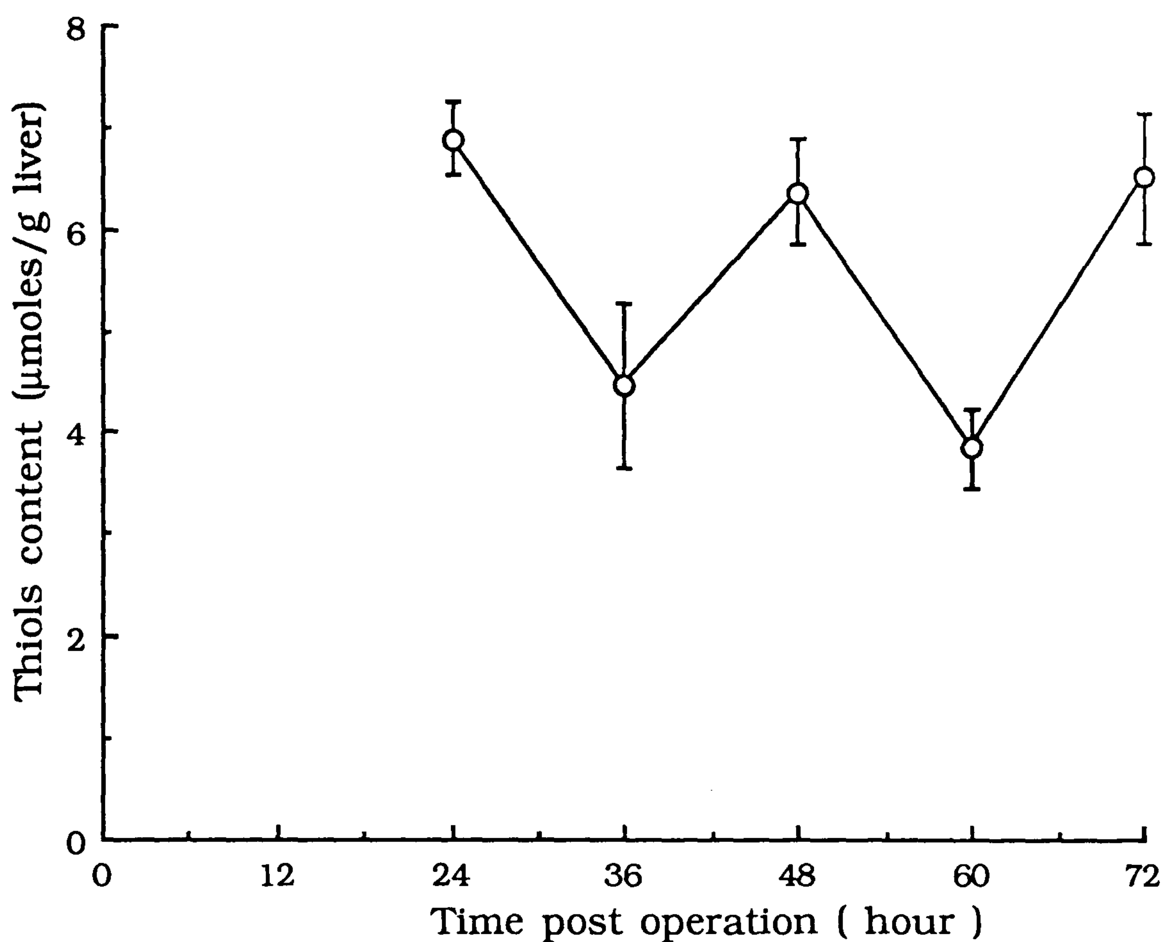


Fig. 3.2.11. Content of acid soluble thiols in homogenate of regenerating rat liver

Homogenates were made from regenerating liver with 0.1M Tris/HCl (pH 7.4). The content of acid soluble thiols was measured as described in "Methods". Mean values are given ± S.E.M. The number of animals was 5 for each point.

ribonucleotide reductase, on activity of CDP reductase from normal juvenile rat liver and 48h and 60h regenerating rat liver was also investigated. All the enzymes investigated displayed a similar sensitivity to hydroxyurea. The activity was inhibited about 60-70% by 4mM hydroxyurea and 10 mM hydroxyurea nearly completely inhibited the enzyme activity (Fig. 3.2.12).

3. 3 PROPERTIES OF 4-HYDROXYNONENAL (HNE) AS AN INHIBITOR OF RIBONUCLEOTIDE REDUCTASE FROM JUVENILE NORMAL RAT LIVER

Previous work has demonstrated that lipid peroxidation products damaged DNA or affected DNA synthesis. In rapidly dividing cells/tissues, for example in tumour cells, the lipid peroxidation levels are very low. Since ribonucleotide reductase is a critical enzyme for DNA synthesis, the effect of the lipid peroxidation product 4-hydroxynonenal (HNE) on ribonucleotide reductase from normal rat liver was investigated. The enzyme preparation used for this work was the 45% ammonium sulphate fraction stored in 0.1M Tris/HCl (pH 7.4) containing 2 mM DTT and 5 mM $MgCl_2$.

3. 3. 1 Inhibition of CDP reductase activity by HNE

The data of Fig. 3.3.1 demonstrate that HNE inhibited the activity of CDP reductase of rat liver when added to the CDP reduction mixture for enzyme activity assay without pre-incubation under standard conditions (incubation time 60 min.). HNE (0.1 mM, final concentration) decreased the activity by 50%.

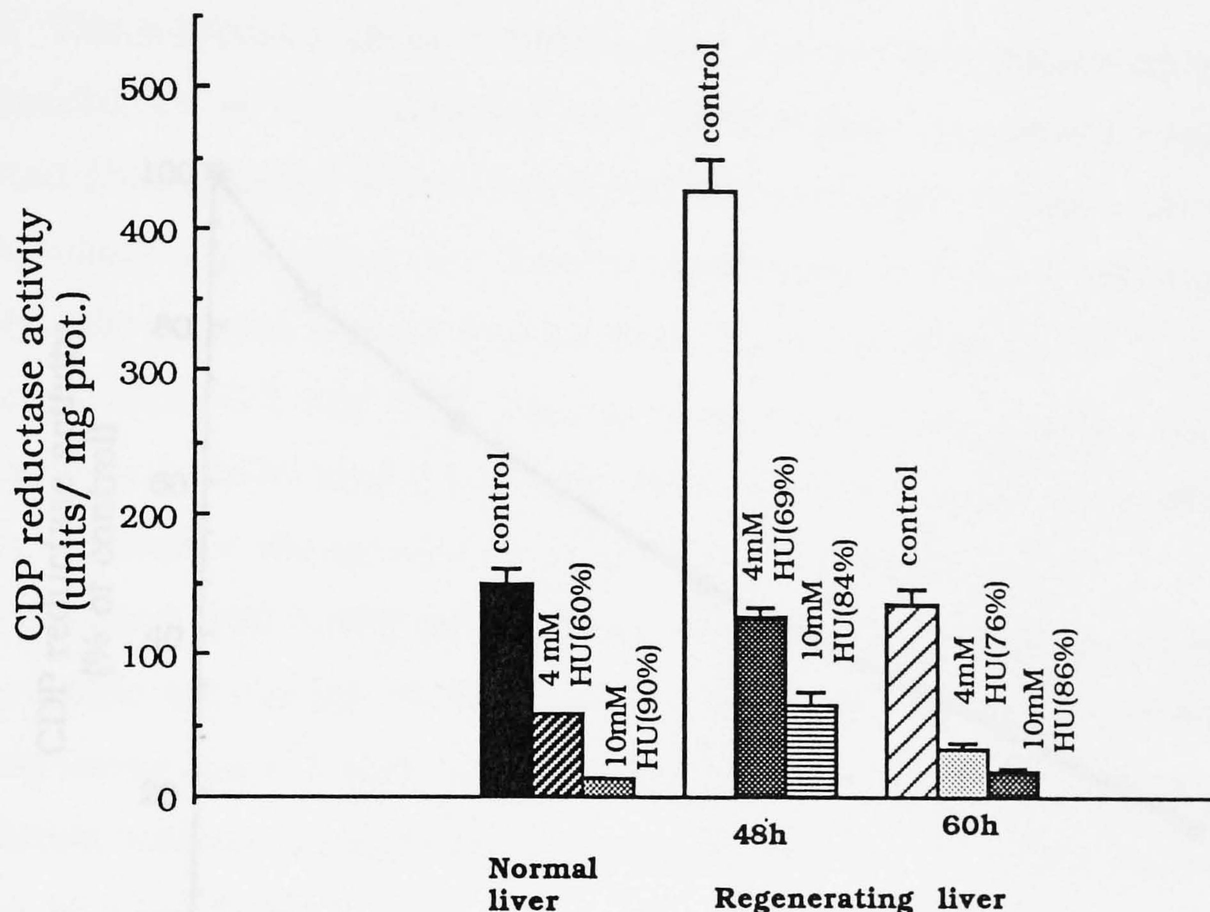


Fig. 3.2.12. Effect of hydroxyurea on activity of CDP reductase from juvenile normal, 48h and 60h regenerating rat liver

CDP reductase activity was measured in the absence or presence of hydroxyurea (4 mM and 10 mM, final concentration) as described in "Methods". The number shown in the figure present the inhibition degree by hydroxyurea compared to control. Mean values are given ± S.D.

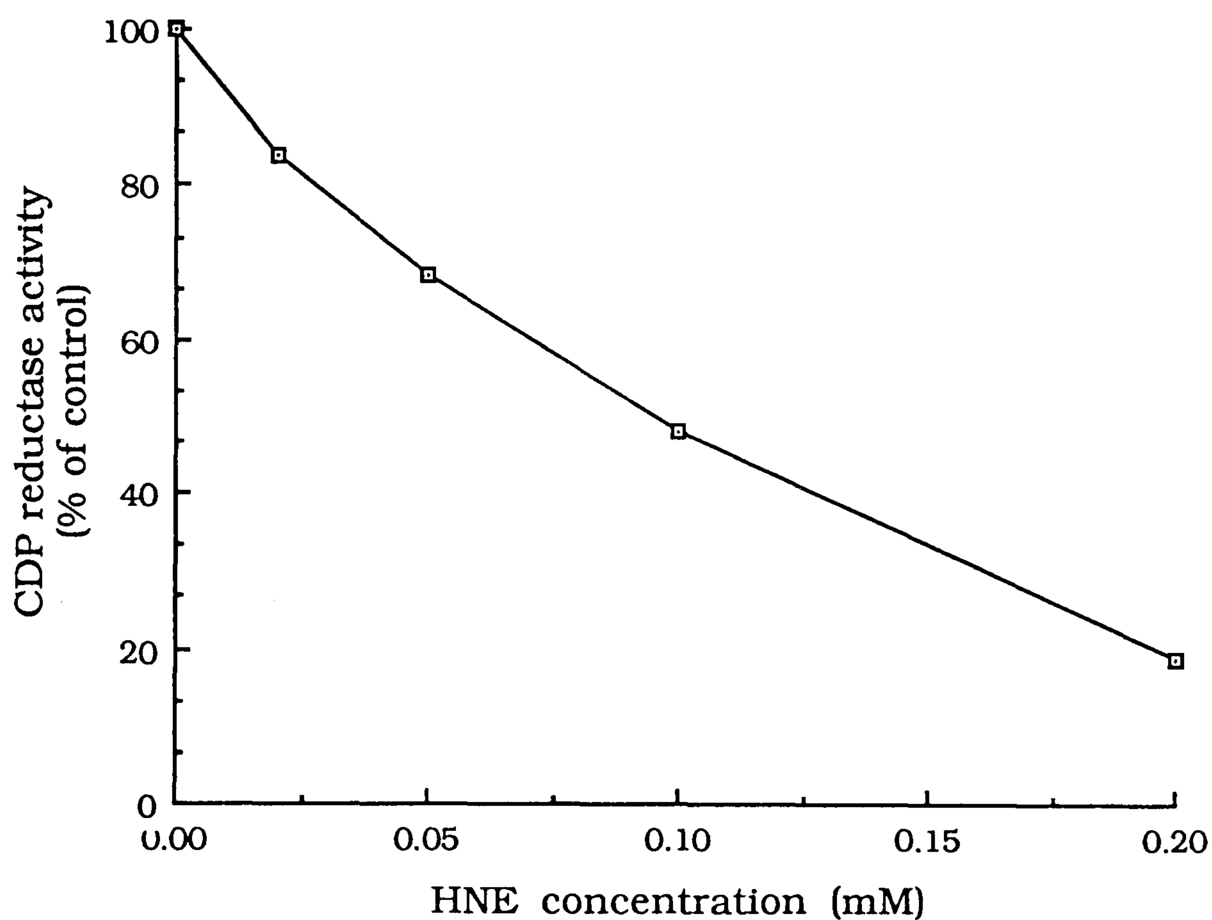


Fig. 3.3.1. Inhibition of CDP reductase by 4-hydroxynonenal (HNE)

The CDP reductase activity was measured in the presence of HNE. The reaction conditions were as described in "Materials and Methods" except the DTT concentration was 1 mM. The activity of control was 204.9 units/mg prot. All assays were carried out in duplicate.

Further investigation showed that the inhibition was related to the incubation time. Fig. 3.3.2 shows the extent of inhibition as a function of the incubation time. Over 60 min. the degree of inhibition increased. The percentage of inhibition was 30.8% at 15 min., 40.7% at 30 min., 48% at 45 min. and 51% at 60 min.

The inhibitory effect of HNE on the enzyme was enhanced by preincubation of the reductase with HNE before the enzymic reaction started (Table 3.3.1). When 0.1mM HNE was present during a 10 min. preincubation (resulting in a final concentration of 0.0174 mM during assay), the enzyme activity was inhibited by 60%. Without preincubation, 0.1 mM HNE (final concentration during assay) reduced the activity by 50% and 0.017 mM HNE (final concentration during assay) inhibited the activity by 15%. When the enzyme was preincubated with 0.575 mM HNE at 30°C for 30 min, there was no measurable activity left. In this case the final concentration of HNE during assay was 0.1 mM. However, the preincubation enhancement of inhibition was not affected significantly by the time of preincubation above 10 min. (Table 3.3.1).

In an attempt to determine if the HNE inhibition was reversible, the enzyme was exposed to HNE at 0°C for 1 hour, then excess HNE was removed using a Sephadex-G25 column. Afterwards the activity of this "treated" enzyme was tested. Two treatments were carried out for this purpose.

(i) the original enzyme preparation containing 2 mM DTT and 5 mM $MgCl_2$ in 0.1M Tris/HCl (pH 7.4) was incubated with HNE (final concentration was 0.1 mM) on ice for 1h. The incubation mixture contained 10 mg protein in a total volume of 400 µl (equivalent 25 mg protein/ml). After removal of HNE, the CDP reductase activity was determined. DTT concentration during

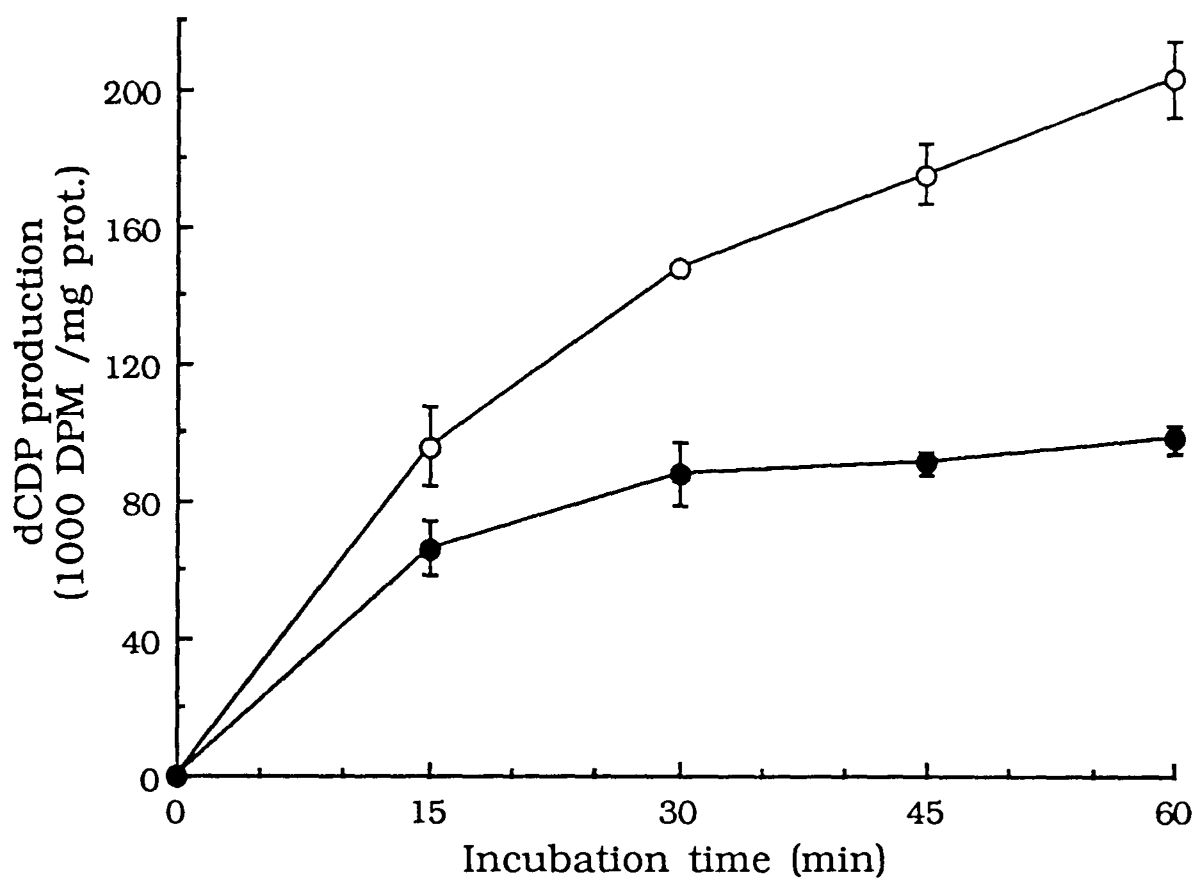


Fig. 3.3.2. The effect of incubation time on inhibition of CDP reductase by HNE (0.1mM).

The CDP reductase activity was determined as describeed in "Materials and Methods" except the DTT concentration was 1 mM. Mean values are given ± S.D. (o) Control; (●) +0.1 mM HNE.

Table 3.3.1 Effect of preincubation of enzyme protein with HNE on the degree of inhibition of CDP reductase activity

	CDP reductase activity (units/mg prot.)				
Preincubation time (min)	Control	0.1mM HNE in preincubation(0.017mM in assay)	Inhibition (%)	0.575 mM HNE in preincubation(0.1mM in assay)	Inhibition (%)
0	204.9±11.0		15*	99.0±4.2	50
10	271.5±1.7	92±0.3	66		
20	238.3±8.6	86±3.2	63		
30	174.9±5.9	71±0.9	60	2.7±0.5	98

The enzyme protein was incubated with HNE at 30°C. At the time indicated aliquots of this mixture were added to the CDP reduction mixture to initiate the regular activity assay under standard reaction conditions except the DTT concentration was 1 mM. Control incubations were carried out in the presence of H_2O during the preincubation. The assays were carried out in triplicate.

* The datum was from Fig. 3.3.1.

assay was 1 mM. The reaction time was 60 min.

(ii) DTT was removed from enzyme preparation firstly by using a Sephadex-G25 column. Then the DTT-free enzyme preparation was incubated with 0.25, 0.57 and 1.0 mM HNE (final concentration) on ice for 1h. The incubation mixture contained 3.8 mg protein in total volume of 235-267 μl (equivalent 16.1-14.2 mg protein/ml). After removal of HNE, the CDP reductase activity was measured under the condition of 1mM DTT and 15 min reaction time.

The results are shown in Fig. 3.3.3 (for treatment (i)) and Fig. 3.3.4 (for treatment (ii)). As seen in Fig. 3.3.3, the enzyme activity was reduced by 33% after HNE treatment compared to the control sample which was incubated with H_2O. Compared with the enzyme without any incubation, the activity of the control sample (incubated with H_2O) was not changed. The activities were 204 and 190 units/mg protein respectively. Fig. 3.3.4 shows that the extent of inhibition of enzyme activity by HNE was dependent on HNE concentration. CDP reductase activity was inhibited 75% by 1.0mM HNE compared to control. Removal of DTT from the enzyme preparation decreased the enzyme activity even without HNE addition. The activity of the original enzyme preparation (without removal of DTT) was 69.3 units/mg protein, the activity of control sample (removal of DTT and then incubated with H_2O) was 49.7 units/mg protein.

Fig. 3.3.5 shows the separation of enzyme and HNE using the Sephadex-G25 column. Fraction nos. 4-5 were collected for enzyme activity assays. In these fractions there was no excess HNE. Thus the inhibition of the enzyme could not be attributed to an inhibition by free HNE being carried through to the enzymic reaction mixture.

The inhibitory effect of HNE against CDP reductase activity in

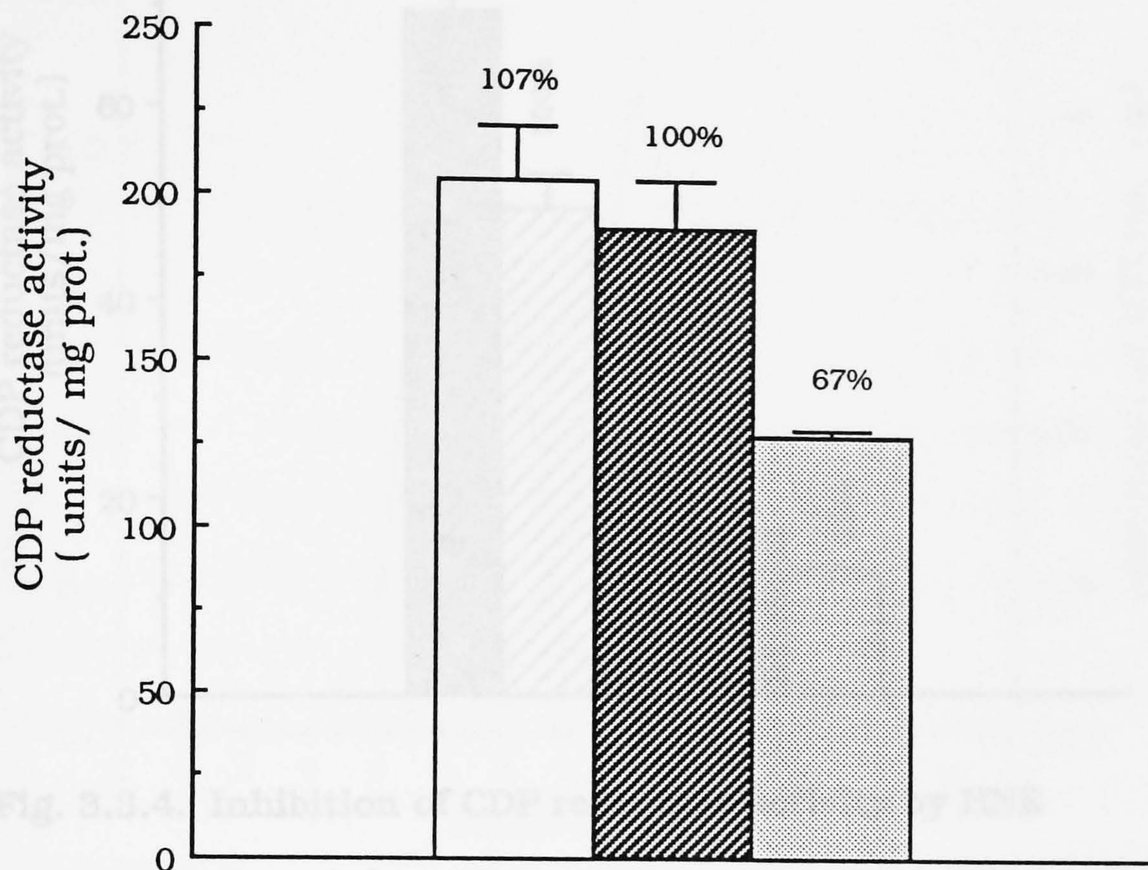


Fig. 3.3.3. Inhibition of CDP reductase activity by HNE

The enzyme protein (10 mg) was incubated at 0°C for 1 hr in the presence or absence (H_2O instead) of 0.1mM HNE (final concentration) in total volume of 400 µl. Then the mixture was passed down a Sephadex G-25 column (21x1 cm) using 0.1M Tris-HCl (pH7.4) containing 2 mM DTT and 5 mM $MgCl_2$ as an eluant at 4°C. In the peak fraction of protein eluting from the column, the CDP reductase activity was determined as described in "Material and Methods" except the DTT concentration was 1mM. (▨): control, the enzyme was incubated with H_2O; (░): the enzyme was incubated with 0.1mM HNE; (□): the enzyme without any incubation. The number in the figure present the activity left compared to control.

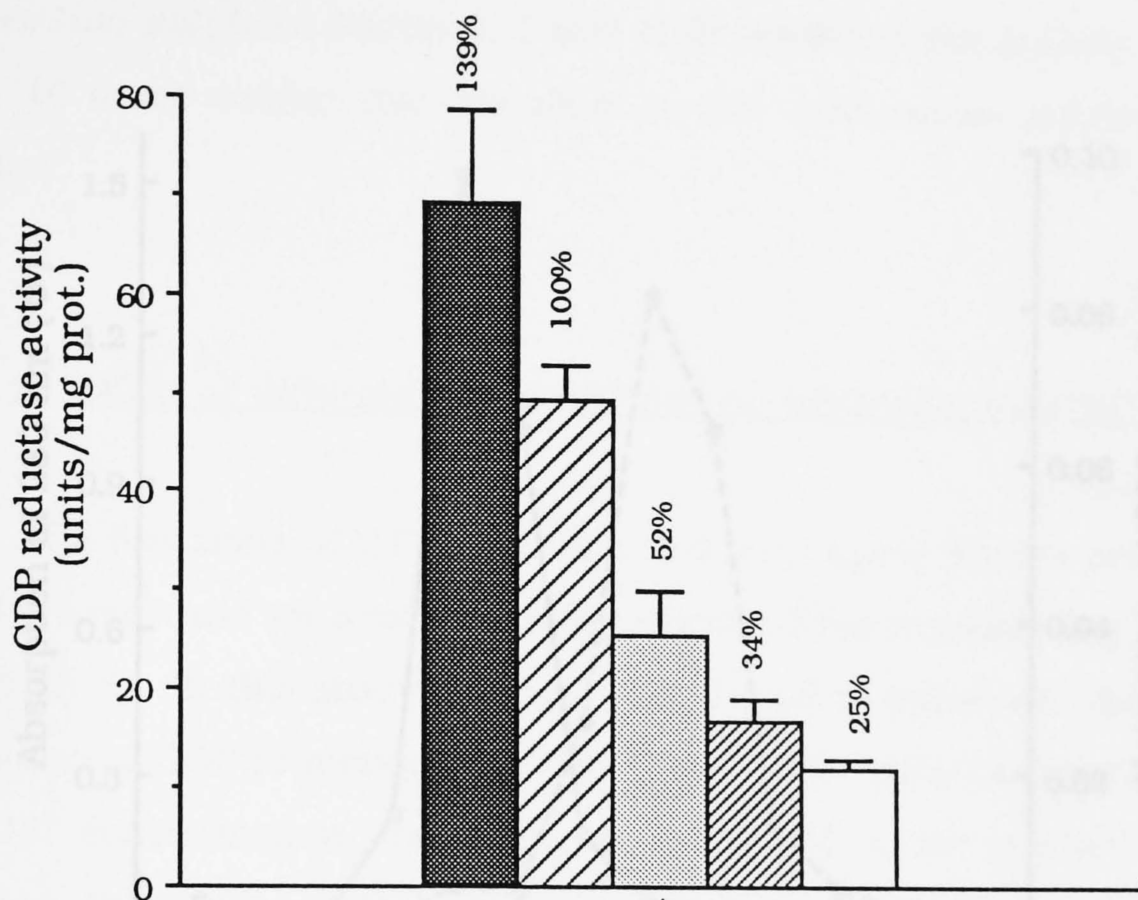


Fig. 3.3.4. Inhibition of CDP reductase activity by HNE

DTT was removed from the enzyme preparation previously by using a Sephadex G-25 column. The protein (3.8 mg) of DTT-free enzyme preparation was incubated at 0°C for 1h with different concentrations of HNE in total volume of 235-267 µl. Then the mixture was passed down another Sephadex G-25 column for removal of HNE. The CDP reductase activity was determined under the condition of 1mM DTT in CDP reduction mixture and 15 min. reaction time. The number in the figure present the activity left compared with control. (▩) without removal of DTT; (▨) control (incubated with H_2O); (▒) incubated with 0.25 mM HNE; (▧) incubated with 0.47mM HNE; (□) incubated with 1.0 mM HNE.

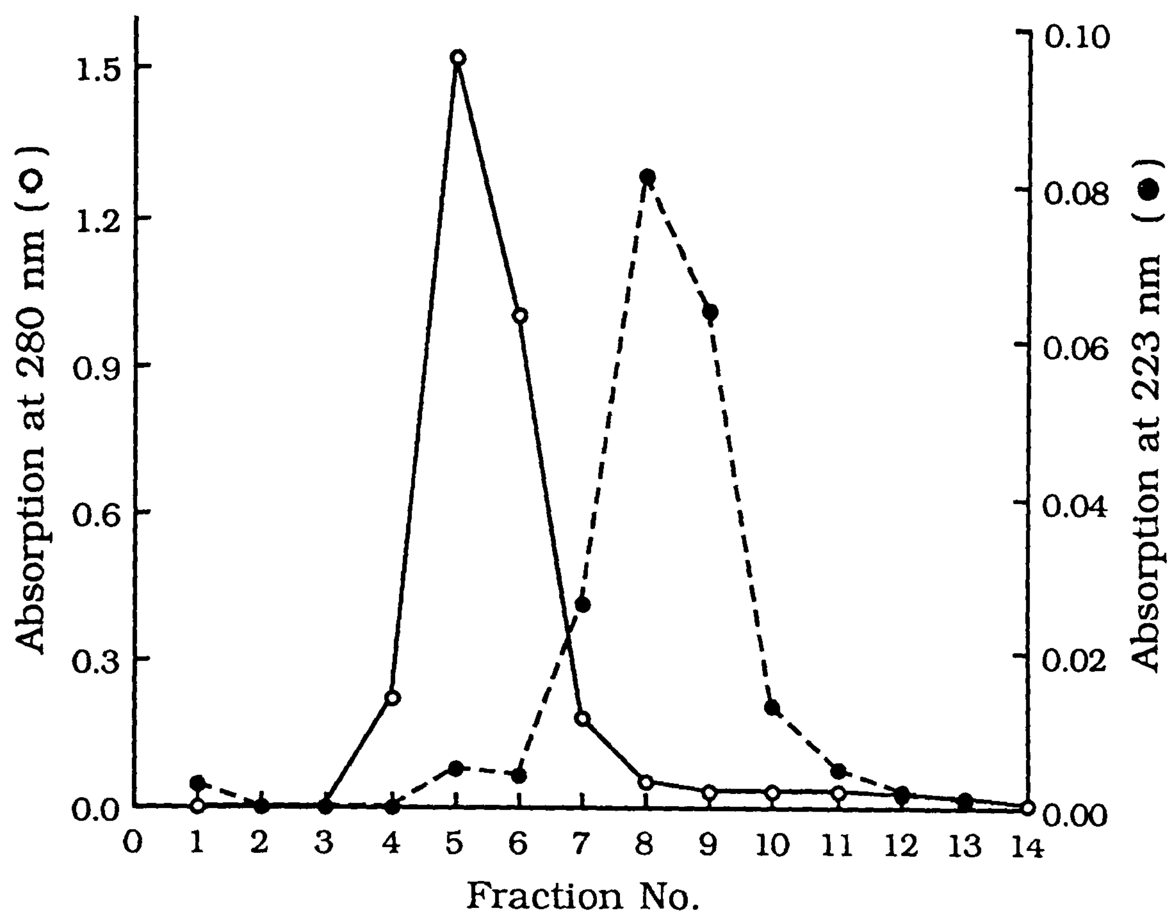


Fig. 3.3.5. Chromatography of separation of enzyme and HNE on Sephadex G-25 column (21x1 cm).

(—o—) 200 μl enzyme (11.8mg protein) + 200μl H_2O; (-●-) 200 μl 0.2mM HNE + 200μl H_2O. The mixtures were passed down the Sephadex G-25 columns (21x1 cm) using 0.1 M Tris/HCl as an eluant. The eluted fractions (1 ml) were collected and 2ml 0.02MTris-HCl was added. The absorbance of this mixture was checked at 280 nm (o) and 223nm (●).

the crude extract of enzyme was also studied. The results are shown in Fig. 3.3.6. HNE had a very weak inhibitory effect on the crude extract of enzyme compared to partially purified enzyme preparation (45% ammonium sulphate fraction). 1 mM HNE inhibited the activity by 50%, 10 times weaker than its effect on the ammonium sulphate fraction.

3. 3. 2 Effect of dithiothreitol (DTT) on the inhibition by HNE

The functions of DTT were as a reducing agent for the reductase activity assay and an activator of the enzyme from regenerating liver (see 3. 2. 3). In this study DTT was also found to influence the inhibition of CDP reduction by HNE. Table 3.3.2 indicated that when the DTT concentration was increased to 15 mM in the reaction solution, the enzyme activity was only inhibited about 24% by 0.2 mM HNE and no significant inhibition was observed by 0.05 mM HNE. However when the DTT concentration was 1 mM, the enzyme activity was inhibited by 30% and 80% at 0.05 mM and 0.2 mM HNE, respectively. To ensure that this decrease of inhibition was due to the direct effect of DTT, and not because of the change of basal reductase activity when a high concentration of DTT was present during assay, the enzyme activities at different concentrations of DTT were studied. The results are shown in Fig. 3.2.6. Using a concentration range of 1 mM to 15 mM DTT, there was no significant difference in the reduction activity.

If HNE was incubated with DTT before exposure to the enzyme, HNE did not inhibit the activity of the enzyme (Table 3.3.3). It can be seen that without preincubation with DTT (preincubation with H_2O

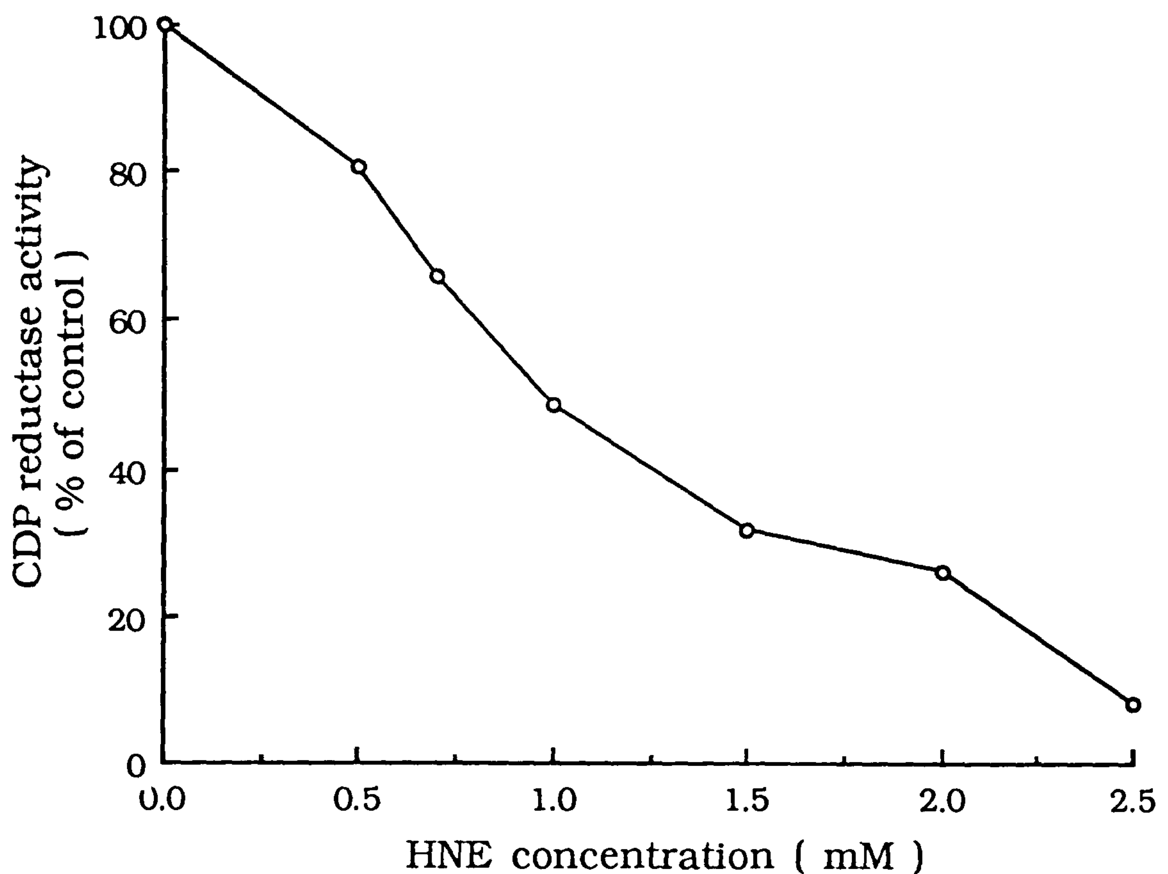


Fig. 3.3.6. Inhibitory effect of HNE on the activity of crude extract of ribonucleotide reductase from rat liver

In the absence or presence of HNE, the CDP reductase activity of crude enzyme extract was measured under standard reaction conditions as described in "Materials and Methods". The activity of the control was 22.8 units/mg protein. Each assay was carried out in at least duplicate.

Table. 3. 3. 2 HNE effect on RNR activity in the presence of a high concentration of DTT

	Concentration of DTT			
	1mM		15mM	
Treatment	Activity (units/mg prot.)	inhibition (%)	Activity (units/mg prot.)	inhibition (%)
Control	204.9±11.0		283.9±5.8	
+HNE				
0.05 mM	140.3±15.0	31.5	274.1±0.1	3.5
0.2 mM	38.7±7.0	81.1	213.9±2.4	24.7

In the presence or absence of HNE the activity of enzyme was measured under standard reaction conditions as described in " Material and Methods ". The concentration of DTT during the reaction was 1 mM or 15 mM as indicated. Mean values are given ± S. D.

Table 3.3.3 Effect of preincubation of HNE with DTT on the inhibition of HNE

Treatment	CDP reductase activity (units/mg protein)
- HNE	205.1±19.4
+0.1 mM HNE (f/c)	
without preincubation with DTT	99.0±4.2
preincubated with DTT	
0.287mM/2.87mM (HNE/DTT)	186.1±25.8
0.575mM/5.75mM (HNE/DTT)	179.7±17.9

HNE was preincubated with DTT (0.287 mM HNE with 2.87mM DTT in one experiment and 0.575 mM HNE with 5.75 mM DTT in another experiment) at 30°C for 30 min. and then the aliquots of this mixture were added to the CDP reduction mixture and the enzyme protein to determine the activity of the enzyme. The final concentration of HNE and DTT in this assay mixture were 0.1 mM and 1 mM respectively. Mean values are given ± S.D.

instead) 0.1 mM (final concentration) HNE inhibited the enzyme activity by about 50%. However, after incubation with DTT at ratio of 1:10 for 30 min at 30°C (0.287 mM HNE with 2.87 mM DTT in one experiment and 0.575 mM HNE with 5.75 mM DTT in another experiments), HNE did not significantly inhibit the activity. If HNE was incubated with enzyme protein in the presence of additional DTT, HNE failed to inhibit the enzyme activity (Fig. 3.3.7). In this experiment the enzyme protein was incubated with 0.1 mM HNE (final concentration) and 2 mM additional DTT together at 0°C for 1h. Then the excess HNE and DTT were removed by using Sephadex-G25 column and the CDP reductase activity was checked. It can be seen in Fig. 3.3.7 that if HNE was incubated with enzyme protein only 0.1 mM HNE produced 33% inhibition; but if HNE was incubated with enzyme protein in the presence of 2 mM DTT, HNE did not damage the enzyme.

In order to study if a high concentration of DTT could reactivate the enzyme activity after the enzyme was damaged by HNE, two treatments were carried out as outlined below,

(i) DTT was removed from the enzyme preparation by using a Sephadex-G25 column. Then the protein of this DTT-free enzyme preparation was incubated with 1 mM HNE at 0°C for 1h. After removal of HNE, the enzyme protein was incubated with 5.4 mM DTT (final concentration) at 30°C for 0.5, 1 and 2h. Afterwards the CDP reductase activity was checked. The DTT concentration in the activity assay was 1 mM and the reaction time was 15 min.

(ii) The original enzyme preparation (without removal of DTT) was incubated with 0.1 mM HNE (final concentration) at 0°C for 1h. CDP reductase activity of this treated enzyme was

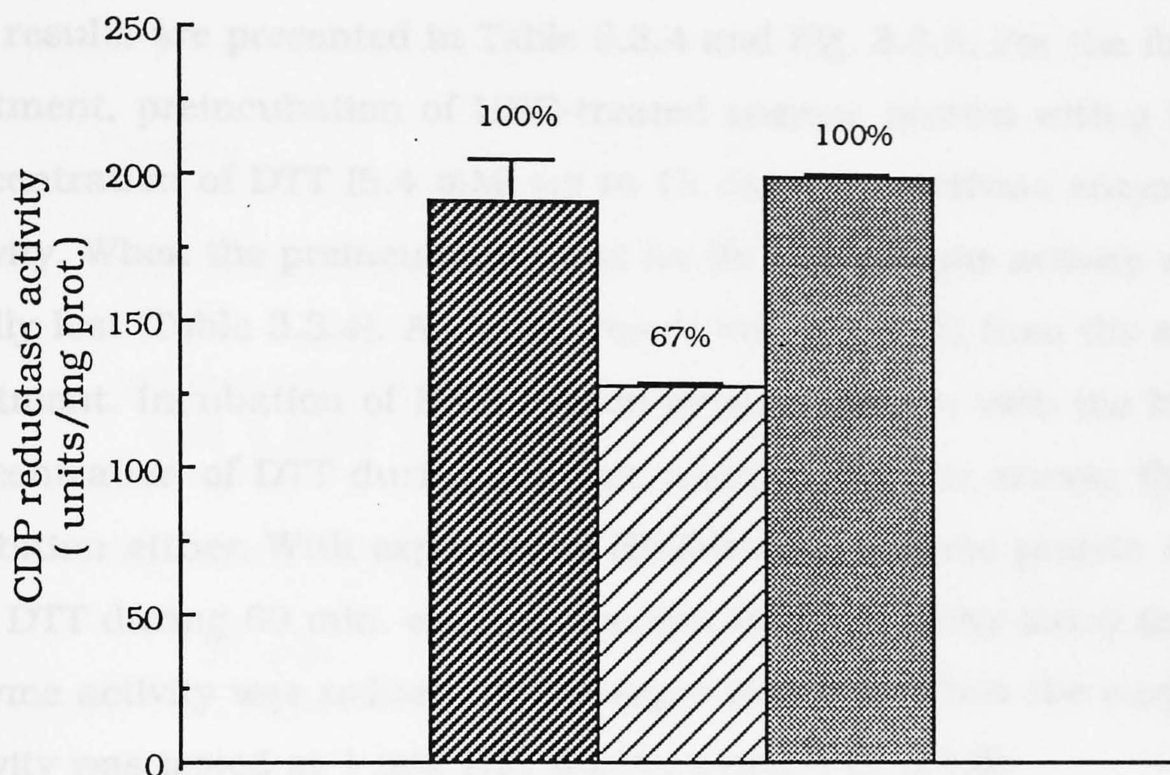


Fig. 3.3.7. Effect of preincubation of HNE with DTT and enzyme protein together on the inhibition of HNE

The protein (10 mg) of original enzyme preparation was incubated at 0°C for 1h with (1) H_2O, as control (▨); (2) 0.1 mM HNE (▨) and (3) 0.1 mM HNE and 2 mM additional DTT (▩) in a total volume of 400 µl. Then the mixture was passed down a Sephadex G-25 columns for removal of HNE and additional DTT. CDP reductase activity was determined under standard conditions except that the DTT concentration was 1 mM. The number in the figure represents the activity left compared to control.

determined following the removal of excess HNE using a Sephadex-G25 column. The concentration of DTT in the activity assay were 1 mM for control and half the HNE-treated samples and 10 mM for the other half of the HNE-treated samples. The assay reaction time was 60 min.

The results are presented in Table 3.3.4 and Fig. 3.3.8. For the first treatment, preincubation of HNE-treated enzyme protein with a higher concentration of DTT (5.4 mM) up to 1h did not reactivate enzyme activity. When the preincubation was for 2h, the enzyme activity was totally lost (Table 3.3.4). A similar result was observed from the second treatment. Incubation of HNE-treated enzyme protein with the higher concentration of DTT during enzymic reaction did not reverse the inhibition either. With exposure of HNE-treated enzyme protein to 10 mM DTT during 60 min. enzyme reaction for the activity assay the enzyme activity was reduced to the same extent as when the enzyme activity was tested at 1 mM DTT during assay (Fig. 3.3.8).

The mechanism of HNE effect on the enzyme activity was also investigated. The results in Fig 3.3.9 shows that after incubation with HNE, the SH content of the enzyme preparation was decreased. The decrease was dependent on the HNE concentration. However incubation time above 5 min did not affect this decrease.

It seems that DTT had a modulating effect on HNE inhibition and afforded protection of the enzyme against HNE. Fig. 3.3.10-12 show that these two compounds can interact. DTT influenced the spectrum of HNE, such that there was decreased absorbance at 223nm(Fig 3.3.10). At the same time HNE decreased the reactive SH content of DTT. The higher the concentration of HNE and the longer the time of incubation, the more the decrease of SH content (Figs 3.3.11,12).

Table 3.3.4 Effect of DTT on CDP reductase activity of HNE-treated enzyme

Treatment	CDP reductase activity (units/mg prot.)	% of control
Control	49.7 ± 3.4	100
1mM HNE treatment		
without incubation with 5.4mM DTT	12.0±1.0	24
incubated with DTT		
30 min.	11.3±1.1	22
60 min.	12.2±1.2	24
120 min.	undetectable	0

DTT was removed from the enzyme preparation first using a Sephadex G-25 column. The protein of the DTT-free enzyme preparation (3.8 mg protein) was incubated with 1 mM HNE at 0°C for 1h in a total volume of 267 μl. Then this incubation mixture was passed down another Sephadex G-25 column for removal of HNE. The HNE-free enzyme protein solution was then incubated with 5.4 mM DTT. At the time indicated aliquots of this incubation mixture were added to the CDP reduction mixture to initiate the regular activity assay. The DTT concentration in the assay was 1 mM. The reaction time was 15 min.

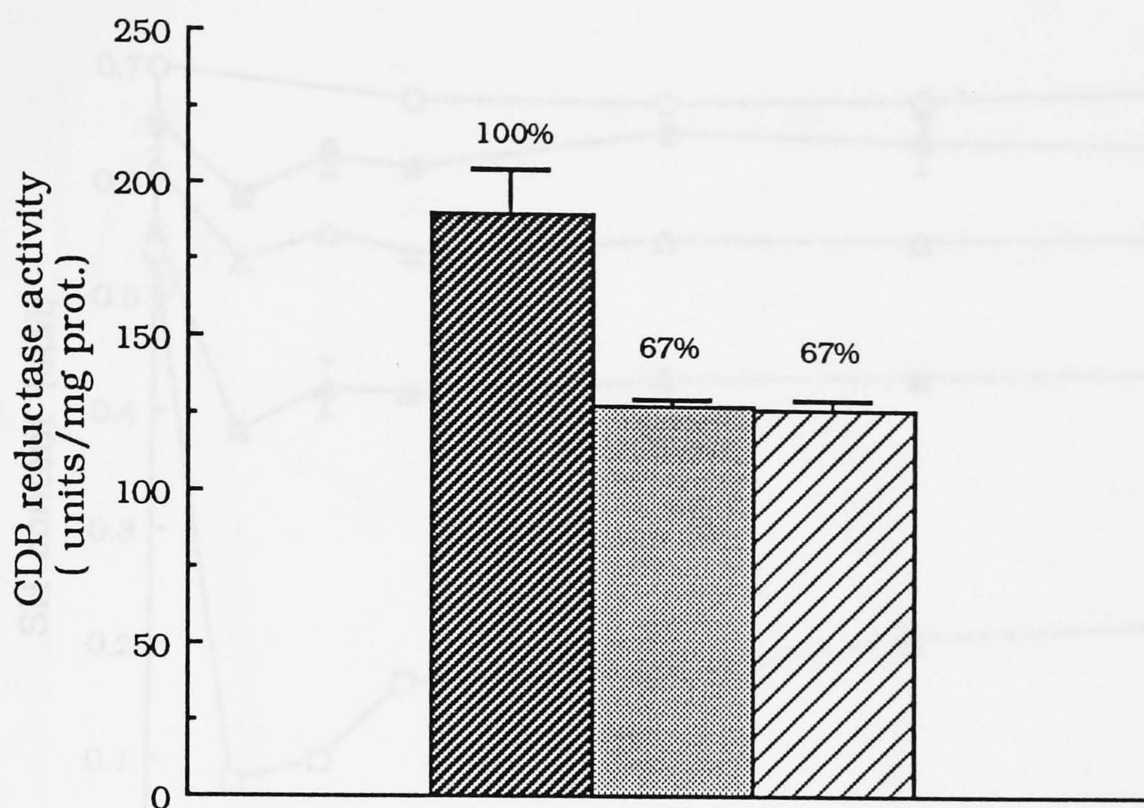


Fig. 3.3.8. Effect of higher concentration of DTT on CDP reductase activity of HNE-treated enzyme

The enzyme protein (10 mg) was incubated at 0°C for 1h in the presence or absence (H_2O instead) of 0.1 mM HNE in total volume of 400 μl. Then the mixture was passed down a Sephadex G-25 column for removal of HNE and CDP reductase activity was determined. (▨) control, the enzyme protein was incubated with H_2O. DTT concentration in assay was 1mM; (▒) the enzyme protein was incubated with 0.1 mM HNE. Then DTT concentration in assay was 1mM; (▨) the enzyme protein was incubated with 0.1 mM HNE. DTT concentration in assay was 10 mM. The numbers in the figure present the enzyme activity left compared to control.

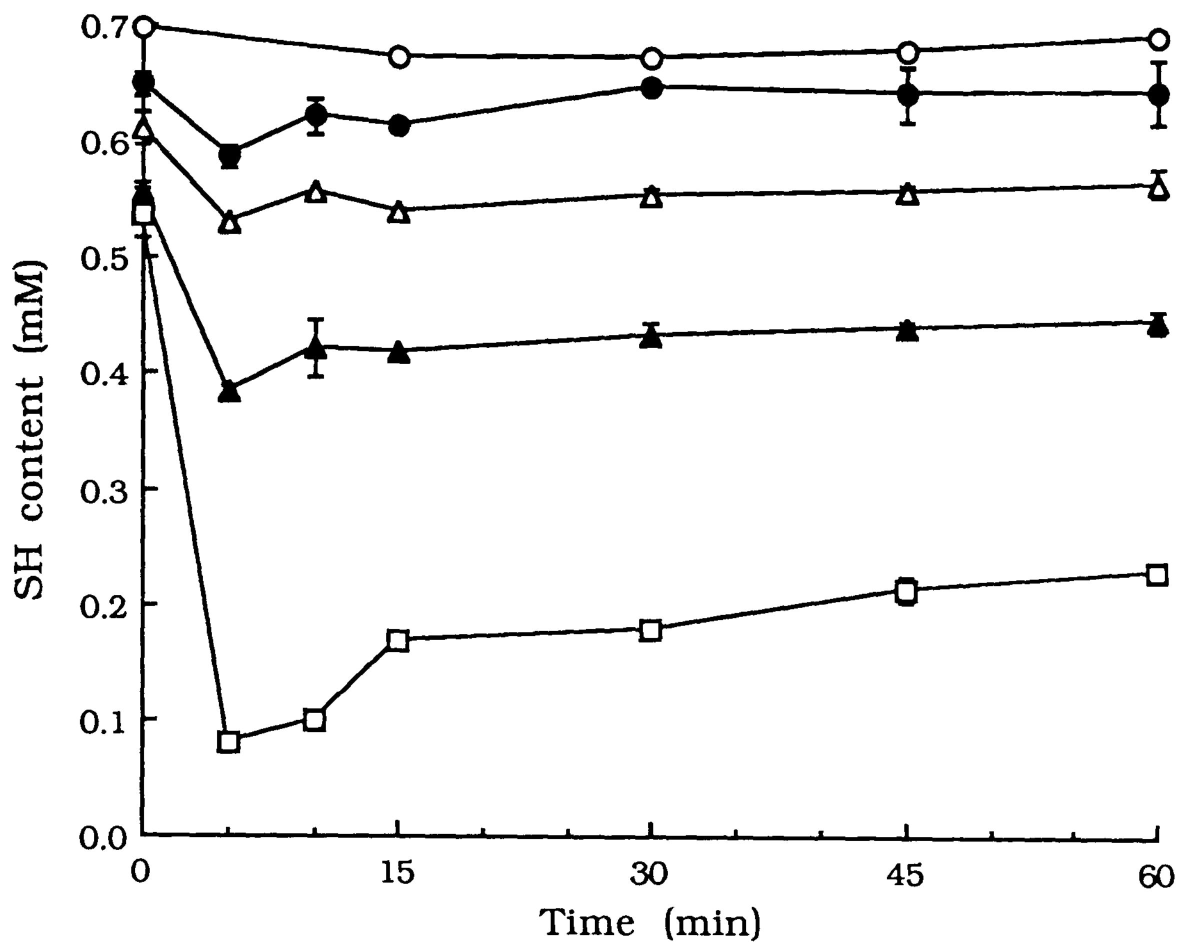


Fig. 3.3.9. Effect of incubation with HNE for various times on the SH content crude extract of the enzyme.

The enzyme was extracted with 0.1M Tris-HCl(pH7.4) containing only 0.25M sucrose. After centrifugation at 100,000 g at 4°C for 30 min, 200 µl supernatant (1.68 mg protein) was incubated with 200 µl HNE or H_2O (as control) at 30°C. The SH content of enzyme was checked as described in "Materials and Methods". Mean values are given ± S.D. (o) control; (●) 0.05 mM HNE; (▵) 0.1 mM HNE; (▲) 0.2 mM HNE; (□) 0.5 mM HNE.

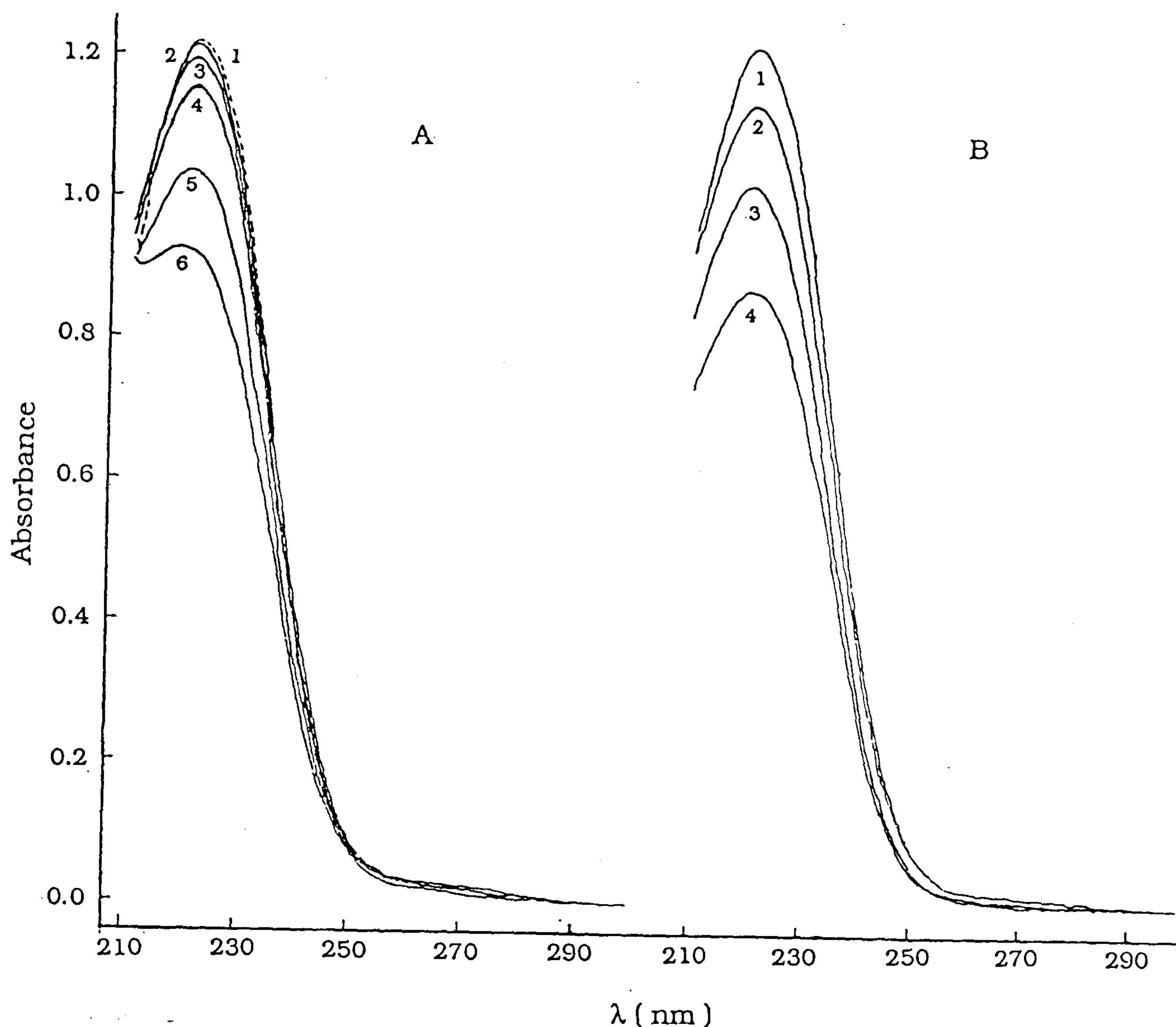


Fig. 3.3.10. Reaction of HNE with DTT in Tris/HCl (36.5 mM pH 7.4)

(A) The spectrum of 0.1 mM HNE after at 30°C for 10 min. in presence of different concentration of DTT. (1) Control: 0.1 mM HNE in Tris buffer; (2) 0.1 mM HNE in Tris buffer at 30°C for 10 min.; (3) + 0.02 mM DTT; (4) + 0.04 mM DTT; (5) + 0.1 mM DTT; 6) + 0.2 mM DTT.
(B) The spectrum of 0.1 mM HNE in presence of 0.04 mM DTT after at 30°C for different time (1) 0; (2) 10 min.; (3) 30 min.; (4) 60 min.

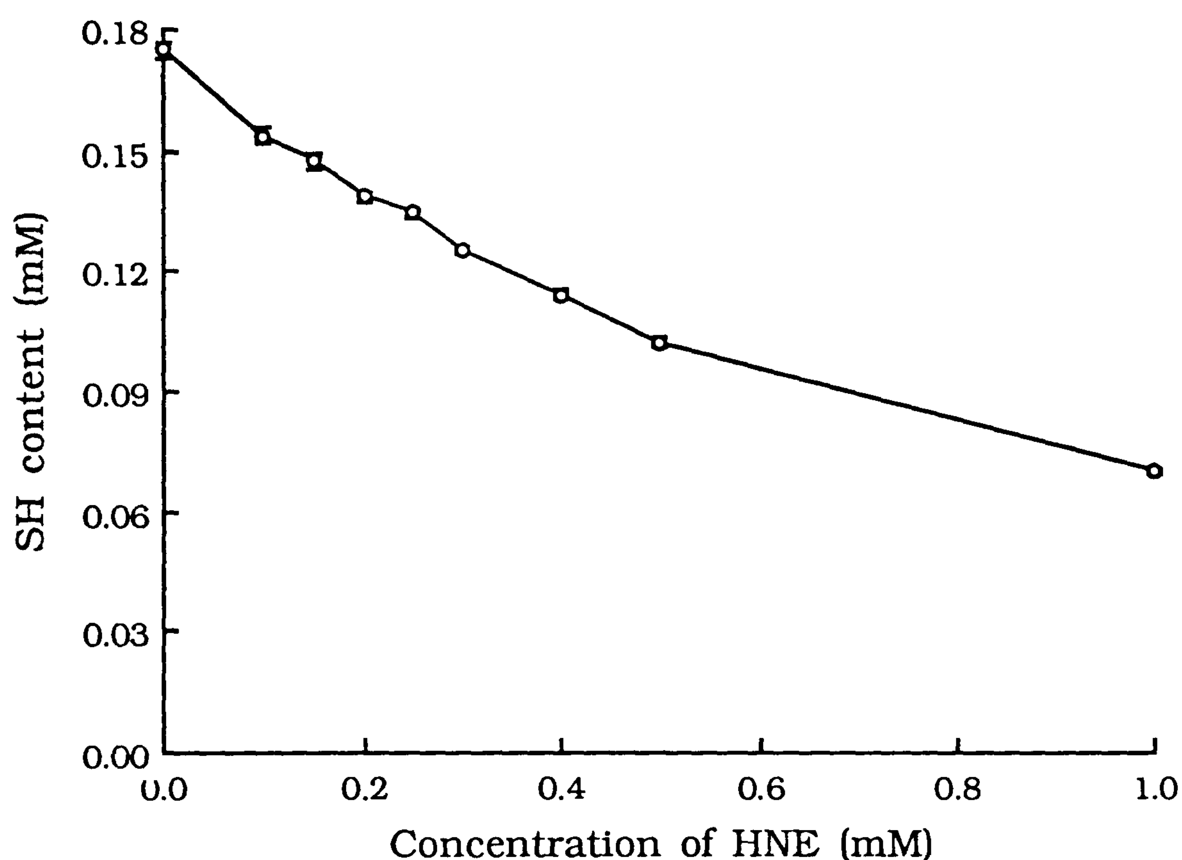


Fig. 3. 3.11. The effect of HNE on the SH content of DTT

0.1 mM DTT was incubated with HNE in 36.5 mM Tris/HCl (pH 7.4) in total volume of 1ml at 30°C for 10 min. The SH content of DTT was measured as described in "Materials and Methods" using GSH as standard. Mean values are given ± S.D.

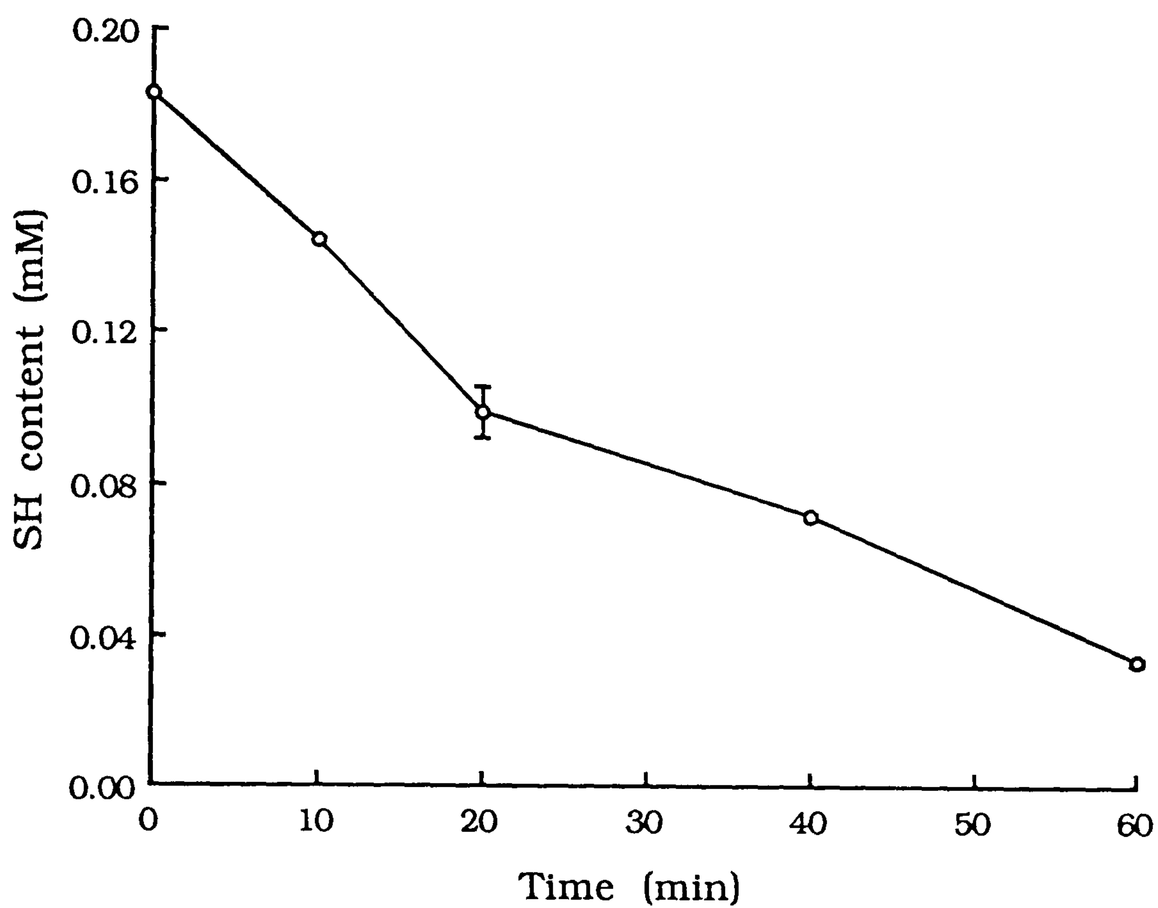


Fig. 3. 3.12. The effect of incubation time of HNE with DTT on the reduction of SH content of DTT

0.1 mM DTT was incubated with 0.5 mM HNE in 36.5 mM Tris/HCl (pH 7.4) in total volume of 1 ml at 30°C. The method of determination of SH content as described in "Materials and Methods". Mean values are given ± S.D.

4. DISCUSSION

4. 1. ACTIVATION OF CCl_4 IN RAT LIVER MICROSOMES

4. 1. 1. Effect of temperature on the activation of CCl_4 in rat liver microsomes

Carbon tetrachloride (CCl_4) can be metabolically activated in liver endoplasmic reticulum to form a reactive intermediate, that was first proposed to be the trichloromethyl free radical ($CCl_3\cdot$) by Slater (1966). A number of reactions including covalent binding of $CCl_3\cdot$ to microsomal macromolecules and CCl_4-induced lipid peroxidation are able to show indirectly the activation of CCl_4 (Fig. 1.1.2). Additionally, $CCl_3\cdot$ can be spin-trapped with phenyl-t-butylnitrone (PBN) to form the PBN-$CCl_3\cdot$ adduct, which is a relatively stable free radical detectable by ESR (Tomasi, et al., 1980; Albano, et al., 1982). The spin trapping of $CCl_3\cdot$ by PBN, covalent binding of radiolabelled CCl_4 to microsomal protein and CCl_4-dependent MDA formation are all indirect methods for measuring the activation of CCl_4 (Cheeseman et al., 1985).

In the present study the temperature (range from 15-40°C) effect on the activation of CCl_4 by rat liver microsomes was studied. Determinations of PBN-$CCl_3\cdot$ formation, CCl_4 covalent binding to protein and CCl_4-dependent MDA production have been used to follow the course of the activation of CCl_4.

The ESR spectrum obtained from incubating liver microsomes with CCl_4 and PBN was a triplet of doublets (Fig. 3.1.1 a). The spectrum was identical with those of the PBN-$CCl_3\cdot$ adduct detected in liver

microsomes observed by Tomasi et al. (1980) and Albano et al. (1982). Although there was a PBN-$CCl_3\cdot$ adduct-like signals in samples incubated without CCl_4 (Fig. 3.1.1. b), the intensity of signals was much lower than that of the samples with CCl_4. These signals may arise from the background free radicals. These free radicals may be lipid free radicals produced during the incubation of microsomes. These radicals can be trapped by PBN to form the lipid-type radical-PBN adduct (Tomasi et al., 1980; Albano et al., 1982).

The incubation temperature was very important for the formation of PBN-$CCl_3\cdot$ adduct. It can be seen from Fig. 3.1.2 that only when the temperature was ≥ 30°C was the level of formation of PBN adduct stimulated by CCl_4 significantly higher than in the control. This result indicated that PBN-$CCl_3\cdot$ was significantly formed in microsomes only when the temperature was ≥ 30°C. Similarly, the production of MDA was only stimulated above baseline values by CCl_4 when the temperature was ≥ 30°C (Fig. 3.1.6). For the covalent binding of CCl_4 to microsomal protein, temperatures higher than 25°C were required to produce specific ^{14}C-CCl_4-protein (Fig. 3.1.4). Taken together, these results indicate that only when the temperature reaches 25-30°C can the activation of CCl_4 start. Figs. 3.1.2, 3.1.4, 3.1.6 also showed that formation of PBN-$CCl_3\cdot$, specific covalent binding of $^{14}CCl_4$ and production of MDA stimulated by CCl_4 were increased as the incubation temperature was increased up to 37-40°C.

The metabolic activation of CCl_4 in rat liver microsomes occurs through the NADPH-cytochrome P_{450} system (Slater, 1984; Slater et al., 1985; Cheeseman et al., 1985). It is possible that any factor which enhances the ability of this system will increase the activation of CCl_4. This point has been demonstrated by the fact that induction of the

microsomal cytochrome P_{450} activity by drugs enhances the activation of CCl_4 (see Introduction 1.1.1.2). Since NADPH:cytochrome P_{450} reductase and cytochrome P_{450} are both involved in the activation it can be considered that the activation of CCl_4 is an enzymic process. Temperature is an important factor in enzymic reactions, so it is understandable that the activation of CCl_4 is increased as the temperature increases and starts only at a certain temperature.

However, the temperature effect on the activation of CCl_4 is more complicated than this simple explanation. Cytochrome P_{450} and NADPH:cytochrome P_{450} reductase are membrane-bound enzymes. Most investigations of the effect of temperature on membrane-bound enzymes have shown that there is a break in the Arrhenius plot (Peterson et al., 1976). The results presented in this thesis are consistent with this statement. It can be seen from Figs 3.1.3, 3.1.5, 3.1.7 that breaks appeared in the Arrhenius plots for the activation of CCl_4. Previous studies have reported the P_{450}-system-dependent metabolism of drugs, like stearic acid nitroxide (Stier and Sackmann, 1973), hexobarbital, ethylmorphine (Peterson et al., 1976), benzphetamine, aminopyrine and ethanol (Puntarulo and Cederbaum, 1989) were dependent on temperature and had breaks in the Arrhenius plots. The break in Arrhenius plot has been assumed to be associated with a phase transition of the lipids in the membrane. In rat liver microsomes the number of P_{450} molecules is about 20-30 times greater than that of the NADPH:cytochrome P_{450} reductase (Depierre and Dallner, 1975). It has been suggested that the whole enzyme system is enclosed by a rather rigid phospholipid halo which undergoes a crystalline-liquid phase transition (Stier and Sackmann, 1973). Cytochrome P_{450} molecules are clustered around the reductase which is envisioned to randomly reduce the cytochrome P_{450} molecules immediately adjacent to it (Peterson et

al., 1976). If P_{450} molecules are not directly associated with the reductase, a translational motion of the reductase or P_{450} molecules through the microsomal membrane is required. In this case the Arrhenius plot will exhibit a break. Below the transition temperature the membrane is presumably in the gel state, the molecule of drug has to penetrate a rigid barrier to reach the reductase and a high activation energy will be needed for the activation. Above the transition temperature, the membrane is in a rather fluid state, therefore the activation energy will decrease (Stier and Sackmann, 1973). Stier and Sackmann reported that transition temperature of the rabbit liver microsomal membrane was 32°C. The transition temperature for aniline and aminopyrine oxidation was 34-36°C and 27-29°C respectively (Stier and Sackmann, 1973), and for P_{450}-dependent ethanol metabolism in liver microsomes it was about 20.3°C (Puntarulo and Cederbaum, 1989). The results in the present study showed the transition temperature was about 26.7-27.5°C or 34.3°C (Fig. 3.1.3; 3.1.5; 3.1.7). Below the transition temperature the energies for PBN-$CCl_3\cdot$ adduct formation, specific $^{14}CCl_4$ binding to protein and CCl_4-dependent MDA production were 6.8 kcal/mol, 11.83 kcal/mol and 9.8 kcal/mol respectively. Above the transition temperature the energies were changed to 1.66 kcal/mol, 3.36 kcal/mol and 2.83 kcal/mol respectively.

Using these different methods to measure the activation of CCl_4 obtained different transition temperatures, it was apparent that the data were very close when PBN-$CCl_3\cdot$ adduct formation and $^{14}CCl_4$ covalent binding to protein were employed as the indices, giving values of 27.5°C and 26.7°C respectively. However, the apparent transition temperature was much higher when CCl_4 metabolism was measured by lipid peroxidation, with a value of 34.3°C being obtained. This phenomenon was consistent with the conclusion that misleading results can be

obtained using only one parameter of CCl_4 metabolism, especially if that parameter is CCl_4-dependent lipid peroxidation (Cheeseman 1982 a, b). Spin trapping of $CCl_3\cdot$, the covalent binding of radiolabelled CCl_4 to microsomal macromolecules and CCl_4-induced lipid peroxidation are all indirect indices of CCl_4 metabolism. Cheeseman (1982 a, b) suggested that the measurement of covalent binding was a better index being more direct than CCl_4-induced lipid peroxidation assay. Using these two methods Cheeseman studied the differences between CCl_4 metabolism and P_{450}-dependent drug metabolism as exemplified by aminopyrene demethylation in response to inhibitors. He found that a better correlation existed between aminopyrene metabolism and $^{14}CCl_4$ binding than with aminopyrene metabolism and CCl_4-induced MDA production.

There are two processes from $CCl_3\cdot$ production to PBN-$CCl_3\cdot$ adduct formation and CCl_4 covalent binding. However there are three processes from $CCl_3\cdot$ production to CCl_4-induced lipid peroxidation (see Fig. 1.1.2) and even more processes to MDA production. Temperature will affect all of these processes. Therefore, the apparent effect of the temperature on CCl_4 activation measured by these three methods is the sum results of the influence of the temperature on these processes together. If there are less indirect processes, the result of the influence of the temperature will be closer to the net effect of temperature on CCl_4 activation. From this point it was suggested that PBN spin trapping of $CCl_3\cdot$ and the covalent binding are the better parameters than CCl_4-induced MDA production. This can explain why the results of the transition temperature were very close when CCl_4 metabolism was measured by PBN-$CCl_3\cdot$ formation and $^{14}CCl_4$ covalent binding whilst the

datum of the transition temperature obtained by CCl_4-dependent MDA assay was very different to the previous two.

In this study the activation of CCl_4 was initiated *in vitro* by using the artificial lipid peroxidation model in which the NADPH was generated from reaction of glucose-6-phosphate with $NADP^+$. The effect of temperature on NADPH generation was also investigated. It can be see from Fig. 3.1.8 that higher temperature enhanced the generation of NADPH.

The activation of CCl_4 in microsomes is found to be absolutely dependent on the addition of NADPH (Slater and Sawyer 1971a; 1977; Kornbrust and Mavis, 1980). Tortoriello et al. (1991) reported that a high concentration of NADPH (300-500 μM) was required for activation of CCl_4 at maximum rate in liver microsomes. An NADPH generator was provided for optimal CCl_4 metabolism. The presence of an NADPH generator in the reaction system increased the rate and extent of CCl_4 metabolism by two-fold compared to the reaction without a NADPH generator system. The NADPH generator can maintain more NADPH in its reduced form and therefore preserves a higher concentration of NADPH for cytochrome P_{450} catalyzed metabolism (Tortoriello et al., 1991).

Therefore, an enhancement of the activity of the electron-transport chain through the changing of membrane lipid phase and an increase in the amount of electron donor (NADPH) may both contribute to the stimulation of the activation of CCl_4 by increasing temperature.

It was observed that the formation of PBN-adduct decreased after the temperature reached 37°C (Fig. 3.1.2) under conditions of both with and without CCl_4. This was probably due to the PBN-spin adduct being unstable at higher temperatures. It has been reported that the PBN-$CCl_3 \cdot$ spectrum changes markedly during warming of the sample from

-196°C to room temperature. Unstable species appear during the warming up (Tomasi, et al., 1980). The instability of PBN-adduct at the high temperature will lead to the underestimation of the activation of CCl_4. Increase in the temperature will increase the rate of CCl_4 activation but also decrease the stability of the PBN-adduct. Therefore, there is a limit to the use of PBN-adduct formation as an index of CCl_4 activation.

4. 1. 2 Effect of selected agents on the activation of CCl_4 in rat liver microsomes

Several agents have been chosen for investigation of their effect on the activation of CCl_4 as evidenced by determination of lipid peroxidation which was measured by MDA production. Two inhibitors of cytochrome P_{450} (pyrazole and imidazole), two sulphydryl reagents, (iodoacetamide and N-ethyl maleimide) and glutathione have been used for this purpose.

This was a continuation and completion of the studies initiated by Slater and Sawyer (1971 a, b, c) and continued by Cheeseman (1982 a, b). In those studies CCl_4-induced lipid peroxidation and $^{14}CCl_4$ covalent binding to microsomal protein were taken as the indices of CCl_4 activation and used to compared with typical mixed-function oxidase (MFO) activity in terms of the effects produced by inhibitors of MFO. The typical MFO activity was exemplified by aminopyrene demethylation. The type of inhibitors used in those studies were classic P_{450} inhibitors, SH reagents, metal-chelating agents and free radical scavengers. It was found in those studies that there were apparent differences between aminopyrene demethylation and CCl_4 metabolism. For instance, the SH reagent pCMB at 0.1 mM moderately inhibited

$^{14}CCl_4$ binding, strongly stimulated CCl_4-dependent MDA production while aminopyrene demethylation was strongly reduced (Cheeseman, 1982 a, b; Slater and Sawyer, 1971 b). CCl_4 is not a typical MFO substance, therefore, it is expected that CCl_4 metabolism respond to inhibitory agents in a different way from aminopyrene demethylation (Cheeseman et al., 1985).

Pyrazole is classically used as a inhibitor of alcohol dehydrogenase (ADH), the principal enzyme responsible for the oxidation of ethanol in liver (Goldberg and Rydberg, 1969). Imidazole, an isomer of pyrazole, is a inhibitor of ADH too (Boiwe and Branden, 1977). Both of these two agents can inhibit P_{450} enzyme activity (Ingelman-Sundberg and Jornvall 1977) as exemplified by aminopyrene demethylase (Cheeseman, 1982 b) and amine oxidase (Terelius and Ingelman-Sundberg, 1986). Pyrazole and imidazole can induce isoenzymes of liver microsomal cytochrome P_{450} and therefore enhance the CCl_4-stimulated lipid peroxidation (see Introduction) but this property is not relevant to investigations *in vitro.*

Pyrazole and imidazole have been used as protective agents *in vivo.* For instance, administration of pyrazole to rats partially prevents the CCl_4-induced loss of microsomal P_{450} (Toranzo et al., 1975). Pretreatment of rats with pyrazole almost completely abolished the ultrastructural effects of CCl_4 on the liver endoplasmic reticulum at 3h after its administration (Bernacchi et al., 1980). Pyrazole protects the animal or hepatocytes against glutathione depletion, lipid peroxidation and cell damage induced by allyl alcohol (Jaeschke et al., 1987; Silva and O'Brien, 1989). Pretreatment of rats with imidazole inhibit the metabolic activation of N-nitrosopyrrolidine (NPYR) resulting in the reduction of NPYR-derived DNA adduct formation. NPYR is a acute hepatoxic and carcinogenic agent to rat liver (Hunt and Shank, 1991).

The effect of pyrazole and imidazole on activation of CCl_4 *in vitro*

has been studied by Cheeseman (1982 b) ten years ago. He found that at 5 mM, pyrazole and imidazole had modest effects on CCl_4-dependent lipid peroxidation and covalent binding of $^{14}CCl_4$ to microsomal protein, although aminopyrene demethylase was strongly inhibited by these two agents at the same concentration.

Since pyrazole and imidazole have protective roles against damage induced by drug metabolism, and particularly as they have been tested as hepatoprotective agents in the CCl_4 model *in vivo* and they can inhibit P_{450} enzyme activity *in vitro,* their effect on CCl_4-dependent lipid peroxidation in liver microsomes at higher concentrations (10-20 mM) was investigated. The results were shown in Fig. 3.1.9 and Table 1.1.1. It can be seen that at the relatively high concentrations these two agents eventually produced small inhibitory effects on the lipid peroxidation stimulated by CCl_4. At 10 mM pyrazole inhibited CCl_4-dependent MDA formation by 30%. Imidazole showed 29% inhibition at 20 mM. These results indicated that the activation of CCl_4 was inhibited by these two compounds at a relatively higher concentration. The mechanism of the inhibition may be attributed to their ability to inhibit of P_{450} enzyme activity.

Iodoacetamide (IDAM) and N-ethyl maleimide (NEM) are sulphydryl reagents that can interact with SH groups of a protein resulting in alkylation of the protein (Haenen, et al., 1987; Chen and Stevens, 1991). In the present study IDAM and NEM had little effect on CCl_4-dependent lipid peroxidation in liver microsomes. IDAM inhibited MDA production by 15% at 10 mM. No increase in inhibition occurred when the concentration of IDAM was increased to more than 10mM (Fig. 3.1.10). NEM exerted biphasic effects on CCl_4-stimulated MDA formation (Fig. 3.1.11). At 0.5-1.0 mM NEM inhibited MDA production by 20 %, but higher concentrations of NEM stimulated the lipid

peroxidation slightly.

These results were similar to those obtained by Cheeseman (1982 b). He observed that IDAM at 10 mM had little effect on MDA production whereas the covalent binding of $^{14}CCl_4$ was inhibited by 29% and aminopyrene demethylase was inhibited to a similar degree. He also found that at 3 mM NEM aminopyrene demethylase was inhibited by 34% whereas 10 mM NEM was required to inhibit covalent binding to a similar degree. At 10 mM NEM CCl_4-dependent MDA production was enhanced.

The effect on CCl_4 metabolism of other SH reagents, pCMB, mersalyl and dithiobisnitrobenzoate (DTNB) have been studied by Slater and Sawyer (1971 b) and Chesseman (1982 a; b). pCMB at the range of 50-300 μM inhibited $^{14}CCl_4$ binding to the microsomal protein. The effect of pCMB on CCl_4-dependent MDA production showed a biphasic effect but this biphasic pattern was different from NEM. At the lower concentration (100 μM) pCMB stimulated CCl_4-dependent MDA production. At 200 μM pCMB strongly inhibited CCl_4-induced lipid peroxidation. Mersalyl and DTNB showed the similar pattern of effects to that found with pCMB (Cheeseman, 1982 b).

SH reagents are believed to inhibit drug metabolism by blocking electron transfer to cytochrome P_{450} and also by denaturing the cytochrome (Franklin and Estabrook, 1971). Since CCl_4 is activated at cytochrome P_{450} it is understandable that IDAM and NEM inhibit CCl_4-mediated lipid peroxidation. However the biphasic effects of NEM is difficult to explain. This effect seems to demonstrate the problem of using MDA production as an indicator of CCl_4 metabolism since, simultaneous with enhanced MDA production, decreased covalent binding occurs (Cheeseman, 1982 b).

GSH is a very important and an effective inhibitor of lipid

peroxidation. In this study, GSH showed inhibitory effects on lipid peroxidation in a concentration-dependent manner. A 50% inhibition was observed at a concentration of GSH less than 0.2 mM and 1mM GSH nearly completely diminished the MDA production (Fig. 3.1.12). GSH not only inhibited the extent and rate of CCl_4-dependent MDA formation, but also prolonged the initiation period of lipid peroxidation, and this effect was concentration-dependent too. In the absence of GSH a 3 min. lag phase was seen before the lipid peroxidation started (see Fig. 3.1.13). GSH prolonged this lag phase. In the presence of 0.08 mM GSH, the induction time of the lipid peroxidation was prolonged to 5 min. The period of induction of lipid peroxidation was further increased to 7 min. when the concentration of GSH was increased to 0.2 mM.

These results are in agreement with previous work. It has been observed that GSH protect hepatocytes (Poli and Gravela, 1982), liver mitochondria (Yonaha and Tampo, 1987), nuclei (Tirmenstein and Reed, 1989) and microsomes (McCay et al., 1981; Burk, 1982, 1983; Burk et al.,1983; Haenen and Bast, 1983; Nagasaka et al., 1989; Wefers and Sies, 1989; Palamanda and Kehrer, 1992) against lipid peroxidation. GSH delays the onset of lipid peroxidation in liver microsomes induced by ascorbate/ADP/Fe^{3+} , NADPH and CCl_4. It also decreases the rate and extent of CCl_4-induced lipid peroxidation in microsomes (Burk et al., 1983).

It was observed that GSH effect on the onset of lipid peroxidation in liver microsomes was dependent on a heat-labile protein (Burk, 1983). Burk (1983) suggested that the GSH-dependent protection against lipid peroxidation was a result of free radical scavenging. Later it was revealed that the protein factor perhaps was vitamin E reductase and the protection by GSH against microsomal lipid peroxidation through a vitamin E-dependent mechanism (Haenen and Bast, 1983;

Wefers and Sies, 1988; Bast and Haenen, 1990). In this mechanism vitamin E radicals in the membrane produced by scavenging of a free radical by vitamin E are regenerated to vitamin E by a free radical reductase at the expense of GSH (Haenen and Bast, 1983; Bast and Haenen, 1990; Shan et al., 1990).

However, these conclusions come from the results obtained by using the non-enzymic dependent lipid peroxidation system. So far there is no evidence to suggest that the inhibitory effect of GSH on CCl_4-induced lipid peroxidation also depends upon a heat-labile factor. This is because CCl_4-induced lipid peroxidation is P_{450} system-dependent and requires the native microsomes. Cheeseman (1982 b) and Burk et al. (1983) reported that GSH inhibited $^{14}CCl_4$ covalent binding to microsomal protein. These observations are consistent with the suggestion that free radical scavenging as the mechanism of protection of GSH (Burk, 1983) since covalent binding is produced by $CCl_3\cdot$ radical. Based on these observations it could be considered that GSH inhibits lipid peroxidation induced by CCl_4 in microsomes through the same mechanism as the GSH dependent inhibition of lipid peroxidation induced by the non-enzymic system.

More evidence is required to determine the mechanism of GSH function in CCl_4-induced lipid peroxidation system, such as

a) the time and concentration courses of GSH effect on $^{14}CCl_4$ covalent binding and PBN-$CCl_3\cdot$ formation for determination of the ability of GSH scavenging $CCl_3\cdot$;

b) requirement of vitamin E for GSH function to check if the inhibitory effect of GSH is vitamin E dependent (for this research vitamin E deficiency microsomes can be employed);

c) the effect of SH reagents on GSH function to test if there is a

protein factor involved in the GSH protection effect;

d) the effect of addition of GSH peroxidase on GSH function.

4. 2. RIBONUCLEOTIDE REDUCTASE

4. 2. 1. Purification of ribonucleotide reductase from juvenile normal rat liver

Mammalian ribonucleotide reductase (RNRase) contains two components, M1 and M2. The two components are easily separated and it is difficult to purify the mammalian RNRase due to its instability (Wright, et al., 1989). So far only subunit M1 of the enzyme from calf thymus and regenerating rat liver has been highly purified (Enstrom, et al.,1979; Thelander et al., 1980; Youdale et al., 1982). In these studies dATP-affinity chromatography was employed as a last or important step since dATP specifically binds the M1 subunit. The liver enzyme is completely dissociated by binding to the dATP-affinity column (Youdale, et al., 1982). A number of reports describe the separation of the mammalian enzyme into two subunits. However, in all cases attempts to recombine the subunits or to purify the subunits separately resulted in very low yields of enzyme activity (Thelander, et al., 1980).

An attempt to purify the intact rat liver enzyme was made in the present work. The purpose was to develop a method of purification for obtaining a protein preparation in which the separation of the two subunits was minimal and the enzyme preparation was pure enough for raising an antibody. Ammonium sulphate fractionation (45% saturation), DEAE-cellulose chromatography, ATP-Agarose affinity chromatography, pH 5.2 precipitation, CM-Sephadex chromatography have all been

utilised for the purification.

Apart from its stability another major problem of purification of the enzyme is the low level of reductase present in most eucaryotic cells. Here the livers of 3-4 week-old rats have been chosen as the source of the enzyme since it is active in the liver at this stage of development and it is available in large quantities.

The protein content and the enzyme activity in different preparations of the enzyme from liver are shown in Table 3.2.1. Up to the step of chromatography on DEAE-cellulose the enzyme protein was purified some 30-fold (Table 3.2.2). This enzyme preparation, however, showed more than 10 bands of protein when it was analysized by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-page) (Fig. 3.2.2).

Chromatography on ATP-Agarose affinity and pH 5.2 precipitation followed by chromatography on CM-Sephadex were employed in an attempt to further purify the enzyme preparation after chromatography on DEAE-cellulose.

ATP-affinity Sepharose has previously been used for purification of the phage T_4 induced ribonucleotide reductase in *E. coli*, but apart from ribonucleotide reductase several other proteins which can interact with ATP were bound to the ATP-Sepharose column (Berglund and Eckstein, 1972). ATP-affinity chromatogtaphy has also been used for a simple purification of ribonucleotide reductase from human cells (Spector and Averett, 1983). In that work the ATP-Agarose column is used for removal of nucleoside diphosphate kinase from the enzyme preparation without binding ribonucleotide reductase. The aim of using ATP-Agarose affinity column in the present purification was to see whether the column could remove other proteins which bind to ATP whilst leaving the intact ribonucleotide reductase to pass through the column under

the experimental conditions. However, this procedure was not successful. The resulting enzyme preparation which was eluted from ATP-Agarose column with buffer containing no ATP lost 80% of the enzyme activity compared to the activity of the DEAE-cellulose fraction (Table 3.2.3). The dissociation of the enzyme may be responsible for this loss of activity. It was impossible that the intact enzyme was bound to the column, like phage T_4-induced ribonucleotide reductase since the protein fraction eluted with 2 mM ATP showed only one band on SDS-page (Fig 3.2.2, line 13). This ATP-Agarose-bound protein was unlikely to be the protein M1 or M2. Comparing the SDS-page of the protein fraction(s) eluted without and with 2 mM ATP (line 12 and line 13 in Fig. 3.2.2) it can be seen that there was no this protein left in the protein fraction eluted without ATP, however, there still 20% of the enzyme activity left in this fraction indicating that the protein which tightly bound to the ATP-Agarose column was not essential for the enzyme activity.

DEAE-cellulose is an anion exchanger; CM-Sephadex is a cation exchanger. The separation of the enzyme on CM-Sephadex after DEAE-cellulose chromatography was not successful either. The resulting enzyme preparation after chromatography on CM-Sephadex had 282 units/mg prot. left compared to a starting activity of 973 units/mg prot. and the purification was not improved (Table 3.2.3 and Fig. 3.2.2).

There was an inaccurate estimation of the recovery of total enzyme activity after the ammonium sulphate precipitation step (Table 3.2.2). This was most probably due to the underestimation of the enzyme activity of the crude enzyme extract. Two factors contributed to this underestimation:

i) <u>RNA</u> RNA inhibits ribonucleotide reductase activity (Cory, et al., 1973) and the crude extract contains a lot of nucleic acids. RNA can be

removed after salt precipitation;

ii) nucleoside kinase (NDP kinase) In the presence of Mg^{2+}, NDP kinase catalyzes the transfer of phosphate from nucleoside triphosphates (NTP) to nucleoside diphosphates (NDP). NDP are the substrates of ribonucleotide reductase and the assay for the activity of ribonucleotide reductase requires Mg^{2+}. Thus the substrate and requirement of Mg^{2+} for NDP kinase are satisfied in the RNR assay mixture and since NDP kinase activity is much higher than RNR in mammalian cells, NDP kinase will quickly convert the radiolabelled NDP (^{3}H-CDP) to NTP (^{3}H-CTP) resulting in the reduction of substrate for RNR. Most of the NDP kinase activity (90%) can be removed from the crude extract by ammonium sulphate precipitation (Spector and Averett 1983).

It is readily apparent that the development of a method for obtaining a highly purified and minimally separated ribonucleotide reductase preparation from juvenile normal rat liver was not successful. Chromatography on DEAE-cellulose is a common method of protein purification, but it is not specific for separation of ribonucleotide reductase from the bulk of proteins, like dATP affinity chromatography for the M1 subunit. ATP-affinity Agarose column did not specifically bind M1 or M2 and caused the loss of the enzyme activity probably due to the separation of the two subunits. Chromatography on CM-Sephadex caused the loss of the enzyme activity too and did not improve the purification situation. It seems that it is impossible to purify both subunits of ribonucleotide reductase together to a very high purity. Purification of M1 and M2 separately perhaps is a better way. In the future it would be worth trying to use monoclonal antibody technique to obtain the M2 or M1 antibody. This protocol includes administration of an M1 or M2-containing preparation to an appropriate recipient animal, production of hybridoma cell lines, cloning of hybrid cell mixtures and screening for

M1 or M2 antibody production (Liddell and Cryer, 1991). This technology has been applied to prepare antibodies against a vast range of antigens including serum proteins, enzymes, cell surface receptors, hormones, drugs, tumour specific antigens and differentiation antigens (Wilson and Goulding, 1986). A major advantage of using monoclonal antibody technology is the extreme confidence with which M1 or M2 antibody can be made. A major disadvantage of using this technology is that it relies on the random fusion of a heterogeneous population of B lymphocytes with myeloma cells, resulting in much time being spent in growing and testing hybrids which secrete M1 or M2 antibody. The other disadvantage is the intensive cost of producing the monoclonal antibodies (Wilson and Goulding, 1986).

4. 2. 2. Activity of ribonucleotide reductase in tissues of different growth states

Ribonucleotide reductase is a key enzyme for DNA synthesis. It catalyzes the conversion of four ribonucleotides into the corresponding deoxyribonucleotides that are the precursors of DNA. The reductase activity is closely related to the growth rate of normal and neoplastic tissues (Thelander and Reichard, 1979; Lammer and Follmann, 1983; Cory, 1989). In the present study, the reductase activity was checked in normal dividing and resting rat liver and hepatomas. The results demonstrated that the activity of the enzyme was higher in proliferating cells. An activity of 215 units/mg prot. existed in juvenile (3-4 weeks old) rat livers while no activity was observed in adult (11-12 weeks old) rat livers (Table 3.2.4). Similarly, an elevated enzyme activity appeared in regenerating liver whilst the enzyme activity was undetectable in

livers from sham-operated animals (Fig. 3.2.5). Hepatomas possessed higher enzymatic activity (Fig. 3.2.3, 3.2.4) and furthermore, the level of the enzyme activity was related to the growth rate of the tissue. The activity of the enzyme in the rapid-growing Yoshida hepatoma was significantly higher than that in the slower-growing Morris tumour (Fig. 3.2.3). All of these results are in agreement with the previous work (King and van Lancker, 1969; Elford et al., 1970; 1972; 1974; Takeda and Weber, 1981; Youdale, et al., 1982).

The time course of the enzyme activity was investigated in cultured Yoshida ascites hepatoma cells during 6 days of culture. The enzyme activity was very low at day 0. It began to increase after day 1 and continued to increase over 4 days. The highest activity was found at day 4, then the activity decreased abruptly (Fig. 3.2.4).

Ribonucleotide reductase is related to cell cycle. The enzyme activity is very high in S phase and very low in G0/G1 phase (see Introduction). When cultured cells are in the exponential phase, there is a large amount of cells in S phase. Therefore, it is expected that ribonucleotide reductase activity is increased. When cultured cells are in the plateau phase, the portion of cells which are in S phase decreases and the portion of cells in G0/G1 phase increases, thus the ribonucleotide reductase activity will decline. According to Carrie (personal communication), the exponential growth phase of cultured Yoshida cells was beginning at day 1 and lasts for 4 days. This growth pattern parallelled the pattern of changes in the activity of CDP reductase (Fig. 3.2.4).

These results are similar to the observation of Chevanne and Caldini (1986). They reported that after the Yoshida cells were transferred from host rats to the culture medium the enzyme activity increased after 2-3h and continued to increase throughout the 18h

period. They concluded that this elevated activity was related to the transition of the cells from G0-G1 to S phase. Takeda and Weber (1981) also observed that when hepatoma cells (3924A) were freshly plated and allowed to grow in lag, log and plateau phases for a 96-hr period the CDP reductase increased at 6 hr. The enzyme activity remained at the increased level (5.5-6 fold) for 48 hours and then declined to a lower level.

For studying changes in the enzyme activity in regenerating liver, the entrained rat model was used here. When the rats are entrained to a 12h light/12h dark regime, with access to food during the first 8h of darkness, liver regeneration is marked by cycles of DNA synthesis and cell division at approx. 24h intervals (Hopkins et al., 1973; Slater et al., 1990). Using this model to study processes of liver regeneration associated with CDP reductase activity, it was found that the enzyme activity in regenerating liver exhibits a periodic cycling in time course following partial hepatectomy (Fig. 3.2.5). There were two peaks of the enzyme activity, one at 48h post operation and another at 72 h post operation. This cyclical change in the reductase activity closely parallelled the time course of thymidine kinase activity in regenerating liver. It has been reported that thymidine kinase activity in regenerating liver is high at 24, 48, and 72h post operation, but very low at 12, 36, 60 and 84h post operation (Cheeseman, et al., 1986; Slater et al., 1990). Several previous studies have reported that the ribonucleotide reductase activity is increased in regenerating liver (King and Lancker, 1969; Elford, 1974; Cory and Fleischerm 1982 b; Takeda and Weber, 1981), but none has been with the entrained rat model used here, therefore neither showed changes in the ribonucleotide reductase activity with cyclical variations. Use of the entrained rat schedule of Hopkins et al. (1973) is better to show the close relationship between DNA synthesis

and ribonucleotide reductase activity and investigate the mechanism of regulation of the enzyme activity in regenerating liver.

What is the mechanism of regulation of ribonucleotide reductase activity in regenerating liver? Earlier work of Cory and Fleischer (1982 b) showed that two components of ribonucleotide reductase, M1 and M2 were not co-ordinately regulated in regenerating liver. M2 increased more rapidly and remains at a high level for a longer period, while the M1 increased later. Cory and Fleischer asked the question: does the non-coordinate increase of the two components result from the non-coordinate synthesis of the protein component or the non-coordinate activation of the newly synthesised components?

Whitfield et al. (1989) believe that non-coordinate synthesis of the protein components is involved in the enzyme activity's regulation. They suggested that one subunit of the enzyme, M1, controls the enzyme activity in regenerating liver. Before the onset of DNA replication, the cells start accumulating a large pool of functional ribonucleotide reductase M2 subunit. When M2 subunits reach a certain level (when the cell is near the end of the G1 phase), the cell begins M1 gene expression, stops inactivating and reduces the degradation of the M1 products. Then M1 and M2 combine to form the active holoenzyme. At the end of the S phase, the cell reduces M1 gene expression and resumes degrading M1 subunits.

The results of this study could not indicate if M1 and M2 were regulated coordinately or not during periodic cycling changes in the enzyme activity. However, the changes in the enzyme activity in the model of regenerating liver used here was the result of the regulation of the enzyme activity *in vivo* and the mechanism of regulation was probably through changing the level of the enzyme protein and/or in- and re-activation of the enzyme activity. This conclusion was from the results

shown in Fig. 3.2.5 and Table 3.2.6. The CDP reductase in regenerating liver had declined at 60h post operation (Fig. 3.2.5). This phenomenon could be explained by three possibilities:

(i) changing the protein level of the enzyme *in vivo,*

(ii) inactivation of the enzyme *in vivo,*

(iii) inhibition of the enzyme activity by inhibitors produced *in vivo* or *in vitro.*

However, it can be seen from Table 3.2.6 that there was only an additive effect when enzymes from 60h and 48h regenerating liver were mixed together. This result indicated that there were no inhibitors in the enzyme preparation from 60h regenerating liver. Therefore, only possibilities of (i) and (ii) would be responsible for the decrease of the enzyme activity in 60h regenerating liver. Of course more direct evidence is required to make the final conclusion about the regulation mechanism in the ribonucleotide reductase activity in the regenerating liver model used here. Examination of the activity and the protein level of M1 and M2 separately at different time of post operation is required for this purpose.

In the present study, it was found that there were differences between the enzyme from juvenile normal rat liver and regenerating liver in the optimal DTT concentration for exerting the full CDP reduction activity (Fig. 3.2.6). A higher concentration of DTT (10 mM) was required for the enzyme from 48h and 60h regenerating rat liver to exert the full CDP reductase activity, while 1 mM DTT was enough for the CDP reductase from juvenile rat liver to express the activity. The enzyme activity of 48h and 60h regenerating liver at 10 mM DTT was about 260% and 181% of the activity at 1 mM DTT, respectively.

The differences in the optimal DTT concentration for the full activity between enzyme preparations from normal and regenerating

liver may due to

(i) different content of low molecular weight thiols in enzyme preparations i.e. higher content of thiols in the enzyme preparation from juvenile normal rat liver and lower content of thiols in the enzyme preparations from regenerating rat liver;

(ii) the enzymes from normal rat liver and regenerating rat liver are different;

(iii) different rate of consumption of the DTT.

The results shown in Table 3.2.6 allow one to rule out the first possibility. There were no significant differences in acid-soluble thiol content between enzyme preparation from 48h regenerating liver and normal rat liver or between 60h regenerating liver and normal rat liver. The content of acid-soluble thiols in enzyme preparations from 48h regenerating liver was significantly higher than that from 60h regenerating liver. However, this difference cannot explain the results that the enzyme activity from both of 48h and 60h regenerating liver was dependent on DTT concentration. In fact, the enzyme from 48h regenerating liver required higher DTT concentration for exerting the full activity than that the enzyme from 60h regenerating liver did.

For the second possibility, it is unlikely that the enzymes from normal and regenerating rat liver have big differences in their properties. Whitfield et al. (1989) reported that both normal and regenerating rat liver contained functional protein M1, 88-90 kDa protein and non-functional fragments of M1, 40 kDa protein; however, normal liver contained only faint traces of the 88-90 kDa protein. This result indicates that normal and regenerating liver contain the same M1 subunit. The experiment here demonstrated that the enzyme from normal and regenerating liver had the same sensitivity to the specific

inhibitor, hydroxyurea. It can be seen from Fig. 3.2.12 that 4 mM hydroxyurea inhibited the enzyme activity form three sources by 60-70%. At 10 mM hydroxyurea the activity of all the enzymes was nearly inhibited by 100%. Hydroxyurea destroys the tyrosine free radical of M2. Therefore, the M2 of normal and regenerating liver have the same properties.

DTT is necessary for CDP reductase activity as a reducing agent in this assay system. As a reducing agent, it would be consumed during the enzymic reaction. The higher the enzyme activity, the more the CDP reduction and the more the DTT consumption. Therefore, the third possibility could explain the results that the enzyme activity from 48h regenerating liver required a higher DTT concentration than the enzyme from normal rat liver was not (Fig. 3.2.6). The CDP reductase activity in 48h regenerating liver was much higher (723 units/mg prot.) than that in normal juvenile rat liver (185 units/mg prot.) (Fig. 3.2.5, Table 3.2.4). It is therefore understandable that 48h regenerating liver enzyme needs more DTT for reducing the CDP. This explanation is supported by the results shown in Fig 3.2.7. The continual presence of a higher DTT concentration was needed to maintain the high activity of the enzyme from 48h regenerating liver (Fig. 3.2.7). At 1 mM DTT, the level of reduction of CDP was lower compared to that at 10 mM DTT. Addition of 10 mM DTT immediately restored the rate of CDP reduction even after the reaction had been going for 20 and 40min. (Fig. 3.2.7). Since the enzyme activity in normal juvenile liver was only 185 units/mg prot., 1 mM DTT was a saturating concentration for the enzyme to catalyse the CDP reduction. Thus there was no significant difference in the normal liver's enzyme activity at the range of 1 mM to 15 mM DTT.

However, the third possibility cannot explain the dependence of

the enzyme activity from 60h regenerating liver on the DTT concentration. The level of the CDP reductase activity in 60h regenerating liver was similar to that in juvenile normal rat liver, that was about 110 units/mg prot. There is another explanation. In this study, it was found that the higher concentration of DTT had the ability to activate the CDP reductase from 48h and 60h regenerating liver (Fig. 3.2.8, 3.2.9). Preincubation of the enzyme protein from 48h and 60h regenerating liver with 10 mM DTT for 20 min enhanced the CDP reductase activity by 29% and 105%, respectively. However, the activity of CDP reductase from normal juvenile rat liver was not activated by high concentration of the DTT (Fig. 3.2.10).

Therefore, DTT had two functions under the present experimental conditions, as a reducing agent that would be continuously consumed in the reaction and as an initial activator of the enzyme activity. The dependence of enzyme activity from 48h regenerating liver on DTT concentration may have been due to these two roles of DTT, reducing agent and activator. The dependence of enzyme activity from 60h regenerating liver on DTT concentration, on the other hand, may have been only due to the function of DTT of activation.

What is the mechanism of the activation of ribonucleotide reductase by DTT? Previous work has been shown that the tyrosine free radical of M2 can be regenerated by DTT and O_2. Under the high concentration of DTT (5-10 mM) and air, tyrosine radical-free M2 can be reactivated to an active M2 containing a M2-specific tyrosine free radical (Thelander, et al., 1983; Graslund et al., 1982; Lassmann, et al., 1989).

From the discussion above and the results shown in Fig. 3.2.8, 3.2.9, it can be suggested that some inactive M2 (specific radical-free M2) exists in regenerating liver. These inactive M2 subunits can be

reactivated by a high concentration of DTT *in vitro.*

Does this phenomenon have some physiological meaning in regenerating liver? A tentative hypothesis for this phenomenon connecting to the regulation of the ribonucleotide reductase activity in regenerating liver is given below. As mentioned already, Whitfield et al. (1989) suggested that in regenerating liver before the onset of DNA replication, the cells start to accumulate a large pool of M2. According to the results of this present study it is supposed that these M2 subunits, all or part, are inactive. When M2 reach a certain level, the cells start to synthesis M1. Then M1 and M2 combine to form a holoenzyme. Before or after the combination, the inactive M2 is reactivated through regeneration of free radicals and the holoenzyme becomes functional. How are these inactive M2 subunits reactivated *in vivo*? They could be reactivated by intracellular thiols, like low molecular weight thiols. Fig. 3.2.11 showed that acid-soluble thiol in homogenates from regenerating liver exhibit a periodic cycle which is the same as the cycle of CDP reductase (Fig. 3.2.5). Possibly, in 48h and 72h regenerating liver there are more M2 subunits than in 36h and 60h regenerating liver, therefore a higher concentration of thiols is required in 48h and 72h regenerating liver for activation of the M2.

It has been suggested that cells may have a mechanism for controlling ribonucleotide reductase activity by generating the free radical of M2 (see Introduction 1. 3. 3. 2).

The activity of CDP reductase from 48h regenerating liver in Figs. 3.2.6-8 was lower, only 170-250 units/mg prot., compared to that in Fig. 3.2.5 which was about 700 units/mg prot.. This was because in the experiments shown in Fig. 3.2.6-8 the content of enzyme in the assay mixture was low, about 0.39 mg in the CDP reduction mixture. Low content of enzyme protein will underestimate the enzyme activity since

the two subunits of the enzyme are easily separated at low protein content. Usually 1 mg enzyme protein is needed for determination of the enzyme activity.

Thymidine kinase is very active in 24h regenerating liver (Cheeseman, et al., 1986). However, no peak of the activity of ribonucleotide reductase was shown at 24h post operation. Perhaps at this time, the dNTP pools in liver is large enough to support the first burst of DNA synthesis. Therefore *de novo* synthesis of M1 and M2 subunits is not necessary.

The present work has confirmed the previous results that ribonucleotide reductase activity is very closely related to the tissue growth and cell division. The CDP reductase activity can only be detected in tissues in which intense cell proliferation occurs, like juvenile rat liver, regenerating rat liver and hepatoma cells. Furthermore, the level of enzyme activity is closely related to the growth rate of these tissues and cells. The enzyme activity in regenerating liver exhibits a periodic cycling in time course following partial hepatectomy. This phenomenon has been found in thymidine kinase. The results in the present study suggested that regulation of M2 activity through the way of regenerating M2 specific free radical in the regenerating liver may play a role in regulation of ribonucleotide reductase activity in regenerating liver. However, more detail and advanced research on this possible regulation mechanism are required in the future. For this purpose M2 subunit from regenerating liver must be highly purified in order to be able to check the amount of M2-specific free radical using ESR spectra and to raise the M2 antibody. Then the time courses of the amount of M2-specific free radical, the protein level of M2 and M2 activity need to be performed.

4. 3. INHIBITORY EFFECT OF HNE ON RIBONUCLEDTIDE REDUCTASE

HNE is one of the most important products of lipid peroxidation having effects at the enzyme, subcellular, and cellular level. HNE has the property of inhibition of cell proliferation, but the mechanism of this inhibition is still not clear. In the present study, the effect of HNE on the activity of ribonucleotide reductase from rat liver and the mechanism of the effect have been investigated.

In this study it was demonstrated the HNE inhibited the activity of rat liver CDP ribonucleotide reductase. This inhibition was related to the concentration of HNE and the time of incubation (Fig. 3.3.1 and 3.3.2). High concentrations of HNE and long incubation produced more inhibition of the activity of the reductase. HNE at 0.1 mM produced 50% inhibition. Preincubation of HNE with the enzyme protein at 30°C for some time before the enzymic reaction started increased the extent of inhibition (Table 3.3.1). The preincubation time had no significant effect on the enhancement of inhibition. If the enzyme protein was exposed to HNE at 0°C for 1 hr, the enzyme activity could not be recovered after removal of HNE by gel filtration (Fig. 3.3.3 and 3.3.4). Since the enzyme preparation contained 2mM DTT and DTT had a protective effect on the enzyme protein against HNE (see below), so two treatments were carried out for this experiment. One was using original enzyme preparation (without removal of 2 mM DTT) and standard enzyme reaction condition (Fig. 3.3.3). Another was using the DTT-free enzyme preparation and employed 15 min of the enzymic reaction time. However, the results from these two treatments were essentially the same. The activity of the HNE-treated enzyme was inhibited and the extent of inhibition was dependent on HNE concentration (Fig. 3.3.3 and 3.3.4). All of

these results indicate that HNE can inhibit the enzyme activity during the enzymic reaction, also it can affect the enzyme protein itself and this effect is irreversible.

Several enzymes have been reported to be inhibited by HNE (see Table 1.2.1). HNE possess high reactivity with SH groups. All inhibitory effects of HNE can be explained by the inactivation of functional SH groups (Benedetti and Comporti, 1987; Esterbauer et al., 1988). Ribonucleotide reductase contains several SH groups which are essential for the enzyme function (see Introduction). Therefore HNE inhibition of CDP reductase activity may be due to the blockage of SH groups on the enzyme protein by the aldehyde.

In this study the following pieces of evidence for this explanation have been given:

(i) DTT has a modulating effect on HNE inhibition and protection of enzyme against HNE

DTT is a reducing agent in the assay for activity in this study. The structure of DTT is as below:

$$HS\text{-}CH_2(CHOH)_2CH_2\text{-}SH$$

The results show that DTT has a modulating effect on HNE inhibition and protection of the enzyme against HNE. In the presence of a high concentration of DTT, the degree of inhibition by HNE was decreased significantly (Table 3.3.2). If HNE was incubated with DTT before exposure to the enzyme, HNE had no inhibitory effect on the CDP reduction (Table 3.3.3). If HNE was incubated with enzyme protein in the presence of DTT, HNE did not damage the protein (Fig. 3.3.7). These results are consistent with previous work that reported that most inhibitory effects of HNE are prevented by the prior addition of thiols (Schauenstein, 1982). It is reported that a 0.1 mM solution of HNE is

completely consumed by 1 mM glutathione within 30 min. (Esterbauer, 1982). Therefore, it is understandable that HNE incubated with DTT (HNE:DTT = 1:10) for 30 min. at 30°C lost the ability to inhibit the enzyme activity (Table 3.3.3). HNE can discriminate between different types of thiols (Schauenstein, 1982), reacting with non-protein thiols most avidly. This fact can explain the result that if HNE was incubated with enzyme protein and DTT together, HNE failed to inhibit the enzyme activity (Fig. 3.3.7). However, DTT did not reactivate the enzyme activity after the enzyme was damaged by HNE. Incubation of HNE-treated enzyme (enzyme exposed to HNE at 0°C for 1 h then the HNE was removal by gel filtration) with higher concentration of DTT pre- or during enzyme reaction, no enzyme activity was recovered (Table 3.3.4, Fig. 3.3.8). The reaction of HNE with SH is an alkylation. The end product of the reaction is a very stable cyclic hemiacetal (see Introduction 1. 2. 1). DTT can reduce S-S bridges. The results here showed that DTT cannot reverse HNE-SH binding.

(ii) <u>HNE decreases the SH content of the enzyme preparation and DTT</u>

As shown in Fig. 3.3.9 HNE decreased the SH content of the enzyme. This effect was dependent on HNE concentration, but independent of the incubation time. As indicated in Fig. 3.3.10 DTT influenced the spectrum of HNE, decreasing the absorbance at 223nm. Fig.3.3.11 and 3.3.12 showed that in presence of HNE the SH content of DTT decreased. The effect of HNE on the content of SH on DTT was proportional to both the HNE concentration and the incubation time.

<u>(iii) The presence of thiols in the enzyme preparation decreases the effect of HNE</u>

Another piece of evidence is from the result of the inhibitory effect of HNE on the enzyme activity of the crude extract. It can be seen

from Fig. 3.3.1and Fig. 3.3.6 that the effect of HNE on crude extract is 10 times weaker than it was on the partially purified enzyme. The explanation is that there is a large amount of thiol in the crude extract enzyme. These thiols react with HNE preferentially and protect the enzyme against HNE (Dianzani et al., 1990).

Therefore, under the present experimental conditions, HNE inhibits the CDP ribonucleotide reductase in two ways. One was that HNE directly affected the enzyme protein. This was the result of blocking the essential SH group of enzyme by HNE. This damage was irreversible (Fig.3.3.3 and 3.3.4). The concentration of HNE influenced this effect (Fig. 3.3.4), but the time of HNE incubation with the enzyme protein was not very important since (a) the preincubation time had no significant effect on the enhancement of inhibition induced by preincubation of HNE with enzyme (Table 3.3.1), and (b) the reduction of the content of SH of enzyme by HNE only related to HNE concentration, the reaction time had no effect (Fig. 3.3.9). The second way was through the interaction of HNE with the reducing agent DTT. HNE reacted with DTT and consumed the amount of the DTT. Finally this effect affected the enzyme activity. This kind of effect of HNE was indirect. It was not only related to the HNE concentration, but was also related to the incubation time (Fig. 3.3.11, and 3.3.12). Therefore, when HNE was present during the enzymic reaction the overall inhibitory effect of HNE on the enzyme activity was related to the incubation time (Fig. 3.3.2).

Ribonucleotide reductase is a key enzyme for DNA synthesis. Its activity is very closely related to growth rate of tissue and cell cycle. HNE is one of the most important products of lipid peroxidation. It has been suggested that HNE has a role for regulation of cell proliferation (see Introduction). It would be very interesting

and worthwhile to investigate the HNE effect on the expression and activity of ribonucleotide reductase *in vivo.* Such research could employ cultured cells for studying the relationship between ribonucleotide reductase activity and cell proliferation in terms of the effects produced by HNE. The effect of HNE on M1 or M2 gene expression and amplification could be investigated by addition of a lower concentration of HNE to the cultured cells at different stages of the cell cycle.

5. CONCLUSION

1. Temperature is very important for CCl_4 metabolism. CCl_4 was activated in rat liver microsomes in a temperature-dependent fashion. The transition temperature for CCl_4 activation was 26.7-27.5°C when the metabolism was measured by PBN-$CCl_3 \cdot$ formation and specific $^{14}CCl_4$ covalent binding to protein. However, the transition temperature was shown to be at 34.3°C when CCl_4-dependent MDA production was taken as an index of CCl_4 metabolism. CCl_4-dependent lipid peroxidation is not a good parameter for the activation of CCl_4.

Two P450 inhibitors (pyrazole and imidazole) and one SH reagent (iodoacetamide) had inhibitory effects on CCl_4-stimulated MDA production only at a relatively high concentrations (10-20 mM). At this concentration level they produced 15-30% inhibition. GSH strongly inhibited lipid peroxidation induced by CCl_4 in a concentration-dependent manner. At 0.2 mM it reduced MDA formation by 50%. GSH also delayed the onset of the lipid peroxidation and this effect was also dependent on the concentration of GSH. The results suggested that GSH inhibited lipid peroxidation possibly by the way of scavenging and interaction with intermediates of the activation of CCl_4. Another SH reagent, N-ethyl maleimide (NEM) showed biphasic effects on CCl_4-stimulated MDA production. NEM at 0.5-1.0 mM inhibited MDA formation, but higher concentrations of NEM stimulated the lipid peroxidation. This phenomenon may be attributed to the fact that CCl_4-dependent lipid peroxidation is a poor index for the activation of CCl_4.

2. It was not possible to successfully purify M1 and M2 subunit of ribonucleotide reductase together to a highly purified state. It was concluded that it would be better to purify M1 and M2 separately.

Ribonucleotide reductase activity was very closely related to growth state and rate of the tissues. The enzyme activity can only be detected in the dividing tissues, such as juvenile normal rat liver, regenerating liver, liver tumour and hepatoma cells. The level of the enzyme activity in fast-growing tissue (hepatoma Yoshida cells) was higher than that in slow-growing tissue (Morris 5123tc tumour). These results have confirmed the previous work. The enzyme activity in regenerating liver exhibits a periodic cycling in time course following partial hepatectomy. This phenomenon has been found in thymidine kinase. The activity of CDP reductase from 48h and 60h regenerating liver could be activated by higher concentration of DTT (10 mM). It is assumed that the regenerating liver's enzyme activity was activated by DTT by regenerating the specific free radical of the M2 subunit. The content of low molecular weight thiols in regenerating liver exhibited the same periodic cycle as the CDP reductase. From these results a possible mechanism of regulation of the ribonucleotide reductase activity in regenerating liver was tentatively suggested. In regenerating liver, before the onset of DNA replication, the cells start to accumulate a large pool of ribonucleotide reductase M2 subunit. These M2, all or part, are inactive. When M2 reach a certain level, the cells start to synthesis M1. Then M1 and M2 combine to form holoenzyme. Before or after the combination, the inactive M2 is reactivated through regeneration of free radicals. Then the holoenzyme becomes functional. It is further suggested that low molecular weight thiols in the regenerating liver may play the role of regenerating free radicals.

3. 4-Hydroxynonenal (HNE) inhibited the CDP reductase activity from juvenile normal rat liver. The inhibition was dependent on the concentration of HNE and incubation time. Under the experimental

conditions used in the present study. HNE achieved its inhibitory effect in two ways. One is direct damage to the enzyme protein by blockage of the functional SH groups in the enzyme protein. This effect was irreversible and only dependent on HNE concentration. The other one is indirect by consuming the DTT in assay mixture through the interaction of HNE with SH groups on DTT, and eventually affected the enzyme activity. This effect was dependent on both the concentration of HNE and the incubation time. Therefore, the overall effect of HNE on CDP reductase activity was dependent on both parameters.

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