Korea J. of Toxicology
Vol. 3, No. 2, pp. 129-141, 1987

PROTECTIVE ACTION OF N-ACETYLCYSTEINE AGAINST HEPATOTOXIC AGENTS IN ISOLATED RAT LIVER CELLS. *-1

Soo-Hee Park, Mi-Sook Dong, Dong-Chul Kang, Kiwan Lee and Young-Nam Cha*-2
Department of Pharmacology and Toxicology and Medicinal Toxicology Research Center, Inha Medical College, Inha University, Inchon, 160, Korea

ABSTRACT: *Hepatocytes isolated from rats which have been pretreated with phenobarbital (80 mg/kg for 3 days), were able to take up N-acetylcysteine from surrounding medium and were able to synthesize the reduced glutathione (GSH *-3) intracellularly. The N-acetylcysteine is quickly deacetylated after the uptake and increases the pool size of cysteine, which was very low initially (5 nmol/10^6 cells). From this increased intracellular cysteine pool, GSH was synthesized. Freshly isolated rat hepatocytes contained a high level of GSH (30 nmol/10^6 cells), but upon incubation with the diethylmaleate, it was markedly decreased (10 nmol/10^6 cells). The hepatocytes with depleted GSH have lost viability upon incubations with acetaminophen (5mM) and paraquat (2 mM). However, when the N-acetylcysteine (1 mM) was added to this incubation condition, these chemical induced hepatocellular necrosis were prevented for longer durations. This N-acetylcysteine dependent protective effect against the hepatotoxic chemicals was lost by adding methionine sulfoximine (10 mM), an inhibitor of GSH biosynthesis. Both the carbontetrachloride (5 mM) and chloroform (5 mM) added to the incubation medium caused rapid losses of GSH and cell viability, even without the prior depletion of cellular GSH. However, again, if the 1 mM N-acetylcysteine was supplemented, the rates of losses of GSH and cell viability were retarded in both cases. Even though large amounts of the added N-acetylcysteine was present in the cell, N-acetylcysteine conjugate of acetaminophen was not formed. Instead, only large amounts of GSH conjugate*

*1; This study was supported by grants from The Korea Science and Engineering Foundation (860401) and The Inha University Research Fund (1987). The technical assistance of Mr. Seung-Woo Kim is gratefully acknowledged.
*2; To whom all correspondences should be directed.
*3; Abbreviations used: GSH, reduced glutathione; GST, glutathione S-transferases · GSSG, glutathione disulfide or oxidized glutathione; DEM, diethylmaleate; MSO, methionine sulfoximine; EGT, ethyleneglycol bis (B-aminoethyl - ether)N,N′-tetraacetic acid; HEPES, N-2-hydroxyethylpiperazine N′-2-ethane sulfonic acid; LDH, lactate dehydrogenase; TCA, trichloroacetic acid; DMSO, dimethyl sulfoxide; FDNB, 1-fluoro-2,4-dinitrobenzene; HPLC, high performance liquid chromatography; CC14, carbontetrachloride; CC13, chloroform.

of the drug was produced. Thus, it is concluded that the added N-acetylcysteine is taken up and utilized for resynthesis of GSH. In turn, this resynthesized GSH contributes to the protection against cytotoxicity inducible with hepatotoxic drugs.

Keywords: *N-acetylcysteine, Isolated rat hepatocytes, GSH biosynthesis, Hepatotoxic agents.*

INTRODUCTION

The N-acetylcysteine is a component of mercapturic acids and the mercapturic acids are conjugates of N-acetylcysteine and toxic metabolites of drugs. The mercapturic acids are the end products of physiologically very important detoxification process for a variety of harmful electrophilic compounds (1). The initial state of mercapturic acid biosynthesis involves conjugation of reactive drug metabolites (electrophilic compounds) with the endogenous intracellular glutathione (GSH). This reaction is usually catalyzed by the glutathione S-transferases (GST) that exist in most aerobic cells which are capable of enzymatic detoxication. Large quantities of both GSH and GST are present in liver cells (2). The GSH conjugates are then converted to mercapturic acids (N-acetylcysteine conjugates) in separate sequential stages by (a) removal of the gamma-glutamyl moiety, (b) removal of the glycine moiety, and (c) addition of acetyl moiety to the N-terminal of cysteine in the remaining drug-cysteine conjugate (1).

The GSH is a major physiological tri-peptide involved in maintaining the redox homeostasis and, as eluded earlier, it is composed of gamma-glutamate, cysteine, and glycine. The thiol (-SH) included in the cysteine moiety of the GSH molecule is very reactive toward molecules having an electrophilic center and donates the hydrogen atom to most carbon-, oxygen-, and nitrogen-centered radicals which are all produced during metabolic oxidation of chemicals. Under physiological conditions, these reactive metabolic products generated from drugs can readily conjugate with the nucleophilic GSH. While the conjugation reaction can proceed non-enzymatically, it is usually catalyzed *in vivo* by the glutathione S-transferases, which make up about 5 to 10% of the cytosolic protein in liver cells (2). As a consequence of the rapid enzyme -catalyzed conjugation with GSH *in vivo,* hepatic levels of GSH are depleted and liver cells are necrotized unless the GSH levels are quickly restored by an active biosynthesis involving the gamma-glutamyl cycle (3). For this to occur, intracellular pool size or the rate of supply of cysteine must be increased. In this connection, it was possible for the N-acetylcysteine moiety cleaved from mercapturic acids could serve as the stable extracellular precursor for the intracellular supply of cysteine.

It is now well established that hepatotoxicity produced by many drugs such as acetaminophen and CCl4 is mediated by the cytochrome P-450 dependent formation of reactive electrophilic metabolites and is caused by the subsequent depletion of intracellular levels of reduced glutathione (GSH) (3). Accordingly, the hepatotoxic effect of such agents may be decreased either by administration of inhibitors of the cytochrome

P-450 dependent mixed function oxidase or by increasing the capacity for removal of the reactive metabolites by administration of sulfur amino acids like cysteine, N-acetylcysteine, and methionine (3). However, it was not certain whether the protective effects provided by these amino acids is due either to facilitated GSH biosynthesis or to direct formation of conjugates with these amino acids.

It has previously been reported by Thor *et al* that the intracellular GSH concentration is decreased when hepatocytes isolated from phenobarbital treated rats were incubated with bromobenzene (4). However, as the hepatocytes are able to resynthesize the GSH quickly and overcome the drug induced depletion of GSH, the bromobenzene dependent cell damage could not be observed with normal hepatocytes and under normal incubating conditions. To observe such damage, hepatocellular levels of cytochrome P-450 had to be increased by pretreatment of rats with phenobarbital, incubations of hepatocytes with bromobenzene had to be performed in an amino acid free medium, or the levels of intracellular GSH had to be lowered either by pretreatment of rats with diethylmaleate (DEM) prior to cell isolation or the isolated normal hepatocytes had to be preincubated with DEM (5).

In any case, the GSH conjugates formed with reactive metabolites of drugs, together with their catabolic metabolites, are excreted generally into the bile in high concentrations (6). Accordingly, the GSH, cysteinylglycine, cysteine, and N-acetylcysteine (mercapturic acid) conjugates of the corresponding reactive compound have been detected in the bile of rats (7). The GSH conjugates and their catabolites excreted into the bile are eventually converted to the original chemical compound not containing the cysteine moiety by the digestive intestinal enzymes (inclusive of those of intestinal microbial flora). Both the regenerated original chemical compound and the cysteinyl metabolites cleaved from the conjugates of these sulfur amino acids and mercapturic acids may then be reabsorbed via the enterohepatic recirculation or they may be excreted in the feces. The cysteinyl moiety of mercapturic acids, as represented by the N-acetylcysteine, may eventually be reabsorbed by the liver cells and be reutilized economically in the gamma-glutamyl cycle for the restorative biosynthesis of GSH. Thus, the present study is designed to determine whether freshly isolated rat hepatocytes can take up N-acetylcysteine and whether it is incorporated into the intracellular GSH by employing ^{14}C labeled N-acetylcysteine. The study is also designed to determine whether a protective effect is observed under conditions of the N-acetylcysteine dependent facilitated GSH synthesis by employing several well-known hepatotoxic agents.

MATERIALS AND METHODS

Pretreatment of animals and preparation of liver cells

Male Sprague-Dawley rats weighing about 180-250 g were obtained from the Animal Breeding Laboratory of Inha Medical College and were acclimatized to the laboratory conditions for 3 days. During this period, food (donated by Korea Purina,

Inc.) and water were supplied ad libitum. Rats were given the intraperitoneal injections of sodium phenobarbital at a daily dose of 80 mg/kg for 3 days, in order to increase the sensitivity of isolated hepatocytes to toxic chemicals, especially those requiring metabolic activation for demonstration of hepatocellular toxicity.

Isolation of hepatocytes was performed by collagenase perfusion of the liver as described by Seglen with minor modification (8). Briefly, the liver was perfused initially for 5 min with EGTA-salt buffer solution and then for another 5 min with the EGTA-free salt buffer containing the enzymes (collagenase Type I obtained from Sigma) and calcium chloride (50 mM). The yield of each preparation was $2\text{-}4 \times 10^8$ cells per liver and the viability of freshly isolated hepatocytes was about 90% as measured by exclusion of trypan blue dye and by leakage of lactate dehydrogenase (LDH).

Incubations of hepatocytes were performed at 37°C in Erlenmeyer flasks under a 95% oxygen and 5% carbondioxide atmosphere at a cell concentration of 10^6 cells per ml in a Krebs-Henseleit buffer, pH 7.4 supplemented with 25 mM HEPES and an amino acid mixture suggested by Seglen (9). All amino acids were present at the concentration specified by Seglen (9), except for the N-acetylcysteine, which was added as indicated in figure legends.

The leakage of LDH test was performed on an aliquot of well-mixed hepatocyte suspension upon 20 fold dilution in Krebs-Henseleit buffer containing 2% albumin (obtained from Sigma). NADH (0.1 mM final concentration) and pyruvate (0.8 mM) were then added. The rate of NADH oxidation dependent on the leaked LDH was recorded at 340 nm, and the 100% LDH activity was obtained after lysis of the cells by addition of Triton X-100 (0.5% final concentration) (10).

The intracellular level of reduced glutathione (GSH) was measured by the fluorometric method of Hissin and Hilf (11). Measurements were performed on $10'' \times 6$ cells after washing once with Krebs-Henseleit buffer by gentle reharvesting centrifugation ($80 \times g$). In order to deplete the intracellular GSH prior to starting the experiment, cells were preincubated for 30 min with 1 mM diethylmaleate (DEM). Also, in order to block the resynthesis of GSH from the added N-acetylcysteine, 10 mM methionine sulfoximine (MSO) was added to the incubation medium. These compounds were dissolved in DMSO prior to addition to hepatocyte suspensions.

The uptake and metabolism of ^{14}C labeled N-acetylcysteine was measured according to Reed and Orrenius (12). Briefly, the hepatocytes were incubated at 37°C for designated durations with the diluted mixtures of labeled (120 mCi/mmol, 97% radiochemical purity) and un-labeled N-acetylcysteine dissolved in acetone (750 dpm /nmol). They were added to the incubation medium at a final concentration of 1 mM. Various metabolites of N-acetylcysteine (e.g., mono-acetylcystine, cystine, cysteine, glutathione, cysteinylglutathione) present in the supernatant of TCA precipitated fraction were coupled with the chromophore, 1-fluoro-2,4-dinitrobenzene (FDNB) to the amine group of cysteine moiety and the separation of different derivatives according to their

polarities were accomplished with a C_{18} reverse phase column employing an HPLC system (Waters Associates, Boston, USA). However, the FDNB will not bind to the amine group of N-acetylcysteine because the available binding position was already occupied by the acetyl group. Thus, the N-acetylcysteine was quantitated by collecting the fractions eluted from the HPLC column and by counting the radioactivity.

Metabolism of acetaminophen and detection for the presence of conjugates of the drug were performed according to the method described by Moldeus (13) utilizing an HPLC system (Waters Associates, USA). While various conjugated metabolites (including sulfate, glucuronide, and glutathione conjugates) have been detected, only the sulfur amino acid conjugates were isolated and quantitated. This has been confirmed by using ^{14}C labeled N-acetylcysteine as the precursor of GSH and the one peak containing radioactivity was identified to be the glutathione conjugate of acetaminophen. Hepatotoxic agents like acetaminophen (5 mM), paraquat (2 mM), CCl4 (5 mM), and CCl3 (5 mM) were all individually dissolved in acetone and each was added to the hepatocyte incubation medium. At the times indicated in Figures, aliquots were withdrawn for determinations of cell viability, metabolite analysis, and intracellular GSH levels. The illustrated experiments are the results of "a typical experiment" and, upon repeating, there were about 25% variability. This method of expression was chosen for the purpose of clear presentation in Figures.

Collagenase Type I, as well as most other biochemicals were purchased from the Sigma Chemical Co., St. Louis, MS, USA. The ^{14}C labeled N-acetylcysteine was from the Radiochemical Center, Amersham, Bucks, England. All other chemicals were of analytical grade and were obtained from local commercial sources.

RESULTS

Uptake and metabolism of N-acetylcysteine

As shown in Fig. 1, freshly isolated hepatocytes obtained from rats pretreated with phenobarbital took up N-acetylcysteine in a linear fashion. Within 30 min, two-thirds of the amino acid taken up was deacetylated within hepatocytes and formed cysteine, mono-acetylcystine, cystine, glutathione, and cysteinylglutathioine (Fig. 2). The order of elution of these metabolites identified with respective standards from the C_{18} reverse column of HPLC was cystine, mono-acetylcystine, cysteine, N-acetylcysteine, cysteinyl S-glutathione, glutathione (GSH), and glutathione disulfide (GSSG). As the chromophore FDNB could not bind the N-acetylcysteine, its quantitation had to rely soly on the scintillation counting of the collected fractions. However, all other metabolites of the amino acid could be quantitated either by UV detector set at 350 nm or by scintillation counting of the collected peak fractions. These metabolites were found mainly from the supernatant portion after the TCA precipitation. There were no or minimal accumulation of the radio-label in the TCA precipitable protein fractions. Also, it was not possible to demonstrate any increased secretion of GSSG or GSH into

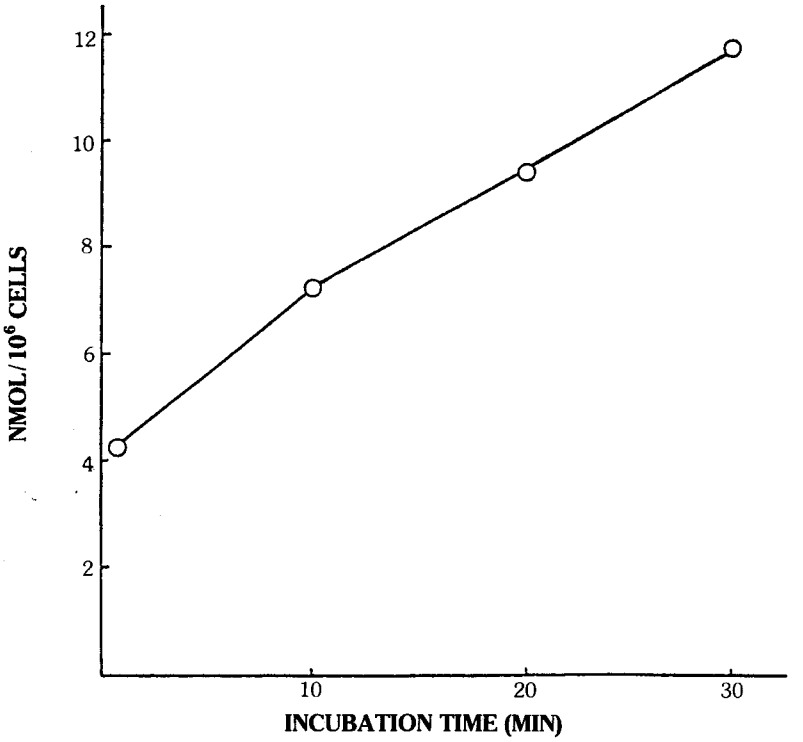

Fig. 1. Uptake of 14-C N-acetylcysteine by isolated rat liver cells.: Rat hepatocytes (10^6 cells1ml) were incubated at 37°C with ^{14}C labeled N-acetylcysteine (1 mM). At the times indicated. 1 ml aliquots were withdrawn and the cells collected on Millipore filters were washed with 10 ml of Krebs-Henseleit buffer. The filters were dried and their radioactive contents were determined.

the surrounding medium by hepatocytes incubated with the ^{14}C labeled N-acetylcysteine.

Effect of N-acetylcysteine on cell necrosis inducible with hepatotoxic chemicals

The possible protective effect of N-acetylcysteine against various well-known hepatotoxic chemicals has been examined using the isolated hepatocytes. Acetaminophen, which by itself is not toxic, causes hepatocellular necrosis only after activation by the cytochrome P-450 dependent microsomal mixed function oxidase. Upon activation, a reactive benzoquinoneimine metabolite is known to be produced from acetaminophen and this reactive metabolite readily binds to GSH. As the consequence, intracellular GSH is depleted and liver cells die (3). Isolated hepatocytes from normal rats are usually not susceptible to necrosis by acetaminophen because, they have inadequate amounts of cytochrome P-450 dependent mixed function oxidase activity, have high levels of intracellular (about 30 nmol/10^6 cells, e.g., Figs. 5,6,7), and have active biosynthetic ability to restore the depleted GSH level.

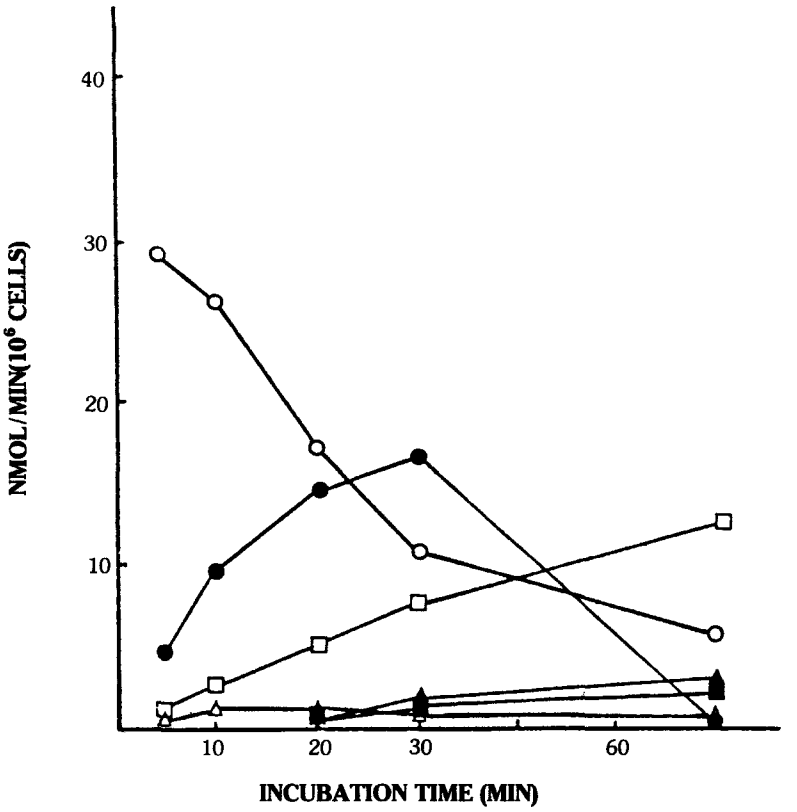

Fig. 2. Intracellular concentration of 14-C labeled sulfur amino acids derived from N-acetylcysteine.: Rat hepatocytes (10^6 cells/ml) were pre-incubated for an hour at 37°C with the radioactive N-acetylcysteine (1 mM) for pre-loading purposes and then, started the experimental incubation with the non-radioactive N-acetylcysteine. At the times indicated after initiating the incubation with the non-radioactive amino acid, 1 ml aliquots were withdrawn and derivatized with FDNB for HPLC determination of the N-acetylcysteine metabolites, the quantitation being by scintillation counting of eluted fractions. ○, N-acetylcysteine; ●, cysteine; □, monoacetylcystine; △, cysteinylglutathione; ▲, glutathione; ■, cystine.

Therefore, rats were pretreated with phenobarbital in order to increase the mixed function oxidase activity, and the isolated hepatocytes were pre-incubated with DEM to deplete the GSH levels. Furthermore, methionine sulfoximine (MSO), an inhibitor of GSH biosynthesis (14), was added to suppress the resynthesis of GSH from the given N-acetylcysteine. Results shown in Fig. 3 have demonstrated that hepatocytes isolated from the phenobarbital treated rats and pre-incubated with DEM could become necrotic when incubated with 5 mM acetaminophen. In the presence of MSO, however, hepatocellular necrosis occurred even faster. Results shown in Fig. 4 were obtained under the same experimental conditions as performed for Fig. 3 and they have demonstrate that when the biosynthesis of GSH was inhibited with MSO, cell viability decreased and less amount of acetaminophen-glutathione conjugates was formed (Fig. 4).

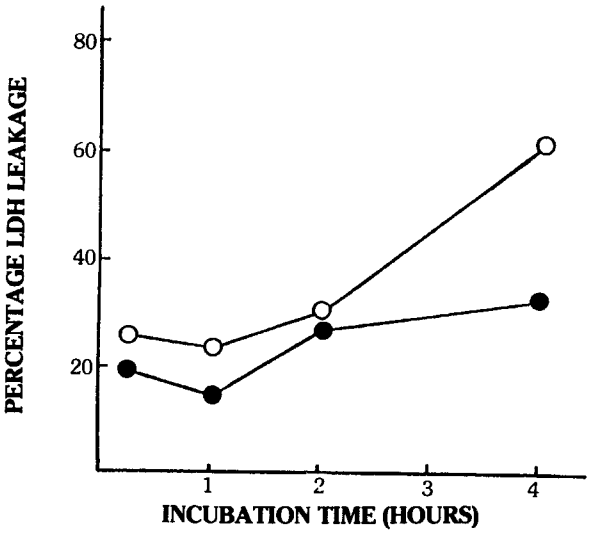

Fig. 3. Effect of methionine sulfoximine on the N-acetylcysteine derived protection against acetaminophen.: Rat hepatocytes (10^6 cells m*l*) were pre-incubated for 30 min with DEM (1 mM). then incubated with acetaminophen (5 mM) and N-acetylcysteine (1 mM), with or without the addition of methionine sulfoximine (10 mM). At the times indicated, aliquots were withdrawn for determination of cell viability, measured as percentage of cells which leaked lactate dehydrogenase. ●, DEM treated hepatocytes incubated with acetaminophen and N-acetylcysteine; ○, as above with the addition of MSO.

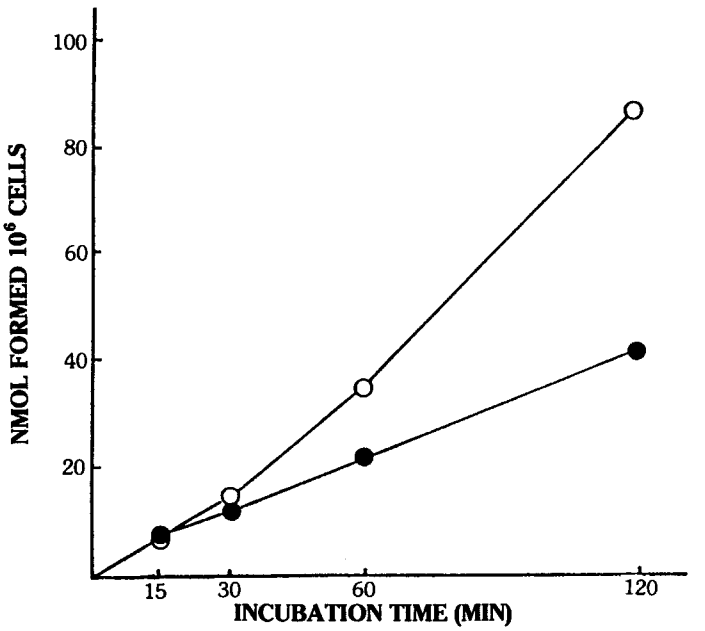

Fig. 4. Effect of methionine sulfoximine on the N-acetylcysteine derived formation of acetaminophen -glutathione conjugate.: Rat hepatocytes (10^6 cells/m*l*) were pre-incubated for 30 min with 1 mM DEM, then incubated with acetaminophen (5 mM) and N-acetylcysteine (1 mM), with and without the addition of MSO (10 mM). At the times indicated, aliquots were withdrawn for HPLC determination of the acetaminophen conjugates. ○, DEM treated hepatocytes incubated with acetaminophen and N-acetylcysteine; ●, as above with the addition of MSO.

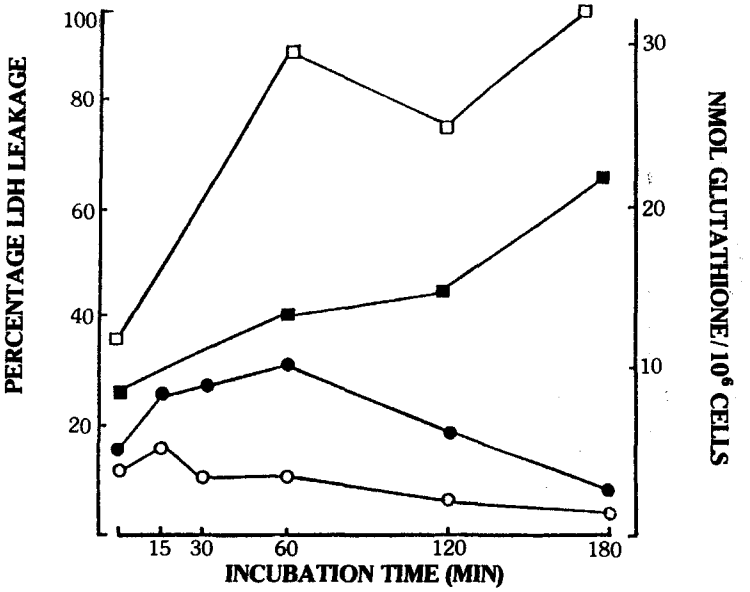

Fig. 5. Effect of paraquat on the loss of cell viability and depletion of glutathione.: Rat hepatocytes (10^6 cells/ml) were pre-incubated for 30 min with 1 mM DEM, then incubated with paraquat (2 mM). At the times indicated, aliquots were withdrawn for determinations of cell viability and intracellular GSH levels. ○, GSH concentration without N-acetylcysteine; ●, with the amino acid; □, cell viability without N-acetylcysteine; ■, with the amino acid.

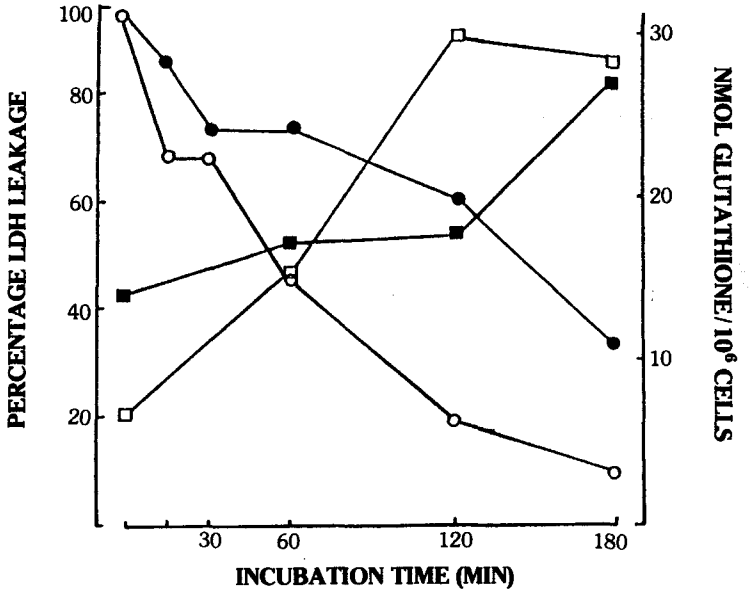

Fig. 6. Effect of carbontetrachloride on the loss of cell viability and depletion of glutathione.: Rat hepatocytes (10^6 cells/ml) were incubated with CC14 (5 mM), in the presence and absence of the added N-acetylcysteine (1 mM). At the times indicated, aliquots were withdrawn for determinations of cell viability and intracellular GSH levels. ○, GSH concentration without N-acetylcysteine; ●, with the amino acid; □, cell viability without N-acetylcysteine; ■, with the amino acid.

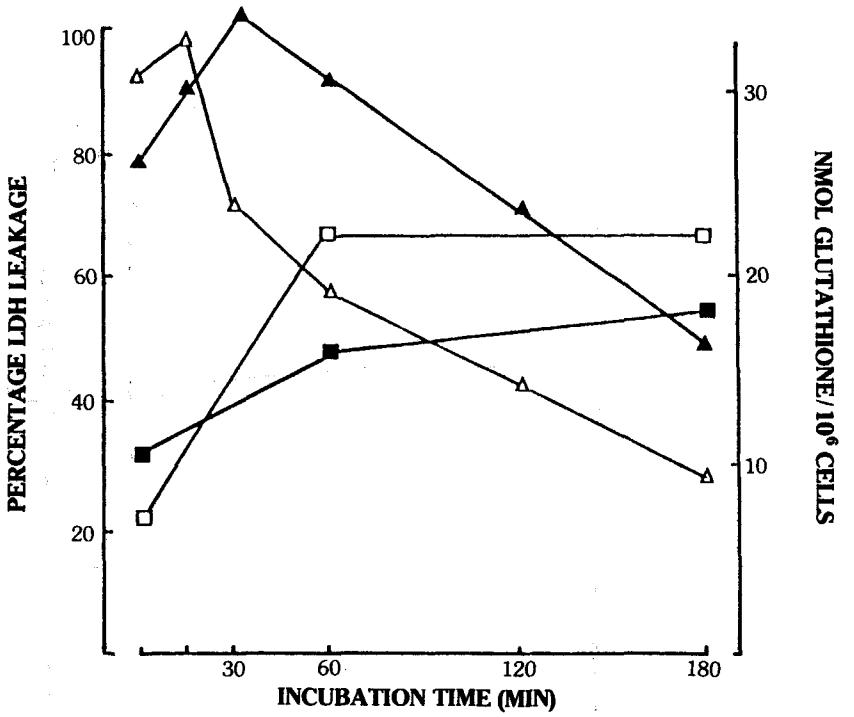

Fig. 7. Effect of chloroform on the loss of cell viability and depletion of glutatione.: Rat hepatocyted (10^6 cells1ml) were incubated with chloroform (5 mM), in the presence and absence of the added N-acetylcysteine (1 mM). At the times indicated, aliquots were withdrawn for determinations of cell viability and intracellular GSH levels. △, GSH concentration without N-acetylcysteine; ▲, with the amino acid; □, cell viability without N-acetylcysteine; ■, with the amino acid.

As with the acetaminophen, paraquat was also toxic to liver cells which have already been depleted of GSH. Cell viability decreased for the hepatocytes not supplemented with N-acetylcysteine and the GSH levels were decreased steadily (Fig. 5). For hepatocytes supplemented with the amino acid, cells can actively synthesize GSH and are protected from the paraquat toxicity for longer durations. Therefore, 1 mM N-acetylcysteine can provide some measure of protection against the paraquat inducible hepatocellular necrosis.

Carbontetrachloride (CCl4, tetrachloromethane) has long been known as a strong hepatotoxin. It depleted cellular GSH rapidly and caused necrosis even for the hepatocytes containing normal levels of GSH (Fig. 6). The presence of N-acetylcysteine in the incubation medium has decreased the rate of GSH depletion and of cell death. Chloroform (CCl3, trichloromethane) was also very toxic to hepatocytes and appeared to have similar necrotic effect as the CCl4. It depleted the cellular GSH rapidly and caused rapid cell death (Fig. 7). The presence of N-acetylcysteine in the incubation medium, however, retarded the rate of GSH depletion and helped maintain cells viable for longer durations.

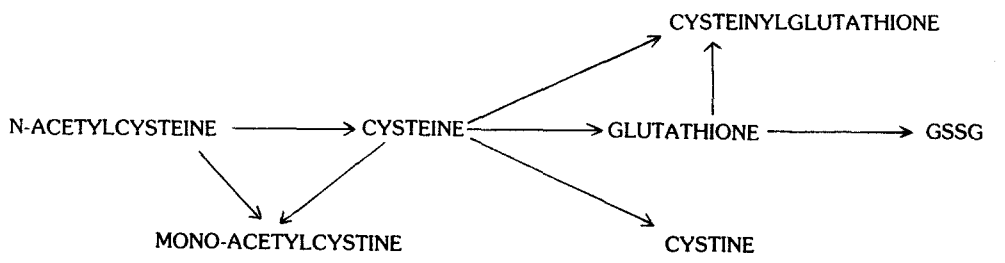

Fig. 8. Schematic diagram for the metabolism of N-acetylcysteine.

DISCUSSION

Freshly isolated rat hepatocytes were able to utilize the N-acetylcysteine added to the medium for biosynthesis of GSH and were able to maintain their intracellular GSH concentration. Following uptake (Fig. 1), the amino acid was quickly deacetylated and provided cysteine (Fig. 2). The initial level of cysteine in the freshly isolated hepatocytes was found to be low (5 nmol/10^6 cells) and this observation supported the previous report on the cysteine content of liver as being very low, about 0.2 to 0.3 mM (15). It has been known that hepatocytes can quickly oxidize the added extracellular cysteine to cystine and, as the exogenous cystine is not taken up, added cysteine is not useful for increasing the intracellular pool size of cysteine (16). In support of this, the depleted GSH from hepatocytes (e.g., upon incubation with DEM) could not be replenished by additions of cysteine to the medium (unpublished observation). Thus, it is of practical and physiological importance that the N-acetylcysteine could serve as a stable extracellular precursor for the intracellular cysteine, which is unstable in the plasma and is not useful for intracellular biosynthesis of GSH (10).

Upon incubation of the hepatocytes with N-acetylcysteine, the intracellular concentration of cysteine was increased by 3 to 4 fold initially and, with the onset of production of GSH and cystine, the cysteine concentration was then decreased. In the meantime, the concentration of mono-acetylcystine continued to increase steadily (Fig. 2). Based on such a sequential changes in the levels of these metabolites, a diagramatic metabolic scheme for the intracellular metabolism of N-acetylcysteine has been constructed (Fig. 8).

The presence of stable extracellular precursor (N-acetylcysteine) for intracellular biosynthesis of GSH in the incubation medium could facilitate the resynthesis of GSH and could protect the hepatocytes from drug induced necrosis (Figs. 5,6,7). Even though the rate of GSH biosynthesis from N-acetylcysteine appeared to be slow (Fig. 2), it was apparently sufficient to demonstrate a significant protection against CCl_4 and CCl_3 induced cell necrosis (Figs. 6,7). The protective effect against paraquat provided by the added amino acid was, however, demonstrable only after preincubation

of isolated cells with DEM for a prior depletion of a significant proportion of intracellular GSH (Fig. 5). Furthermore, the protective effect against the acetaminophen inducible toxicity provided by the added amino acid was lost by including methionine sulfoximine (MSO), a well-known inhibitor of GSH biosynthesis from cysteine (Fig. 3) (14).

Although N-acetylcysteine is known to form direct chemical conjugates with reactive electrophilic compounds *in vitro*, evidences obtained with acetaminophen (Ref. 13 and Fig. 4) have indicated that the N-acetylcysteine conjugate of the drug was not formed, or at least, it could not be detected. Instead, only the glutathione conjugate was formed in large amounts. In this experimental system, it appeared that the added N-acetylcysteine facilitated the biosynthesis of GSH, which in turn, formed the GSH-conjugate of the drug. In support of this, the obtained result (Fig. 4) also showed that when the biosynthesis of GSH from the added amino acid was suppressed with MSO, less of the GSH-acetaminophen conjugate was formed. This and other results obtained with acetaminophen (Fig. 4 and Ref. 18) supported the suggestion made from microsomal experiments (19), which indicated that the reactive metabolites of drugs produced intracellularly can interact only with GSH. Furthermore, within the cell, this reaction is mediated by the glutathione S-transferases, which is specific for GSH.

In summary, N-acetylcysteine is utilized by the liver cells to facilitate the GSH synthesis. This, in turn, protects hepatocytes from necrosis inducible with hepatotoxic drugs. In support of this, both cysteine and N-acetylcysteine have been used clinically to decrease the liver damage caused by accidental overdose of therapeutic drugs (20). However, the N-acetylcysteine has been reported to be more effective than the cysteine (21). This may be due to the fact that cysteine is unstable within gastro-intestinal tract or in plasma. Therefore, the N-acetylcysteine moiety cleaved from mercapturic acids by the intestinal digestive enzymes may eventually become reabsorbed by the liver cells via the enterohepatic recirculation *in vivo*. Thereupon, it is economically reutilized for restoration of GSH levels in the liver. This pathway may provide an efficient conservation mechanism for the physiologically important sulfur amino acids.

REFERENCES

1. Boyland, E. and Chasseaud, L.F. (1969): The role of glutathione and glutathione S-transferases in mercapturic acid biosynthesis. *Adv. Enzymol.*, 32: 173-219.
2. Chasseaud, L.F. (1973): The nature and distribution of enzymes catalyzing the conjugation of glutathione with foreign compounds. *Drug Metab. Rev.*, 2: 185-220.
3. Gillette, J.R., Mitchell, J.R. and Brodie, B.B. (1974): Biochemical basis for drug toxicity. *Ann. Rev. Pharmacol.*, 14: 271-288.
4. Thor, H., Moldeus, P., Kristoferson, A., Hogberg, J., Reed, D.J. and Orrenius, S. (1978): Metabolic activation and hepatotoxicity. Toxicity of bromobenzene in hepatocytes isolated from phenobarbital- and diethylmaleate- treated rats. *Arch. Biochem. Biophys.*, 188: 122-129.
5. Boyland, E. and Chasseaud, L.F. (1967): Enzyme-catalyzed conjugations of glu-

tathione with unsaturated compounds. *Biochem. J.*, 104: 95-102.
6. Chasseaud, L.F. (1973): Glutathione S-transferases. *Hoppe Seylers Z. Physiol. Chem.*, 354: 829-830.
7. Boyland, E. and Sims, P. (1962): The metabolism of phenanthrene in rabbits and rats: Mercapturic acids and related compounds. *Biochem. J.*, 84: 564-570.
8. Seglen, P.O. (1972): Preparation of rat liver cells. I. Effect of Ca^{++} on enzymatic dispersion of isolated perfused liver. *Exp. Cell Res.*, 74: 450-454.
9. Seglen, P.O. (1976): Incorporation of radioactive amino acids into protein in isolated rate hepatocytes. *Biochem. Biophys. Acta*, 442: 391-404.
10. Thor, J., Moldeus, P. and Orrenius, S. (1979): Metabolic activation and hepatotoxicity. Effect of cysteine, N-acetylcysteine, and methionine on glutathione biosynthesis and bromobenzene toxicity in isolated rat hepatocytes. *Arch. Biochem. Biophys.*, 192: 405-413.
11. Hissin, P.J. and Hilf, R. (1976): A fluorometric method for determination of oxidized and reduced glutathione in tissues. *Anal. Biochem.*, 74: 214-226.
12. Reed, D.J. and Orrenius, S. (1977): The role of methionine in glutathione biosynthesis by isolated hepatocytes. *Biochem. Biophys. Res. Commun.*, 77: 1257-1264.
13. Moldeus, P. (1978): Paracetamol metabolism and toxicity in isolated hepatocytes from rat and mouse. *Biochem. Pharmacol.* 27: 2859-2863.
14. Palekar, A.G., Tate, S.S. and Meister, A. (1975): Decrease in glutathione levels of kidney and liver after injection of methionine sulfoximine into rats. *Biochem. Biophys. Res. Commun.*, 62: 561-567.
15. Meister, A. and Tate, S.S. (1976): Glutathione and related gamma-glutamyl compounds: Biosynthesis and utilization. *Ann. Rev. Biochem.*, 45: 559-604.
16. Vina, J., Hems, R. and Krebs, H.A. (1978): Maintenance of glutathione content in isolated hepatocytes. *Biochem. J.*, 170: 627-630.
17. Kosower, E.M. (1976): Chemical properties of glutathione. In; Glutathione: Metabolism and function. Ed. by I.M. Arias and W.B. Jakoby; pp 1-15; Raven Press, N.Y.
18. Buckpitt, A.R., Rollins, D.E., Nelson, S.D., Franklin, R.B. and Mitchell, J.R. (1977): Quantitative determination of the glutathione, cysteine, and N-acetylcysteine conjugates of acetaminophen by high pressure liquid chromatography. *Anal. Biochem.*, 83: 168-177.
19. Mitchell, J.R., Jollow, D.J., Potter, W.Z., Gillette, J.R. and Brodie, B.B. (1973): Acetaminophen-induced hepatic necrosis IV. Protective role of glutathione. *J. Pharmacol. Exp. Ther.*, 187: 211-217.
20. Rumack, B.H., Peterson, R.C., Koch, G.G., and Amara, I.A. (1981): Acetaminophen overdose. *Arch. Intern. Med.*, 141: 380-385.
21. McLean, A.E.M. and Day, P.A. (1974): The effect of diet on the toxicity of paracetamol and safety of paracetamol-methionine mixtures. *Biochem. Pharmacol.*, 24: 37-42.