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Mechanisms of Carnitine-Mediated Modulation of Aflatoxin B_1-DNA Adduct Formation

A Dissertation

Presented for the

Doctor of Philosophy

Degree

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Ayub Mohd Yatim

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In memory of my dad,

Mohd Yatim Yaacob

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ABSTRACT

The main purpose of this study was to investigate the possible mechanism(s) of L-carnitine-mediated modulation of AFB_1-DNA adducts formation. The first part of the study investigated the effect of increased doses of carnitine on AFB_1 binding to macromolecules, AFB_1 uptake, activity of glutathione S-transferase (GST), and total glutathione concentrations in freshly isolated hepatocytes. Carnitine dose was significantly correlated with the formation of AFB_1-DNA adduct ($r = -0.68$; $p = 0.0002$), AFB_1-protein adduct ($r = 0.58$; $p = 0.017$), but not AFB_1-RNA adduct ($r = 0.14$; $p = 0.604$). There was no effect of carnitine on the uptake of AFB_1 by the hepatocytes or on the activity of GST. Carnitine protected AFB_1-induced reduction of total glutathione concentration in the hepatocytes, which may contribute to the overall protective effect of carnitine. The second part of the study investigated the effect of carnitine on the binding of microsomal-activated AFB_1 to exogenous DNA and protein. Carnitine significantly decreased the binding of activated AFB_1 to DNA. It is postulated that carnitine reduced this binding by inhibiting the AFB_1-epoxide formation and/or binding of AFB_1-epoxide to DNA. This effect of carnitine was not very specific because acetylcarnitine and γ-aminobutyric acid were equally effective in reducing AFB_1-DNA adduct formation; however, choline and glycine had no effect. Carnitine enhanced the binding of AFB_1 as well as microsomal-activated AFB_1 to rat plasma proteins and bovine serum albumin. This mode of carnitine actions is proposed to be another mechanism of carnitine-mediated reduction of AFB_1-DNA adduct formation.

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CHAPTER 1

Introduction

We have reported earlier that carnitine supplementation reduced aflatoxin B_1 (AFB_1)-DNA adducts formation in rat liver (Sachan & Yatim 1992). Since AFB_1-DNA binding is an important step for the carcinogenic effect of AFB_1, the attenuation of the binding by carnitine may decrease AFB_1-induced carcinogenesis. However, the mechanism by which carnitine modulates AFB_1-DNA binding is still unknown. Therefore, the main focus of this dissertation research is to explore the mechanism(s) for carnitine-mediated decrease in AFB_1-DNA adducts formation.

The in vitro experiments will be conducted employing freshly isolated liver cells (hepatocytes), microsomes, and other biomolecules instead of intact animals. Advantages of conducting an in vitro experiment are that the experimental conditions can be easily controlled and, more importantly, that the use of laboratory animals can be minimized. Furthermore, since the activation of AFB_1 is in the microsome and the GSH conjugation is in the cytosol, experimentation using hepatocytes offers special advantages, such as the ability to monitor both activation and detoxification pathways of AFB_1 could be monitored in the same preparation (Metcalfe and Neal 1983). Freshly isolated hepatocytes closely resembled the in vivo cells and their capacity for AFB_1 biotransformation and thus allows direct monitoring of intracellular DNA adduct production (Bailey et al. 1982). The concern, however, was whether the carnitine effects found in the intact animals could be

reproduced in this in vitro system. Therefore, the first objective was to study the effect of carnitine on the AFB_1-macromolecule adducts formation in the isolated hepatocytes. The results of these studies indicated that carnitine incubated in freshly isolated hepatocytes reduced AFB_1-DNA binding as we had found in intact animals (Sachan & Yatim 1992).

There are several possible mechanisms for carnitine inhibition of the AFB_1-DNA formation. Since carnitine has a positively charged N^+, an electrophilic center, AFB_1-epoxide may bind to carnitine as the epoxide binds to DNA at the N^7 position of guanine of DNA. As a result, the DNA may be spared from the electrophilic attack of AFB_1-epoxide.

Carnitine may also influence the activation and/or detoxification processes of AFB_1. AFB_1 is first metabolized (Phase I metabolism) by the cytochrome P-450 enzyme system found in the microsome. This metabolism will produce a variety of metabolites such as AFB_1-epoxide and hydroxylated metabolites (AFM_1, AFP_1, AFQ_1, and aflatoxicol). AFB_1-epoxide is a very reactive and unstable metabolite of AFB_1 that will bind to cellular DNA, RNA, and protein. The effect of carnitine on microsomal AFB_1 activation and on the binding of activated AFB_1 (AFB_1-epoxide) to exogenous DNA will be investigated.

Carnitine may affect the Phase II biotransformation enzymes that are involved in detoxification of many xenobiotics and reactive molecules. Glutathione-S-transferase (GST) is the main enzyme that catalyzes the conjugation of AFB_1-epoxide to glutathione, resulting in detoxicification of AFB_1-epoxide. Therefore, the influence of carnitine on GST activity and total glutathione content in the hepatocyte will be studied.

We have reported that rats supplemented with carnitine had a higher plasma concentration of AFB_1 than the unsupplemented animals (Sachan and Yatim 1992). Therefore, the effects of carnitine on AFB_1-protein binding will also be studied.

Also, AFB_1 toxicity and carcinogenicity to cells will not have occurred if AFB_1 cannot permeate the plasma and nuclear membranes. Therefore, the role of carnitine in the uptake of AFB_1 by the hepatocytes will be investigated.

It is important to know the specificity of carnitine effect since there are a number of carnitine analogs (acylcarnitine) and carnitine-like substances (choline, gamma amino butyric acid). The carnitine-like substances may also affect AFB_1-DNA adducts formation and biotransformation of AFB_1.

In summary, the questions that this research will address are:

1. Can the modulation of AFB_1-DNA adducts formation by carnitine in vivo be duplicated in vitro?
2. What is the effect of carnitine on AFB_1-calf thymus DNA binding mediated by microsome?
3. What is the effect of carnitine on GST activity and total glutathione concentration?
4. Does carnitine have an effect on cellular uptake of AFB_1?
5. Is the inhibitory effect of AFB_1-DNA binding specific for carnitine?

CHAPTER 2

Review of Literature

Aflatoxins

History

Aflatoxins are a group of mycotoxins produced by the molds *Aspergillus flavus* and *A. parasiticus*. They are commonly found to contaminate food and feeds, such as milk, corn, peanuts, cottonseed, rice, and barley, grain-fermented beverages and edible animal tissues (Park & Pohland 1986). The current technology and knowledge can neither totally inhibit aflatoxins synthesis by the molds nor completely eliminate them once they are produced. As a result, the significance of aflatoxins contamination has long been recognized, and limits in agricultural commodities have been set since 1965.

Aflatoxins were first discovered in 1960 when a series of outbreaks in poultry and fish occurred in different parts of the world. One of the worst outbreaks was the "Turkey-X" disease that caused the deaths of many turkies, ducklings, and chicks in Britain (Blount 1961). Consumption of aflatoxin-contaminated Brazilian groundnut meal was implicated in the disease. At the same time, feeding of contaminated corn, peas and cottonseed to farm animals and fish were reported to cause outbreaks analogous to the "Turkey-X" disease elsewhere (Palmgren & Ceigler 1983).

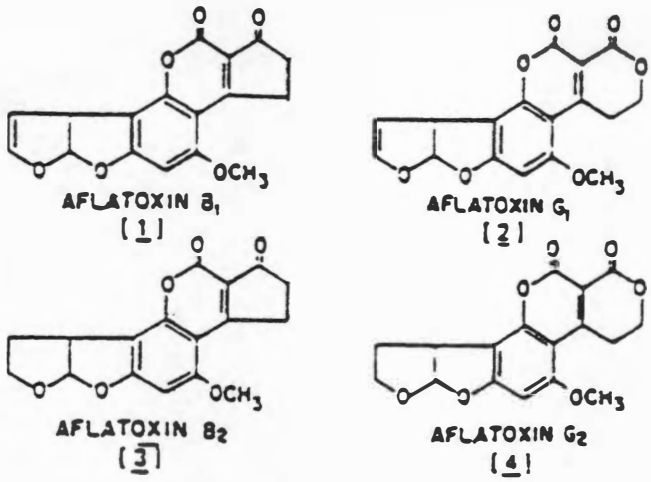
Experiments conducted on the contaminated Brazilian peanut meal resulted in the

isolation of *A. flavus*, and when the fungus was inoculated into untainted peanut meal, the fungus produced toxins similar to those found in the contaminated meal (Sargeant 1961). The isolated toxins were named "aflatoxin."

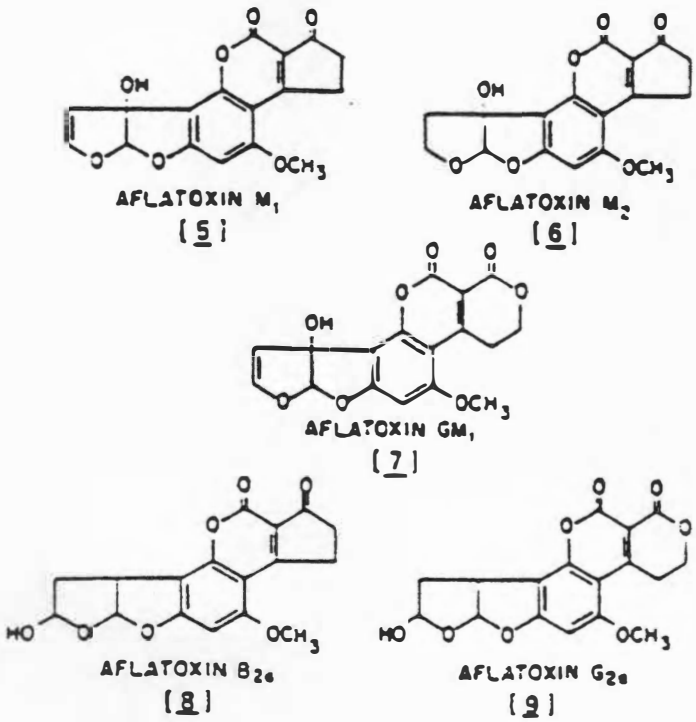
Structure and Toxicity

Aflatoxin B_1 (AFB_1), B_2 (AFB_2), G_1 (AFG_1), and G_2 (AFG_2) are the four main naturally occurring aflatoxins. The letters B and G refer to the aflatoxins' color under UV light (B for Blue; G for Green), and the subscripts 1 and 2 refer to their relative positions on a developed thin-layer chromatography plate. The structure of aflatoxins consists of a coumarin nucleus attached to a bifuran and either pentanone (AFB_1 and AFB_2) or a six-membered lactone (AFG_1 and AFG_2) (**Figure 2.1**). AFB_1 and AFG_1 are more toxic to rats and ducklings as compared to AFB_2 and AFG_2 (Wogan et al. 1971). As for carcinogenicity, AFB_1 is more carcinogenic than AFG_1, while AFG_1 is more carcinogenic than AFB_2 (Shoenhard et al. 1981).

The metabolites of aflatoxins produced in microbial or animal system include AFM_1, AFM_2, $AFGM_1$, AFB_{2a}, AFG_{2a}, AFP_1, AFQ_1, parasiticol and aflatoxicol. Although AFM_1 is less carcinogenic than AFB_1, their toxicities are about similar. AFG_{2a} (also identified as AFB_1 hemiacetal, aflatoxin W and hydroxydihydroaflatoxin) is relatively non-toxic (Palmgren & Ceigler 1983). Aflatoxicol is the degradation product of AFB_1 and is very toxic and carcinogenic to trout (Shoenhard et al. 1981). This is because aflatoxicol can be converted back to AFB_1 by reductase and dehydrogenase enzymes (Loveland et al. 1987). However, Gallagher & Eaton (1995) reported that the high rate of AFB_1



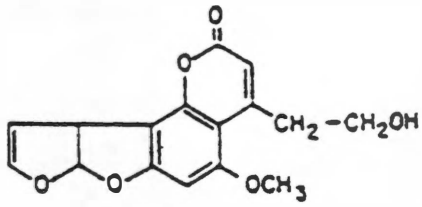
Structures of aflatoxins B_1, B_2, G_1, and G_2.



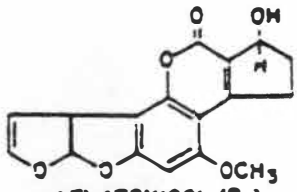
Hydroxylated aflatoxin derivatives.

FIGURE 2.1 Structure of aflatoxins.

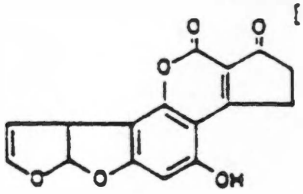
Source: Palmgreen et al. (1983). Aflatoxins. In: Handbook of Natural Toxins: Plant and Fungal Toxins (Keeler, R.F. & Tu, A.T., eds.), pp 299-323. Marcel Dekker, New York.



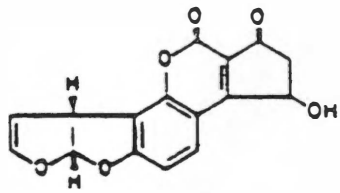
AFLATOXIN B_3
(Parasiticol)
[10]



AFLATOXICOL (R_0)
[11]

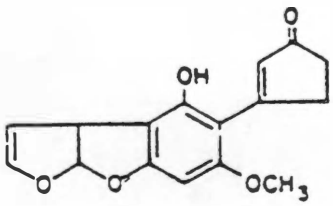


AFLATOXIN P_1
[12]

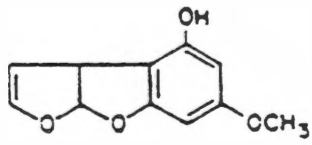


AFLATOXIN Q_1
[13]

Metabolized forms of aflatoxin B_1.



Compound D_1 (mol wt 286)
[14]



Compound D_2 (mol wt 206)
[15]

Degradation products of aflatoxin B_1.

FIGURE 2.1 (Continued).

biotransformation to aflatoxicol is one reason for Channel catfish being resistant to AFB_1 hepatocarcinogenesis. AFP_1 is about 15 times less toxic than AFB_1 (Buchi et al. 1973).

AFB_1 Toxicity and Carcinogenicity

AFB_1 is the most abundant and toxic form of all naturally occurring aflatoxins. It is hepatotoxic (O'Brien et al. 1983), hepatocarcinogenic (Adamson et al. 1976), and teratogenic (Bassir & Adekunle 1970) to various animal species. Rainbow trout (first) and rat (second) are the two animals most susceptible to the carcinogenic effects of AFB_1 as compared to other experimental animals (reviewed by Hendricks 1994). The Channel catfish is resistant to AFB_1 toxicity and hepatocarcinogenicity mainly because AFB_1 is not rapidly oxidized to form AFB_1-epoxide but instead is rapidly metabolized to aflatoxicol (Gallager & Eaton 1995). AFB_1 represents 75% of all aflatoxins found in contaminated food and feeds. The main target organs for AFB_1 toxic and carcinogenic effects are the liver and kidney.

Severe morbidity and deaths attributed to acute toxicity of AFB_1 have been well documented in laboratory (O'Brien et al. 1983) and farm animals (Palmgren & Ceigler 1983), and humans (Krishnamachari et al. 1975). The AFB_1-DNA adducts formation has a strong correlation to animal and human cancer cases (Thabrew & Bababumi 1980; Wogan et al. 1971; Alpert et al. 1971; Groopman et al. 1988). The "Virtually Safe Dose" of AFB_1 is estimated at 0.016 ng/kg/day (reviewed by Eaton & Gallagher 1994).

Hepatocellular carcinoma (HCC) is a major health problem in China where each year approximately 110,000 patients are diagnosed with it. The HCC cases in China

account for almost 45% of HCC incidences in the world (Parkin et al. 1984). The mortality rate for HCC is more than 95%. Excluding other risk factors, the consumption of AFB_1-contaminated food such as corn, soya-based products, and peanut oil was correlated ($r = 0.55$) to the HCC fatality rates in people living in ten Chinese villages that were studied. See Yu (1995) for a current review of HCC in China.

AFB_1 Absorption

The absorption of AFB_1 is quite efficient and fast as it has a low molecular weight and is lipophilic (Hsieh & Wong 1994). AFB_1 is absorbed in the small intestine, and the duodenum is the most efficient site (Kumagai 1989). Since the absorption is roughly proportional to AFB_1 concentration, this may indicate a passive absorption of AFB_1. Once absorbed, AFB_1 goes into the mesenteric venous blood, into the hepatic portal vein, and finally into the liver. In an experiment involving ligated small intestines, the absorbed AFB_1 found in the mesenteric venous blood was in the form of free AFB_1, protein bound, and water-soluble metabolites (Hsieh & Wong 1994). At 65 min post-AFB_1 treatment, 45% of the AFB_1 dose was found in the ligated intestines, and 55% was found in the whole blood. About 32% of the dose present in the small intestines was bound to protein, and the rest was as free AFB_1, and water-soluble and organic-soluble metabolites (**Figure 2.2**).

AFB_1 Metabolism

Similar to other xenobiotics (foreign substances), AFB_1 undergoes phase I and

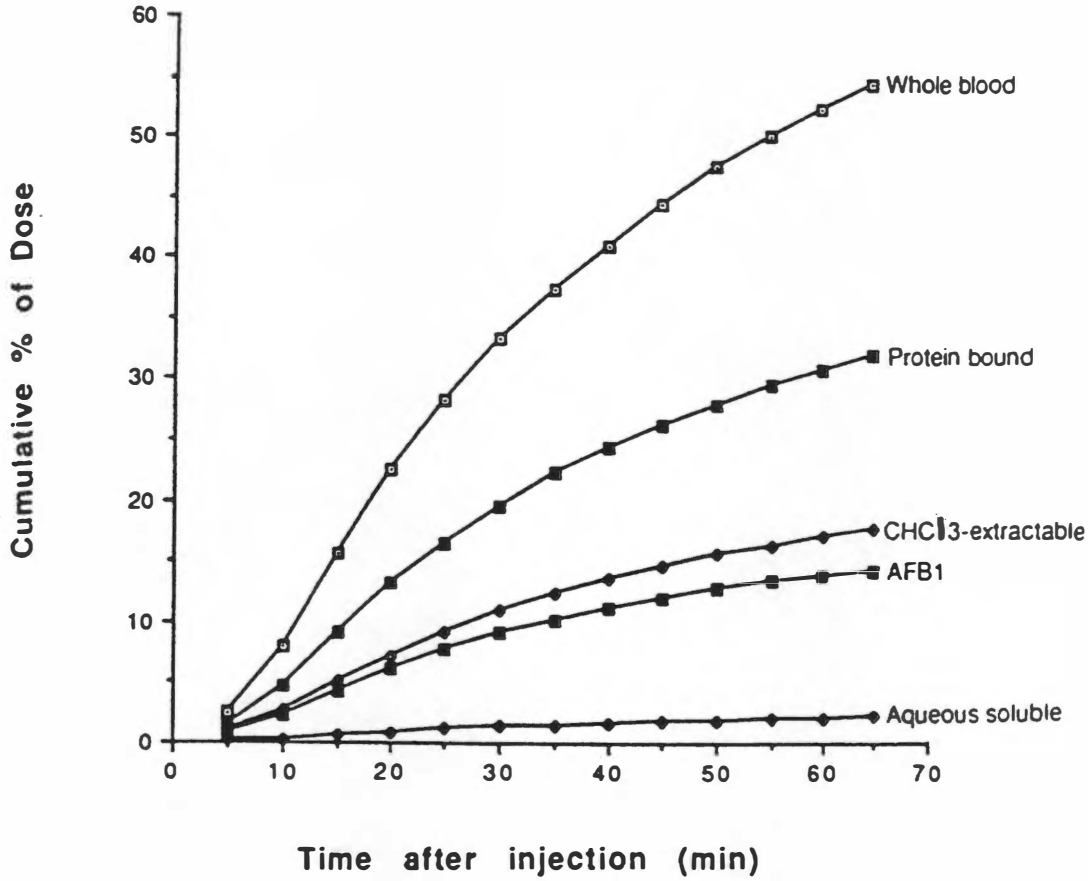


FIGURE 2.2 The percentage of [^{14}C]AFB$_1$ dose absorbed from ligated duodenum of rats.

Source: Hseih, D.P.H. & Wong, J.J. (1994) Pharmacokinetics and excretion of aflatoxins. In: The Toxicology of Aflatoxins: Human Health, Veterinary and Agricultural Significance (Eaton, D. L. & Groopman, J. D., eds.), pp. 73-88. Academic Press, New York, NY.

phase II biotransformations or metabolisms (reviewed by Eaton et al. 1994). The metabolism in vivo or in vitro may results in several different derivatives of the mycotoxin. The liver and kidney are the two main organs where AFB_1 is metabolized. In vitro, AFB_1 undergoes at least five different types of metabolic reactions with liver enzyme preparations, which are hydroxylation, hydration, reduction, O-demethylation, and epoxidation (**Figure 2.3**). During the phase I biotransformation, depending on the pathways, AFB_1 can either be activated or transformed into less toxic compounds. Phase II pathways involve different transferase enzymes that catalyze the detoxification (conjugation) reactions of the Phase I metabolites. Species differences, health status, nutritional manipulations, drugs, and chemicals treatments affect the biotransformations, and thus, the potency of AFB_1.

Phase I metabolism

AFB_1 has to be activated for it to possess toxic and carcinogenic effects. The phase I biotransformation pathways of AFB_1 with microsomal cytocrome P450 (CYP) system produced metabolites, such as the reactive AFB_1-8,9-epoxide, the more polar (hydroxylated) AFM_1 and AFQ_1, the O-demethylation AFP_1, and the reduced aflatoxicol. The biotransformation rates of AFB_1 to AFM_1, AFP_1, and AFQ_1 in human liver slices were comparable to those found in rat liver slices. Nevertheless, the production of AFB_1-epoxide and bindings of AFB_1 to cellular DNA, RNA, and protein were much lower in human liver slices than in rat liver samples (Heinonen et al. 1996). The AFB_1-8,9-epoxide may covalently bind to cellular DNA and other macromolecules, form AFB_1-glutathione

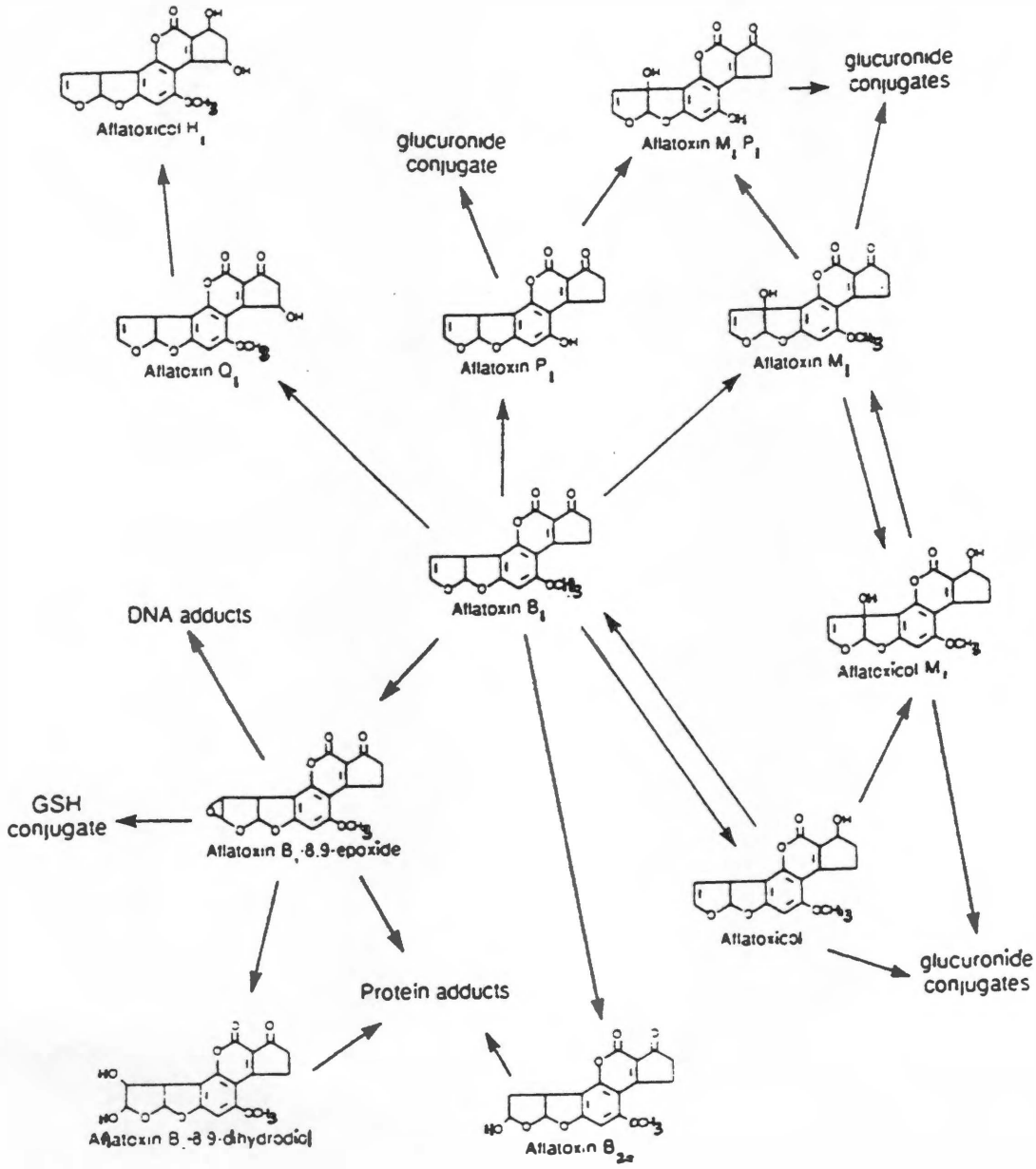


FIGURE 2.3 Metabolism of AFB_1.

Source: Eaton et al. (1994) Biotransformation of aflatoxins. In: The Toxicology of Aflatoxins: Human Health, Veterinary and Agricultural Significance (Eaton, D.L. & Groopman, J.D., eds.), pp. 45-72. Academic Press, New York, NY.

conjugate, hydrolyze to form AFB_1-8, 9- dihydrodiol, and/or reduced to a hemiacetal form. In rats, CYP1A2, CYP2C11, and CYP3A4 isoforms or genes have been found to activate AFB_1. Whereas in human, CYP1A2, 2A6, 2B7, 3A3 and 3A4 are involved in AFB_1-epoxide production (Forrester et al 1990; Eaton & Gallagher 1994; Eaton et al. 1994).

The formation of AFB_1-DNA adduct is highly correlated to the carcinogenic effect of AFB_1 in both in vivo and in vitro studies (Dragan & Pitot 1994,). The major AFB_1-DNA adduct formed with liver DNA is aflatoxin B_1-N^7-guanine (AFB_1-N^7-gua) (Essigmann et al. 1977). This adduct is unstable and subjected to decomposition. The major decomposed derivatives of AFB_1-N^7-gua in rat liver are the imadozole ring-opened AFB_1 formamido-pyrimidine adducts, namely AFB_1-N^7-FAPY major and minor (Croy & Wogan 1981). AFB_1-N^7-FAPY adducts are more stable, and their accumulation in liver DNA is related to the subsequent reduction of AFB_1-N^7-gua adduct level.

AFB_1 can also be activated by other reactions not involving cytochrome P450 system. Prostaglandin H synthase (Liu et al. 1990), lipoxygenase (Liu & Massey 1992), and UV light (Stark et al. 1990) have been shown to activate AFB_1.

Phase II metabolism

The phase I AFB_1 metabolites may undergo phase II biotransformation involving the enzymes glutathione S-transferase (GST), β-glucuronidase, and/or sulfate transferase which produce conjugates of AFB_1-glutathione, AFB_1-glucuronide, and AFB_1-sulfate,

respectively. The major conjugate of AFB_1-epoxide identified is the AFB_1-glutathione conjugate (Monroe & Eaton 1987; O'Brien et al. 1983). This conjugation is the principal detoxification pathway of activated AFB_1 in many mammals (**Figure 2.4**). It has been accepted that cytosolic GST activity is inversely correlated to susceptibility of the several animal species to AFB_1 carcinogenicity (Eaton & Gallager 1994, Neal 1987).

The hydoxylated (AFM_1 and AFQ_1) and O-demethylated (AFP_1) metabolites of AFB_1 can undergo glucuronidation and sulfation. Glucuronidation, catalyzed by liver microsomal UDP-glucuronyl transferase (UDPGT), has been reported for a variety of endogenous and foreign compounds (Burchell & Coughtrie 1989). In rat hepatocytes, AFP_1 is a better substrate for glucuronide conjugation than AFM_1 and AFQ_1 (Metcalfe & Neal 1983). AFP_1-glucuronide conjugate is the only significant glucuronide or sulfate conjugate other than the other two AFB_1 metabolites (AFM_1, and AFQ_1) in rat liver cells (Ch'ih et al. 1983). These conjugations results in formations of water-soluble aflatoxin esters that are excreted in the urine or bile (Hseih & Wong 1982). Refer to **Figure 2.5** for an overview of AFB_1 metabolism pathways that lead to its toxic and carcinogenic effects.

AFB_1 Metabolism in the Small Intestine and Blood, and Binding to Proteins

Although the major sites for AFB_1 metabolism are in the liver and kidney, there are suggestions that some biotransformation may also occur in the small intestine. (Hartiala 1977; Vainio & Hietanen 1979). The intestinal mucosa contains cytochrome P450 enzymes and has been suggested to be the site for the metabolism of AFB_1. The intestinal

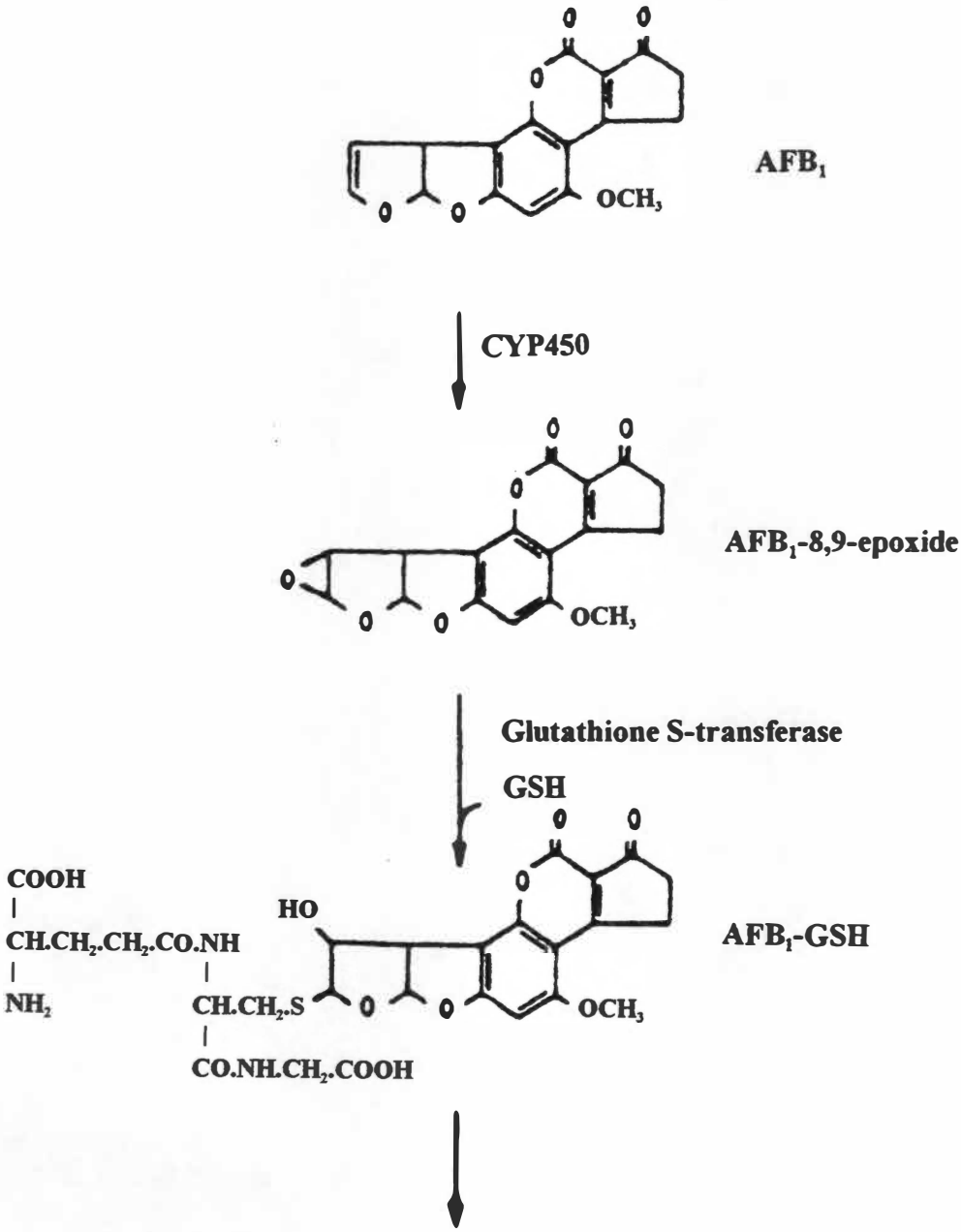


FIGURE 2.4 AFB_1-glutathione conjugation pathway.

Source: Neal, G.E. (1987) Influences of metabolism: aflatoxin metabolism and its possible relationship with disease. In : Natural Toxicants in Food: Progress and Prospects (Watson, D.H., ed.), pp. 225-168. VCH Publishers, New York, NY.

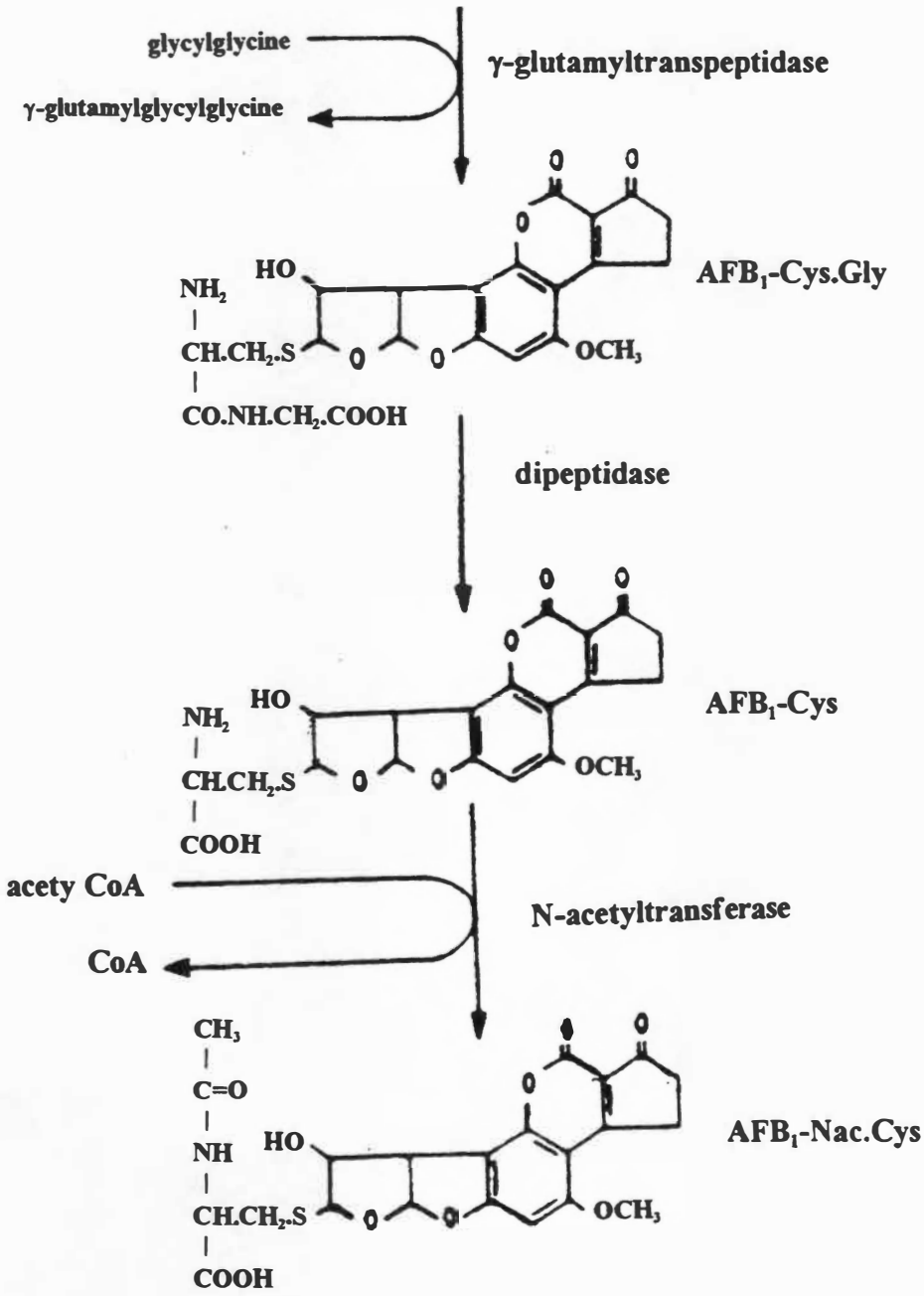


FIGURE 2.4 (Continued).

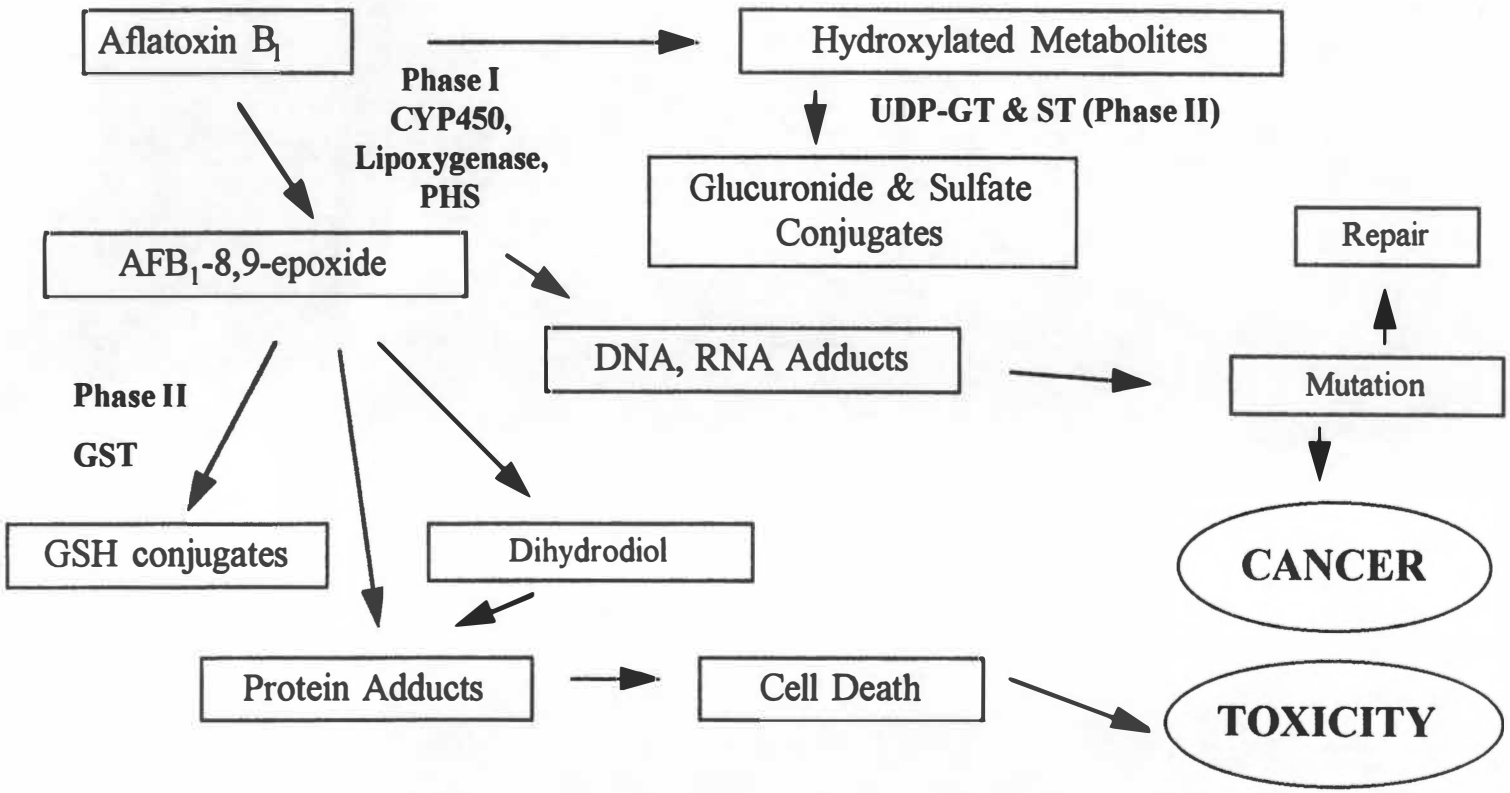


FIGURE 2.5 Overview of aflatoxin B_1 biotranformational pathways that lead to toxicity and carcinogenicity.

Source: Eaton, D.L. & Gallagher, E.P. (1994) Mechanisms of aflatoxin carcinogenicity. Ann.Rev. Pharmacol. 34:135-172.

metabolism produces metabolites, such as AFB_1-epoxide, AFB_{2a}, and AFB_1 dihydrodiol, that may bind covalently to proteins.

It has also been suggested that there is some detoxification of AFB_1 metabolites in the gut by the phase II detoxification enzymes (Hsieh & Wong 1994). This suggestion was made due to the absence of organic-extractable AFB_1 metabolites in the blood. The authors concluded that since the liver has high capability to draw free AFB_1 from the blood, AFB_1 binding to serum albumin after absorption from the intestine could be one of the major detoxification processes of AFB_1. At low doses of AFB_1, they found no free AFB_1 in the blood. This is because the absorbed AFB_1 readily binds to albumin. Albumin serves as the major transporter of AFB_1 in the blood since very little AFB_1 was bound to other blood proteins (Dirr & Scabort 1987). Because of the high levels of accumulation and occurrence, serum albumin adduct is currently used as a biomarker for AFB_1 exposure in humans (Chang et al. 1994).

The presence of AFB_1-albumin adducts in serum (Sabbioni et al. 1987) indicates that AFB_1 is metabolized either in the intestine or in blood compartments. Blood platelets contain cytochrome P450, prostaglandin synthase (Hodgston & Levi 1994) and lipoxygenase (Liu & Massey 1992), which are capable of activating AFB_1. Therefore, it may be that these enzymes catalyze the formation of AFB_1-epoxide found in the blood, and that epoxide then binds to albumin. However, another study reported that a very high percentage of AFB_1 bound to rat plasma albumin noncovalently (more than 95%) (Ewaskiewicz et al. 1991). This indicates that AFB_1 does not have to be metabolized first for it to bind to albumin and that albumin serves as major transporter of AFB_1 in the blood

to the liver.

Hirano et al. (1994) studied the probable role of bovine serum albumin (BSA) in inhibiting the toxicity and absorption of AFB_1 in one-day-old chicks. The BSA was given intragastrically prior to AFB_1 administration. BSA treated chicks showed less AFB_1-induced liver damages such as bile duct proliferation and necrosis than did the non-BSA treated animals. AFB_1 concentrations in the liver and plasma were also lowered in the BSA treated animals 6-h post-AFB_1 treatment, at which time the AFB_1 level was at the maximum. These phenomena might be due to an increased binding of AFB_1 to BSA in the intestine. Since macromolecules (which include AFB_1-BSA adduct) cannot be absorbed from the small intestine of a one-day-old chick, it was postulated that the BSA adduct was rapidly excreted without absorption.

AFB_1 and Diet Interactions

There are many reports on the effects of various food or nutrients and xenobiotics on AFB_1-macromolecule adducts formation. Obviously, the major objectives of these studies have been to determine if and how those nutrients or xenobiotics could affect adducts formation, especially DNA adduct (see **Tables 2.1** and **2.2**).

Fat-soluble vitamins

A number of vitamins and vitamin analogs have been tested on AFB_1-macromolecule adduct formation. Bhattacharya et al. (1984; 1987) reported comprehensive studies on the effects of various vitamins on in vitro adducts formation.

TABLE 2.1

Influences of dietary nutrients on AFB_1-DNA adducts formation.

Compound*	Test System	Increase (↑)/Decrease (↓)	Reference
Low Protein	Rat liver	↑	Mandell et al. (1992)
Low fat (high carbohydrate)	Rat liver	↑	Nyathi et al. (1993)
Fat (Saturated or unsaturated)	Rat liver	NSE*	Marzuki & Norred (1984)
Essential oils	Rat liver microsome	↓	Hashim et al. (1994)
Vitamin A	Rat liver	↓	Bhattacharya et al. (1989)
	Rat liver	NSE	Chen et al. (1982)
	Woodchuck hepatocyte	↓	Yu et al. (1994)
β-carotene	Woodchuck hepatocyte	↑	Yu et al. (1994)
Vitamine E	Woodchuck hepatocyte	↑	Yu et al. (1994)
Riboflavin	Rat liver	↓	Webster et al. (1996)
Vitamin B_6	Rat liver microsome	NSE	Bhattacharya et al. (1984)
Thiamin (1984)	Rat liver microsome	NSE	Bhattacharya et al.
Vitamin C	Woodchuck hepatocyte	↓	Yu et al. (1994)
Lipotropes (deficient)	Rat liver	↓	Campbell et al. (1978)

Continued

TABLE 2.1: *Continued*

Compound*	Test System	Increase (↑)/Decrease (↓)	Reference
Carnitine	Rat liver	↓	Sachan & Yatim (1992)
Choline (deficient)	Rat liver		
	- Single AFB_1 dose	NSE	Schrager et al. (1990)
	- Multiple AFB_1 doses	↑	Schrager et al. (1990)
Copper	Rat liver microsome	↓	Bhattacharya et al. (1984)
Selenium (Excess or deficient)	Rat liver	↓	Chen et al. (1982)
Selenium	Chick liver	NSE	Chen et al. (1982)
Selenium	Hamster ovary cells	NSE	Shi et al. (1995)
Feed restriction	Rat liver	↓	Pegram et al. (1989) Gao & Chou (1992) Chen et al. (1995)
Indole-3-carbinol	Trout liver	↓	Dashwood et al. (1989)
	Trout liver microsome	↓	Takahasi et al (1995)
R-goitrin	Rat liver	↓	Chang & Bjeldanes (1987)
Curcumin	Rat liver microsome	↓	Firozi et al. (1996)

*NSE, no significant effect

TABLE 2.2

Influences of xenobiotics on AFB_1-DNA adducts formation.

Compound*	Test System	Increase (↑)/Decrease (↓)	Reference
BHA	Rat liver microsome	↓	Bhattacharya et al. (1984)
	Rat liver	↓	Chang & Bjeldanes (1987)
	Trout liver	NSE**	Goeger et al. (1988)
	Rat and mouse livers	↓	Monroe & (Eaton 1987)
BHT	Rat liver microsome	↓	Bhattacharya et al. (1984)
Cortisol	Rat liver	↑	Chentanez et al. (1988)
Crocetin	Fibroblast cell	↓	Wang et al. (1991a)
DDB	Rat liver	↓	Liu et al. (1995)
Ethoxyquin	Rat liver	↓	Kensler et al. (1986)
Ethanol	Rat liver	↓	Toskulkoa & Glinsukon (1986)
Geniposide	Rat Liver	↓	Wang et al. (1991b)
	Rat liver microsome	↓	Wang et al. (1992)
Phenobarbital	Rat liver	↓	Lotlikar et al. 1989)

*BHA, butylated hydroxyanisole; BHT, butylated hydroxytoluene; DDB; dimethyl-4,4' dimethoxy-5,6,5'6'-dimethylenedioxy biphenyl-2,2' dicarboxylate.

**NSE, no significant effect

Vitamin A supplemention in rats inhibited AFB_1-DNA binding (Bhattacharya et al 1989). The protective effects of retinoids such as retinol, retinal, retinoic acid, and retinal esters on AFB_1 carcinogenicity were due to inhibition of AFB_1-DNA adduct formation by affecting the CYP450 systems resulting in less epoxide being formed (Bhattacharya et al. 1984). Retinal had the same inhibitory effect on the formation of AFB_1-protein adducts (Bhattacharya et al. 1989). Vitamin A has been shown to induce the activity of glutathione S-transferase, thereby enhancing the detoxification of AFB_1-epoxide (Bhattacharya et al. 1989). On the other hand, vitamin A deficiency decreased glutathione S-transferase activity.

However, another study reported that a vitamin A supplement had no significant effect on AFB_1-macromolecules binding in rats. On the contrary, a combined deficiency of vitamin E and selenium decreased AFB_1 binding to DNA, RNA, and protein (Chen et al. 1982). Vitamin E and menadione (a water-soluble synthetic vitamin K) have been found to prevent AFB_1-induced mutagenesis in the Ames bacterial system (Raina & Gurto 1985). β-carotene and vitamin E increased DNA adduct formation in woodchuck hepatocytes (Yu et al. 1994).

Water-soluble vitamins

Riboflavin, riboflavin-5'-phosphate (FMN), and flavin adenine dinucleotide (FAD) inhibited AFB_1-DNA adduct formation in vitro. Riboflavin was reported as the most effective of the three vitamins (Bhattacharya et al. 1984). It has been recently suggested that the mechanism for the riboflavin effect is its ability to induce the enzymes involved in

repairing damaged DNA (Webster et al. 1996).

Vitamin C, vitamin B_6 and thiamin had no significant effect on DNA adduct production (Bhattacharya et al. 1984). However, vitamin C, B_6, and folic acid inhibited mutagenesis in bacterial systems (Bhattacharya et al. 1984; Bhattacharya et al. 1987). The inhibition by vitamin C was not as great as with the fat-soluble vitamins.

In a study employing woodchuck hepatocytes to find the role of vitamins A, C, and E, and β-carotene on the initiation of AFB_1-induced carcinogenesis, the workers found that vitamin A was more effective than vitamin C in inhibiting DNA adduct formation. In contrast, vitamin E and β-carotene enhanced the binding (Yu et al. 1994). Therefore, these results suggest that different antioxidant vitamins may effect AFB_1-DNA binding differently.

Amino acids

There are conflicting reports on the effects of different amino acids on AFB_1 carcinogenesis. A diet marginally deficient in methionine (which was also deficient in choline and lacking in folacin) depressed DNA and RNA adducts formation in rat liver. Protein adduct formation was not affected by the diet. The inhibition of AFB_1-nucleic acid adducts in the marginally lipotrope-deficient diet was due to the decrease in the activation of AFB_1 and not due to an increase in glutathione levels (Campbell et al. 1978). A choline and methionine-deficient diet fed rats showed no significant AFB_1 dose-response changes in serum biochemical parameters or liver pathology compared to the complete amino acid diet (Mehta et al. 1993). These two studies treated the rats with a single dose of AFB_1.

Schrager et al. (1990) found that a single dose of AFB_1 did not affect the DNA adduct concentration in both choline-deficient and control animals. However, when multiple doses of AFB_1 were administered, the AFB_1-DNA adduct levels were significantly higher in the rats fed a choline-deficient diet than in the rats fed with a control diet. An earlier report also found that marginally deficient lipotrope diets induced AFB_1 tumorigenesis in rats (Rogers & Newberne 1969). L-carnitine supplementation in rats has been found to decrease AFB_1-DNA binding (Sachan & Yatim 1992).

Sulfur-containing amino-acids such as cysteine, N-acetylcysteine, cystine, methionine, and glutathione inhibited AFB_1 mutagenicity in microbial systems. Cysteine and N-acetylcysteine were more potent inhibitors than glutathione. The investigators suggested that the inhibition was due to amino acids affecting the synthesis of AFB_1-epoxide (Shetty et al. 1989).

Protein

Weanling rats fed a low protein diet (5% casein) had fewer AFB_1-induced preneoplastic foci in their livers than rats fed a high protein diet (20% casein) (Youngman & Campbell 1992). The enhanced development of the preneoplastic foci (γ-glutamyl transpeptidase-positive foci) by the high protein diet was reversed when the animals were put on a low protein diet. Thus, it was concluded that the low protein diet prevented lesions caused by AFB_1.

Mandell et al. (1992) reported that AFB_1-induced hepatocarcinogenicity can occur in both low- and high-protein fed weanling animals. Moreover, the low protein diet (5%

lactalbumin) also caused severe liver histopathological changes or sub-acute toxicity symptoms such as necrosis and bile duct proliferation due to AFB_1. Also, the protein-deficient animals had a more rapid decrease in glutathione S-transferase activity than the protein-sufficient (20% lactalbumin) animals. High protein fed animals did not show the sub-acute toxicity responses induced by AFB_1. Therefore, the authors suggested that protein deficiency is more likely to enhance, rather than protect the liver against, AFB_1 toxicity and carcinogenicity.

Fat and essential oils

Different types and amounts of fat may have different effects on the carcinogenesis of AFB_1. High polyunsaturated oil (corn oil) increased the incidence of liver cancer in rats caused by AFB_1 as compared to rats fed with saturated oil (beef fat). The cancer incidence was higher when the corn oil was fed with or after the exposure to AFB_1 than when the oil was fed before the AFB_1 dose. An increase in induction of AFB_1 activation by corn oil was suggested for the high cancer incidence (Newberne et al. 1979). However, in a similar study, saturated (coconut oil) and unsaturated dietary (corn oil) fats were found to have no significant effect on the adduct formation and the production of AFB_1-epoxide in rat livers (Marzuki & Norred 1984).

In another report, the metabolism and mutagenicity of AFB_1 were not significantly different between mice fed with beef fat and olive oil diets (Brennan-Craddock et al. 1990). With respect to the levels of dietary fat, a low-fat (high carbohydrate) diet increased the AFB_1-DNA binding more than a high fat diet (Nyathi et al. 1993). The

protective effect of a high fat diet may be due to a decrease in the uptake of AFB_1 into hepatocytes or a reduction of AFB_1-epoxide production.

Hashim et al. (1994) investigated the capability of essential oils extracted from nutmeg, ginger, cardamom, celery, xanthoxylum, coriander, cumin, and black pepper to inhibit AFB_1-DNA adducts formation mediated by liver microsomal enzymes. All the essential oils tested were suppressive to the adducts formation, and the inhibition was dose-dependent. The modulating effect of these oils was through their ability to inhibit the activation of AFB_1.

The presence of long chain fatty acids at their physiological concentrations increased the strength of AFB_1-albumin binding considerably (Dirr 1987). The binding affinity (ka) was increased by 3-fold when the pH was raised from 6 to 9. The presence of palmitic, stearic, and oleic acids (the most abundant plasma fatty acids) at the concentration range of 0.5 to 2.0 mol/mol albumin, further increased the albumin binding of AFB_1 to greater than 4 times at pH 7.4 (Dirr 1987).

Trace elements

Copper inhibited AFB_1-DNA binding in vitro (Bhattacharya et al. 1984). A deficiency and an excess of selenium decreased the adduct formation in rats. In chicks, however, excess of selenium did not change the concentration of adducts formed (Chen et al. 1982). Recently, selenium was demonstrated to have no effect on DNA adduct formation in ovary cells and did not effect AFB_1 mutagenesis (Shi et al. 1995).

Copper, manganese, zinc, and selenium were effective in preventing in vitro AFB_1-

induced mutagenesis. Copper was the most potent among the elements tested. To a lesser extent, iodine, molybdenum, cobalt, and iron were antimutagenic. The investigators suggested that the inhibition was due to interaction of trace elements with the microsomal enzymes (Francis et al. 1988).

Feed restriction

The potentially protective effects of caloric restriction on cancer-causing compounds has promoted considerable interest and investigation. It has been reported that rats fed with 60% of the food consumed by ad libitum animals had lower AFB_1 microsomal activation, lower AFB_1-adducts, faster plasma clearance, and increased urinary excretion of AFB_1 than the ad libitum fed animals. The authors concluded that 40% feed restriction may decrease AFB_1 carcinogenicity (Pegram et al. 1989).

Similarly, about more than 50% reduction in AFB_1-DNA binding was found when rats were fed 40% caloric restricted diet (Gao & Chou 1992). The restriction also decreased the hepatic DNA double strand damage induced by AFB_1. Induction in glutathione S-transferase activity in feed restriction will enhance AFB_1-epoxide conjugation to GSH and thus reduce AFB_1-DNA adducts formation (Chen et al. 1995).

Cruciferous vegetables

Cruciferous vegetables have been shown to enhance detoxification of xenobiotics by inducing xenobiotic-metabolizing enzymes in animals and humans (Salbe & Bjeldanes 1989). High consumption of vegetables such as broccoli, cabbage, cauliflower, and

Brussels sprouts has been related to a reduced risk of bladder, colon, and rectum cancers (Grahams 1983).

Brussels sprouts significantly decreased AFB_1-DNA binding and increased the GST activity in rats. Indole-3-carbinol, a compound found in cruciferous vegetables, did not have much effect on the DNA binding and GST activity (Salbe & Bjeldanes 1989). The same investigators also found that the route of administration, intragastric or intraperitoneal, did not have a different effect on AFB_1-DNA binding. Thus, they concluded that the small intestine did not play an important role in AFB_1 metabolism. However, in another study that utilized trout, 1000 and 2000 ppm of indole-3-carbinol were shown to strongly depress AFB_1-DNA adducts formation (Dashwood et al. 1989). R-goitrin, another compound found in cruciferous vegetables, also exhibited anticarcinogenic properties such as inhibition of AFB_1-DNA binding, induction of GST activity, and enhancement of biliary excretion of AFB_1 in rats (Chang & Bjeldanes 1987).

Plant flavonoids and phenolic compounds

Five major derivatives of plant flavonoids, namely flavone, flavonol, isoflavone, and flavonol, have been tested on activation of AFB_1 and AFB_1-DNA adducts formation (Bhattacharya & Firozi 1988). Most of the flavonoid derivatives significantly inhibited adduct formation, and flavonols being the most potent. Flavonols also showed greater inhibition of AFB_1 mutagenicity in bacterial system (Goeger et al. 1988).

Phenolic compounds may have protective effects against AFB_1-induced mutagenicity. Gallic acid, chlorogenic acid, caffeic acid, dopamine, p-hydroxybenzoic

acid, and salicyclic acid decreased mutation caused by AFB_1 in bacterial system containing rat-liver microsomes. The inhibition occurred when the compounds and AFB_1 were administered concurrently (San & Chan 1987). Using the S9 liver fraction that contains the metabolic enzymes, ellagic acid (a compound found in strawberries, grapes, and walnuts) has been shown to be antimutagenic against AFB_1 in bacterial assay (Loarca-Pina et al. 1996). The inhibition was greatest when the acid was incubated together with AFB_1.

Curcumin, a phenolic compound extracted from tumeric, was recently reported to inhibit the production of AFB_1-epoxide by affecting CYP enzyme function (Firozi et al. 1996). The inhibition became higher as the curcumin concentration was increased in the incubation mixture. However, the suppression was reversed when the CYP level in the mixture was higher.

AFB_1 and Drugs/Xenobiotics Interactions

Antioxidants

Rats fed a butylated hydroxyanisole (BHA)-containing diet had lower AFB_1-DNA binding, higher GST activity, and higher biliary excretion of AFB_1 (Chang & Bjeldanes 1987). Animals treated with butylated hydroxytoluene (BHT) before or together with AFB_1 had lower cancer incidences than the animals administered AFB_1 alone (Dragon and Pitot 1994). However, in trout, BHA did not effect liver tumor incidence, AFB_1-DNA binding, or AFB_1-glutathione conjugation (Goeger et al. 1988). In in vitro system, both BHA and BHT inhibited AFB_1-DNA binding (Bhattacharya et al. 1984). Another antioxidant, ethoxyquin, also suppressed AFB_1 carcinogenesis by inducing the activity of

glutathione S-transferase activity (Kensler et al. 1986).

Ethanol

Ethanol is a known carcinogen or procarcinogen (Yirmaya & Taylor 1993). Ethanol is metabolized in the liver by alcohol dehydrogenase, microsomal ethanol-metabolizing system (MEOS), and catalase (Odeleye & Watson, 1992). CYP2E1 enzyme (also known as rat P450j) is the major MEOS that is influenced by ethanol. The induction of CYP2E1 by ethanol consumption was implicated in alcohol-induced liver diseases (Nanji et al. 1994). CYP2E1 has also been reported to activate certain liver toxins and chemicals (Ingelman-Sunberg et al. 1988).

Ethanol, when given to animals together with or prior to aflatoxin, increased the aflatoxin hepatotoxicity and DNA binding (Toskulkoa & Glinsukon 1986; Toskulkoa et al. 1991; Sahaphong et al. 1992). The alcohol pretreatment increased the activation of AFB_1 but not the GST activity. This explains the increased binding of AFB_1 to DNA. On the other hand, when given after AFB_1 administration, ethanol showed no influence on AFB_1-DNA binding (Messlbeck et al. 1984).

Other Drugs/Xenobiotics

The activities of CYP, GST, and UDPGT enzymes can be induced by several drugs or xenobiotics. Enzyme inducing drugs such as phenobarbital (anti-seizure drug) and Aroclor 1254, given before or together with AFB_1, reduced the number of neoplasms as compared to animals given AFB_1 only (Dragan and Pitot 1994). Although phenobarbital

enhanced AFB_1 activation, it also induced GST activity and thus increased AFB_1-glutathione conjugation. Therefore, the overall hepatic binding of AFB_1 to DNA is reduced (Loury et al. 1984; Lotlikar et al. 1989). Other inducers such as ethoxyquin (Kensler et al. 1986), and oltipraz (Primiano et al. 1995), have also been shown to inhibit AFB_1-induced carcinogenesis by inducing GST activity. In a study employing human hepatocytes, oltipraz was also reported to lower the the production of AFB_1-epoxide by inhibiting the CYP1A2 and CYP3A4 activities (Longouet et al. 1995).

A compound isolated from a Chinese herb, dimethyl-4,4'dimethoxy-5,6,5',6'-dimethylenedioxy biphenyl-2,2'-dicarboxylate (DDB), is a drug used for its liver protective effects. Pretreatment of rats with DDB inhibited liver damage caused by AFB_1. DDB also induced the activity of glutathione S-transferase and therefore enhanced detoxification of AFB_1-epoxide (Liu et al. 1995). Crocetin, a carotenoid isolated from the seeds of Cape jasmine, has been reported to elevate the cytosolic glutathione S-transferase activity and glutathione concentration in a fibroblast cell line treated with AFB_1 (Wang et al. 1991a). Another Chinese herbal drug, geniposide, isolated from a fruit of a species of gardenia, can also inhibit AFB_1-induced DNA binding. Induction of glutathione S-transferase and gamma glutamyl cysteine synthase (involved in glutathione synthesis) activities, and suppression of AFB_1-induced unscheduled DNA synthesis were the suggested mechanisms of action of geniposide (Wang et al. 1991b; Wang et al. 1992).

Cortisol pretreatment in rats markedly increases the acute hepatoxicity of AFB_1 (Chentanez et al. 1988). The toxicity effects, such as higher mortality rates, increased in liver triacylglycerol, and elevated AFB_1 binding to DNA and protein, were dose-

dependent. These cortisol effects may by due to increased metabolism of AFB_1 to its epoxide derivative.

Carnitine

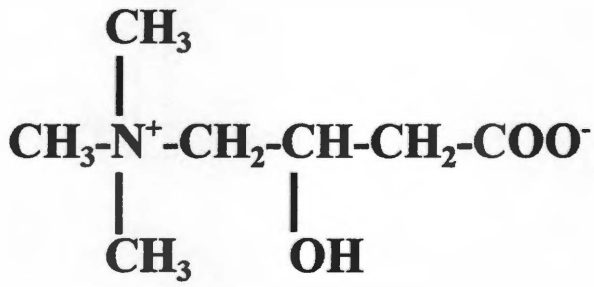
History

Carnitine was discovered from muscle extracts by two Russian scientists in 1905 (reviewed by Leibovitz 1984). It was named *carnis*, which is a Latin word for flesh or meat. In the 1940's, carnitine was found to be essential for the mealworm by Fraenkel and was named Vitamin B_t. In the late 1950's and early 60's, it was discovered that carnitine is involved in the transportation of fatty acids into the mitochondria for β-oxidation (Fritz 1959). However, it was not until 1973 that carnitine gained much attention and investigation, when the first carnitine deficient patient was described (Engel & Angelini 1973).

Chemistry and Dietary Sources

Carnitine is a quarternary amine and is very similar to choline and amino acids. However, unlike amino acids, carnitine does not involve in protein synthesis. Chemically, it is known as 3-hydroxy-4-N-trimethylamino butyric acid. It exists as D and L isomers, but only the L-isomer is biologically active. The D-isomer is not only inactive, it can also depress L-carnitine utilization (reviewed by Leibovitz 1987). Carnitine is present in the body as free carnitine and as acylcarnitine (fatty acyl-carnitine ester).

Carnitine can be produced in the body, and therefore it is not essential to humans and is not considered to be a vitamin. Muscle, liver, yeast, and milk are very good sources of dietary carnitine. Most vegetables and grains such as cauliflower, cabbage, spinach, barley, and rice have very little or no carnitine. The detailed reviews of carnitine history, biosynthesis, dietary sources and functions are available (Leibovitz 1984; Leibovitz 1987; Borum 1983).



Structure of L-Carnitine

Carnitine Biosynthesis

The biosynthesis of carnitine starts from two essential amino acids, lysine and methionine, and involves five different enzymes in a five step pathway (**Figure 2.6**). Niacin, vitamin B_6, vitamin C and iron are also required for for the biosynthesis of carnitine in animals (Borum 1983).

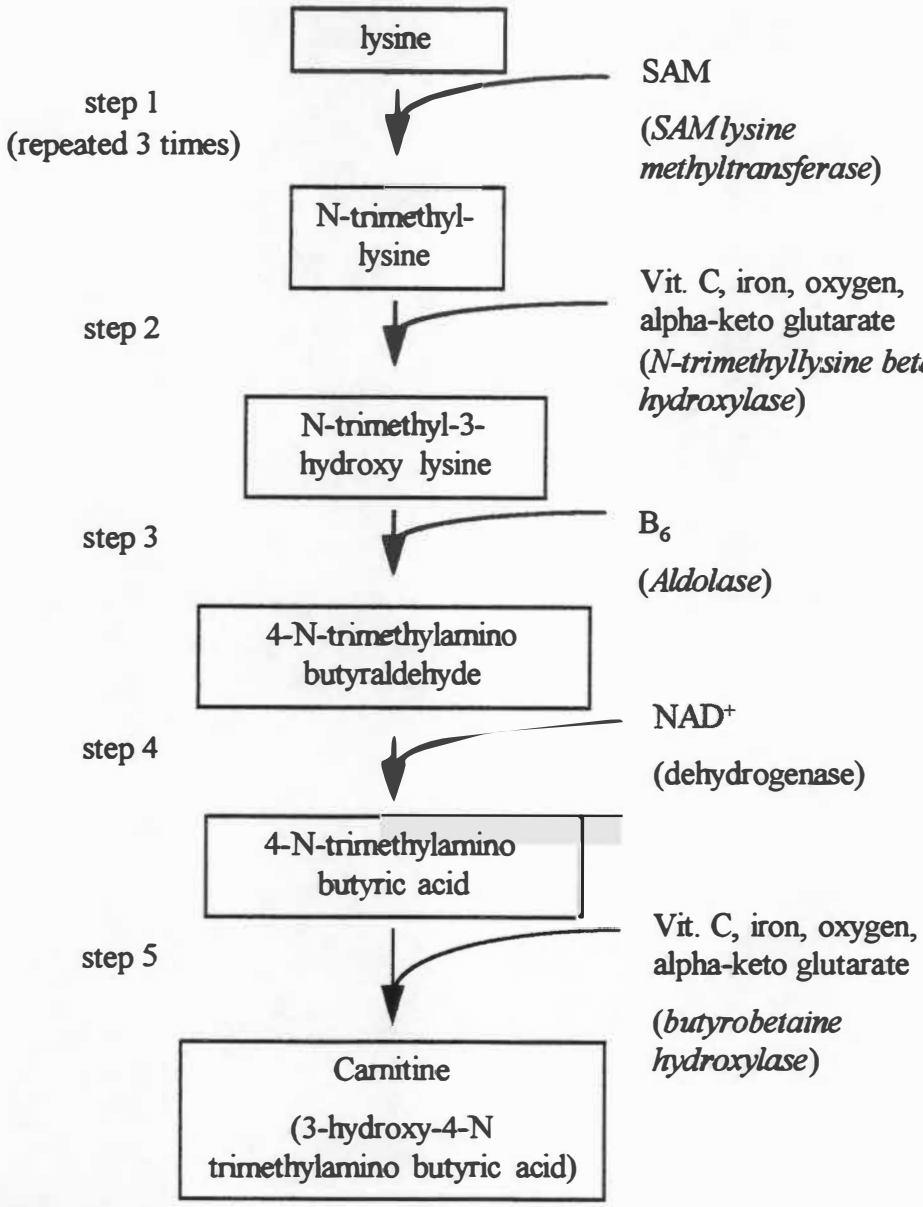


FIGURE 2.6 Biosynthesis of carnitine.

Source: Leibovitz (1984) Carnitine: How it functions in your body. In: Carnitine: The Vitamin Bt Phenomena, pp 24-38. Dell Publishing Co., Inc., New York.

In rats, the first 4 steps of the pathway occur in the liver, kidney, skeletal muscle, cardiac muscle, testis, and epididymis. However, the final step occurs only in the liver. In humans, the liver, kidney, brain, heart, and skeletal muscle can convert N-trimethyllysine to 4-N-trimethyllyamino butyric acid (Rebouche & Engel 1980). However, only the liver, kidney, and brain can convert 4-N-trimethyllyamino butyric acid to carnitine. This is because the enzyme that catalyzes the final step, butyrobetaine hydroxylase, is only present in these three tissues. Therefore, carnitine must be transported to those tisues that do not have the final enzyme.

Carnitine Functions

The main function of carnitine is the transport of long-chain fatty acids from the cytosol into the mitochondria for β-oxidation to take place. In brief, other possible roles of carnitine include the regulation of cellular concentration of free CoA, metabolism of branched chain amino acids, removal of short-chain and medium-chain acyl residues accumulated in the peroxisomes, initiation of ketogenesis, interaction with membranes to alter their physiological functions, prevention of fatty liver development due to xenobiotics, and detoxification of some drugs (for reviews on carnitine roles refer to Borum 1983; Carter et al. 1995; Frenkel & McGarry 1980; Leibovitz 1984; Leibovitz 1987; and Famularo, G. & De Simone, C. 1995).

Carnitine and Drug/Xenobiotic Interactions

Alcohol

It is widely known that chronic alcohol consumption inhibited β-oxidation and thus causes fatty infiltration in human and experimental animal livers (Leiber et al 1987). Impaired oxidation and/or increased lipogenesis are suggested mechanisms for the malady. In addition, ethanol depresses carnitine palmitoyltransferase 1 activity, the rate-limiting enzyme in the translocation of long-chain fatty acids across the mitochondrial membrane (Guzman & Geelen 1988). This inhibition may also contribute to the fat accumulation in the liver due to alcohol consumption. Supplementary L-carnitine has been found to inhibit alcohol-induced fatty liver (Sachan et al. 1984; Sachan & Rhew 1983). Retardation of ethanol oxidation found in rats may be one of the mechanisms for the ameliorating effect of carnitine (Mynatt & Sachan 1992). Carnitine also delayed ethanol oxidation and therefore augmented the half-life of ethanol in broilers (Smith et al. 1994). L-acetylcarnitine and L-carnitine reduced the rate of ethanol oxidation in rat hepatocytes (Cha & Sachan 1995). The underlying mechanism for these effects of carnitine is that carnitine competitively inhibits alcohol dehydrogenase when the NAD:acetylcarnitine ratio is less than 1 (Sachan and Cha 1994).

Carnitine appears to offer protection against the toxicity of industrial alcohols such as methanol, isopropanol, and ethylene glycol (a common antifreeze). This is because carnitine has been shown to prevent accumulation of these alcohols by enhancing their excretion in the urine (Brothers 1989). Carbon tetrachloride administration causes fat accumulation in rat liver, which is ameliorated by carnitine supplementation (Sachan &

Dodson 1992).

Drugs

Carnitine may also alleviate the side-effects of adriamycin (doxorubicin hydrochloride), a widely used anti-cancer drug. It has been reported that carnitine treatment decreased histopathological changes and ameliorated irregular heart performance due to adriamycin (Senekowitsch et al. 1987). Furthermore, carnitine prolonged the survival rate of mice administered with adriamycin (Strohm et al. 1982).

Valproic acid, an anticonvulsant drug, has produced very serious side-effects similar to Reye's syndrome (stupor, hyperammonia, coma, and hepatic dysfunction) (Gerber et al. 1979). Valproic acid treatment in epileptics lowers the serum free carnitine and increases the short-chain acylcarnitine concentrations (Rodriguez-Segade et al. 1989). Carnitine has been found to conjugate with valproic acid and other drugs, such as cyclopropanecarboxylic acid and pivalic acid. These conjugates are excreted in the urine (Quistad et al. 1986; Vickers et al. 1985). However, in another study with rat hepatocytes, glycine, but not L-carnitine, prevented lactic acid dehydrogenase leakages (an indicator of cytotoxic effect) induced by valproate (Vance et al. 1994)

Carnitine also decreased the toxicity of ifosfamide therapy. Ifosfamide, a drug used for cancer chemotheraphy, has been known to cause symptoms such as severe decreased in the concentrations of tricarboxylic acid cycle intermediates (succinate, fumarate, citrate, and α-ketoglutarate) in the urine. When rats were supplemented with L-carnitine and later injected with this drug, the rats showed better health and had a lower

decrease in intermediary products in the urine. The authors suggested that carnitine may form a complex with ifosfamide, known as chloroacetylcarnitine, that reduces the toxicity of the drug (Schlenzig et al 1995).

AFB$_1$ and Lipopolysaccharide (LPS)

The effects of L-carnitine on AFB$_1$ binding to hepatic DNA, RNA, and protein are shown in **Table 2.3**. L-carnitine supplemented diet fed to rats decreased the binding of AFB$_1$ to liver DNA and RNA 6-h post AFB$_1$ administration (Sachan & Yatim 1992). The total amounts of AFB$_1$ present in the liver and kidney were not significantly different between the carnitine supplemented and control animals (**Table 2.4**). However, the concentrations of AFB$_1$ were higher in the plasma of carnitine supplemented rats than the non-supplemented rats. We had also shown that carnitine ameliorated the earlier signs of acute toxicity of AFB$_1$, such as the elevation of total lipid concentration in the liver and the decrease in total lipids and triacylglycerol concentrations in the plasma (Sachan & Yatim 1992).

LPS or endotoxin is a constituent of the cell wall of Gram-negative bacteria. Injection or infiltration of LPS into the body triggers the prodution of cytokines that may result in increased lipogenesis, hypertriglyceridemia, and in severe cases, septic shock. Carnitine-treated animals exposed to endotoxin have been shown to have higher survival rates (Takeyama et al. 1989), lower plasma triacylglycerol, inflammatory cytokines levels, and fat synthesis in their livers (Gallo et al. 1993; Winter et al. 1995).

TABLE 2.3

Effects of L-carnitine supplement on aflatoxin B_1-macromolecules adducts formation in rat liver 6-h post-aflatoxin B_1 administration[1]

Parameter	Group		
	Control	L-Carnitine[2]	Different (%)
	pmol/mg macromolecules[3]		
AFB_1-DNA	6.8 ± 2.8^a	4.7 ± 2.9^b	30.9
AFB_1-RNA	21.6 ± 0.3^a	14.3 ± 0.5^b	33.8
AFB_1-Protein	1.2 ± 0.1^a	1.2 ± 0.1^a	0

[1]Values are mean ± SEM, n = 5.

[2]Diet contained 0.4% L-carnitine (w/w) and given to animals for 6 weeks.

[3]Different letters indicate significant difference between groups ($p < 0.05$).

TABLE 2.4

Tissues and plasma [^{3}H] aflatoxin B_1 concentrations in rats supplemented with L-carnitine 6-h post-aflatoxin B_1 administration[1]

	Group		
	Control	L-Carnitine[2]	Different (%)
Liver, *nmol* AFB_1 */g*[3]	3.81 ± 0.28[a]	3.25 ± 0.28[a]	14.7
Kidney, *nmol* AFB_1 */g*	0.68 ± 0.08[a]	0.66 ± 0.07[a]	2.5
Plasma, *nmol* AFB_1 */dl*	247 ± 14[a]	310 ± 41[b]	25.8

[1]Values are mean ± SEM, n = 5.

[2]Diet contained 0.4% L-carnitine (w/w) and given to animals for 6 weeks.

[3]Different letters indicate significant difference at $p < 0.05$

Apoptosis

The effect of carnitine on "programmed" cell death (apoptosis) has been studied. When carnitine and its analogs were incubated in C2.8 hepatocytic cell line incubation mixture, the survival and growth rates of the cell were enhanced (Revoltella et al. 1994). Carnitine also delays the onset of apoptosis induced by the deprivation of hepatocyte growth factor, a factor necessary for the cell growth and survival. In another study involving P19 teratoma cells, acetylcarnitine prolonged cell survival time and delayed DNA fragmentation and nuclear condensation caused by serum deprivation (Galli & Fratelli 1993). These findings suggest that carnitine may serve as a cytoprotective biomolecule; that is, it buffers the impact of certain harmful agents or conditions.

Cytochrome P450 (CYP)

CYP is a group of iron-containing enzymes important in the metabolism (oxidation) of various endogenous (fatty acids, bile acids, steroids, biogenic amines, prostaglandins, and leukotrienes) and foreign compounds (drugs, environmental pollutants, alcohol, and natural plant products). Its name derives from the maximum absorption wavelength (450 nm) produced when CYP is reacted with carbon monoxide. It is found in the microsomes or the endoplasmic reticulum (ER) of the cell. CYP is isolated by homogenization of the liver and followed by ultracentrifugation of the postmitochondrial supernatant fraction. There are two types of ER, rough and smooth, which are differentiated by the presence or absence of ribosomes, respectively. The

smooth ER has higher oxidative activity of CYP than does rough ER. The organs where CYP is found include skin, liver, kidney, testes, ovaries, bladder, aorta, placenta, lung, nasal mucosa, blood platelets, gastrointestinal cells, and lymphocytes (Hodgston & Levi 1994).

There are about 67 different isoenzymes of CYP families 1, 2, and 3 (CYP1, CYP2, and CYP3) that participate in the biotransformation of toxic materials (Netter 1994). The metabolism of xenobiotics by CYP enzymes can either activate the compounds to become carcinogens or detoxify the compounds. The reactions that CYP catalyzes include epoxidation, hydroxylation, dealkylation or O-demethylation, oxidative deamination, oxidation, and desulfuration.

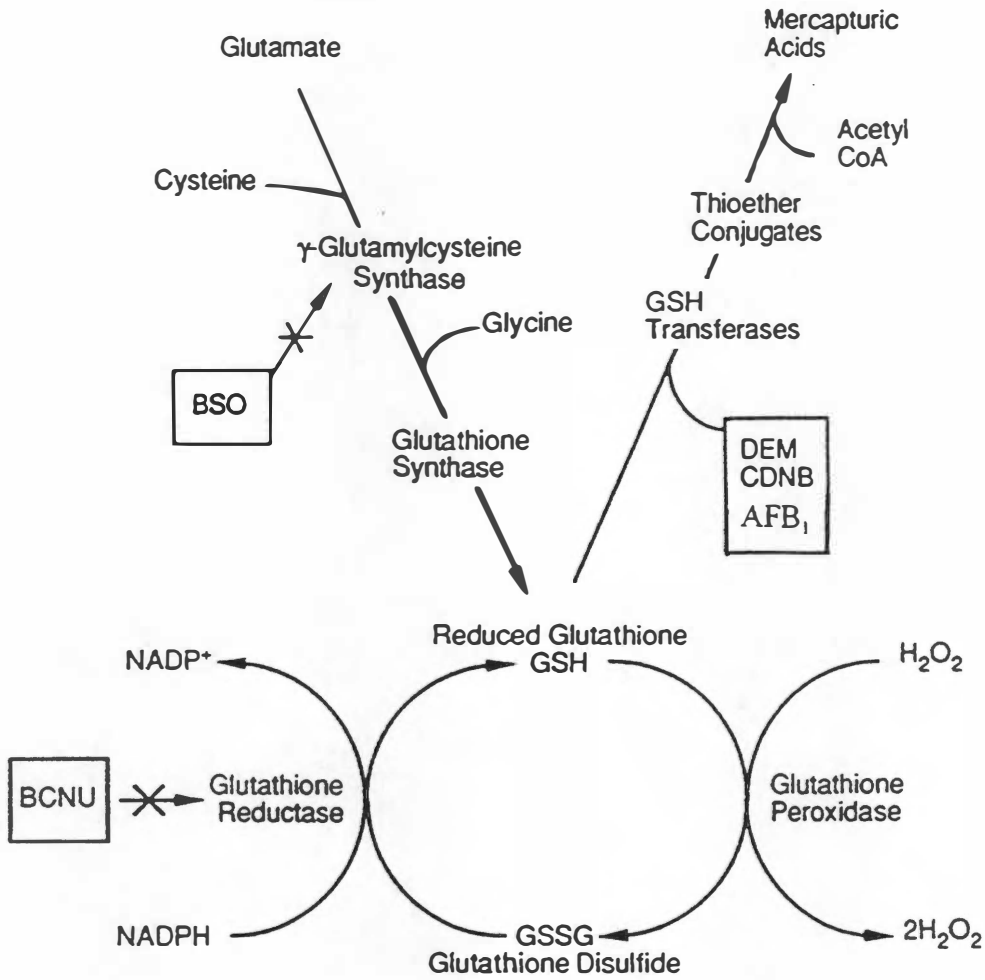
Glutathione S-Transferase and Glutathione

Glutathione S-transferases (GST) are a group of enzymes found in liver, kidney, lung, spleen, small intestine, brain, heart, ovary and testes (Kraus & Kloft 1980). One of GST's primary functions is the metabolism of drugs and xenobiotics, that is, catalyzing the conjugation of reduced glutathione (GSH) to the electrophilic center of the foreign compounds. This conjugation is the initial step in the synthesis of mercapturic acid. GST has many isozymes, and in mammalian species the isozymes are being classified as alpha (intermediate molecular weight), mu (highest molecular weight), and pi (lowest molecular weight) (Dauterman 1994).

Glutathione is the most abundant (> 90%) intracellular non-protein thiol in animal

organs. It is a tripeptide made-up of glutamate, glycine, and cysteine. In mammalian livers, GSH represents more than 95% of total glutathione concentration (4 to 8 mM), the rest being oxidized glutathione (GSSH). GSH plays an important role in regulation of enzyme activities by disulfide (thiol) exchange and binding of proteins. In liver cells, most of GSH is found in the cytosol and about 10-15% is found in the mitochondria.

Besides conjugating to reactive xenobiotics, glutathione is also involved in other cellular defense mechanisms such as detoxification of lipid peroxides and free radicals, regulation of protein synthesis, and maintenance of immune function (Vina 1990). Depletion of GSH can be directly due to conjugation to electrophiles or inhibition of GSH synthesis. Decreased level of GSH has been correlated to the pathogenesis of liver disease, AIDS, and cataracts. Low cellular concentration of GSH is dangerous since that increases the susceptibility of the cell to injury due to toxic challenge (see **Figure 2.7** for GSH homeostasis). Carbon tetrachloride, bromobenzene, and acetaminophen are a few examples of compounds that can deplete tissue levels of GSH (Reed 1994).



AFB_1 = Aflatoxin B_1
GSH = Reduced glutathione
GSSH = Oxidized glutathione
BSO = Buthionine sulfoxide
DEM = Diethyl maleate
BCNU = 1,3-bis(2-chloroethyl)-1-nitrourea
CDNB = 1-Chloro-2,4, dinitrobenzene

FIGURE 2.7 Changes in glutathione homeostasis by chemical compounds and AFB_1.

Source: Reed, D.J. (1994) Mechanisms of chemically induced cell injury and cellular protection mechanisms. In: Introduction to Biochemical Toxicology (Hodson, E. & Levi, P.E., eds.), pp. 265-296. Appleton & Lance, Norwalk, CT.

CHAPTER 3

Effects of L-Carnitine Dosage on Aflatoxin B_1-Macromolecule Adducts Formation, Glutathione S-Transferase Activity, Glutathione Contents, and Aflatoxin B_1 Uptake in Freshly Isolated Hepatocytes

Introduction

Aflatoxin B_1(AFB_1) is a well known potent hepatotoxic (O'Brien et al. 1983) and hepatocarcinogenic (Adamson et al. 1976) mycotoxin. It has been frequently found to contaminate many foods such as corn, barley, rice, peanuts, and peanut butter. Once AFB_1 is consumed, it is easily absorbed in the small intestine and goes into the liver via the portal vein. In the liver it then undergoes several biotransformational pathways that produce the reactive AFB_1-8,9-epoxide or the less toxic hydroxylated metabolites (reviewed by Eaton et al. 1994).

AFB_1-epoxide is the ultimate carcinogen that covalently binds to cellular macromolecules, namely protein, DNA, and RNA. The covalent bindings are the means by which AFB_1 exerts its toxic and carcinogenic effects. AFB_1-epoxide may also undergo detoxification by conjugating to reduced glutathione (GSH), a process catalysed by glutahione S-transferase (GST). This conjugation is the principal detoxification pathway of AFB_1-epoxide in many mammals. The cytosolic GST activity has been correlated to susceptibility of several animal species to AFB_1 carcinogenicity (Eaton & Gallagher

1994).

L-carnitine, 3-hydroxy-4-N-trimethylamino butyric acid, is an important biomolecule for the intramitochondrial translocation of long chain fatty acids and many other emerging roles in health and disease (Ferrari et al. 1992). We reported earlier that a 0.4% carnitine supplemented diet fed to rats decreased the binding of AFB_1 to hepatic DNA and RNA 6 h and 24 h post-AFB_1 administration (Sachan & Yatim 1992). The AFB_1 dose (1 mg/kg) was given orally, 6 week after the animals were started on the diet. Carnitine significantly reduced AFB_1-protein adduct formation 24 h after the AFB_1 dose. Carnitine supplementation also prevented hepatic steatosis and hypolipidemia caused by AFB_1.

Therefore, the primary objective of this study was to understand the mechanisms by which carnitine modulated the AFB_1-DNA adducts formation. Since it was decided to use freshly isolated rat hepatocytes, we first investigated the effect of carnitine on AFB_1-macromolecules adducts formation in these hepatocytes. The possible role of carnitine in inducing the activity of GST and increasing total glutathione concentration were studied since GST and glutathione are involved in the detoxification of activated AFB_1. The uptake of AFB_1 by hepatocytes in the presence of carnitine was also investigated because if carnitine could reduce the cellular uptake of AFB_1, that would reduce the exposure of macromolecules to AFB_1.

Materials and Methods

Animals

The research protocol was approved by the Animal Care and Use Committee of the University of Tennessee, Knoxville. Male Sprague Dawley rats weighing 300-350 g were housed individually in suspended stainless steel cages in a cubicle of an AAALAC approved animal facility. The animals were given free access to Teklad 22/5 Rodent Diet (W) 8640 (Harlan, Indianapolis, IN) and chlorinated water.

Liver Perfusion and Isolation of Hepatocytes

Buffers

The buffers used for preperfusion, collagenase perfusion and incubation were modified Hank's balanced salt solutions as described by Lotlikar et al. (1989). All buffers were saturated with 95% O_2 + 5 % CO_2 gas before use. (Refer to **Appendix 1** for detailed procedures for liver perfusion and isolation of hepatocytes). The pre-perfusion modified Hank's buffer contained 0.5 mM EGTA, 25 mM HEPES, and 0.5% BSA (added prior to perfusion) but had no Ca^{++} or Mg^{++}. The perfusion buffer was a modified Hank's buffer having 2 mM $CaCl_2$, 0.5 mM EGTA, 25 mM HEPES, 0.5% BSA, and 0.05% collagenase (type IV). The solution was saturated with 95% O_2 + 5% CO_2 gas before the addition of BSA and collagenase just prior to perfusion. The incubation buffer was similar to the collagenase buffer except it had no collagenase.

Liver perfusion

The perfusion apparatus and procedures used were according to the modified methods of Seglen (1976). Rats were anesthetized with metofane (Pitman-Moore, Inc. Mundelein, IL) by the open-drop method in a closed jar. The portal vein was cannulated and Ca^{++}- and Mg^{++}-free (preperfusion) buffer was injected at a flow rate of 30 ml/min for about 1 min while the cannula was secured with ligature. The buffer flow was then increased to 50 ml/min, and the anterior vena cava was cut to let the perfusate escape. After starting the perfusion (2-3 min), the intact liver was carefully removed from the rat and placed flat on the perfusion dish for continous perfusion with 500 ml of the preperfusion buffer for about 10 min. Then, the collagenase buffer was introduced at the flow rate of 50 ml/min for about 10 min. One hundred ml of 0.05% collagenase buffer was used and recycled during the course of the perfusion. The liver swelled to about twice its original size at the end of the collagenase perfusion. The liver was placed on a watch glass containing 5 ml of the incubation buffer (containing 0.5% BSA), and was carefully raked with a stainless steel fork to release the cells from the connective tissue and vascular stroma.

Hepatocyte isolation and viability test

The liberated cells were suspended in 10 ml of ice-cold incubation buffer containing 0.5% BSA and were centrifuged at 50 x *g* for 3 min at 4 °C. The loose cells and supernatant were removed. After this time, the cells were placed in an ice-bucket (temperature at about 4 °C). The heavy cell pellet was resuspended with 20 ml of incubation buffer containing 0.5% BSA and centrifuged as above. The supernatant and loose cells were discarded. These steps were repeated two more times to ensure maximal removal of dead cells and other cells (macrophage and fat cells). The hepatocyte pellet was suspended and made up to a 20 ml suspension with the incubation buffer containing

0.5% BSA. For viability test, the hepatocyte suspension was diluted 1:10, and 100 µl of the diluted cells were added to 100 µl of 0.6% trypan blue and counted using a hemacytometer (see details in **APPENDIX 1**).

Hepatocytes incubation

The hepatocytes incubation was done according to the method by Lotlikar et al. (1987). In brief, the incubation mixtures contained 4 x 10^6 cells/ml, modified Hank's buffer (pH 7.4) containing 2 mM $CaCl_2$, 25 mM HEPES, 0.5% bovine serum albumin (fatty acid-free), various levels of L-carnitine (0 - 1.5 mM), and 0.5 µM [3H]AFB_1 (0.5 µCi/ml). [^{3}H]AFB_1 was dissolved in dimethylsulfoxide (DMSO), and the final concentration of DMSO was 2%. The incubation medium was saturated with 95% O_2 + 5% CO_2 gas before addition to the mixtures. The incubation volume was 5 ml, and incubation was conducted in duplicates or triplicates in Erlenmeyer flasks. Carnitine was preincubated for 15 min before adding the [^{3}H]AFB_1 and incubated further for 60 min. At the end of incubation time, the flasks were immediately dipped into an ice-water bath before further manipulations were carrired out.

Extraction and Isolation of Macromolecules

The extraction and isolation of DNA, RNA, and protein were carried out according to Glazer's & Weber's (1971) method (**Appendix 2**). Briefly, the homogenized hepatocytes were treateded with perchloic acid (PCA) to precipitate the macromolecules, and were centrifuged at 2700 x *g* for 15 min at 2 ^{0}C. The pellet was treated with potassium acetate, chloroform:ethanol, and absolute ethanol to remove lipids. The pellet was resuspended with PCA, left overnight, and centrifuged. The supernatant contained RNA. The pellet was resuspended in PCA and heated at 70 ^{0}C for 20 min. After centrifuging, the supernatant (DNA fraction) was collected. The pellet was washed with

double deionized water (DDW) and was centrifuged. The pellet containing the protein fraction was suspended by adding potassium hydroxide and heating at 70 ^{0}C for 15 min.

In experiments where only DNA was extracted, the procedures of Gross-Bellard et al. (1973) were followed (**Appendix 3**). In short, at the end of the incubation period, the hepatocytes were centrifuged at 500 x *g* for 5 min to sediment cells. The supernatant was discarded. The cells pellet was suspended with 0.3 ml digestion buffer (containing 100mM NaCl, 10 mM Tris-Cl, pH 8.0, 25 mM EDTA, pH 8.0, 0.5% SDS, and 0.1 mg/ml proteinase K) and incubated at 50 ^{0}C in a shaking water-bath for 12-18h. Then, 0.3 ml digestion buffer (without proteinase K) was added and mixed. DNA was extracted with 0.6 ml of phenol:chloroform:isoamyl alchohol (25:24:1), and was centrifuged at 1700 x *g* for 10 min. To the aqueous layer 250 µl of 3.0 M sodium acetate and 1 ml of 100% alcohol were added. After centrifuging at 1700 x *g* for 2 min, the supernatant was discarded, and the pellet was washed with 70% EtOH. The pellet (crude DNA) was dried in a "Speedvac Concentrator" for 1 min, suspended in 1 ml of 10 mM Tris-Cl (pH 7.4) containing 1mM EDTA, and was shaken to aid solubilization at room temperature or at 65 ^{0}C for several hours.

RNA was removed by adding 0.1% SDS and 1 µg/ml DNAase-free RNase and incubated at 37 ^{0}C for 1 h. DNA was again extracted as described above and solubilized in 1 ml of 10 mM Tris-Cl (pH 7.4) containing 1mM EDTA.

The content of DNA was determined colorimetrically using calf thymus DNA as standard (Ceriotti 1952) (**APPENDIX4**). The RNA was measured spectrophotometrically using yeast RNA as standard at 260 nm (Glazer & Weber 1971). Protein was measured by the method of Lowry et al. (1951) (**APPENDIX 5**).

[^{3}H]AFB$_1$ radioactivity measurement

Radioactivity or disintergration per minute (DPM) was measured by transfering

0.5 ml of DNA, RNA, and protein extracts into scintillation vials, added with 5 ml Aquasol-2 (Dupont-NEN Research Products, Boston, MA) and counted in a liquid scintillation counter (LS-3801 Beckman Instrument, Irving, CA) for 10 min. For protein, 0.1 ml of glacial acetic acid was added to decrease random coincidence monitor (RCM) before counting.

Determination of GST activity

Cytosolic GST activity was determined according to the method by Habig et al. (1974) (see **Appendix 6** for complete procedures). In brief, the incubation mixture was homogenized in 3 complete strokes in a Potter-Elvehjem homogenizer. The cell homogenate was centrifuged at 9,000 x *g* for 15 min in a swinging bucket. The supernatant was centrifuged at 100,000 x g for 60 min and the resulting supernatant (cytosol) was assayed for GST activity. The changes in absorbance of the reaction mixture containing the cytosol minus blank were monitored at 340 nm at 25 ^{0}C for 3 min in an Hitachi U-2000 double-beam spectrophotometer. The results were reported as μmol S-(2,4-dinitro-1-benzyl)glutathione formed /mg cytosolic protein/min. The cytosolic protein content was assayed according to Lowry's method (1951), using BSA as standard.

Determination of total glutathione concentration

Total glutathione was assayed according to the method of Griffith (1985) as described by Anderson (1985) (**Appendix 7**). After incubation, 100 μl of incubation mixture was transferred into microcentrifuge tubes containing 100 μl of 10 mM HCl. The cells were lysed by freezing in liquid nitrogen and thawing four times. After centrifuging at 10,000 x *g* for 5 min, 100 μl of the supernatant was added with 50 μl 10% sulfosalicylic acid to precipitate protein. The protein-free supernatant (25 μl) was mixed with 0.2 mM

NADPH, and 0.6 mM 5,5'-dithiobis-(2-nitrobenzoic acid (DTNB), and preincubated at 37 ^{0}C for 3 min. Then 1.3 - 2.6 U glutathione reductase was added to start the reaction. The rate of change of absorbance was monitored for 3 min. The concentration of total glutathione was calculated from a standard curve using known concentrations of GSH.

Determination of uptake of AFB_1 by hepatocyes

After incubation with carnitine and [^{3}H]AFB_1, the hepatocytes were separated from the incubation medium by centrifugation at 500 x *g*. After washing the cells with incubation buffer (no BSA), the cells were again centrifuged, resuspended, homogenized, and centrifuged at 600 x *g* for 10 min to pellet the nuclei. The post-nuclear supernatant was separated from the nuclear pellet. The nuclear pellet was suspended in incubation buffer. The incubation medium, washed cells suspension, nuclear pellet suspension, and post-nuclear supernatant were extracted with chloroform:ethylacetate (1:1) to remove free AFB_1. Aliquots of these extracted fractions were measured for radioactivity (DPM) in a liquid scintillation counter for 10 min.

Statistics

All data were expressed as group mean ± SEM. The data were analyzed using regression procedure of SAS (SAS Institute, Inc., Cary, NC). The regression curve was plotted using Excel 5.0 software for Windows. The minimum level of significance was set at $p \leq 0.05$.

Results

AFB_1-Macromolecules Adducts Formation

Regression analyses were used to examine the correlations of L-carnitine

concentrations on AFB_1 binding to DNA, RNA and protein of isolated hepatocytes. As shown in **Figure 3.1**, as the concentrations of carnitine increased from 0-400 μM, the concentrations of AFB_1-DNA adducts were linearly decreased in the cells ($r = -0.52$; $p = 0.039$). In the next experiment, a higher range of carnitine concentrations (0-1500 μM) was preincubated with the hepatocytes to raise the intracellular carnitine concentrations that would affect the DNA adducts formation. When higher levels of carnitine were tested, the correlation of carnitine and DNA adducts was stronger ($r = -0.681$), and the significance level was better ($p = 0.0002$) (**Figure 3.2**). The carnitine effect on the DNA adducts formation seems to have flattened at the carnitine concentrations from 1200 μM to 1500 μM. As a result, 1200 μM concentration was used in experiments where a single concentration of carnitine was required, such as experiments described in **Chapter 4**.

Carnitine did not affect the AFB_1-RNA adducts formation (**Figure 3.3**) in the hepatocytes. The concentrations of AFB_1-RNA adducts remained unchanged as the concentrations of carnitine were increased. However, as for AFB_1-protein adducts, regression analysis shows that carnitine had a significant positive correlation ($r = 0.5845$; $p = 0.017$) on the protein adducts production (**Figure 3.4**). These RNA and protein adducts results are different from what were found in the in vivo study (Sachan & Yatim 1992). In rats, carnitine supplementation significantly decreased the binding of AFB_1 to liver RNA (and DNA), but did not affect on binding to liver protein at 6-h post AFB_1 administeration.

Effect of carnitine on glutathione S-transferase activity (GST) and total glutathione (GSH + GSSH)

GST is an important detoxification enzyme that catalyses the conjugation of AFB_1-epoxide to GSH. **Figure 3.5** illustrates the effects of carnitine on GST activity toward 1-

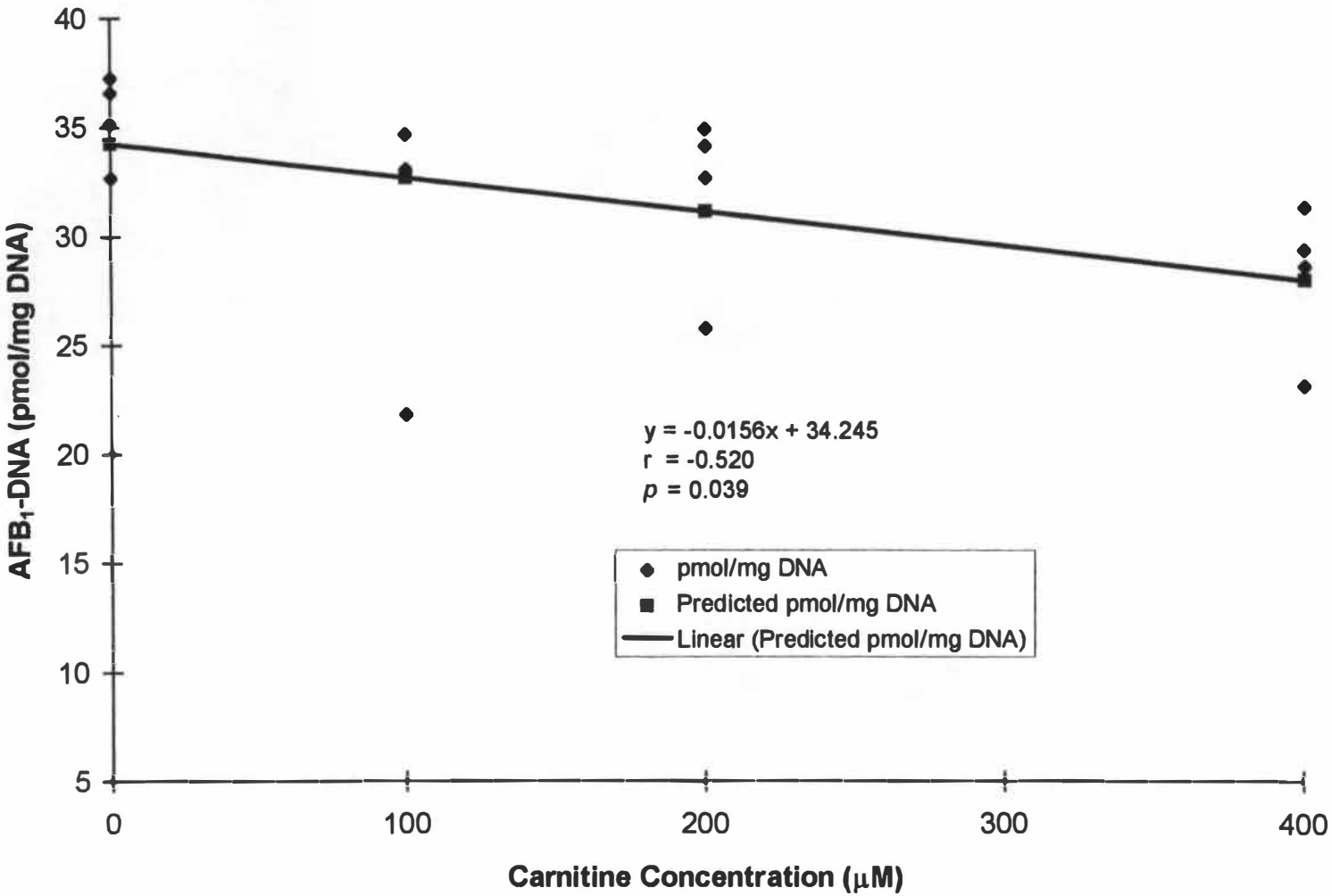


FIGURE 3.1 Effect of L-carnitine on AFB_1-DNA adducts formation in isolated hepatocytes (n = 4).

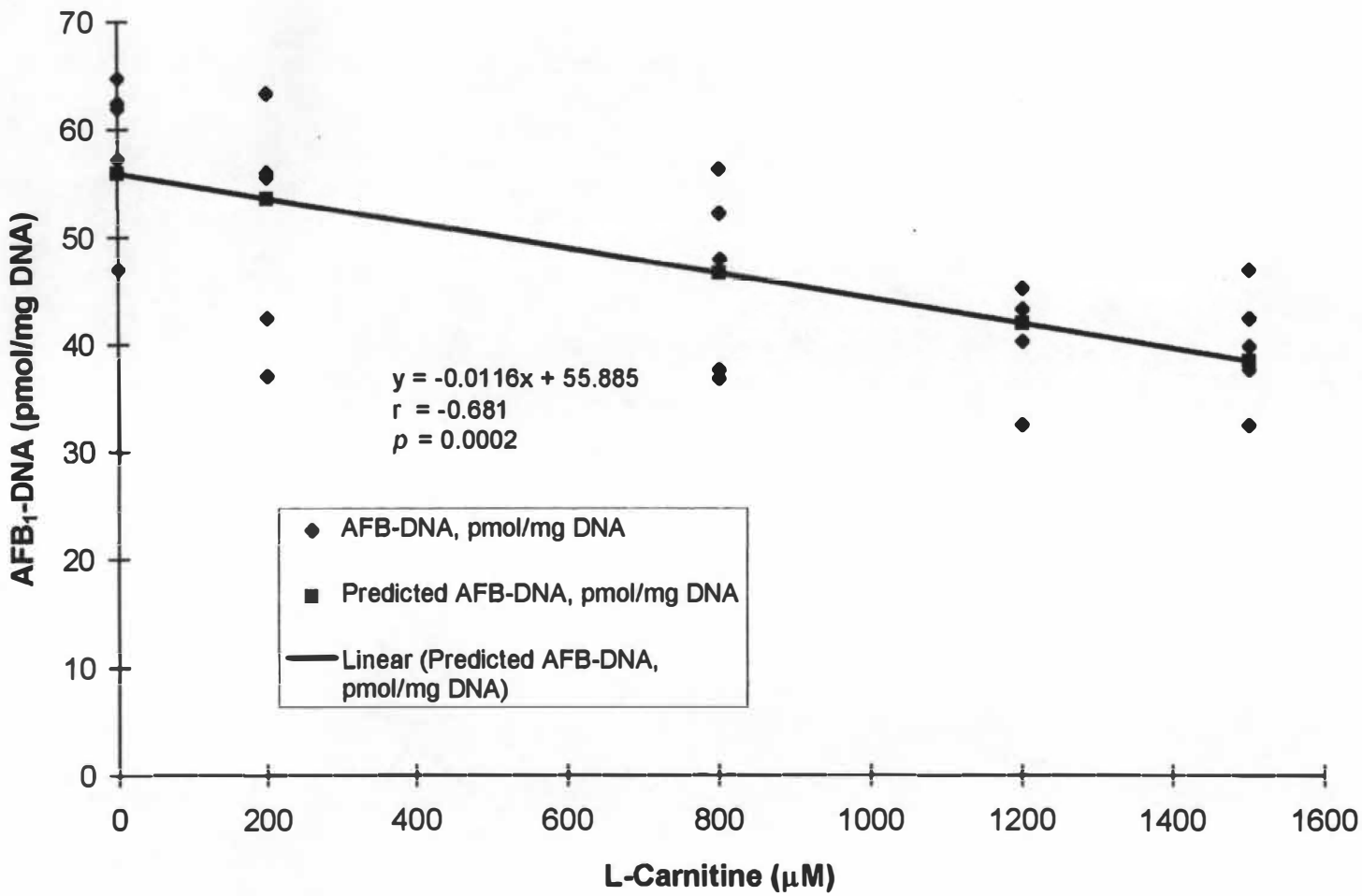


FIGURE 3.2 Effect of L-carnitine (1.2 mM) on AFB_1-DNA adducts formation in freshly isolated hepatocytes (n = 5).

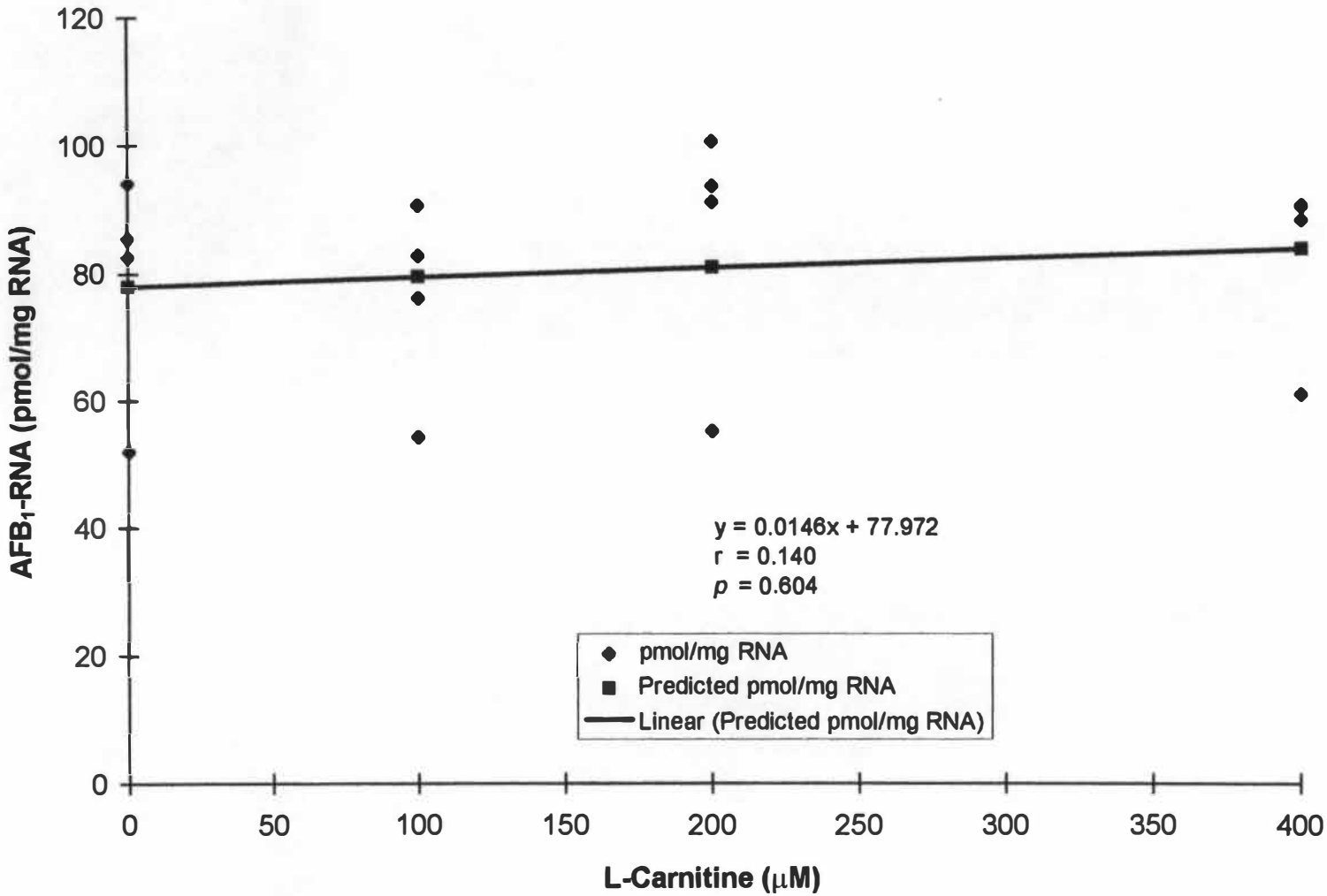


FIGURE 3.3 Effect of L-carnitine on AFB_1-RNA adducts formation in isolated hepatocytes (n = 4).

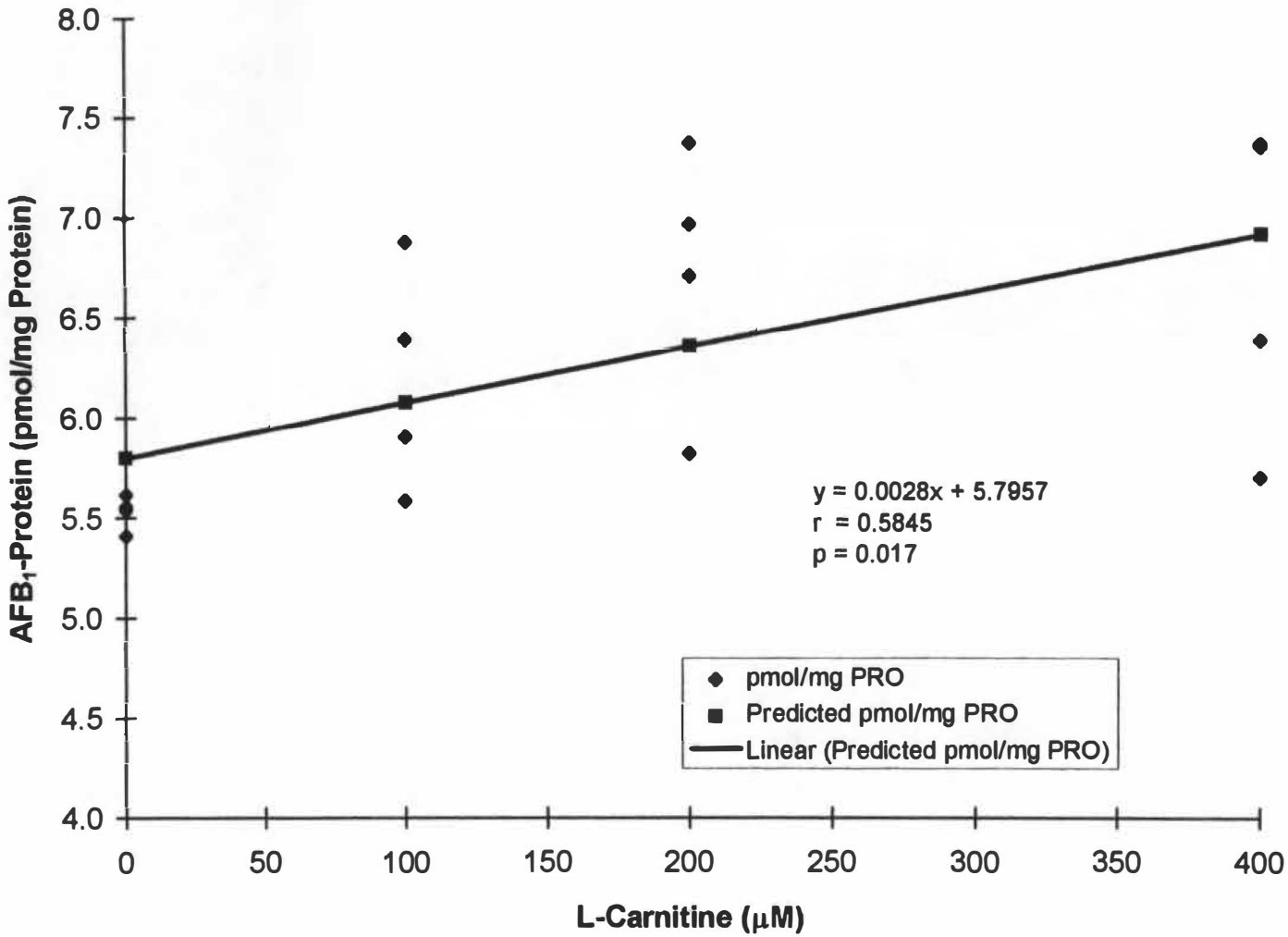


FIGURE 3.4 Effect of L-carnitine on AFB_1-protein adducts formation in isolated hepatocytes (n = 4)

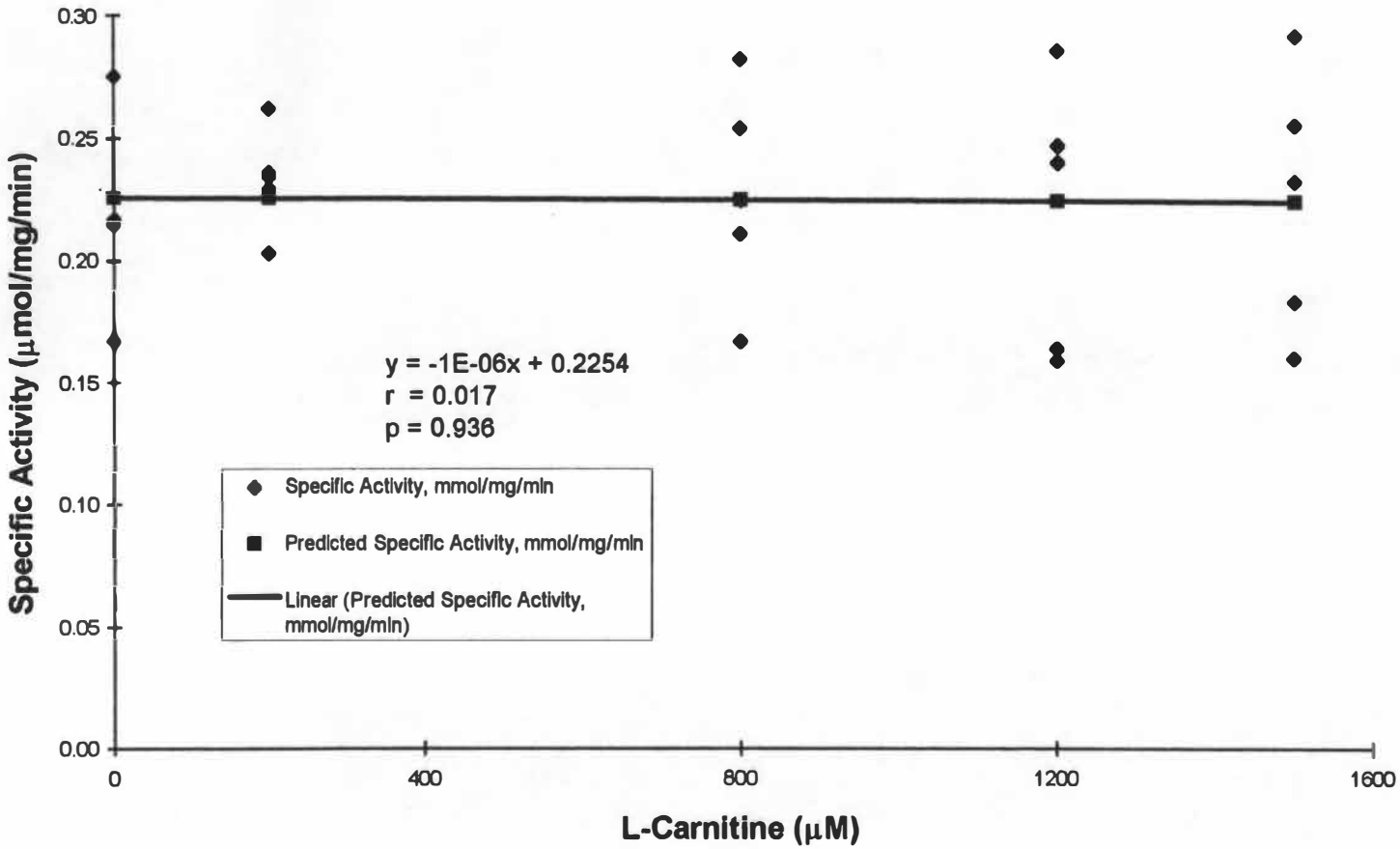


FIGURE 3.5 Effect of L-carnitine on glutathione S-transferase activity toward 1-chloro-2,4 dinitrobenzene in isolated hepatocytes treated with AFB_1 (n = 5).

chloro-2,4 dinitrobenzene in hepatocytes exposed to AFB_1. The activity of GST was not affected by carnitine. However, the total glutathione concentrations were positively correlated ($r = 0.41$; $p = 0.041$) to carnitine levels added to the hepatocytes (**Figure 3.6**). This indicates that carnitine has a protective effect on the decrease in the cellular total glutathione contents caused by AFB_1.

Effect of carnitine on uptake of AFB_1 by hepatocytes and cellular compartments

The entry of AFB_1 into hepatocytes and consequently into the nuclei is an important determinant for the amounts of AFB_1 available for bioactivation and binding to macromolecules. Therefore, inhibition of AFB_1 entry into the cells may account for the lower concentrations of AFB_1-DNA adducts in carnitine treated animals and hepatocytes. However, as shown in **Figure 3.7**, carnitine had no significant effect on the entry of AFB_1 into hepatocytes, nuclei, or post-nuclear supernatant.

Discussion

AFB_1-macromolecule adducts formation

Our previous studies with intact rats showed that carnitine supplementation decreased hepatic covalent binding of AFB_1 to DNA, RNA, and protein 24-h post AFB_1 treatment. (Sachan & Yatim 1992). The present investigation with freshly isolated hepatocytes also showed that an increasing dosage of carnitine linearly decreased AFB_1-DNA adduct formation. When higher range of carnitine concentrations (0 to 1.5 mM) were present in the hepatocytes' incubation system, the correlation was stronger than when lower range (0 to 0.4 mM) of carnitine concentrations was used. In contrast to the in vivo study, carnitine linearly increased the AFB_1-protein adducts concentration, and had no significant effect on AFB_1-RNA adduct concentration in isolated hepatocyes.

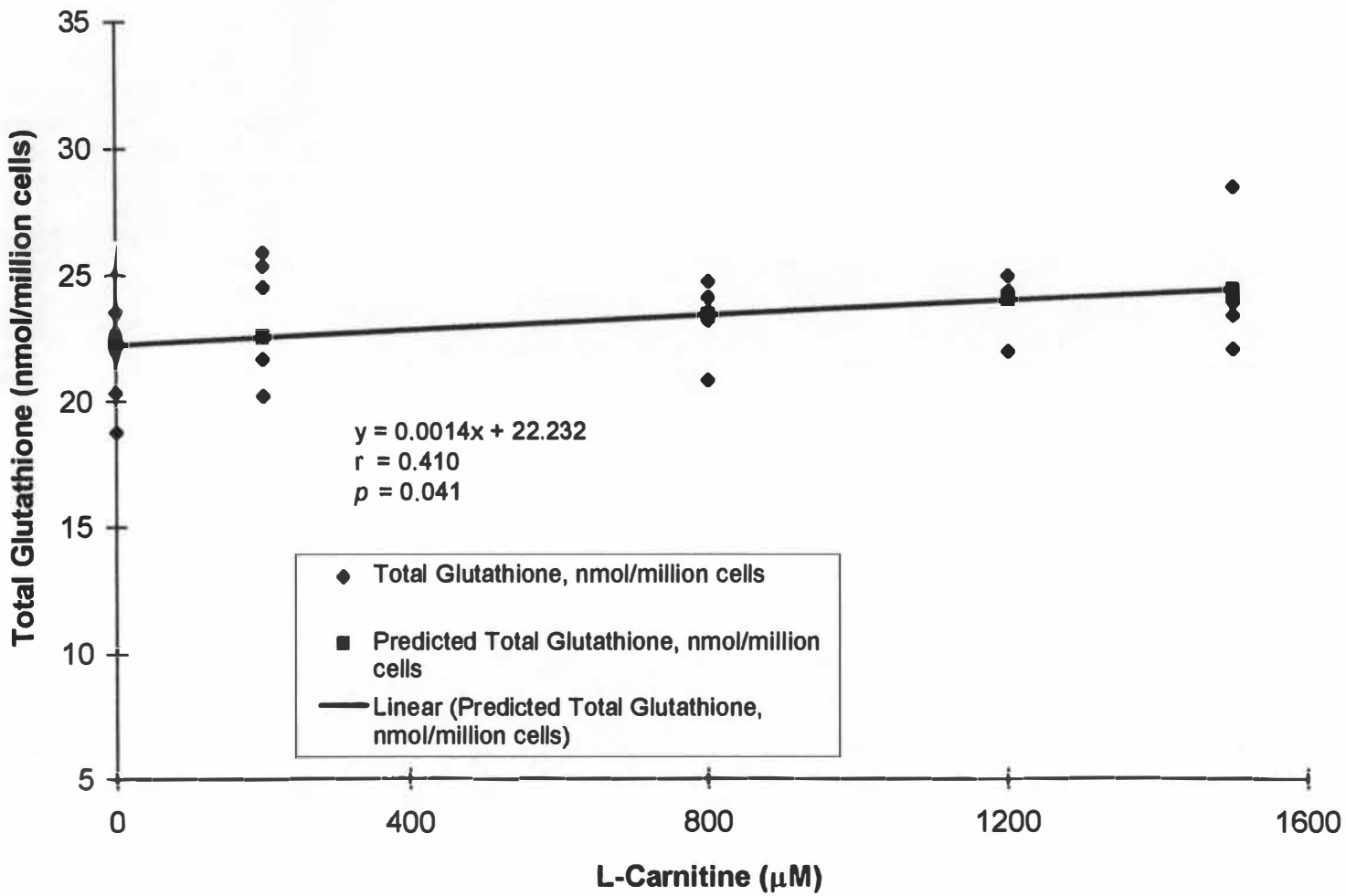


FIGURE 3.6 Effect of L-carnitine on total glutathione concentration in freshly isolated hepatocytes treated with AFB_1 (n = 5).

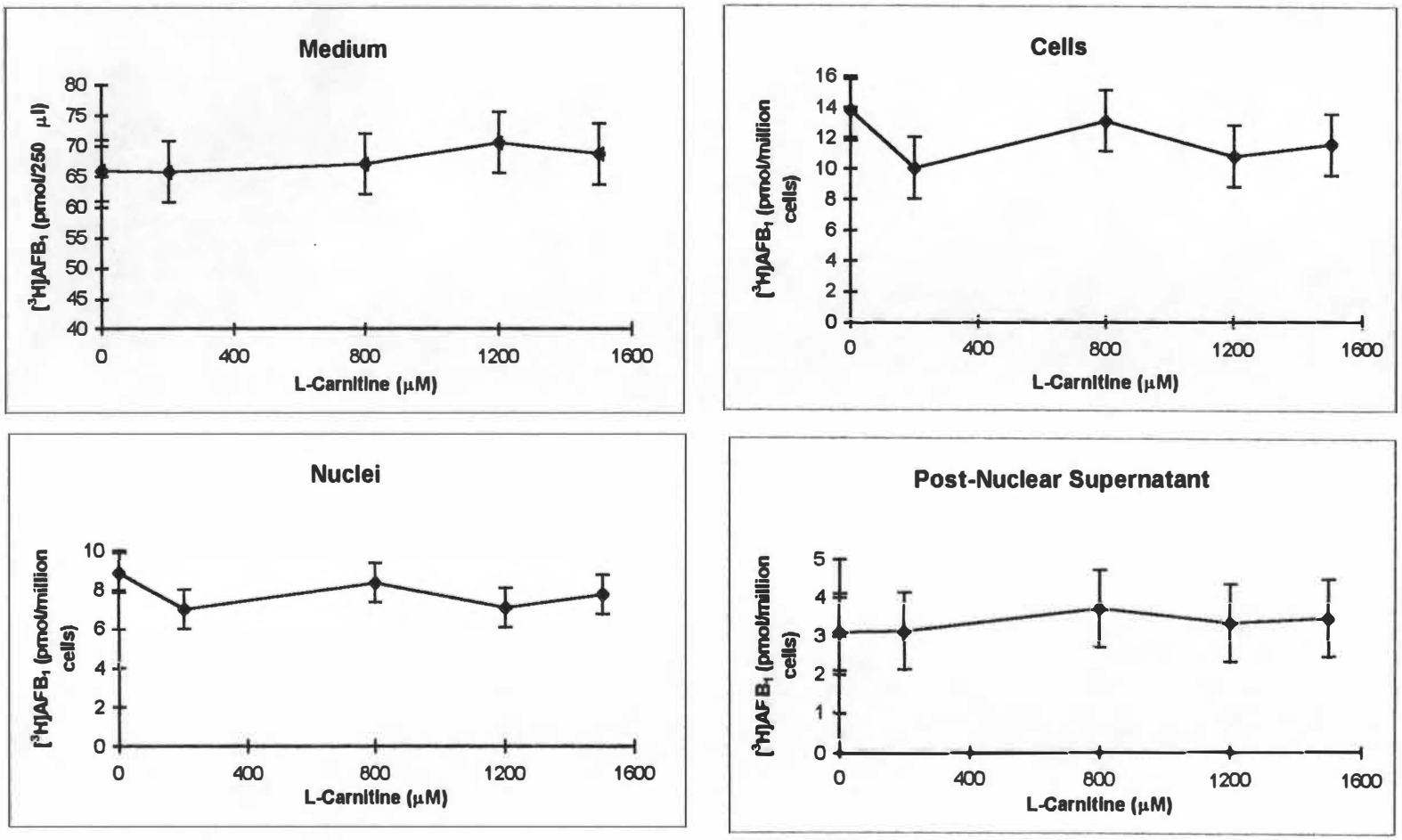


FIGURE 3.7 Effects of L-carnitine on [^{3}H]AFB$_1$ entry into isolated hepatocytes and distribution into cellular compartments (n = 5).

The covalent binding of AFB_1 to DNA is an important step in AFB_1 carcinogenesis (Eaton & Gallager 1994), and AFB_1-protein adduct is currently used to assess human exposure to AFB_1 (Sheabar et al. 1993; Chang et al. 1994). Therefore, several follow-up experiments were conducted to study the biochemical basis for the carnitine's effects on the formation of these two adducts (see **CHAPTER 4**).

The protective mechanisms suggested for crocetin (a carotenoid) on the AFB_1-DNA adduct formation (Wang et al. 1991a) may also apply for carnitine, that is, carnitine may suppress the bioactivation of AFB_1, induce the detoxification of activated AFB_1, and act as an antioxidant that scavenges AFB_1 free radicals or peroxides that are produced when AFB_1 is metabolized by prostaglandin H synthase and lipoxygenase. Prostaglandin H synthase (Lui et al. 1990) and lipoxygenase (Lui & Massey 1992) are the other metabolic pathways not involving the cytochrome P450 system that can bioactivate AFB_1. Carnitine increased glutathione concentration in the cells treated with AFB_1, which is similar to the effect of crocetin (and will be discussed later in this Chapter). Carnitine has been shown to inhibit formation of oxygen-free radicals and increase the repair rate of DNA in human cells (Marini et al. 1990; Monti et al, 1992).

The elevation of AFB_1 binding to protein in the hepatocytes system is an important observation. In intact animals, carnitine had no effect on AFB_1-protein interaction when measured at 6-h after AFB_1 administration. However, at 24-h post AFB_1 treatment, carnitine decreased the AFB_1-protein concentration (Sachan & Yatim 1992). One reason for this difference may be that in the hepatocytes' incubation medium, 0.5% BSA was added to keep the cells intact longer when incubating with noxious agents (Berry et al. 1991). We have found that carnitine enhances the binding of AFB_1 to BSA (in the presence or absence of microsomes) and to rat plasma protein (**CHAPTER 4**). The higher amount of microsomal-activated AFB_1 binding to BSA mediated by carnitine would be expected to reduce the amount of activated AFB_1 available for binding to DNA.

This may be a very important mechanism by which carnitine decreases the AFB_1-DNA adduct concentration in the hepatocytes. Unfortunately, from this experiment we are unable to differentiate the protein adducts formed with cellular protein and those formed with added BSA.

GST activity

The present study found no significant effect of carnitine on GST activity toward 1-chloro-2,4 dinitrobenzene. This indicates that carnitine has no influence on this detoxification enzyme activity. Several dietary nutrients, nutritional status, drugs, and xenobiotics have been demonstrated to affect GST activity. Vitamin A has been shown to induce the activity of GST in liver fractions and thereby enhance the detoxification of AFB_1-epoxide (Bhattacharya et al. 1989). However, no difference in the GST M1 protein concentrations was found in woodchuck hepatocytes treated with AFB_1 in the presence or absence of vitamin A (Yu et al. 1994). Dietary restriction has been shown to inhibit AFB_1-DNA adduct because of induction of GST activity toward AFB_1-epoxide (Chen et al. 1995). Protein-deficient animals has lower GST activity and AFB_1-GSH which resulted in higher AFB_1-DNA adduct formation (Mandell et al. 1992).

Ethoxyquin, an antioxidant, added to the diet (0.4%) and fed to rats inhibited AFB_1-DNA adduct formation because of induction of GST activity (Kensler et al. 1989). Another antioxidant, BHA, has been shown to increase the activity of GST, resulting in the reduction of DNA adduct formation (Monroe & Eaton, 1987). Oltipraz, a chemotherapy drug, has been reported to induce GST activity, which leads to a reduction of putative preneoplastic lesions caused by AFB_1 (Primiano et al. 1995). In another study the protective role of oltipraz has been shown to be a result of both inhibition of AFB_1 bioactivation and the induction of GST activity in human hepatocyte culture (Langouet et al. 1995). A traditional Chinese herbal drug, dimethyl-4,4'dimethoxy-5, 6, 5', 6'-

dimethylenedioxy biphenyl-2, 2'-dicarboxylate (DDB), also has an inhibitory effect on AFB_1 binding to DNA. In this case, the effect is due to inhibition of cytocrome P-450 system by DDB, which results in the production of more hydroxylated metabolites which are less genotoxic than the epoxide (Guengerich & Shimada 1991). A more recent study has demonstrated a similar effect of DDB-mediated reduction of DNA adduct, where the mode of action was through the induction of the GST activity and not the increased in the production of hydroxylated AFB_1 metabolites (Liu et al. 1995).

Total glutathione concentration

GSH is important in cellular defense mechanisms such as detoxification of reactive xenobiotics and free radicals, and maintenance of immune function (Vina 1990). About 97.5 - 98.5% (Griffith 1988) or more than 99.5% (Anderson 1985) of the total glutathione (oxidized + reduced glutathione) in tissues is in the form of reduced glutathione (GSH). GSH can be formed from oxidised glutathione (GSSG) by the catalytic action of glutathione reductase. Depletion of GSH concentration can be due to conjugation to electrophiles (e.g. AFB_1-epoxide) or inhibition of GSH synthesis. Low levels of GSH are detrimental since that increases the susceptibility of cells to injury due to toxic attack.

The novel finding of the present study is the ability of carnitine to prevent the decrease in total glutathione content due to AFB_1 in the hepatocytes. This observation is important since the availability of GSH is essential for GST to promote AFB_1-epoxide binding to GSH and therefore spare binding to macromolecules. An increase in total glutathione concentration may be indicative of reduced amounts of activated AFB_1 formation in the presence of carnitine. If carnitine decreased the amount of AFB_1-epoxide, there would be decreased need of GSH for detoxification of the epoxide. It is also possible that carnitine induces the biosynthesis of glutathione. Additionally, carnitine itself may conjugate to the activated AFB_1 and therefore spare the glutathione. The data

to support these hypotheses remain to be determined by additional experiments.

An anology to the carnitine effect on AFB_1-DNA adduct formation is the effect of crocetin on the same. Crocetin, a carotenoid isolated from the seeds of Cape jasmine, has been reported to decrease AFB_1-DNA adduct production in both microsomal and cultured cell systems treated with AFB_1 (Wang et al. 1991a). The crocetin concentrations of 0.1 and 0.5 mM decreased DNA binding by aproximately 17 to 38%, respectively. Crocetin had no effect on AFB_1-epoxide production in the microsomal system as quantified by AFB_1-8,9-dihydrodiol concentration. The protective effects of crocetin on DNA adduct formation were related to its role in elevation of cytosolic GSH concentration and GST activity. As for carnitine, it increased total glutathione concentration but had no effect on GST activity.

The protective mechanism of geniposide, a compound isolated from the Rubiaceae plant family, is different than that of crocetin. Geniposide decreased cellular GSH and GSSG concentrations but induced the activity of of γ-glutamyltranspeptidase (the rate-limiting enzyme for GSH synthesis) and GST (Wang et al. 1991b). Carbon tetrachloride, bromobenzene, and acetaminophen have been found to deplete tissue levels of GSH (Reed 1994). In hepatocytes culture, acetominophen and AFB_1 both deplete GSH concentrations (Hayes et al. 1986). In light of these observations, the GSH-sparing effect of carnitine is important and is a new function of carnitine.

AFB_1 uptake by hepatocytes

This study found no significant difference in the uptake of AFB_1 by the hepatocytes or their nuclei in the absence or presence of different concentrations of carnitine. However, it is possible that the dose response to carnitine was at the lower end of the carnitine concentration used in the experiments. This is because there was a steep decline in the AFB_1 concentration between 0 and 200 μM carnitine doses (**FIGURE 3.7**).

Additional experiments using 0 to 400 μM carnitine with an increment of 50 μM need to be carried out for clarification.

Conclusion

The results of this study lead us to conclude that carnitine enhances total glutathione concentration and promotes the binding of activated AFB_1 to plasma albumin, and that it therefore spares DNA from covalently binding to the activated AFB_1.

CHAPTER 4

Effects of L-Carnitine on Aflatoxin B_1 Bindings to Exogenous DNA and Proteins

Introduction

Aflatoxins, a group of secondary metabolites of *Aspergillus flavus* and *A. parasiticus*, are commonly found to contaminate food and feed supplies. Consumptions of aflatoxins contaminated food have been linked to incidences of human liver cancer (Alpert 1971; Yu 1995). Due to mounting evidence, the International Agency for Research on Cancer (IARC) has upgraded AFB_1 from a Group II to a Group I human carcinogen in 1993 (IARC 1993).

Aflatoxin B_1 (AFB_1) is the most toxic and carcinogenic of all naturally occuring aflatoxins. Its metabolite, AFB_1-epoxide, possesses hepatocarcinogenic and hepatotoxic properties through its ability to covalently bind to cellular DNA and protein respectively. The AFB_1-DNA adducts formation is a crucial step for AFB_1-induced carcinogenesis. The DNA adducts concentrations have been correlated to incidences of liver cancer in animals (Bechtel 1989) and humans (Groopman et al. 1988).

L-Carnitine, 3-hydroxy-4-N-trimethylamino butyric acid, is an important co-factor for mitochondrial long chain fatty acid oxidation. We have reported that carnitine supplement reduced the AFB_1 binding to liver DNA and RNA, 6-h after a single oral dose of AFB_1, in intact rats (Sachan & Yatim 1992). Carnitine has no effect on AFB_1-protein

adducts formation in the liver. In isolated hepatocytes, increasing concentrations of carnitine linearly decreased the binding of AFB_1 to DNA (**Chapter 3**). Therefore, one of the objectives of this study was to determine if the effects of carnitine on AFB_1-DNA adduct formation were a result of microsomal metabolism of AFB_1. Since microsomes contain the enzymes that bioactivate AFB_1, the findings of this study were expected to elucidate the mechanisms of carnitine-mediated decrease of AFB_1-DNA adduct formation. Furthermore, it was deemed necessary to determine the specificity of carnitine effect by testing the effect of carnitine analog (acetylcarnitine), and carnitine-like substances, (choline and glycine) on AFB_1-DNA adducts formation in microsomal systems.

We had observed that the total amounts of AFB_1, 6-h post-dosage, present in the liver and kidney were not significantly different between the carnitine supplemented and control animals. (**Table 2.3**). However, the concentration of AFB_1 was higher in the plasma of carnitine supplemented rats. Further, carnitine ameliorated the earlier signs of acute toxic effects of AFB_1, such as the increase in total lipids concentrations in the liver and the decrease in total lipids and triacylglycerol concentrations in the plasma (Sachan & Yatim 1992). The higher amounts of AFB_1 in the plasma of carnitine supplemented rats were interpreted to indicate that carnitine may inhibit the uptake of AFB_1 into the hepatocytes.

AFB_1 can readily bind to plasma albumin once it is absorbed from the small intestine, and that albumin serves as a major transporter of AFB_1 in blood (Dirr & Scabort 1987). Since it has been postulated that some bioactivation of AFB_1 occurs in the intestinal mucosa (Hartiala 1977; Vainio & Hietanen 1979) and blood (Sabbioni et al.

1987), albumin could be expected to bind to metabolites of AFB_1 as well. The binding of AFB_1 to albumin after being absorbed has been postulated to be one of the detoxicification processes of AFB_1 (Hseih & Wong 1994). It was hypothesized, therefore, that carnitine may increase the binding of AFB_1 to proteins in the mesentric veins and portal vein blood, resulting in decreased availability of AFB_1 and its metabolites to bind to hepatocellular components. Therefore, the other objectives of this study were to determine the effects of carnitine on binding of AFB_1 to plasma proteins and bovine serum albumin with and without microsome.

Materials and Methods

Animals

The research protocol was approved by the Animal Care and Use Committee of the University of Tennessee, Knoxville. Male Sprague Dawley rats weighing 300-350 g were individually housed in suspended stainless steel cages in a cubicle of an AAALAC approved animal facility. The animals were given free access to Teklad 22/5 Rodent Diet (W) 8640 (Harlan, Indianapolis, IN) and water.

Liver Perfusion and Microsome Preparation

Rats were anesthetized with metofane (Pitman-Moore, Inc. Mundelein, IL) by the open-drop method in a closed jar. The portal vein was cannulated and and the liver was perfused with 100 ml ice-cold physiological saline. The details of the procedure are

described in **Appendix 8**. The perfused liver was mixed with 0.154 M KCl buffer containing 0.01 M KH_2PO_4 (pH 7.4) and homogenized with 3 complete strokes in a teflon-glass homogenizer to produce a 25% (w/v) homogenate. The homogenate was centrifuged at 10,000 x *g* for 20 min at 4 ^{0}C. The supernatant was centrifuged at 100,000 x g for 60 min at 4 ^{0}C using a Sorvall ultracentrifuge. The microsomal pellet was suspended in glycerol:phosphate buffer (1:1, glycerol:0.15 M KH_2PO_4 (pH 7.4). Microsomal protein concentration was determined by the method of Lowry et al. (1951) decribed in **Appendix 5**.

[^{3}H]AFB$_1$ Binding to Calf Thymus DNA

The DNA binding method of Allameh et al. (1988) with modifications (Hasler et al. 1994) was followed (**Appendix 9**). There were four groups in this experiment, Group 1 had no microsome and no carnitine; Group 2 had no microsome but had carnitine (1.2 mM); Group 3 had microsome but no carnitine; and Group 4 had both microsome and carnitine. Briefly, the microsomal incubation mixtures contained 0.1 M phosphate buffer, microsome equivalent to 1.0 mg protein, 1.2 mM L-carnitine or other tested compounds (pH 7.0), 2 M NADPH, 2 nmol [^{3}H]AFB$_1$ dissolved in dimethylsulfoxide (DMSO), 0.1 mg calf thymus DNA, and double deionized water (DDW) to make-up a total volume of 1.0 ml. The samples were incubated at 37 ^{0}C for 30 min in triplicates and repeated 5 times using freshly prepared microsome from a rat each time.

At the end of the incubation time, a volume of 5 M NaCl was added to produce a mixture containing 1 M NaCl. Two ml of chloroform:isoamyl alcohol (24:1, v/v) and 0.9 mg of calf thymus DNA (as a carrier) were added. The tubes were shaken and centrifuged at 10,000 x *g* for 10 min. The DNA fraction (top layer) was again extracted with chloroform:isoamyl alcohol, shaken and centrifuged 10,000 x *g* for 10 min. The DNA fraction was centrifuged at 100,000 x *g* for 1 h. The supernatant was mixed with an

equal volume of 95% ethanol and centrifuged at 10,000 x *g* for 10 min to pellet the DNA. The DNA pellet was resuspended in 1 ml of 0.1 M NaCl (pH 7.0). The DNA concentration was determined colorimetrically according to Ceriotti (1952), **Appendix 4**. An aliquot of 200 µl of DNA solution was added to 5 ml Aquasol-2 (Dupont-NEN Research Products, Boston, MA), and DPM were determined for 10 min in a liquid scintillation counter (LS-3801 Beckman Instrument, Irving, CA).

[^{3}H]AFB$_1$ Binding to Bovine Serum Albumin

For this study, similar incubation medium and experimental design were used as in the AFB$_1$-exogenous DNA adducts formation as described above except that fatty-acid free bovine serum albumin (BSA) was added in placed of DNA. In brief, after incubation, the mixtures were quickly chilled in an ice-water bath and then centrifuged at 100,000 x g for 1 h at 4 ^{0}C to pellet the microsome. The supernatant was extracted with 2 ml chloroform:ethylacetate (1:1, v/v), and centrifuged at 2,000 x *g* at 4 ^{0}C for 10 min to remove free AFB$_1$. The aqueous (top layer) was transferred to new tubes and extracted with chloroform:ethylacetate one more time. Protein concentrations were determined in the aqueous (protein) fraction according to Lowry et al. (1951) method. Radioactivity or DPM was measured by adding 0.2 ml of the protein fraction in 5 ml Aquasol-2 (Dupont-NEN Research Products, Boston, MA), with 0.04 ml of glacial acetic acid to decrease random coincidence monitor (RCM).

[^{3}H]AFB$_1$ Binding to Microsome

The incubation medium and procedure were similar to that used in the AFB$_1$ binding to BSA described above. There were two groups in this experiment, control and carnitine groups. The medium contained 0.1 M phosphate buffer, microsome equivalent to 1.0 mg protein, 1.2 mM L-carnitine (pH 7.0) for the carnitine group, 2 M NADPH, 2

nmol [^{3}H]AFB$_1$ dissolved in dimethysulfoxide (DMSO), and DDW to make-up a total volume of 1.0 ml.

At the end of the incubation period, the mixture was quickly chilled in an ice-water bath and centrifuged at 100,000 x g for 1 h at 4 ^{0}C to pellet the microsome. The supernant was removed, and the microsomes were then suspended in 1 ml of 1 M KOH. The microsomal suspension was extracted with 2 ml chloroform:ethylacetate (1:1, v/v), and centrifuged at 2,000 x *g* for 10 min to remove free AFB$_1$. The top (aqueous layer) was transferred to new tubes and extracted with chloroform:ethylacetate one more time. Protein concentration and radioactivity were measured as described in the earlier experiments.

AFB$_1$ Non-Covalent Binding to Proteins as Measured by Membrane Ultrafiltration Method

The incubation was done in glass culture tubes. The mixture contained BSA (0.8 µg) or rat plasma (20 µl equivalent to 0.8 µg albumin), with or without 1.2 mM L-carnitine (pH7.0), 2 nmol [^{3}H]AFB$_1$ dissolved in DMSO and was made-up to a total volume of 300 µl with DDW. The mixture was allowed to stand at room temperature (approximately 25 ^{0}C) for 10 min and then transferred into the upper chamber of a prewashed Ultrafree-MC filter unit (Millipore Corporation, Bedford, MA). The nominal molecular weight limit of the Ultrafree-MC regenerated cellulose membrane used was 30,000. Refer to **Figure 4.1** for the flow diagram of separation of bound versus unbound [^{3}H]AFB$_1$ to proteins by the modified method of Lipford et al. (1990). The filter unit was then centrifuged at 2000 x *g* for 20 min. The filtrate was removed, and the concentrate in the upper chamber was washed once with 400 µl Hank's buffer and centrifuged again at 2000 µl for 30 min. The upper chamber that retained the protein was transferred into scintillation vials and radioactivity was determined.

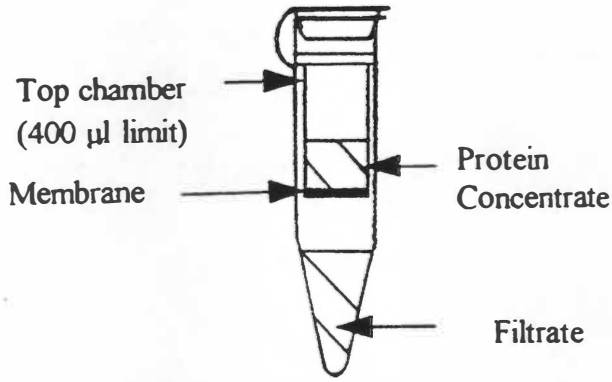
Prewashed Ultrafree filter with 400 µl DDW

↓

Transfer incubation mixture to top chamber of Ultrafree filter unit

↓

Centrifuge at 2,000 x *g* for 20 min at 25 °C



Remove filtrate

↓

Add 400 µl of buffer to top chamber

↓

Centrifuge at 2,000 x *g* for 30 min at 25 °C

↓

Measure radioactivity in the top chamber that contains the protein concentrate

FIGURE 4.1 Ultrafiltration method of determining bound [^{3}H]AFB$_1$ to proteins

Statistics

The values are reported as means ± SEM. Statistical analysis of the data was performed using the SAS statistical program (SAS version 6.11, SAS Institute Inc., Cary, NC). The differences between two groups were analyzed by Student's *t* test. For comparison of several groups, general linear model (GLM) procedures or two-way crossed ANOVA were used when appropriate. When significant, Duncan's Multiple Range test or Contrast statement were used to compare between means. The critical level of significance was set at $p < 0.05$.

Results

AFB_1-calf thymus DNA adducts formation

The bindings of AFB_1 to calf thymus DNA in the presence or absence of carnitine and microsome are shown in **Figure 4.2**. There were none or very little AFB_1-DNA adducts formed in the absence of microsome in the incubation mixture, and carnitine has no significant effect. In the presence of microsome, there was significantly higher AFB_1-DNA adducts formation in the control (250 pmol/mg DNA) and carnitine (200 pmol/mg DNA) groups. This is consistent with the fact that AFB_1 has to be metabolized for it to bind to DNA. This microsome-mediated AFB_1 binding to DNA was significantly lower in the presence of carnitine. This indicates that carnitine may alter the bioactivation of AFB_1 (produces less epoxide) and/or affect the binding of AFB_1-epoxide to DNA (inhibition of AFB_1-epoxide electrophilic binding to guanine of DNA).

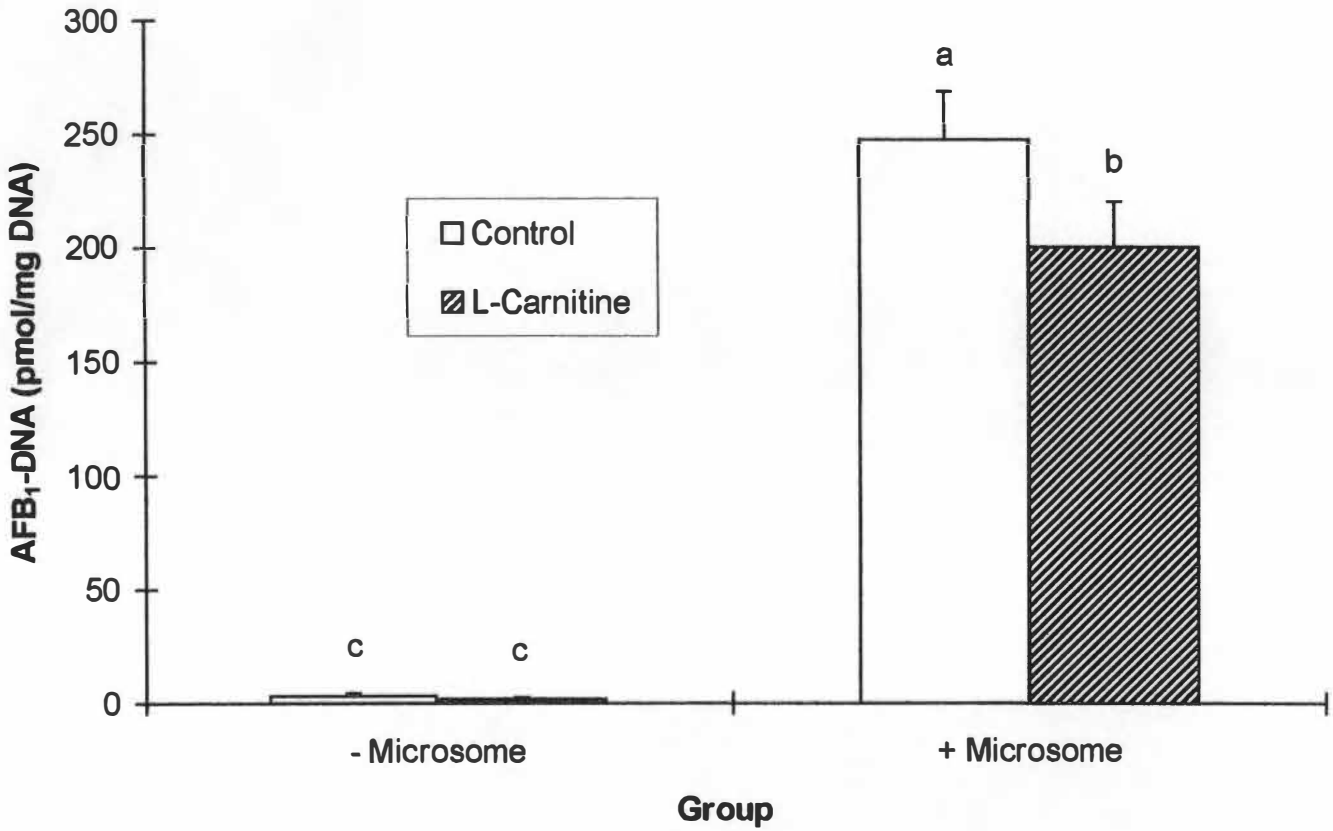


FIGURE 4.2 Effects of L-carnitine (1.2 mM) on AFB_1-calf thymus DNA adducts formation mediated by microsomal enzymes (n =5). Different letters indicate significant difference among groups ($p < 0.05$).

Specificity of carnitine

The effects of carnitine, acetylcarnitine, and carnitine-like substances on the DNA adducts formation are shown in **Figure 4.3**. The concentration of AFB_1-DNA adducts in the control group was 216 pmol/mg DNA. In the carnitine, acetylcarnitine, and GABA groups, the DNA adduct concentrations were 170, 154, and 165 pmol/mg DNA respectively. The decreases in DNA adduct in these three groups were significantly lower than the control group. This shows that the reduction in the DNA-adducts formation is not very specific to carnitine. Choline and glycine had no significant effect on the AFB_1-DNA adduct formation.

AFB_1-BSA Adducts Formation

The binding of AFB_1 to proteins (BSA and microsomal protein) are presented in **Figure 4.4**. In contrast to DNA (**Figure 4.2**), there was some binding of unmetabolized AFB_1 to BSA (13.4 pmol/mg BSA in Group 1, and 16.2 pmol/mg BSA in Group 2). These are noncovalent bindings since no microsome was added into the incubation mixture to produce the AFB_1-epoxide that can bind covalently to macromolecules. Carnitine had no significant effect (contrast p value = 0.171) on these non-covalent AFB_1-BSA bindings (Group 1 vs Group 2).

In the presence of microsome, there were markedly higher amounts of AFB_1-BSA adduct formed (Group 3 and 4). The BSA adducts formed were 5.8 times higher in Group 3 than in Group 1, and 5.5 times higher in Group 4 than in Group 2. The higher concentrations of AFB-BSA adducts in the presence of microsome were due to both covalent and non-covalent bindings. The average amount of covalent AFB_1-BSA bindings in Group 3 was 64.1 pmol/mg BSA, and in Group 4 was 72.7 pmol/mg BSA (calculated by subtracting the adduct concentrations of Group 3 from Group 1, and Group 4 from 2). Therefore, covalent bindings constitute the higher portion of the AFB_1-BSA adduct shown

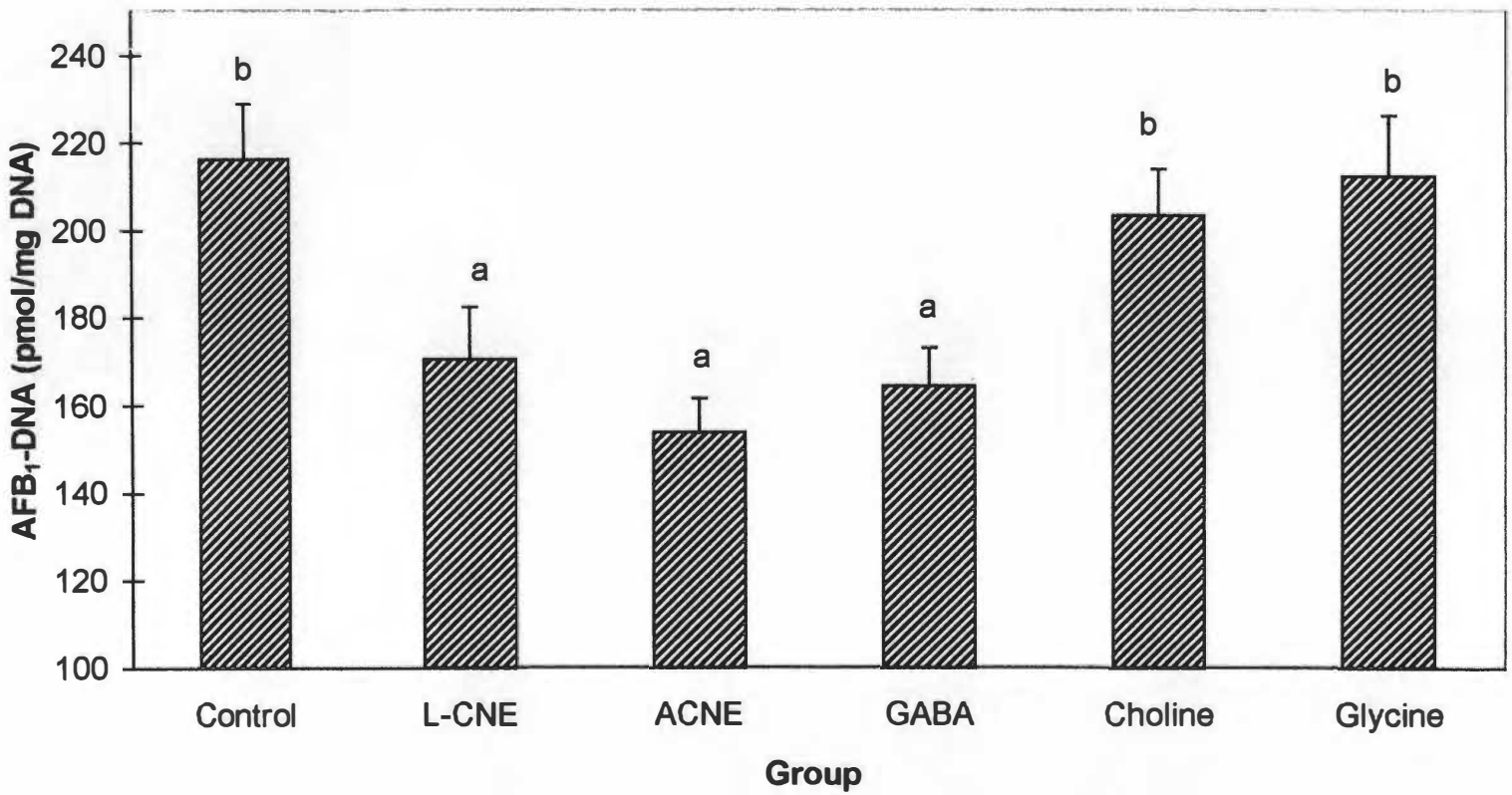


FIGURE 4.3 Effects of L-carnitine, acetylcarnitine, and structurally-related compounds on AFB_1-DNA adducts formation (n = 5). The concentrations of the compounds were 1.2 mM. Different letters indicate significant difference among groups ($p < 0.05$).

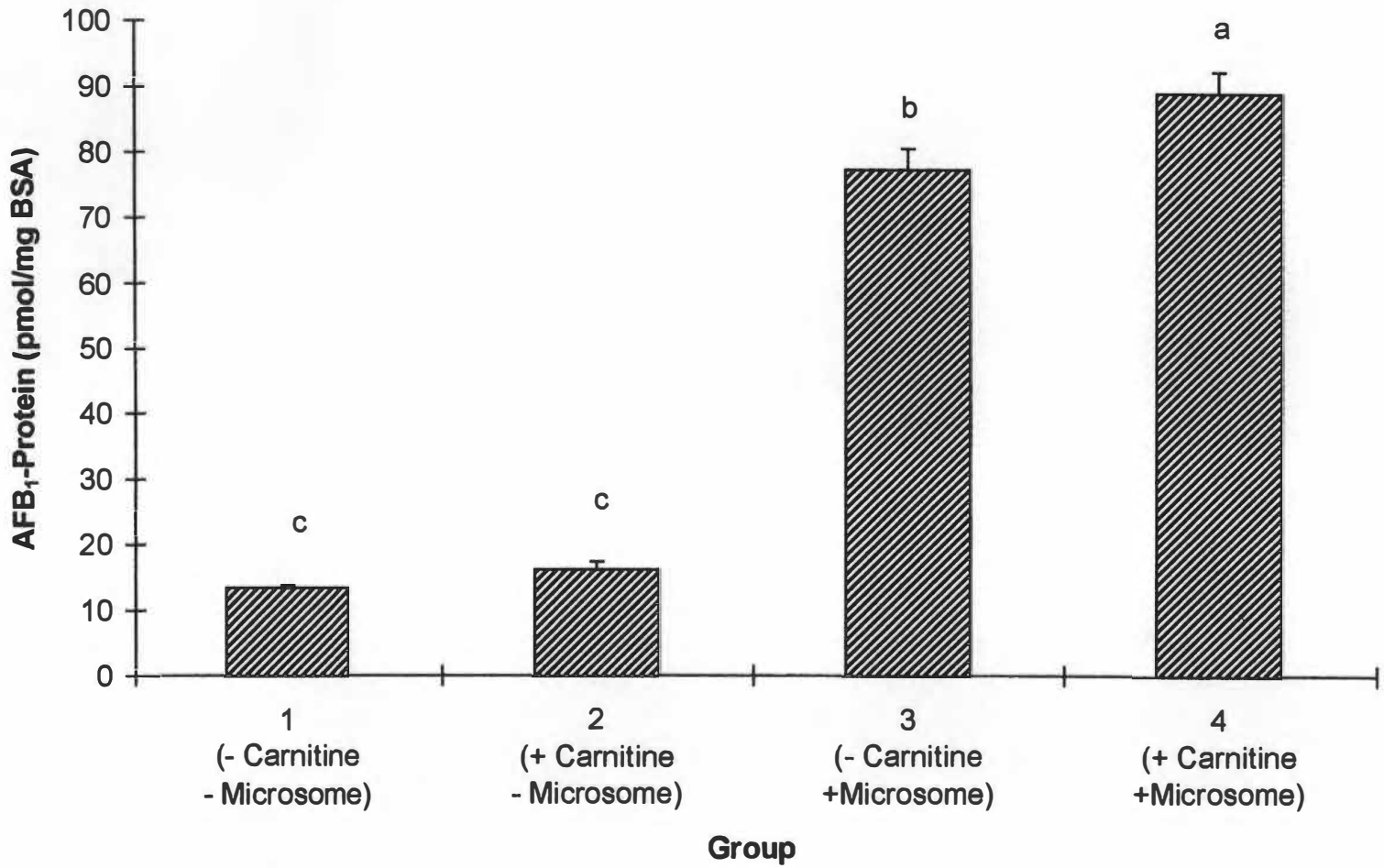


FIGURE 4.4 Effects of L-carnitine (1.2 mM) on the binding of AFB_1 to BSA. The bars represent means ± SEM of n = 5. Different letters indicate significant difference among groups ($p < 0.05$).

in Groups 3 and 4. The presence of carnitine in the microsome-containing incubation mixture significantly increased ($p = 0.0005$) the AFB_1-BSA adducts formation (Group 4 vs Group 3). This is an indication that carnitine increased the covalent binding of AFB_1-epoxide to BSA.

AFB_1-Microsomal Binding

The concentrations of AFB_1 binding to microsomal proteins in the absence or presence of carnitine are shown in **Figure 4.5**. Obviously, there was some binding of AFB_1 to the microsomal component, and carnitine significantly increased ($p = 0.007$) the binding compared to the control. This AFB_1 binding to microsomal protein was about two times higher than the binding of AFB_1 to BSA (**Figure 4.4**; Groups 1 and 2).

AFB_1 Non-Covalent Binding to Proteins as Measured by Ultrafiltration Method

The data on the binding of unmetabolized AFB_1 to plasma proteins and BSA are presented in **Figures 4.6** and **4.7**, respectively. The carnitine group had significantly higher binding of AFB_1 to plasma proteins than the control group ($p = 0.022$). The AFB_1-BSA adducts formation was also higher in the carnitine group than the control group ($p = 0.018$). Compared to the rat plasma, higher concentrations of the protein adducts were formed (approximately 159% more in the control groups and 175% more in the carnitine groups) when the protein was BSA. This is most likely related to the purity of BSA protein as plasma has components other than albumin.

Discussion

The experiments in this Chapter were aimed exploring other possible mechanisms of carnitine-mediated attenuation of AFB_1-DNA adduct formation. The data suggest that

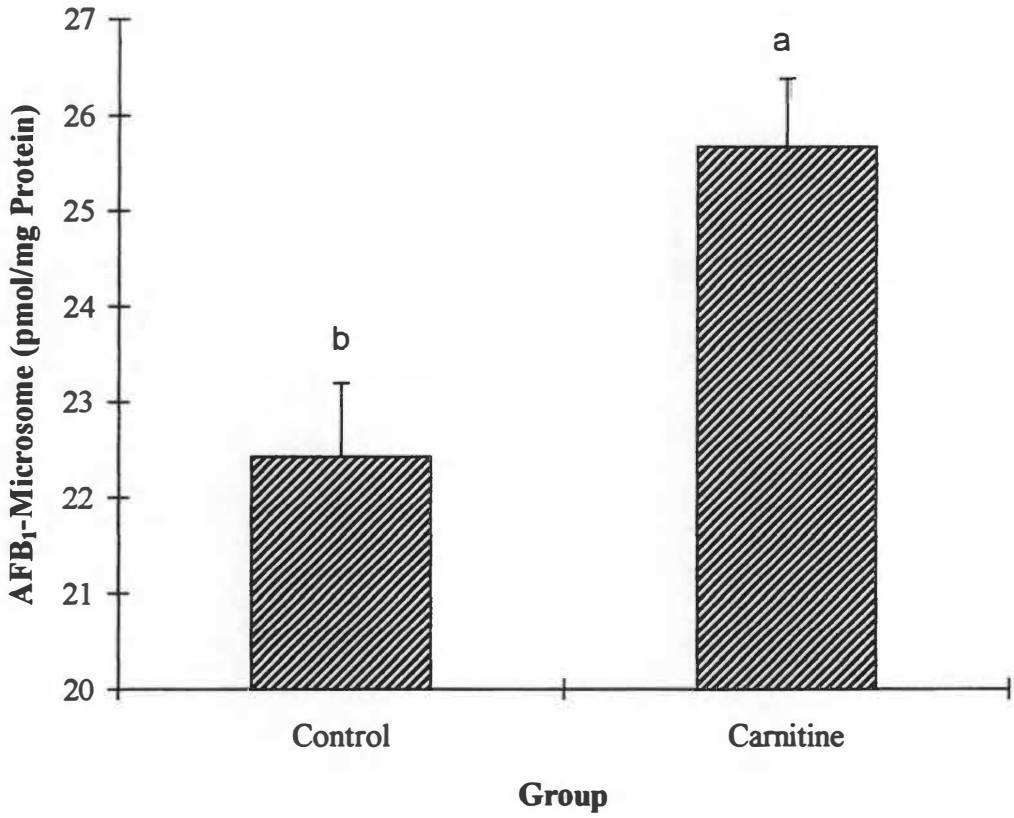


FIGURE 4.5 Effects of L-carnitine (1.2 mM) on AFB_1 binding to microsome. The bars represent means ± SEM of n = 5. Different letters indicate significant difference between groups ($p < 0.05$).

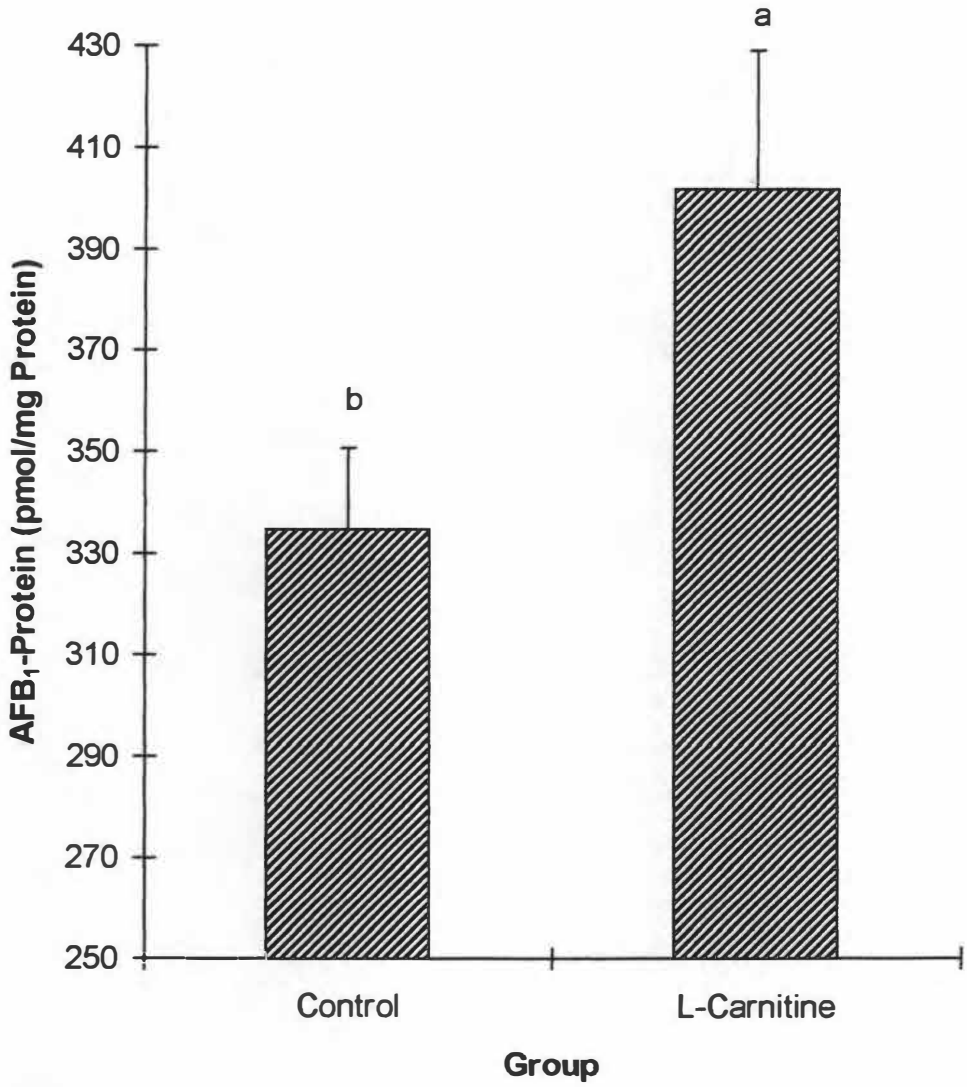


FIGURE 4.6 Effect of L-carnitine on AFB_1 binding to plasma protein as determined by an ultrafiltration separation technique. The bars represent means $\pm$ SEM of n = 8. Different letters indicate significant difference between groups ($p < 0.05$).

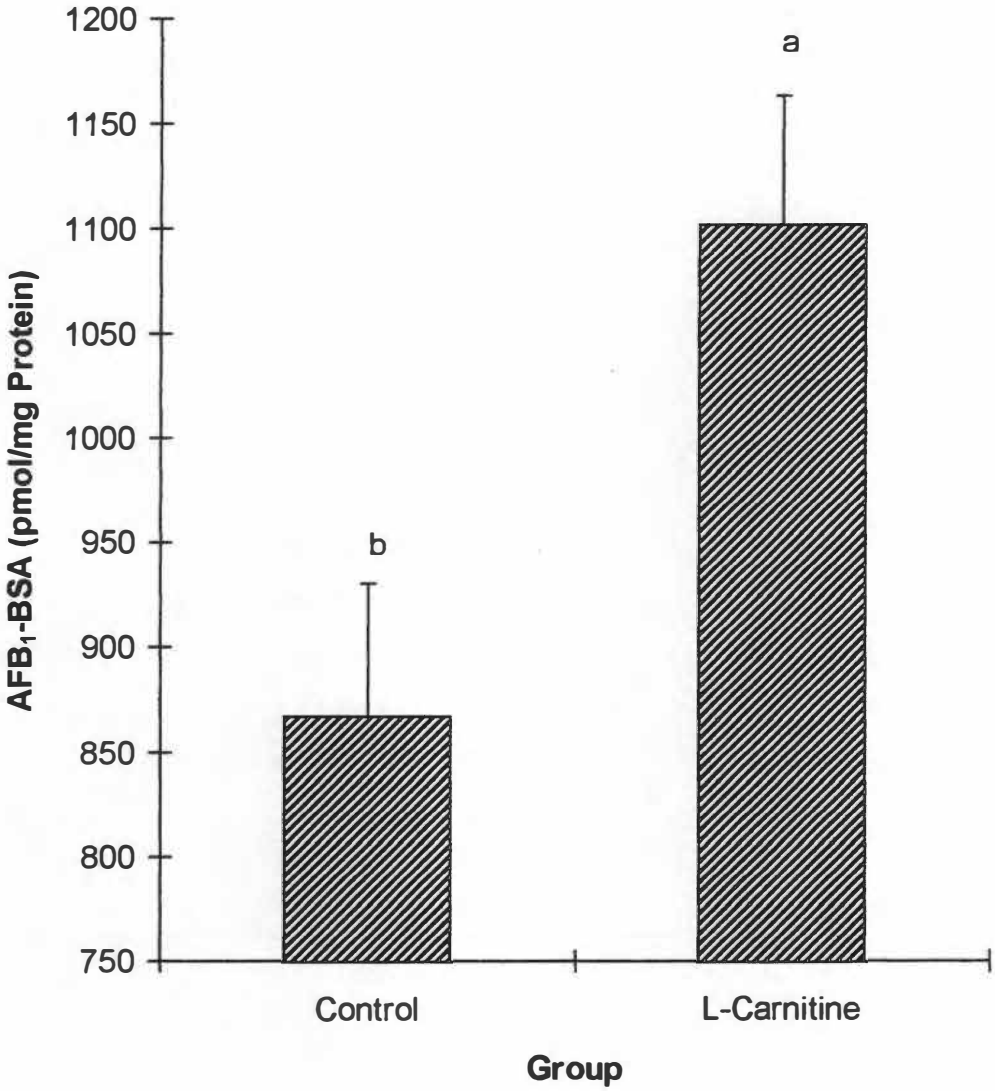


FIGURE 4.7 Effect of L-carnitine on AFB_1 binding to BSA as determined by an ultrafiltration separation technique. The bars represent means $\pm$ SEM of n = 10. Different letters indicate significant difference between groups ($p < 0.05$).

at least three processes are affected by carnitine. These are: 1) bioactivation of AFB_1 and binding of activated AFB_1 to DNA; 2) binding of AFB_1 to plasma proteins; and 3) binding of AFB_1 to microsomal proteins.

Bioactivation of AFB_1 and binding of activated AFB_1 to DNA

AFB_1 needs to be activated by microsomal enzymes to form the AFB_1-epoxide which covalently binds to the electrophilic center (N, O, and S atoms) of cellular macromolecules such as DNA, RNA, and protein (Stark 1986). The covalent binding, especially to DNA, is the crucial initial step of AFB_1 carcinogenesis.

First of all, the current study, confirms the role of microsomal enzymes in producing the metabolites that can bind to DNA. More importantly, this study shows that in the presence of carnitine, the binding of microsomal activated AFB_1 to DNA was decreased. This indicates that carnitine interferes with the bioactivation of AFB_1. Microsomes contain the enzymes that biotransformed AFB_1 to its activated form (AFB_1-epoxide) and the less toxic hydroxylated metabolites. Therefore, if carnitine can affect the metabolism of AFB_1 that results in lesser production of AFB_1-epoxide, then there will be lower amount of epoxide for covalent binding to DNA. On the other hand, it may be that carnitine has no effect on the metabolism of AFB_1 whatsoever, but it simply prevents the epoxide produced from binding to DNA. This postulation is made on the basis that carnitine itself may bind the AFB_1-epoxide and therefore limit the amount of epoxide for binding to DNA. Carnitine has the quarternary nitrogen group similar to the electrophilic center of guanine of DNA and is available for electrophilic attack by AFB_1-epoxide. More studies are needed to specify which one of the above two pathways is operational.

The effect of carnitine is not highly specific as acetylcarnitine and GABA were also found to reduce the AFB_1-DNA adduct formation to about the same degree as carnitine. Two other compounds structurally related to carnitine, choline and glycine, had

no significant effect on the binding of AFB_1-epoxide to DNA. The analogy with GABA in this regard is quite interesting for studying the receptor-mediated mechanism of carnitine effect.

The lack of choline effect is not entirely surprising as choline effect is highly controversial. It has been reported that choline-deficiency had no effect on the liver AFB_1-DNA adduct concentration in rats given a single dose of AFB_1. However, when multiple doses of AFB_1 were given, the DNA adduct formation was markedly elevated in the choline-deficient animals (Schrager et al. 1990). On the contrary, an earlier report indicates that a diet marginally deficient in lipotropes (methionine, choline, and folacin) fed to rats which were given a single dose of AFB_1 suppressed DNA adduct formation in the liver (Campbell et al. 1978). Yet another recent study demonstrated that a choline-methionine deficient diet, fed to rats, had no significant effects on serum biochemical parameters and liver pathology due to a dose of AFB_1 (Mehta et al. 1993).

Binding of AFB_1 to proteins

AFB_1 and its metabolites, particularly AFB_1-epoxide, are known to bind to plasma protein (Chu 1994), and this binding may be altered by pH and fatty acid concentrations (Dirr 1989). Carnitine significantly increased the covalent binding of microsomal activated AFB_1 to BSA, a purified form of serum protein (**FIGURE 4.4**). It also increased the binding of non-metabolized AFB_1 to BSA and plasma proteins (**FIGURES 4.6 and 4.7**). The binding of AFB_1 was nearly six times higher in the presence of microsome than in absence of microsome, suggesting that AFB_1 metabolites have a higher affinity for BSA and that the effect of carnitine is significant in both cases.

There are two hypotheses for how carnitine elevation of AFB_1-protein binding can lead to a reduction in AFB_1-DNA binding. First, it is postulated that if carnitine can somehow increase the AFB_1 retention by plasma proteins, less will be available for uptake

by hepatocytes. When the uptake of AFB_1 is reduced, less AFB_1 will be available for metabolism in the liver cells to cause harmful physiological changes. It has been suggested that since the liver has high capability to draw free AFB_1 from the blood, AFB_1 binding to plasma albumin once it is absorbed from the intestine can be one of the detoxification processes of AFB_1 (Hsieh & Wong 1994). Plasma contains about 7-8% proteins, and albumin constitutes about 50-60% of plasma proteins (Anderson & Lunden 1979). Albumin has been shown to be the main plasma protein that binds AFB_1 and therefore to serve as the major transporter of AFB_1 in the blood (Dirr & Schabort 1987). It was demonstrated that almost all (> 95%) of the AFB_1 found in rat plasma proteins was noncovalently bound, and about 80% of AFB_1 was found in the albumin fraction (Ewaskiewicz et al. 1991). So the albumin serves as the transporter of AFB_1 in the plasma, and carnitine is a positive effector.

Second, since carnitine increases the covalent binding of AFB_1 to plasma protein, it is possible that carnitine can also increase the covalent binding of AFB_1 to cellular protein and therefore reduce the binding of activated AFB_1 to cellular DNA. We were unable to study this for technical reasons.

The above AFB_1-albumin data raise an important implication, since AFB_1-albumin adduct concentration in plasma has been widely used as an acceptable biomarker in evaluating exposure of AFB_1 in humans (Chang et al. 1994). Our findings invoke a special caution that should be taken when interpretating the results of AFB_1-albumin data. Higher concentrations of AFB_1-albumin adduct in the blood may not always indicate higher intake of AFB_1 but may be a consequence of modulation by dietary nutrients such as carnitine and others yet to be identified. Dirr (1987) reported that the increase in plasma concentrations of long chain fatty acids considerably increased the AFB_1-plasma albumin concentration. This is besides the fact that AFB_1 binds to serum albumin and hepatic DNA in a dose-dependent manner (Sabbioni et al. 1990).

Binding of AFB_1 to microsomal proteins

From this rather indirect determination, it is extrapolated that carnitine increases binding of AFB_1 to the microsome. This is supported by the fact that AFB_1 has been shown to have highest affinity for liver microsome followed by the cytosol, mitochondria and nuclei (Ewaskiewicz et al. 1991).

Conclusion

The protective mechanisms of carnitine against the AFB_1-DNA adduct formation are related to 1) reduction of AFB_1 bioactivation; 2) sequestration of AFB_1-epoxide; and 3) enhancing of the binding of AFB_1-epoxide to cellular protein and microsome.

CHAPTER 5

Future Research

The following are some areas for future studies:

a) Identify the cytochrome P-450 isozyme(s) and quantify the metabolites of AFB_1 that may be affected by carnitine. These findings will show if carnitine has an effect on AFB_1 metabolism, especially the production of the AFB_1-epoxide.

b) Determine if carnitine can bind to activated AFB_1. The results of this experiment will further explain the mechanisms of carnitine reducing the AFB_1-DNA adduct formation.

c) Determine the effect of carnitine on AFB_1-glutathione conjugate formation, concentrations of GSH and GSSH, and GSH:GSSH ratio. These data will explain how carnitine ameliorated the reduction in total glutathione concentration caused by AFB_1.

d) Determine the effect of carnitine on the AFB_1 binding kinetics (for examples, maximum number of binding sites (Bmax) and equilibrium dissociation constant (Kd)) to plasma protein (albumin) and cytosolic protein.

e) Determine the effect of carnitine on the bindings of AFB_1 to DNA, RNA, and protein when the macromolecules are incubated together in a microsomal system. This will provide information about the changes in the binding affinity of activated AFB_1 to macromolecules in presence of carnitine.

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Appendixes

APPENDIX 1

Isolation and Preparation of Hepatocytes

Buffers for Pre-Perfusion, Perfusion and Incubation

Table 1 shows the buffers used for preperfusion, collagenase perfusion and incubation. The buffers were modified Hank's balanced salt solutions as described by Lotlikar et al. (1989). The pre-perfusion modified Hank's buffer contained 0.5 mM EGTA, 25 mM HEPES, and 0.5% BSA (added prior to perfusion) but had no Ca^{++} and Mg^{++}. The solution was saturated with 95% O_2 + 5 CO_2% gas before used. The perfusion buffer contained modified Hank's buffer having 2 mM $CaCl_2$, 0.5 mM EGTA, 25 mM HEPES, 0.5% BSA, and 0.05% collagenase (type IV). The solution was saturated with 95% O_2 + 5 CO_2% gas before the addition of BSA and collagenase just prior to perfusion. The incubation buffer was similar to the collagenase buffer except it had no collagenase.

Perfusion Apparatus

The perfusion apparatus utilized were similar to that described by Seglen (1976). It consisted of :

a) a water-jacketed coiled glass tube (2 x 4 mm glass tube with 22-24 coil (coil diameter 30 mm; coil length 100mm) connected to a water bath that maintained the buffer temperature at 37°C.

b) a filter and bubble trapping unit that prevented any bubbles from entering the liver during perfusion.

c) an adjustable flow pump unit that pumped the buffer from the buffer flask through the coiled glass tube and flowed into the filter and bubble trapping at certain flow rate.

d) a canula tube that connected to the filter and bubble trapping unit.

Table 1 The compositions of the buffers were as follows :

Chemicals (mg/L)	*Ca^{++}- and Mg^{++}-free buffer*	*Collagenase buffer*	*Incubation buffer*
$CaCl_2$	-	**222**	**222**
$MgSO_4.7H_2O$	-	**200**	**200**
KCl	**400**	**400**	**400**
KH_2PO_4	**60**	**60**	**60**
$NaHCO_3$	**350**	**350**	**350**
NaCl	**8000**	**8000**	**8000**
$Na_2HPO_4.H_2O$	**60**	**60**	**60**
Glucose	**1000**	**1000**	**1000**
EGTA	**190**	-	-
HEPES	**5,958**	**5,958**	**5,958**
pH	**7.4**	**7.4**	**7.4**
10% BSA (ml/L)	-	**50**	**50**
Collagenase	-	**500**	-
Needed (ml/perfusion)	**500**	**100**	**250**

e) a 95% O_2 and 5% CO_2 gas tank.

Surgery and Perfusion Procedure

a) Rat was anesthetized with metofane (Pitman-Moore, Inc. Mundelein, IL) by open-drop method in a closed jar.

b) The anesthetized rat was placed on an operating tray on its back. When needed, metofane in a 10 ml scintillation glass bottle was placed to cover the nose of the rats to continue anesthesia of the rat.

c) A cut was made from the middle of lower end of the abdomen to the upper end of the abdomen just below the diaphragm.

d) A V-shaped cut was made below the ribs that went to the left and right ribs.

e) The intestine was carefully moved to the left side of the abdomen to exposed the portal vein.

f) Two loose ligatures (about 5 mm apart) were placed around the portal vein about 1-2 mm away from the liver.

g) The Ca^{++}- and Mg^{++}-free buffer was turn on at a flow rate of 30 ml/min. This buffer was continually saturated with O_2 gas and the temperature were set at 37 °C.

h) A small cut was made just below the furthest ligature from the liver, cannula tube (with the buffer flowing) was inserted through the cut, and the ligatures were tightened.

i) The perfusion flow was increased to 50 ml/min and the posterior vena cava was cut to

the let the perfusate to escape.

j) After about 2-3 min, while the perfusate still flowing the liver was removed from the rat by cutting all the attachments to the liver and placed flat as the in situ position on a perfusion dish.

k) When the 500 ml of Ca^{++}-free buffer was about finished (10 min.), the buffer flow was stopped, changed the buffer reservoir to the collagenase buffer in a 250 ml beaker, placed the liver dish on top of the 250 ml beaker, and started the buffer flow at 50 ml/min for about 10 min. The liver swelled to about twice its original size.

l) The liver was placed on a watch glass, added with 5 ml of the incubation buffer containing 0.5% BSA, and the liver was carefully raked with a stainless steel fork to release the cells from the connective tissue and vascular stroma.

Cell Purification and Viability Test

a) The liberated cells were suspended in 10 ml of ice-cold incubation buffer containing 0.5% BSA, and was centrifuged at 50 x *g* for 3 min at 4 °C. The loose cells and supernatant were removed. After this time, the cells were placed in a ice-bucket (temperature at about 4 °C) when they were not being manipulated.

b) The heavy cell pellet was resuspended with 20 ml of incubation buffer containing 0.5% BSA, and centrifuged as above. The supernatant and loose cells were discarded.

c) Step 4 b was repeated 2 more times to ensure maximal removal of dead cells and other cells (macrophage and fat cells).

d) The hepatocytes pellet was suspended and made-up to 20 ml suspension with the

incubation buffer containing 0.5% BSA.

e) For viability test, the hepatocyte suspension was diluted 1:10, and 100 ml of this diluted cells was addded to 100 ml of 0.6% trypan blue (therefore, the dilution factor =20). The cells were counted from 4 outer squares in a hemacytometer, by an Olympus TO41 microsope.

f) Calculation:

Cells/ml = Average # cell x D.F. x Factor for vol. of cell suspension under cover slip (10^{-4} ml)

$\therefore$ Cells/ml = (Total # cells/4) x 20 x 10^4/ml.

APPENDIX 2

Composition of Hepatocyte Incubation Mixtures and Detemination of AFB_1-Macromolecule Adducts Formation

A. Incubation Mixture (in duplicate or triplicate)

		Group 1 0 CNE	Group 2 0.2 mM CNE	Group 3 0.8 mM CNE	Group 4 1.2 mM CNE	Group 5 1.5 mM CNE
1	10×10^6 cell/ml, ml	2	2	2	2	2
2	L-Carnitine (10 mM, pH 7.0), µl	--	100	400	600	750
3	Hank's Buffer (+ 0.5%BSA), µl	2130	2130	2130	2130	2130
4	DDW, µl	750	650	350	150	0
5	DMSO, µl	100	100	100	100	100
6	$[H^3]AFB_1$ (0.1mCi/umol), µl	20	20	20	20	20
	TOTAL, ml	5	5	5	5	5

B. Incubation Procedures

1. Add hepatocytes, L-carnitine, HB, and DDW (but not **DMSO and $[H^3]AFB_1$**) in 25

ml Erlenmeyer flasks.

2. Mix the mixtures by swirling the flasks.
3. Flush the flasks (without touching the incubation mixture) with O_2:CO_2 (95%:5%) for 30 sec, and seal with rubber stoppers.
4. Incubate at 37 ^{0}C for 10 min a shaking water bath (speed 75-80 rpm which is about 3.5 of the water bath knob scale).
5. Mix [H^3]AFB_1 and DMSO together, and add them to the incubation mixture.
6. Mix by swirling the flask.
7. Flush the flasks (without touching the incubation mixture) with O_2:CO_2 (95%:5%) for 1 min, and seal with stopper.
8. Incubate at 37 ^{0}C for 60 min in a shaking water bath (speed 75-80 rpm).
9. Place the flasks immediately into an ice-water bath to stop the reaction.
10. Transfer **100** µl of IM into microcentrifuge tubes containing 100 µl of 10 mM HCl for Total GSH determination, **0.5 ml** into another tubes for GST activity assay (2 x 10^6 cells contain 5-8 mg protein; needed 1 mg protein), **3 ml** into another tubes for DNA determination, and **300** µl into another tube for polar and non-polar metabolites extraction and quantitation. Transfer also the remaining of IM into new tubes.
11. Quickly freeze all tubes in liquid nitrogen, and store tubes at -80 ^{0}C until use.

C. Macromolecules Extraction, Quantitation, and AFB_1-macromolecules Determination

1. Centrifuge the tubes (from step B10) at 500 x *g* for 5 min to sediment cells. Discard SN.

2. Wash the cells with 1ml of ice-cold incubation buffer (no BSA). Suspend cells by gentle mixing. Centrifuge. Discard SN.
3. Repeat step 2.
4. Resuspend cell in 4 ml of incubation buffer.
5. Homogenized cells with 3 complte strokes in a teflon-glass homogenizer.
6. Transfer 3 ml of homogenized cells and add 3 ml of ice-cold 1.0 PCA.
7. Vortex and centrifuge at 600 x *g* for 10 min at 2 ^{0}C. Discard the supernatant.
8. Wash pellet with 1.5 ml 0.2N PCA and centrifuge at 600 x *g* for 10 min. Remove supernatant.
9. Repeat Step 8 for 3 more times.
11. Add 1.5 ml 0.1N KAc prepared in absolute ethanol. Vortex, centrifuge at 2000 x *g* and discard supernatant
12. Add 1.0 ml chloroform:absolute ethanol (1:1, v/v). Vortex, centrifuge at 2000 x *g* and discard supernatant. Repeat once.
13. Add 1 ml absolute ethanol. Vortex, centrifuge at 2000 x *g* and discard supernatant <u>completely</u>. Air dry or vacuum dry the pellet.
14. Add 1.0 ml of 1.5N PCA, resuspend pellet by vortexing, and leave overnight in refrigerator (4 ^{0}C).
15. Centrifuge at 2000 x *g*, collect supernatant.
16. Wash pellet 2 times with 0.5 ml of 0.5N PCA. Vortex, centrifuge, and pool supernatant each time into Step 15 tubes. The combined supernatants contain the RNA fraction.

17. Add 1 ml of 0.5N PCA to pellet. Vortex and incubate at 70 ^{0}C water bath for 20 min. Centrifuge and collect supernatant.
18. Repeat Step 18. The combined supernatant contains the DNA fraction.
19. Wash pellet with DDW twice.
20. Add 1.0 ml of 1.0N KOH to pellet. Vortex and incubate at 70 ^{0}C water bath for 15 min. Centrifuge and collect supernatant. The supernatant contains the protein fraction.
21. RNA is determined the same day spectrophometrically at 260 nm using yeast RNA as standard.
22. DNA is determined by Ceriotti method (1952) (**APPENDIX 4**).
23. Protein is determined by Lowry et al. (1951) (**APPENDIX 5**).
24. To measure radioactivity, transfer 0.5 ml of each RNA, DNA, and protein fractions into scintillation vials, add 5 ml of scintillation fluid. For protein samples, add 0.1ml glacial acetic acid. Mix by turning vials, and count radioactivity (DPM) for 10 min.

APPENDIX 3

DNA Extraction and Isolation (Genomic DNA Procedure), Quantitation, and AFB_1-DNA Determination

1. At the end of incubation time, centrifuge the tubes at 500 x g for 5 min to sediment cells. Discard SN.
2. Wash the cells with ice-cold 1ml of Hank's buffer (no BSA). Suspend cells by gentle mixing. Centrifuge. Discard SN.
3. Repeat step 2.
4. Add 0.3 ml digestion buffer (100 mM NaCl, 10 mM Tris-Cl (pH 8.0), 25 mM EDTA, pH 8.0, 0.5% SDS, and added fresh 0.1 mg/ml proteinase K) to pellet. Suspend cells by gentle mixing. Tightly cap tubes.
5. Incubate at 50°C in a shaking water-bath for 12-18h (80-90 oscillations/min).
6. Add 0.3 ml digestion buffer (without proteinase K), and mix.
7. Add 0.6 ml of phenol:chloroform:isoamyl alchohol (25:24:1) to extract DNA.
8. Vortex, centrifuge at 1700 x g for 10 min.
9. Transfer 500 ul of the top (aqueous) layer to new tubes.
10. Add 250 ul of 3.0 M sodium acetate and 1000 ul of 100% alcohol. Vortex.
11. Centrifuge at 1700 x g for 2 min. Discard SN.
12. Wash pellet thoroughly with 1 ml of 70% EtOH. Centrifuge at 1700 x g for 2 min. Discard SN. Dry in a 'Speedvac Concentrator' for 1 min.

13. Suspend DNA pellet in 1 ml of 10 mM Tris-Cl (pH 7.4) containing 1mM EDTA (TE buffer) until dissolve.
14. Remove RNA by adding 0.1% SDS (10 ul of 10 % SDS) and 1 ug/ml DNAase-free RNase (20 ul of 50 ug/ml RNase), and incubate at 37 ^{0}C for 1 h.
15. Add 1 ml of chloroform:isoamyl alchohol (10:2), mix thoroughly.
16. Centrifuge at 1700 x g for 10 min. Transfer 800 ul of the top layer (aqueous layer) into new tubes.
17. Add 400 ul of 3M sodium acetate and 1.6 ml 100% EtOH. Vortex. Centrifuge at 1700 x g for 2 min. Discard the SN.
18. Wash pellet thoroughly with 1 ml of 70% EtOH. Centrifuge at 1700 x g for 2 min. Discard SN. Dry in a 'Speedvac Concentrator' for 1 min.
19. Resuspend DNA in 1 ml of 10 mM Tris-Cl (pH 7.4) containing 1mM EDTA. (DNA may be shaken gently at R.T. or at 65 ^{0}C for several h to aid solubilization).
20. Determine DNA content spectrophotometrically using calf thymus DNA dissolved in Tris-EDTA buffer as standards (0.2 to 5 ug/ml) and by Ceriotti method (**APPENDIX 4**).

(Concentration of DNA is approximately **2 mg/g of cells**; 1 g of hepatocytes contain approximately 10^9 cells)

21. Transfer 0.2 ml of pure DNA extracts into scintillation vials, add 5 ml scintillation fluid, and measure radioactivity (DPM) for 10 min.
22. Store DNA at 4 ^{0}C for short-term storage, and at -80^{0}C for long term storage.

APPENDIX 4

DNA Assay

Procedures:

1. Set the DNA standards and sample tubes as shown below:

Tube	1 mg/ml DNA	TE Buffer	0.04% Indole	c. HCl	$CHCl_3$	mgDNA
Blank	0 μl	1000 ul	0.5 ml	0.5 ml	2 ml	0
S1	5	995	0.5	0.5	2	5
S2	10	990	0.5	0.5	2	10
S3	15	985	0.5	0.5	2	15
S4	20	980	0.5	0.5	2	20
S5	25	975	0.5	0.5	2	25
S6	30	970	0.5	0.5	2	30
Sample	100-200 μl	800-900	0.5	0.5	2	?
Sample	20 μl	980	0.5	0.5	2	?

2. Add DNA standards or sample, TE buffer (see **APPENDIX 4**), and 0.5 ml of 0.04% indole. Mix.3. Add 0.5 ml concentrated HCl. Mix.

4. Cover tunes with cap tightly and place in 100°C water-bath for exactly 10 min.

5. Immediately cool tubes by dipping them in a bucket of tap water.

6. Add chloroform (do this under the hood) and vortex thoroughly.

7. Centrifuge at 1700 x g for 10 min.

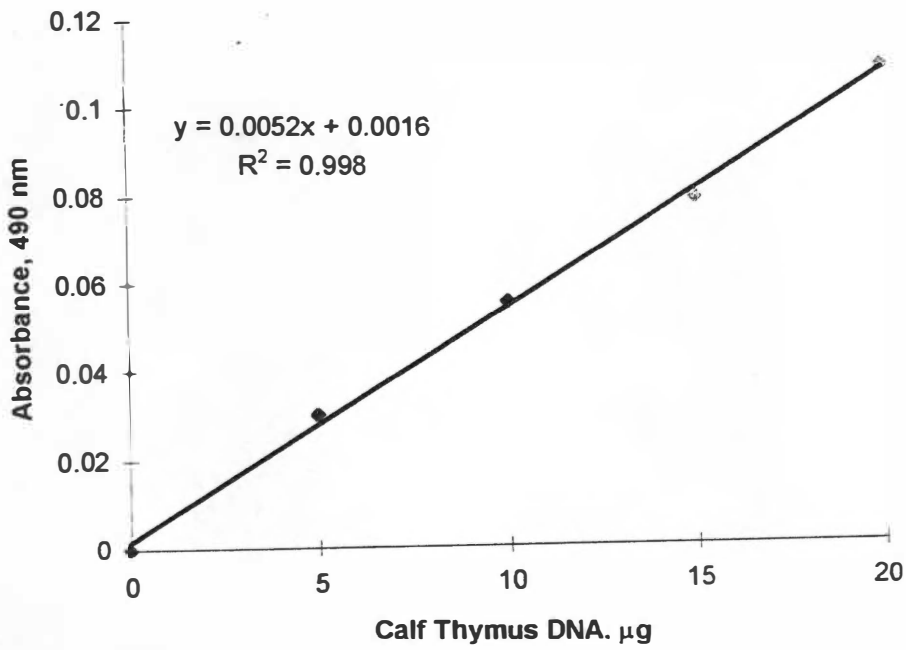
8. Using clean disposable transfer pipet each time, remove the lower ($CHCl_3$) layer.

9. Repeat steps 6 and 8.

10. Transfer the top layer into 1 ml disposable cuvet (do not take the lower layer).

11. Read absorbance at 490 nm against the blank.

12. Construct a standard curve get the linear regression equation.



DNA Standard Curve

Calculation : μg DNA = Absorbance/Slope x D.F.

Reference: Ceriotti, G. (1952) A microchemical determination of DNA. J. Biol. Chem. 198:297-303.

APPPENDIX 5

Protein Determination (Lowry's Method)

Reagents

1. 2 % (w/v) sodium carbonate (Na_2CO_3) in 0.1 N sodium hydroxide (NaOH).

   Dissolve 6 g Na_2CO_3 in 0.1N NaOH and mark-up to 300 ml with DDW.

2. 0.1N NaOH.

   Dissolve 2g NaOH in 450 ml DDW and mark-up to 500 ml volume.

3. 1% (w/v) cupric sulfate ($CuS0_4.5H_20$).

   Dissolve 0.5g $CuS0_4.5H_20$ in DDW and bring up to 50 ml volume.

4. 2% Sodium potassium tartarate ($NAKC_4H_4O_6.4H_2O$).

   Dissolve 1g $NAKC_4H_4O_6.4H_2O$ in DDW and bring up to 50 ml volume.

5. Diluted Folin reagent.

   Dilute Folin & Ciocalteu's phenol reagent 1:1 (v/v) with DDW and store in an amber bottle at 4°C.

6. Alkaline copper reagent.

   Add 0.5 ml of 2% $NAKC_4H_4O_6.4H_2O$ and 1% $CuS0_4.5H_20$ solutions to 50 ml of 2% Na_2CO_3 in 0.1 N sodium hydroxide solution (enough for 25 samples). Mix solutions thoroughly to avoid precipitation of $Cu(OH)_2$. Prepare enough just prior to assay daily.

7. 10% and 0.5 mg/ml bovine serum albumin (BSA).

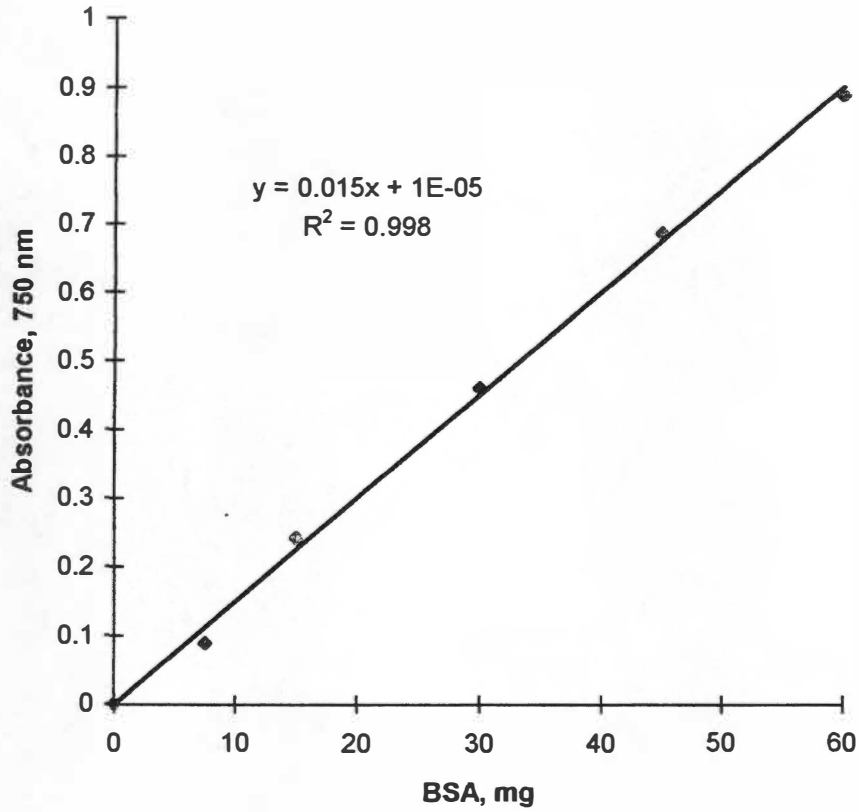
   Dissolve 10g of crystalline BSA in 100 ml of saline. A working satndard is made by diluting the 10% BSA with DDW to give 0.5 mg/ml BSA exactly. Store at less than 0°C.

Procedure

1. Set the protein standards and sample tubes as shown below:

Tube	0.5 mg/ml BSA	DDW	Alk. Cu_2SO_4	Folin Reagent	μg PRO
Blank (2 tubes)	0 μl	800 ul	2 ml	0.2 ml	0
S1	15	785	2	0.2	7
S2	30	770	2	0.2	15
S3	60	740	2	0.2	30
S4	90	710	2	0.2	45
S5	120	680	2	0.2	60
S6	150	650	2	0.2	75
Sample	20-50	750-780	2	0.2	?

2. Add BSA standards or sample and DDW. Mix.
3. Add 2.0 ml alkaline Cu_2SO_4 solution. Mix. Stand at room temperature for 15 min.
4. Add 0.2 ml diluted Folin reagent. Mix **immediately and thoroughly**.
5. Stand for 30 min at room temperature.
6. Read absorbance at 750 nm against blank.
7. Plot the regression curve, and get the linear regression equation.



Protein Standard Curve

Calculation:

μg Protein = Absorbance/Slope x Dilution Factor.

APPENDIX 6

Glutathione S-Transferase Assay of Rat Hepatocytes

Principle

Glutathione S-Transferase (GST) catalyzes the conjugation of glutathione (GSH) with 1-chloro-2,4, dinitrobenzene (CDNB) which can be monitored spectrophotometrically at 340 nm. (Caution: **CDNB is very toxic**. Avoid skin contact and inhalation. Prepare under the hood).

Reagents

1. 0.1M sodium-phosphate buffer, pH 6.5 containing 1 mM EDTA.

   2.84g Na_2HPO_4 + 0.0584g EDTA in 180 ml DDW. Adjust pH to 6.5. Make-up to 200 ml with DDW. Store at -4°C.

2. 20 mM GSH in DDW.

   0.0615g GSH dissolve in 10 ml DDW. Store at -20°C. Stable for about 1 week.

3. 20 mM CDNB in 95% ethanol.

   0.0405g CDNB dissolve in 10 ml of 95% EtOH. Store at 4 °C.

4. Hank's buffer.

   (or any other buffer that was used to suspend the cells)

Method

Cytosol preparation from hepatocyte incubation mixture

1. Prepare supernatant (SN) fraction from the incubation mixture immediately at the end of incubation, or after thawing the hepatocytes at -80 °C. All subsequent steps are done at 4 °C.
2. Centrifuge 0.5 ml of incubation mixture (about 2 x 10^6 cells/ml) at 500 x g for 10 min. Remove supernatant.
3. Wash and suspend pellet with 0.5 ml Hank's Buffer (HB). Centrifuge and remove supernatant.
4. Resuspend cells with 0.3 ml HB. Homogenize cell with a pellet pestle for 20 -30 sec.
3. Transfer homogenized cells into a thick-wall Nalgene® ultratube (13 x 51 mm).
4. Centrifuge homogenate at 9,000 x *g* for 15 min in a swinging bucket.
5. Transfer SN to a new ultratube, and centrifuge at 100,000 x *g* for 60 min.
6. Collect the SN (cytosol) and determine protein content by Lowry method (**APPENDIX**) using BSA as standard.

   (Cytosol could be stored at -80 °C, but it must be centrifuged at 14000 rpm for 20 min after thawing)

Assay

	Control	Sample
0.1M sodium-phosphate buffer	850-895 μl	850-895 μl
20 mM GSH in DDW	50 μl	50 μl
20 mM CDNB	50 μl	50 μl
Hank's Buffer	5-50 μl	--
(Mix)		
Cell cytosol	--	5-50 μl
Total	1 ml	1 ml

1. Prewarm the reagents and cytosol at 30 ^{0}C for 5-10 min. or keep them at room temperature (25 ^{0}C)
2. To two 1-ml quartz cuvettte add the buffer, GSH, and CDNB, and for the Control cuvette add Hank's buffer.
3. Cap the cuvette with parafilm and mix the reaction mixture by inverting the cuvette 2-3 times.
4. Measure spontaneous reaction by monitoring the rate of absorbance (A) at 340 nm (UV) for 3 min at 30 °C in a Hitachi U-2000 spectophometer with UV and visible light on.
5. Cap the cuvette with parafilm and mix the reaction mixture by inverting the cuvette 2-3 times.
6. Monitor and plot the increased in A rate for 3 min.

Calculation

$$\text{Specific activity } (\mu\text{mol/mg protein/min}) = \frac{\Delta A * V}{\epsilon * MP}$$

$$= \frac{\Delta A * 1}{9.6 * MP}$$

ΔA = Rate of A change with enzyme - Rate of A change without enzyme (per min at 340 nm)

V = Volume of reaction mixture = 1 ml

ϵ = Extinction coefficient of CDNB = 9.6 mM^{-1} cm^{-1}

MP = mg of cytosolic protein

Definition:

Specific activity of GST is defined as the amount of the enzyme catalyzes the formation of one unit or 1 μmol of 2,4-dinitrophenylglutathione per min at 30 °C using 1 mM of GSH and CDNB per mg of protein.

Reference:

Habig et al. (1974) J Biol Chem 249:7130-7139 as described by Warholm et al. (1985) Methods in Enzymology 113: 499-507.

APPENDIX 7

Total Glutathione Assay in Hepatocytes

Principal

I) 2GSH + DTNB → GSSG + 2TNB

2) GSSG + NADPH + H^+ →* 2GSH + $NADP^+$

*GSSH Reductase

GSH is oxidised non-enzymatically with DTNB to produce GSSG and 2TNB. GSSG is then reduced to GSH enzymatically by GSSH reductase and NADPH. Total GSH is calculated by the rate of color change of DTNB at 412 nm for 3-6* min minus the rate of reduction of DTNB in absence of GSH or GSSH (blank). The rate of TNB formation is proportional to the total amount of GSH and GSSH. The rates are also monitored in known amount of GSH solutions (standards) to plot the caliberation curve.
(*3 min monitoring of the rate of change is sufficient to get the linear portion of the curve.)

Reagents (for about 100 assays)

1. 100 ml of 10 mM HCl.

2. 10 ml of 10 % 5-sulfosalicyclic acid.

3. 143 mM sodium phosphate and 6.3 mM EDTA (stock buffer).

   Mix 5.077g Na_2HPO and 0.59 g EDTA-$Na_2.2H_2O$, flask and make-up to 250 ml with DDW; final pH 7.5. Keep at room temperature or at 4 °C.

4. 0.3 mM NADPH buffer

   26.6 mg NADPH tetrasodium salt in 100 ml of stock buffer. Prepare daily and keep at 4 °C.

5. 266 U/ml GSSH reductase Type III (Bakers yeast).

   Dilute yeast enzyme to get 266 U/ml with stock buffer. Keep at 4 °C; stable for 2 weeks.

6. 6 mM 5,5'-dithiobis-(2-nitrobenzoic acid)(DTNB) in stock buffer.

   23.8 mg DTNB in 10 ml stock buffer. Store at 4 °C; stable for 2 weeks.

7. 20 mM GSH Standard solution (stock standard solution).

   Dissolve 61.4 mg GSH in 10 ml DDW. Store frozen; stable for 1 weeks.

   Dilute stock standard solution with 5-sulfosalicylic acid to give 0.5, 1, 2, 3, and 4 nmol of GSH. Prepare daily.

Hepatocyte or Blood Sample Preparations

1. Hepatocyte or whole blood sample (20 μl) is added to 100 μl of 10 mM HCL in a 400 μl or 1.5 ml microcentrifuge tube.

2. Lyse cell by freezing in liquid nitrogen or dry-ice acetone bath, and thaw.

3. Repeat Step 2 for 3 more times.

4. Centrifuge in a Beckman microfuge at 10,000 g for 5 min.
5. Transfer 100 μl of the supernatant into a new microcentrifuge tube.
6. Add 50 μl of 10% 5-sulfosalicylic acid and mix to deproteinized the supernatant.
7. Centrifuge in a Beckman microfuge at 10,000 g for 5 min.
8. Up to 25 μl of the protein-free supernatant can be used for assay.

Assay

	Reference/Blank	Standard/Sample
NADPH buffer	700 μl	700 μl
DTNB	100 μl	100 μl
DDW	200 μl	175 μl
(Cap with parafilm, mix, and warm at 30 °C for 5 min)		
Protein-free sample	--	**25** μl
(Cap, mix, and zero the Abs)		
GSSG reductase	**5**-10 μl	**5**-10 μl
Cap, mix, zero Abs., and read change in absorbance at 412 nm for 3 min.		

1. Add the NADPH, DBNB, and DDW into two 1.5 microcentrifuge tubes.
2. Vortex and incubate at 37 °C for 3 min.

3. Add 25 μl sample (or blank and standards) to sample cuvet, and mix.
4. Add GSSH reductase to both reference and sample cuvets, and mix gently.
5. Transfer the reaction mixtures into 1.0 ml quartz cuvets.
6. Zero absorbance, and start monitoring change in absorbances for 3 min.
7. Run the assay for GSH standards containing 0, 0.5, 1, 2, 3, and 4 nmol of GSH equivalent.
8. Plot rate of absorbance change against GSH equivalent.
9. The amount of total glutathione in sample is calculated from the regression equation obtained from standard curve graph.

(For other samples, other extraction procedures are outlined by Griffith (1985) and Anderson (1985). Use the equations in Griffith (1985) to calculate for blood, plasma, and tissues glutathione contents)

References

1. Griffith (1985) Methods in Enzymology VIII: 521-529.
2. Anderson (1985) Methods in Enzymology 113: 548-555.

APPENDIX 8

Isolation of Microsomes from Rat Liver

Method

1. Anesthetize rat with metofane in a jar.
2. Perfuse rat with about 100 ml ice-cold physiological saline.
3. Add 20 ml of 0.154 M KCl containing 0.01 M KH_2PO_4 (pH 7.4) to 4 g liver (25% w/v), and homogenize(6 complete strokes) in a Teflon-glass homogenizer.
4. Centrifuge homogenate at 10,000 x *g* for 10 min at 4 °C.
5. Transfer supernatant (SN) into clean centrifuge tubes and centrifuge at 105,000 x *g* for 60 min at 4 °C.
6. Collect the SN (cytosol) into new tubes.
7. Resuspend microsomal pellet in 4 ml (1 ml/g tissue) of glycerol-phosphate buffer (1:1, glycerol:0.15M KH_2PO_4, pH7.8).
8. Determine protein content by the method of Lowry et al. (1951)
9. Store at -80 °C .

Reference

Kato, R. (1966) P450 and microsomal oxidation of drugs. J. Biochem. 59: 574-579.

APPENDIX 9

Binding of AFB_1 to Exogenous DNA in a Microsomal System

Method

1. Triplicate samples are prepared as follows:

	CONTROL	1.2 mM L-CNE
0.5 M Phosphate Buffer	200 µl	200 µl
100 M NADPH	20 µl	20 µl
[3H]AFB1, 2 nmol/20 ml DMSO	20 µl	20 µl
Microsomes, 1 mg protein equivalent	200 µl	200 µl
4 mg/ml Calf Thymus DNA	25 µl	50 µl
10 mM L-CNE, pH 7.0	0 µl	120 µl
DDW	535 µl	415 µl
TOTAL	**1000 µl**	**1000 µl**

2. Incubate the incubation mixtures at 37 °C for 30 min.
3. Add 0.245 ml 5M NaCl to produce a mixture containing 1M NaCl, 2 ml of chloroform:isoamyl alcohol (24:1 ; v/v), and 0.9 mg calf thymus DNA (0.225 ml of 4 mg/ml DNA) as carrier.
4. Shake the tubes for 10 min, and centrifuge at 10,000 x *g* for 10 min.

5. Transfer all the top layer (DNA fraction) to new tubes, add 2 ml chloroform:isoamyl alcohol, shake for 10 min., and centrifuge at 10,000 x *g* for 10 min.

6. Transfer the top layer to new tubes.

7. Add equal vol. of 95% ethanol to precipitate DNA. Vortex. Centrifuge at 10,000 x *g* for 10 min. Remove SN.

8. Dissolve DNA in 1 ml of 0.1M NaCl (pH 7.0).

9. Determine DNA content by Ceriotti (1952) (**APPENDIX 4**).

10. Transfer 200 ul of DNA solution into scintillation vials, add 5 ml scintillation fluid, and DPM for 10 min.

References

1. Allemeh, A., Saxena, M., & Raj, H.G. (1988) Differential effects of butylated hydroxyanisole on metabolism of aflatoxin B_1 in vitro by liver and lung microsomes. Cancer Lett. 40:49-57.

2. Hasler, J.A., Dube, N., Nyathi, C.B., Furmann, H., & Sallmann, H.P. (1994) The influence of dietary fat on hepatic bioactivation of aflatoxin B_1 in rats. Res. Commun. Chem. Pathol. Pharmacol. 83:279-287.

Vita

Ayub Mohd Yatim was born in Klang, Selangor, Malaysia in June 6, 1959. He received his elementary and high school educations at the Anglo-Chinese Methodist School located in Klang. Following high school, he entered MARA Institute of Technology and graduated with a Diploma in Food Technology in August, 1981. He later worked at Malaysian Agricultural Research and Development Institute (MARDI) at Serdang, Selangor, as an Assistant Research Officer. After working for 4 years, he attended the University of Tennessee, Knoxville, and graduated with a Bachelor of Science degree majoring in Nutrition and Food Science in March 1988, and with a Master of Science degree majoring in Nutrition in May, 1990. Upon returning to Malaysia, he then continued working with MARDI, and later worked with the Universiti Kebangsaan Malaysia (National University of Malaysia), Bangi, Selangor in January, 1993. In August, 1993 he came back to the University of Tennessee, Knoxville to pursue his doctoral degree in Nutritional Science with the sponsorship from the Malaysian government and Universiti Kebangsaan Malaysia. He graduated in December, 1996 and returned to his country to teach and to do research.