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Effects of L-Carnitine on Aflatoxin B_1 Toxicity

Ayub Mohd Yatim
University of Tennessee, Knoxville

To the Graduate Council:

I am submitting herewith a thesis written by Ayub Mohd Yatim entitled "Effects of L-Carnitine on Aflatoxin B_1 Toxicity." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Nutrition.

Dileep S. Sachan, Major Professor

We have read this thesis and recommend its acceptance:

James W. Bailey, Frances A. Draughon

Accepted for the Council:
Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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EFFECTS OF L-CARNITINE ON AFLATOXIN B_1 TOXICITY

A Thesis

Presented for the Master of Science

Degree

The University of Tennessee, Knoxville

Ayub Mohd Yatim

May 1990

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ABSTRACT

The effects of carnitine supplementation on AFB1 toxicity in male Sprague-Dawley rats were studied. Rats were fed Purina Rat Chow with or without carnitine supplement for 6 weeks. In Experiment 1, the rats (n=20) were divided into 4 groups, namely nonsupplemented control (NSC), nonsupplemented AFB1 (NSA), carnitine supplemented control (CSC) and carnitine supplemented AFB1 (CSA) groups. The NSA and CSA groups were given an oral dose of [^{3}H]AFB1 (1 mg/kg) 6 h before sacrificing. In Experiment 2, the rats (n=10) were divided into NSA and CSA groups and were sacrificed 24 h post-AFB1 administration. Concentrations of total lipid, triglycerides, carnitine and AFB1-macromolecules adduct formation, and urinary excretion of AFB1 were measured. Carnitine supplementation prevented increase in hepatic total lipid and triglyceride concentrations caused by AFB1. In the plasma, supplemental carnitine prevented the AFB1-induced decrease in the amount of total lipid and triglyceride. Carnitine supplementation reduced AFB1 covalent binding to hepatic macromolecules, namely RNA, DNA, and protein. The concentrations of RNA, DNA, and protein in the CSA rats were significantly higher than in the NSA animals 24 h post-AFB1 treatment. [^{3}H]AFB1 radioactivity was found in the acid insoluble acylcarnitine (AIAC) fraction. Carnitine supplement, however, had no significant effect on the amount of [^{3}H]AFB1 associated with AIAC. AFB1 treatment of rats 6 h prior to sacrifice caused significant increase in the liver nonesterified carnitine (NEC), acid soluble acylcarnitine (ASAC), and total carnitine. The amounts of AFB1 in the liver, kidney and plasma were not significantly different in the NSA and CSA animals. The concentration of AFB1 excreted in the urine was also not statistically different in the CSA and NSA rats. In conclusion, carnitine supplemention offered protection against AFB1-induced fatty liver and AFB1-macromolecule adducts formation.

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CHAPTER 1

INTRODUCTION

Aflatoxins, a group of secondary metabolites of Aspergillus flavus and A. parasiticus, are often found to contaminate food and feed supplies. Epidemiological studies and clinical findings especially in developing countries such as Uganda (1), Kenya, Swaziland, Mozambique, and Thailand (quoted from 2) showed incidences of liver cancer correlated to consumption of aflatoxin-contaminated food. In India, acute toxicity of aflatoxins due to consumption of aflatoxins-contaminated maize affected 397 patients, of which, 106 died (3). Analysis on the maize samples showed that the patients might have consumed 2 to 6 mg of aflatoxins per day for several weeks. Recently, acute toxicity of aflatoxins have been reported as the cause of deaths of eleven children in Malaysia who ate rice noodles that were contaminated with aflatoxins (4).

Aflatoxin B_1 (AFB_1) is the most abundant and toxic of all aflatoxins. It has been found to be hepatotoxic (5), hepatocarcinogenic (6), and teratogenic (7). AFB_1 in combination with other aflatoxins has been reported to be immunosuppresive (8). The liver is the main target organ of aflatoxin toxicity. The signs of liver damage caused by AFB_1 include fatty liver, bile duct hyperplasia, and finally necrosis (9).

Fatty liver is also caused by acute and chronic alcohol intoxication in animals and humans (10, 11, 12) which has been attributed to increase lipogenesis and/or impaired fatty acid oxidation. Carnitine, a vitamin-like substance whose main function is in intramitochondrial transport of fatty acids for beta-oxidation, has been shown to ameliorate

ethanol-induced fatty livers in rats (13, 14, 15). Therefore, it was thought desirable to examine if carnitine supplement could prevent the fatty liver caused by AFB_1.

AFB_1 needs to be activated by mixed function oxygenase system to form an AFB_1-epoxide which can covalently bind to electrophilic centers (N, O, and S atoms) of macromolecules such as DNA, RNA, and protein (2). These covalent bindings, especially to the DNA have been linked to AFB_1 carcinogenicity. Since carnitine has a electrophilic N center, it is possible that AFB_1-epoxide could bind to carnitine, and in doing so, spare the macromolecules from the electrophilic attack of AFB_1. Carnitine has been reported to lessen the side-effects of therapeutic drugs (16) via conjugation to the drugs (17).

In light of these observations, the following objectives were setforth for this study:

1. To determine the effects of supplementary carnitine on AFB_1-induced fatty liver;
2. To study the effects of supplementary carnitine on AFB_1-macromolecules binding;
3. To determine of effects of AFB_1 on plasma and liver carnitine concentrations and $[^3H]AFB_1$ associated with carnitine.

CHAPTER 2

REVIEW OF LITERATURE

2.1 AFLATOXINS

2.1.1 History

Aflatoxins are a group of secondary metabolites of Aspergillus flavus and A. parasiticus. These mycotoxins are often found to contaminate food and feeds (such as milk, corn, peanuts, cottonseed, rice, barley, and many others) as well as grain-fermented beverages and edible animal tissues (18). These toxins have been considered as unavoidable contaminants since they cannot be totally eliminated or prevented from contaminating food or feeds even with the most modern technology available today (19). The problem of aflatoxin contamination has long been recognized and limits or action levels for the toxin content in agricultural commodities have been set since 1965.

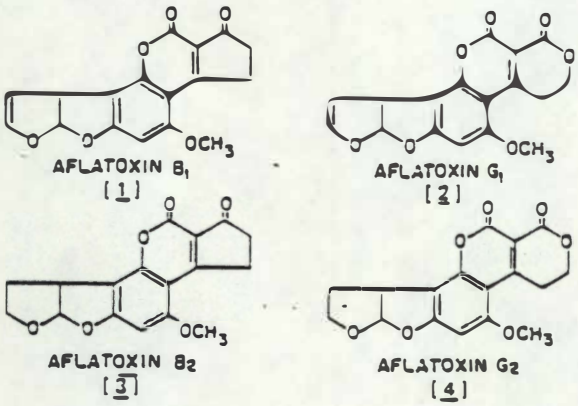
Aflatoxins were first discovered in 1960 when a series of outbreaks in poultry and fish occurred in different parts of the world. One of the worst outbreaks was known as a "turkey X" disease which had caused many deaths of turkeys and ducklings in the United Kingdom due to consumption of contaminated Brazilian peanut meal (quoted from 9). The acute symptoms of the disease included weakness, loss of appetite, lethargy, and "distinctive attitude of the head and neck" at the time of death. Histologically, the disease caused an acute liver necrosis and bile duct proliferation. Consumption of contaminated corn, peas and cotton-seed had also been reported as the cause of outbreaks analogous to the turkey X disease in farm animals and fish (quoted from 9).

In 1961, Sargeant et al. (20) isolated A. flavus from the contaminated Brazilian peanut meal. The investigators found that when the fungus was reinoculated into peanut meal, it produced toxins similar to those found in the contaminated Brazilian meal. The name "aflatoxins" (AF) was given to the toxins that had been extracted and isolated. The letters B and G refer to the toxin's color under UV light (B--Blue; G--Green), and the subscripts relate to their position on thin-layer chromatography plates. Currently, over a dozen aflatoxins have been identified.

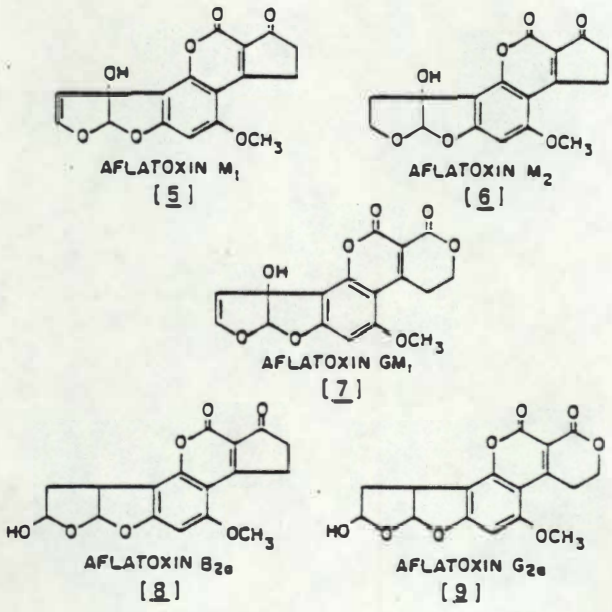
2.1.2 Structure and Toxicity Differences of Aflatoxins

Aflatoxin B_1(AFB_1), B_2 (AFB_2), G_1 (AFG_1) and G_2 (AFG_2) are the four major naturally occuring aflatoxins. The structure of aflatoxins consists of a coumarin nucleus attached to a bifuran and either a pentanone (AFB_1 and AFB_2) or a six-membered lactone (AFG_1 and AFG_2) (refer to Figure 2.1). AFM_1, AFM_2, $AFGM_1$, and $AFGM_2$ are the metabolites produced in microbial and/or animal systems when they are exposed to the above naturally occuring aflatoxins (22). AFB_1 and AFG_1 were found to be more toxic to rats and ducklings than AFB_2 and AFG_2 (23). As for carcinogenicity, AFB_1 was more potent than AFG_1, which in turn, was more carcinogenic than AFB_2 (24).

Hydroxylated derivatives of aflatoxins are AFM_1, AFM_2, $AFGM_1$, AFB_{2a}, AFG_{2a}, parasiticol, aflatoxicol, AFP_1, and AFQ_1. The toxicity of AFM_1 is similar to AFB_1 but less carcinogenic. AFG_{2a} is relatively non-toxic, and it has been identified as AFB_1 hemiacetal, aflatoxin W (AFW) and hydroxydihydroaflatoxin (9). Parasiticol (AFB_3) has an ethanol moiety in place of the terminal pentanone ring. It is as toxic as AFB_1 to duckling but less toxic than AFB_1 to chick embryos . Aflatoxicol (AFR_0) is a degradation product of AFB_1 and has been found to be highly toxic and carcinogenic to fish (24).



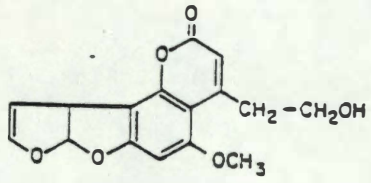
Structures of aflatoxins B_1, B_2, G_1, and G_2.



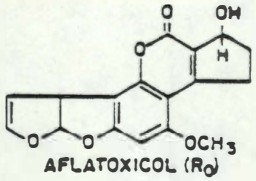
Hydroxylated aflatoxin derivatives.

Figure 2.1 Structure of Aflatoxins.

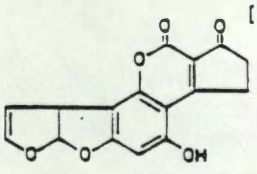
Source: Palmgreen et al. (1983). Aflatoxins. In: Handbook of Natural Toxins: Plant and Fungal Toxins. Keeler, R.F. and Tu, A.T., eds.) pp. 299-323, Marcel Dekker, New York.



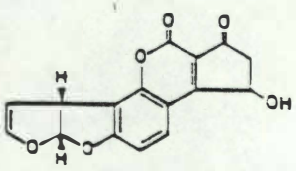
AFLATOXIN B_3
(Parasiticol)
[10]



AFLATOXICOL (R_0)
[11]

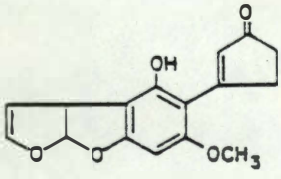


AFLATOXIN P_1
[12]

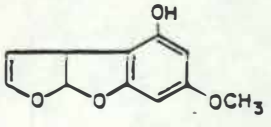


AFLATOXIN Q_1
[13]

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).

AFP_1 is produced when AFB_1 undergoes O-demethylation in animals. AFP_1 LD_{50} value to mice is about 15 times less than that of AFB_1 (25). AFQ_1, which contains a hydoxyl group at the C-3 position, has been isolated in vitro using monkey and human liver microsomes (25,26). Aflatoxin D_1 and D_2 are two major degradation products of AFB_1 when detoxified with ammonia.

2.1.3 AFB_1 Metabolism

The metabolism of AFB_1, in vivo or in vitro, may result in many different derivatives of the toxins. In vitro, AFB_1 undergoes at least five types of metabolic reactions with liver enzyme preparations, namely reduction, hydroxylation, hydration, O-demethylation and epoxidation (see Figure 2.2) (22,27). The products of the respective reactions are aflatoxicol, AFM_1, AFQ_1, AFB_{2a}, AFP_1, and AFB_1-2-3-oxide. Except for the epoxide, all the metabolites contain an OH^- group which can be conjugated with glucuronic acid and sulfate. This conjugation results in detoxification of the aflatoxins by the formation of water-soluble aflatoxin esters which can be excreted in the urine or bile (22).

AFB_1 requires metabolic activation by mixed-function oxygenase (MFO) system to form the epoxide derivative that is responsible for AFB_1 toxicity and carcinogenicity (22). Once formed, the epoxide can either cause an electophilic binding on nucleophiles (such as nucleic acids and proteins); or be conjugated with reduced gluthathione and be detoxified; or be hydrolyzed to form the 2, 3-dihydrodiol of AFB_1; or be reduced to the hemiacetal form (22).

The major adduct formed between AFB_1 and DNA and RNA has been identified as the 2, 3-dihydro-2-(N7-guanyl)-3-hydroxy-AFB_1 (28). The covalent binding of AFB_1 to

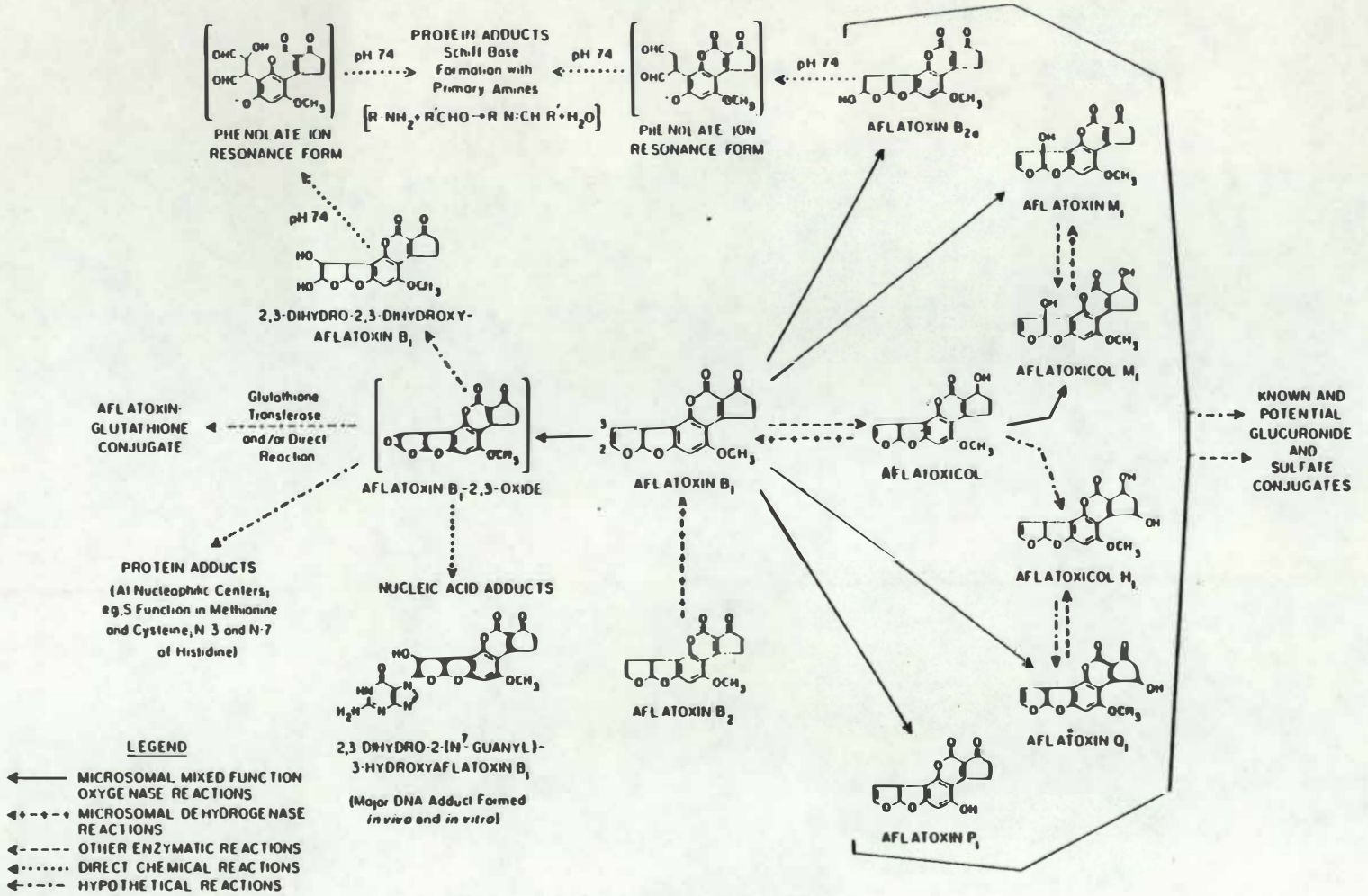


Figure 2.2 Metabolism of AFB_1.

Source: Busby et al. (1979). Mycotoxins and alimentary mycotoxicosis. In: Foodborne Infections and Intoxications (Reimann, H. and Bryan, F.L., eds.) pp. 519-610, Academic Press, New York.

macromolecules DNA, RNA, protein) has been suggested as the main mode of acute toxicity and carcinogenicity of AFB_1(22). The half-life of AFB_1-DNA adducts is reported to be approximately 12 h, after which they are rapidly excreted in the urine (29).

2.1.4 Toxicity and Carcinogenicity of AFB_1

AFB_1 is the most toxic, most abundant, and therefore, the most studied of all aflatoxins. It constitutes about 75% of the aflatoxins found in naturally contaminated agricultural commodities (22). It has been reported to be hepatotoxic (5), hepatocarcinogenic (6), teratogenic (7), mutagenic (30), and immunosuppressive (8). The liver is the main target organ for AFB_1 toxicity and carcinogenicity. However, AFB_1 has been found to cause tumors in kidneys of rats (31).

The acute toxicity of AFB_1 has been shown to cause severe morbidity and deaths in laboratory (5) and farm (9) animals, and humans (3,4) (refer to Table 2.1 for LD_{50} of AFB1 on different animals). The covalent binding of AFB_1 with DNA was linearly correlated with the susceptibility of animals such as rats and trout to liver cancer (32). It has been documented to have carcinogenenic effects on mice, rats (23), ducks (33), trout (24) and primates (6). A number of epidemiological studies have also shown a strong correlation between dietary aflatoxin consumption and liver cancer rates in humans (1, 2, 29, 32) (see Table 2.2.) Virtual safe dose of aflatoxin in human was estimated to be 0.264 ng/kg/d or less (32).

2.1.4.a Species and Gender Differences

Metabolism of AFB_1 in different species of animals may produce different metabolites of AFB_1. As a result, the susceptibility to acute toxicity and carcinogenicity of

Table 2.1 Median Lethal Dose of AFB1.

Species	mg/kg, oral
Rabbit	0.3
Duckling	0.3
Cat	0.6
Pig	0.6
Trout	0.8
Dog	1.0
Guinea Pig	1.4
Sheep	2.0
Monkey	2.2
Mouse	9.0
Hamster	10.2
Chick	11.5
Male Rat	7.2
Female Rat	17.2

Source: Marasas et al. (1987). Aflatoxicosis. In: Mycotoxicology: Introduction to the Mycology, Plant Pathology, Chemistry, Toxicity, and Pathology of Natural Occuring Mycotoxicosis in Animals and Man. pp. 25-31, Pennsylvania State University Press, University Park, PA.

Table 2.2 Aflatoxin Ingestion and Liver Cancer Incidence in Humans.

Population	Dietary aflatoxin intake (ng/kg of body weight per day)	Cases of liver cancer in adults (>15 years old)			
		Men		Women	
		No./100,000 population per year	Incidence	No./100,000 population per year	Incidence
Kenya					
High altitude	3—5	1	3.1	0	0
Medium altitude	6—8	13	10.8	6	3.3
Low altitude	10—15	16	12.9	9	5.4
Swaziland					
Highveld	5—9	9	7.0	2	1.4
Middleveld	9—14	24	14.8	5	2.2
Lebombo	15—20	4	18.7	0	0
Lowveld	43—53	35	26.7	7	5.6
Thailand[a]					
Songkhala	5—8	—	—	—	—
Ratburi	45—77	—	—	—	—
Mozambique[b]	222	—	35.0	—	15.7

Note: Data were compiled for 1 year in Thailand, 3 years in Mozambique, and 4 years in Kenya and Swaziland.

[a] Statistics for the total population were cases per 100,000 population with an incidence of 6.0. In Ratburi, Thailand, statistics for the total population were two cases per 100,000 population per year with an incidence of 2.0.

[b] Incidence for the total population was 25.4 cases per 100,000 population per year.

Source: Groopman et al. (1988). Aflatoxin exposure in human populations: Measurements and relationships to cancer. In: CRC Critical Reviews of Toxicology, Volume 19, Issue 2, pp. 113-145.

AFB1 varies from species to species. Also, the liability of acute toxicity and carcinogenicity are not dependent on one another (9).

Rats were found to be highly susceptible to carcinogenic effects but moderately susceptible to acute toxicity of AFB_1. On the other hand, guinea pigs were more susceptible to acute effects but less susceptible to carcinogenic effects (34). Monkeys were relatively resistant to carcinogenic effects of AFB_1 but were quite sensitive to acute effects compared to rats and mice. Also, of the three species, monkeys had the lowest AFB_1 excretion rate, and mice were quite resistant to both acute and carcinogenic effects (35).

AFB_1-binding to liver RNA and protein was higher in guinea pigs than rats. This corresponds with the higher susceptibility of guinea pigs than rats to acute toxicity of AFB_1. In addition, the lower AFB_1-DNA binding in guinea pigs than in rats indicates that rats are more susceptible to the carcinogenic action of AFB_1. Mice had the lowest AFB_1-macromolecule adducts formation which corresponded to their resistance to both acute and carcinogenic effects (36).

Male rats have higher AFB_1-DNA binding than female rats which indicates that female rats are relatively resistant to carcinogenic action of AFB_1 (37). It was suggested that the increase in induction of detoxification enzymes in females rats, may have a protective effect on AFB_1 toxicity. In humans, cases of acute aflatoxicosis were twice as many in males than in females (3).

2.1.4.b Symptoms of Acute Aflatoxicosis

The general symptoms of acute aflatoxicosis in cattle, dogs, pigs, and poultry were decrease in weight, general unthriftiness and inappetence (38). Signs of liver injury include icterus, edema, hemorrhage, and ascites. Death rates are usually high and may be preceeded by convulsions and ataxia. Histopathological studies on livers of laboratory

animals showed fatty degeneration, necrosis, fibrosis, and extensive bile-duct proliferation (38). In humans, symptoms of acute aflatoxicosis due to consumption of contaminated maize included vomitting, anorexia, icterus, ascites, and sometimes edema. Death usually occurred after massive gastrointestinal hemorrhage (3). Similar symptoms were also seen in dogs that had eaten the contaminated maize.

2.1.4.c AFB_1 and Lipids Metabolism

AFB1 has often been reported to interfere with lipid metabolism in both animals and humans. Lipid synthesis was inhibited in ducklings (39) and chickens (40) due to AFB_1. In ducklings, the toxin significantly suppressed growth and lowered liver weight, but it increased total lipids content in the liver. The toxin showed a dose-dependent inhibition of lipids synthesis by reducing the incorporation of ^{14}C-acetate into long-chain fatty acids in chickens (40). Triglycerides, total phospholipids, and free and esterified cholesterol in chicken's blood serum were significantly lowered with a dose of aflatoxin that was too small to inhibit growth. However, liver lipids were significantly increased (41).

In rat liver, AFB_1 increased cholesterol levels while inhibiting cholesterol synthesis (42). The investigators suggested that the inhibition of cholesterol synthesis was specific and not the result of general hepatic injury. Serum cholesterol levels were significantly decreased. Phospholipids synthesis was also inhibited in liver preparation of rats previously injected with aflatoxin (43).

Aflatoxins have been found to cause steatorrhea in chickens. Pancreatic lipase, primary fat digestive enzyme, and bile, important for lipid digestion and absorption, were decreased significantly in this case. The authors concluded that the steotorhea was due to impairment in lipid digestion produced by the aflatoxins added to the diet (44).

AFB_1 had also attenuated lipids synthesis in humans. Using human skin specimens, Black et al. (45) found that incorporation of radio-labeled acetate into total lipids, phospholipids, free sterol, and neutral fat was significantly inhibited. The authors also suggested that the inhibition was specific and not due to general liver toxicity.

Overall, the investigators that studied the effects of aflatoxins on lipid metabolism concluded or suggested that i) the increase in liver lipids and decrease in serum lipids was due to inhibition of lipid transport from the liver to the blood and peripheral tissues and not due to increase in lipogenesis (39,42); ii) the inhibition was general and not specific to lipid fractions (41); iii) the impairment in lipogenesis was due to a decrease in the formation of enzymes involved in fatty acid biosynthesis such as fatty acid synthetase (40).

2.1.5 AFB1-Macromolecule Adducts and Diet/Drug Interactions

Many studies have been carried out to determine the effects of various food/nutrients and drugs/xenobiotics on the AFB_1-macromolecule adducts formation. The major objective of these investigations was to find if the nutrients and drugs/xenobiotics could inhibit the adducts formation, and thus, preventing the carcinogenic effects of AFB_1.

2.1.5.a Food Nutrients

Fat-Soluble Vitamins

Quite a range of vitamins and vitamin analogs that have been tested on AFB_1-macromolecule adducts formation. Bhattacharya et al. have reported comprehensive studies on the effects of various vitamins on in vitro adducts formation (46) and AFB_1 mutagenicity (47).

Vitamin A supplementations in rats inhibited AFB1-DNA adducts formation (48). The protective effects of retinoids such as retinol, retinal, retinoic acid, and retinyl esters on

AFB_1 carcinogenicity was due to inhibition of DNA adducts formation mediated by microsomal MFO enzymes (46,49). Retinol had the same inhibitory effect on the formation of AFB_1-protein adducts (49). As for mutagenicity, retinol, and retinoic acid were shown to inhibit mutagenesis due to AFB_1 in the Salmonella typhimurium system (47,50). It was suggested that the protective effects of vitamin A were the result of interaction with microsomal enzymes; resulting in interference with the activation of AFB_1 (47,49). Recently, vitamin A has been shown to be important in regulating gluthathione-S-transferase (GST) activity involved in the detoxification process. Vitamin A supplementation increased the GST activity and the reverse occurred when vitamin A was deficient (48).

Vitamin E supplementation had no significant effect on in vivo covalent binding of AFB_1 to liver macromolecules in rats. However, a combined deficiency of vitamin E and selenium decreased DNA, RNA, and protein adducts in the rats (51). Yet, the same investigators reported that the combined deficiency of vitamin E and selenium increased the DNA and RNA adducts in chicks (quoted from 51). Vitamin E, as well as menadione, a water soluble synthetic vitamin K, both inhibit AFB_1-induced mutagenisis in Ames bacterial system (50).

Water-Soluble Vitamins

Riboflavin, rifboflavin-5'-phosphate (FMN), and flavin adenine dinucleotide (FAD) inhibited AFB1-DNA adduct formation in vitro (46). Riboflavin being more effective than FMN and FAD. Vitamin C, vitamin B6 and thiamin had no significant effect on the DNA adducts production (46). However, vitamins C, B6, B12 and folic acid significantly inhibit mutagenisis in bacterial systems (46,47). The inhibition by vitamin C; however, was not as great compared to fat-soluble vitamins.

Amino Acids and Protein

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 was not affected by the diet. The inhibition of AFB_1-nucleic acid adducts by the marginally lipotrope-deficient diet was due to the decrease in MFO activities and not due to an increase in gluthathione levels (52). The authors concluded that the earlier reported AFB_1-induced tumorigenesis caused by marginally deficient lipotrope diets (53) was not due to an increased in activation of AFB_1.

Sulfur-containing amino acids such as cysteine, N-acetylcysteine, cystine, methionine, and reduced or oxidized gluthathione inhibited AFB_1 mutagenicity in microbial systems. Cysteine and N-acetylcysteine were more potent inhibitors than gluthathione. The investigators suggested that the inhibition was due to the amino acids interfering with the activation of the AFB_1 (54).

Fat

Both saturated and unsaturated dietary fats did not significantly affect the adduct formation and the production of the AFB_1-epoxide in rat livers (55). In a similar study, high polyunsaturated oil (corn oil) increased the incidence of liver cancer in rats exposed to AFB_1 as compared to rats fed with saturated oil (beef fat). The cancer incidence was higher when the corn oil was fed together with or after the exposure to AFB_1 than when corn oil was fed after the AFB_1 dose. An increase in induction of AFB_1 activation by corn oil was suggested for the high incidence of cancer (56).

The presence of long chain fatty acids at their physiological concentrations increased the strength of AFB_1-albumin binding considerably (57). Albumin serves as the major

transport mechanism of AFB_1 in blood since very little AFB_1 was bound to other blood proteins (58). This binding affinity (Ka) is increased 3 fold by increasing the pH 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 moles/mole albumin further increased the albumin binding of AFB1 to greater than 4 fold at pH 7.4 (57).

Trace elements

Copper decreased AFB_1-DNA binding in vitro (46). Deficiency and excess of selenium decreased the adducts formation in rats. In chicks, however, excess of selenium did not affect the amount of adducts formed (51).

Copper, manganese, zinc, and selenium were effective in depressing in vitro mutagenesis induced by AFB_1. Copper was the most potent of the elements tested. To a lower extend, iodine, molybdenum, cobalt, and iron were antimutagenic. It was suggested that the inhibition was due to interaction of the trace elements with the microsomal enzymes (59).

2.1.5.b Caloric Restriction

There has been considerable interest on the effect of caloric restriction on cancer promoting chemicals and compounds. It has been reported that rats fed with 60% of the food consumed by the ad libitum animals had lower AFB_1 microsomal activation, lower AFB_1-DNA adducts, faster plasma clearance, and increased excretion of AFB_1 in urine than the ad libitum fed animals. From these results, the authors concluded that 40% food restriction promoted a more efficient AFB_1 metabolism (60).

2.1.5.c Cruciferous Vegetables

Cruciferous vegetables have been shown to enhance detoxification of xenobiotics by inducing xenobiotic-metabolizing enzymes in animals and humans (quoted from 61). Increased consumption of these vegetables, for example broccoli, cabbage, cauliflower, and Brussel sprouts has been related to a reduced risk of bladder, colon and rectum cancers (62).

Brussel sprouts may have a protective factor against the AFB_1 carcinogenicity. This vegetable significantly decreased AFB_1-DNA binding and increased GST activities in rats. Indole-3-carbinol, a compound found in cruciferous vegetables did not have much effect on the DNA binding to AFB_1 or the GST activity (61). The same investigators also found that the route of administration of AFB_1, intragastric or intraperitoneal, did not change the inhibition of AFB_1-DNA binding. Thus, they concluded that the small intestine may not play an important role in the 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 (63). R-Goitrin, another compound common in cruciferous vegetables, also exhibited anticarcinogenic role by inhibiting AFB_1-DNA binding, increasing GST activity, and enhancing biliary excretion of AFB_1 in rats (64).

2.1.5.d Plant Flavonoids and Phenolic Compounds

Several derivatives of five major plant flavonoids, namedly, flavone, flavonol, isoflavone, and flavonol have been tested on AFB_1-DNA binding and activation of AFB_1 (65). Most of the flavonoid derivatives significantly inhibited the adducts formation. However, flavonols had the most potent effect on the inhibition of the binding and activation of AFB_1. Flavonols also showed greater inhibition of AFB_1 mutagenicity in bacterial systems (66).

Phenolic compounds have also been shown to have protective effects on mutagenicity caused by AFB_1. Gallic acid, chlorogenic acid, caffeic acid, dopamine, p-hydroxybenzoic acid, and salicyclic acid depressed AFB_1-induced mutagenicity in a bacterial system containing rat-liver microsomes. The inhibition occurred when the compounds and AFB_1 were administered concurrently (67).

2.1.5.e Antioxidants

Antioxidants, such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), are chemicals commonly added to food to prevent spoilage due to oxidation. Rats fed a BHA added diet (7500 ppm) had significantly lower AFB_1-DNA binding, higher GST activities, and higher biliary excretion of AFB_1 (64). In trout; however, BHA had no effect on liver tumor incidence, AFB_1-DNA binding, and AFB_1-gluthathione conjugation (68). These results have led to suggestion that in mammals, an increase in the gluthathione conjugation is the basis for BHA inhibition of AFB_1 carcinogenicity. In in vitro system; however, BHA as well as BHT markedly inhibited AFB_1-DNA binding (46).

2.1.5.f Drugs and Other Compounds

Pretreatment of an anti-seizure drug, phenobarbital, in rats increased AFB_1 activation in vivo and in vitro (69,70). However, it also induced GST activity and enhanced the excretion of biliary AFB_1-gluthathione conjugate; therefore, the overall hepatic binding of AFB_1 to DNA is reduced. Ethanol pretreatment on the other hand, increased the activation of AFB_1 but not the activity of GST. Thus, ethanol pretreatment increased binding of AFB_1 to DNA and RNA (71).

Cortisol pretreatment in female rats significantly increase the acute hepatotoxicity induced by AFB_1. This effect of cortisol was dose dependent and supported by increased mortality rate, hepatic triglycerides, and binding of AFB_1 to DNA and protein (72). This effect was perhaps mediated via increased metabolism of AFB_1 to its epoxide derivative as well as hydroxylated derivatives (AFM_1 and AFQ_1) in microsomes since activity of aniline hydroxylase was elevated in cortisol pretreated rats.

2.2 CARNITINE

2.2.1 History

Carnitine was first discovered by two Russian scientists from muscle extracts in 1905 (quoted from 73). 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 ß-oxidation (74). However, not until 1973 did carnitine gain much attention and investigation, that is, when the first carnitine deficient patient was described by Engel and Angelini (75).

2.2.2 Chemistry and Dietary Sources

Carnitine is a quarternary amine and is very similar to choline and amino acids. However, unlike amino acids, it is not involved in protein synthesis. Chemically, it is known as 3-hydroxy-4-N-trimethylamino butyric acid. It exists in two forms or isomers, L-carnitine and D-carnitine. Only the L-carnitine is biologically active. The D-isomer is not only inactive, it has been reported to depress L-carnitine utilization (76). Carnitine is not essential for humans since it can be produced in the body, and therefore, is not

considered a vitamin. Muscle tissue of animals, lamb liver, yeast, and milk are very good sources of dietary carnitine. Most vegetables and grains such as cauliflower, cabbage, spinach, barley, and rice contain very little or no carnitine. More detailed reviews are available on carnitine history, biosynthesis, dietary sources and functions (73,76,77).

2.2.3 Carnitine Biosynthesis

The biosynthesis of carnitine starts from two essential amino acids, lysine and methionine, in a five step pathway involving five different enzymes. Niacin, vitamin B6, vitamin C, and iron are also required for biosynthesis of carnitine in animals (quoted from 77). (Refer to Figure 2.3 for the complete pathway of carnitine biosynthesis.)

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 rat liver (78). The human brain, liver, kidney, heart, and skeletal muscle can convert N-trimethyllysine to 4-N-trimethylamino butyric acid; but only the liver, kidney, and brain can convert 4-N-trimethylamino butyric acid to carnitine (79). This is because the enzyme catalyzing the final step, butyrobetaine hydroxylase, is present only in the tissues where carnitine is finally synthesized. Therefore, carnitine must be transported to those tissues that do not have the final enzyme.

2.2.4 Functions of Carnitine

The main function of carnitine is the transportation of long-chain fatty acids from the cytosol into the mitochondria to allow beta-oxidation to take place. In brief, other functions of carnitine include metabolism of branched-chain amino acid, initiation of ketogenesis, thermogenesis in brown adipose tissue of infant rats, stimulation of gluconeogenesis, facilitation of urea removal from blood and tissue, the removal of short-chain and medium-

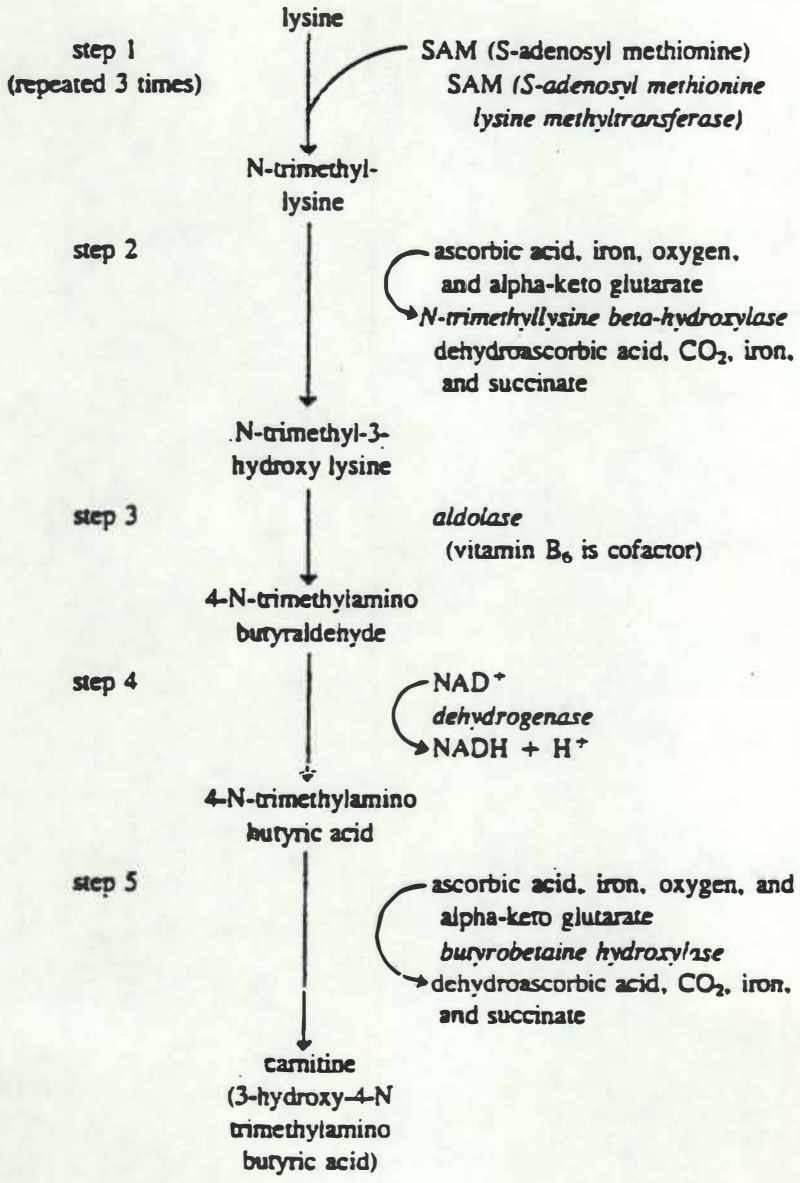


Figure 2.3 The Biosynthesis of Carnitine.

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.

chain acyl residues accumulated in the peroxisomes, prevention of fatty liver development, and detoxicification of drugs (refer to 73,76,77, 80, and 81 for complete review of the above functions).

2.3.5 Carnitine and Drug/Xenobiotic Interactions

Chronic consumption of ethanol is widely known to inhibit beta-oxidation which leads to fatty infiltration in human and animal livers (10,12). Impaired oxidation and/or increased lipogenesis are the suggested mechanisms of this malady (12). In addition, ethanol depresses the activity of carnitine palmitoyltransferase I, the rate limiting enzyme in the transportation of long-chain fatty acids into the mitochondria (82). This inhibition may also contribute to accumulation of fat in the liver. Supplementary carnitine has been reported to inhibit this fatty liver phenomena caused by ethanol (13,14,15). Carnitine may also offer protection against the toxicity of other alcohols such as methanol, isopropanol and ethylene glycol (a common anti-freeze.) This is because carnitine prevents accumulation of these alcohols by enhancing their excretion in the urine (83).

Carnitine may also alleviate the toxicity of adriamycin (doxorubicin hydrochloride), a widely used anti-cancer drug. Adriamycin treatment has resulted in a series of acute and chronic side-effects and cardiomyopathy in rats and humans and (quoted from 16,84). It was found that carnitine administration decreased histophatological changes and ameliorated irregular heart performance due to adriamycin (16,84). Furthermore, carnitine lengthened the survival rate of mice following chronic adriamycin administration than the mice that were administered with adriamycin only (85).

Valproic acid, a short chain fatty acid, is a frequently used anticonvulsant drug. This drug has produced very serious side effects similar to Reye's syndrome (i.e. stupor, hyperammonia, coma, and hepatic dysfunction) (86). Vaproic acid treatment in epileptics

lowers the serum free carnitine but increases the short-chain acylcarnitine concentrations in the patients (87,88). When vaproic acid was treated together with other drug such as primodone, phenobarbital, ethosuximide or phenytoin, total carnitine was markedly reduced.

Lately, carnitine has been found to conjugate with certain drugs/xenobiotics. For example, vaproic, cycopropanecarboxylic (CPCA), and pivalic acid have been found to conjugate with carnitine and are then excreted in the urine by humans (quoted from 89). Prolonged high doses of CPCA decrease free and total carnitine in the hearts of female rats.

CHAPTER 3

MATERIALS AND METHODS

3.1 ANIMALS AND HOUSING

Male Sprague-Dawley rats (Harlan, Indianapolis, ID) were used in this experiment. They were acclimatized to the animal facility for 7 d upon arrival and had free access to food and water. All rats were housed in individual wire-meshed bottom, suspended, stainless steel cages in a cubicle of an AAALAC approved animal facility. The room temperature was maintained at 23 $\pm$ 1 ^{o}C, relative humidity around 45%, and with a 12-h light/dark cycle.

3.2 DIETS

During the acclimatization period, the animals were fed Purina Rodent Chow pellets #5001 (Ralston Purina Co., St. Louis, MO). For the experiment, the diets were either ground Purina Rodent Chow, or the same ground Purina Rodent Chow supplemented with 0.5% (w/w) L-carnitine-HCl (Sigma Chemical Co., St Louis, MO) which contained about 0.4% L-carnitine. The diets and water were provided ad libitum .

3.3 EXPERIMENTAL DESIGN AND TREATMENTS

In Experiment 1, twenty Sprague-Dawley rats were divided into 4 groups (b.w. about 270-273 g) as shown in Table 3.1. NSC and NSA groups consisted of the non-

Table 3.1 Design of Experiment 1.

	GROUPS[1]			
	NSC	NSA	CSC	CSA
# Animals	5	5	5	5
Body Weight, (g ± SD)	271 ± 11	270 ± 13	274 ± 9.2	273 ± 9.8
L-Carnitine.HCL (%,w/w)	-	-	0.5	0.5
AFB_1 (mg/kg b.w.)	-	1.0	-	1.0

[1]NSC = Nonsupplemented Control
NSA = Nonsupplemented Aflatoxin
CSC = Carnitine-supplemented Control
CSA = Carnitine-supplemented Aflatoxin

carnitine supplemented rats and CSC and CSA were the carnitine-supplemented animals. For Experiment 2, 10 rats were divided into 2 groups (Table 3.2). NSA group was fed with the regular diet, while CSA group received the carnitine-supplemented diet. Body weights were monitored weekly.

After 6 weeks on the diet, a single oral dose of 1 mg/kg [^{3}H]AFB$_1$ (Moravek Biochemicals, Brea, CA) was administered by gavage to NSA and CSA groups in Experiment 1 and to both groups in Experiment 2. Unlabelled AFB$_1$ (Sigma Chemical Co., St. Louis, MO) was dissolved in methanol while the [^{3}H]AFB$_1$ came already dissolved in methanol. The cold AFB$_1$ was mixed with [^{3}H]AFB$_1$ to get the spec. act. of 8 μCi/μmol. The above AFB$_1$ solution was dissolved in corn oil to give a final concentration of 1.0073 mg of diluted [^{3}H]AFB$_1$ in 1 ml of corn oil. NSC and CSC groups were given 1 ml/kg suspension of methanol:corn oil (1:9, v/v) by gavage.

Six hours in Experiment 1 and 24 h in Experiment 2 after the [^{3}H]AFB$_1$ administration, the animals were anesthetized with methoxyflurane (Pitman-Moore, Inc., Washington Crossing, NJ) and blood samples were collected by cardiac puncture. The animals were euthanized by exsanguination, and the livers and kidneys were removed. The livers were rinsed with cold 0.9% NaCl to remove blood, blotted, dipped in liquid nitrogen, weighed, and stored immediatedly at -70 °C. The kidneys were also dipped into liquid nitrogen, weighed and stored at -70 °C.

3.4 TISSUE HOMOGENATE PREPARATIONS

Whole livers and kidneys were allowed to thaw. The livers were added to 1 volume of cold 0.2M Tris buffer containing 1.15% KCl in a teflon glass homogenizer. The kidneys were placed in a teflon glass homogenizer containing 2 volumes of the Tris buffer.

Table 3.2 Design of Experiment 2.

	GROUPS[1]	
	NSA	CSA
# Animals	5	5
Body Weight, (g ± SD)	338 ± 8	336 ± 7
L-Carnitine.HCL (%,w/w)	-	0.5
AFB_1 (mg/kg b.w.)	1.0	1.0

[1]NSA = Nonsupplemented Aflatoxin

CSA = Carnitine-supplemented Aflatoxin

The tissues were homogenized by 6 complete strokes according to the method of Campbell et al. (52). The livers and kidneys were rinsed with 1 volume of the buffer, vortexed and each homogenate was placed into 7 separate 5 ml capacity culture tubes. The tissue homogenate were prepared at 0-5 °C and stored at -70 °C. For analysis, only one tube of each tissue homogenate was thawed and used for one assay. Thus, repeated thawing and freezing were avoided and homogenate samples in different tubes were used for determination of the tissue radioactivity, carnitines, lipids, macromolecules, and AFB_1-macromolecules adducts.

3.5 BLOOD PLASMA PREPARATION

The blood samples collected by cardiac puncture were placed into heparin-containing tubes (143 USP units of sodium heparin, Becton-Dickinson, Rutherford, New Jersey). They were later centrifuged at 1500 x g at 4 °C for 10 min. The plasma was transferred into culture tubes and stored at -70 °C. The plasma was analysed for all the assays as carried out in the tissue homogenates except for the quantitation of macromolecules and AFB1-macromolecules adducts.

3.6 ANALYTICAL PROCEDURES

3.6.1 Tissue Radioactivity

The measurement of tissue radioactivity was done following the method of Petroff et al. (90). 100 µg of the tissues (livers or kidneys) was added with 2 ml 2N methanolic KOH in scintillation vials. The tissues were digested at 70 °C in a water bath for 30 min, cooled to room temperature, and 15 ml Aquasol-2 (NEN® Research Products, Boston,

MA) was added to each tubes. Approximately 300 µl glacial acetic acid was added to decrease the random coincidence monitor (RCM) to less than 5%. The radioactivity was counted in a liquid scintillation counter (LS-3801Beckman Instrument, Irving, CA.)

3.6.2 Carnitine

Tissue and plasma carnitines were determined according to the radioisotopic method of Cederblad and Lindstedt (91) as described by Sachan et al. (13). Three different fractions of carnitine were measured, namely, free carnitine or non-esterified carnitine (NEC), short-chain acylcarnitine or acid soluble acylcarnitine (ASAC), and long chain acylcarnitine or acid insoluble acylcarnitine (AIAC). Summation of these three fractions gave the total carnitine (TC) of the tissue or plasma samples.

Perchloric acid was used to extract the ASAC, while potassium hydroxide and heating were used to hydolyze and extract AIAC. The NEC was assayed by the addition of carnitine acetyltransferase (CAT) and sodium tetrathionate which drove the following reaction to the right by binding of CoASH.

$$\text{L-Carnitine} + [1\text{-}^{14}\text{C}]\text{Acetyl CoA} \xrightarrow{\text{CAT}} [1\text{-}^{14}\text{C}]\text{Acetyl Carnitine} + \text{CoASH}$$

The reaction mixture was passed over an ion-exchange resin column where 1-^{14}C acetyl carnitine was eluded and unreacted-labeled acetyl CoA was trapped. Scintillation solution was added to the eluate and radioactivity was determined in a Beckman scintillation counter. Tissue carnitine was expressed in µmol/g wet wt of tissue, and plasma carnitine as nmol/ml. The details of carnitine determination procedures used in Sachan's laboratory (13) are described as follows:

3.6.2.a Reagents

1. 0.5 mM carnitine standard solution (stock): 9.88 mg L-carnitine HCL (Sigma Chemical C0., St. Louis, MO) was dissolved in cold glass distilled water (GDW) and diluted to 100 ml in a volumetric flask. This stock standard was diluted 1:2 to give a working standard concentration of 0.25 mM.
2. 22.9 mM L-palmitoylcarnitine standard (stock): 1.0 ml of GDW was added to a vial containing 10ml of L-palmityl carnitine (Sigma Chemical Co., St. Loius, MO). This stock standard was diluted 1:100 to give a working standard concentration of 0.229 mM.
3. Carnitine standard mixture: Working standard were prepared by mixing equal volumes of L-carnitine and L-palmitoyl carnitine.
4. 0.5 M Potassium hydroxide: 28.05 g of KOH was dissolved in some GDW and diluted to a volume of 1 liter.
5. 4 M Potassium hydroxide: 56.11 g of KOH was dissolved in some GDW and diluted to a volume of 250 ml.
6. 0.6 M Perchloric acid (PCA): 51.26 ml of 70% PCA was added in some GDW and diluted to a volume of 1 liter.
7. 0.1 M Sodium tetrathionate: 0.6756 g of anhydrous sodium tetrathionate (J.T. Baker Chem. Co., Phillipsburg, NJ) was dissolved in GDW and diluted to a volume of 25 ml.
8. 0.1% Phenol red: 100 mg of phenol red (Sigma Chemical Co., St. Loius, MO) was dissolved in absolute ethanol and made to a volume of 100ml. Phenol red tubes were prepared by adding one drop of phenol red to 12 x 75 mm Pyrex glass tubes and allowed to dry.

9. [1-^{14}C] acetyl Coenzyme A: 50 µCi of [1-^{14}C] acetyl Coenzyme A (Amerstam Corp., Chicago, IL) was dissolved in cold GDW and made to a volume of 300 ml. 5.0 ml aliquots were dispensed into plastic vials and frozen at -70 °C.
10. 1M Potassium bicarbonate: 1 g potassium bicarbonate (Certified ASC, Fisher Scientific Co., Fair Lawn, NJ) was dissolved and made to a volume of 10 ml with GDW. Solution was stored at room temperature and made daily.
11. 0.1 M acetic anhydride: 0.5 ml of acetic anhydride (Fisher Scientific Co., Fair Lawn, NJ) was added to 4.95 ml of cold GDW and used immediatedly for acetyl CoA solution.
12. 0.1 mM acetyl Coenzyme A: 10 mg of acetyl Coenzyme A (Sigma Chemical Co., St. Louis, MO) was added to 0.5 ml cold GDW and mixed. Then, 100 µl of 1 M potassium bicarbonate was added and mixed, and followed by the addition of 200 µl of 0.1 M acetic anhydride. The solution was made up to 80 ml with cold GDW, mixed thoroughly, and dispensed into plastic vials and frozen at -70 °C.
13. Carnitine acetyltransferase (CAT): CAT from pigeon breast muscle (Sigma Chemical Co., St. Louis, MO) was diluted with GDW to give an enzyme activity of 50 units/ml.
14. 1 M [3-(4-morpholino)propanesulfonic acid] MOPS: 20.92 g of MOPS (Sigma Chemical Co., St. Louis, MO) was added to about 80 ml of GDW. The pH was adjusted 7.4 with 4 M potassium hydroxide and was diluted to a volume of 100ml with GDW.
15. PCA/MOPS-I: 20.9 g of MOPS was added to 50 ml of 0.6 M PCA and brought up to a volume of 100 ml with GDW. The solution was stored at 4 °C.
16. PCA/MOPS -II: 20.9 g of MOPS was added to 20 ml of 0.6 M PCA and brought up to a volume of 100 ml with GDW. The solution was stored at 4 °C.

17. 0.1 M ethylene glycol-bis (beta-amino-ethylether) N, N-tetraacetic (EGTA), pH 7: 1.902 g EGTA (Sigma Chemical Co., St. Louis, MO) was added in 30 ml GDW, adjusted to pH 7.0 with 4 M potassium hydroxide, and diluted to 50 ml with GDW.
18. 0.1 mM [1-^{14}C] acetyl Coenzyme A solution: 2:1 volumes of [1-^{14}C] acetyl CoA and 0.1 mM acetyl CoA were mixed and stored at 4 °C.
19. Reagent mixture for one assay: 1 M MOPS, pH 7.4, 120 µl; 0.1 M EGTA, pH 7.0, 20 µl; 0.1 M sodium tetrathionate, 20 µl; 0.1 mM [1-14C]-acetyl CoA solution, 200 µl; GDW, 40 µl; to a volume of 400 µl.
20. 8% bovine serum albumin (BSA): 4 g of BSA (Fatty acid poor, Fraction V, ICN Pharmaceuticals Inc., Life Sci. Group, Cleveland, OH) was dusted into about 20 ml of GDW, stirred gently, and diluted to 50 ml.
21. Scintillation fluid: Ready-Solv® CP (Beckman Instruments, Palo Alto, CA).

3.6.2.b Procedure

Aliqouts of 200 µl of tissue homogenates and blood serum were placed in 200 µl of PCA in a 12 x 75 mm Pyrex glass test tubes. The contents were vortexed thoroughly and centrifuged (Model TJ-6, Beckman Instruments, Palo Alto, CA) at 1500 x g for 10 minutes at 4 °C. Nonesterified carnitine (NEC) and acid soluble acylcarnitine (ASAC) were contained in the supernatant, while the acid insoluble carnitine (AIAC) was contained in the pellet.

Carnitine standards were made by adding 10, 20, 30, 40, and 60 µl of carnitine standard mixture to 200 µl of PCA in 12 x 75 mm Pyrex glass test tubes (Refer to Table 3.3). Blank was made by mixing 100 µl of GDW, 100 µl of BSA, and 200 µ of PCA . The standards and blank underwent the same procedures as in the samples.

Table 3.3 Carnitine Standard Preparations.

0.25 mM Carnitine		0.23 mM P-Carnitine[1]		GDW[2]	8% BSA[3]	6% PCA[4]	Total	Total Carnitine
(µl)	(nm)	(µl)	(nm)	(µl)	(µl)	(µl)	(µl)	(nm)
0	0	0	0	100	100	200	400	0
5	1.25	5	1.15	90	100	200	400	2.40
10	2.50	10	2.30	80	100	200	400	4.80
15	3.75	15	3.45	70	100	200	400	7.20
20	5.00	20	4.60	60	100	200	400	9.60
30	7.50	30	6.90	40	100	200	400	14.40

[1]L-Palmityl Carnitine

[2]Glass distilled water

[3]Bovine serum albumin

[4]Perchloric acid

150 µl and 100 µl of the supernatant from the tissue samples, blood, blank and standards were transferred to 12 x 75 mm phenol red tubes that were labeled NEC and ASAC respectively. The pellets were drained of the supernatant by inverting the tubes.

Nonesterified Carnitine Determination

35 µl of 1 M potassium bicarbonate was added to the 150 µl of the supernatant in the phenol red test tube and vortexed. The contents were slightly acidic as indicated by a golden yellow color. The mixtures were held for 30 min on ice.

Acid Soluble Acylcarnitine Determination

75 µl of 0.5 M potassium hydroxide was added to the 100 µl of the supernatant in the phenol red test tube and vortexed. The contents were slightly alkaline as indicated by a purple-red color which was essential for hydrolysis. The contents were incubated in a 37 ^{o}C rotating water bath (100 shakes/min) for 30 min for complete hydrolysis. The mixtures were then neutralized by adding 30 µl of PCA/MOPS-II, and vortexed. They were held on ice for 30 min.

Acid Insoluble Acylcarnitine Determination

The pellet was washed twice with 200 µl of 0.6 M PCA and the washes were drained by inverting the tube. One drop of phenol red indicator and 200 µl of 0.5 M potassium hydroxide were added to the tube and vortexed vigorously for several min until the pellet was completely dissolved. The contents were alkaline indicated by purple-red color and were hydrolyzed in a 65 ^{o}C shaking water bath (100 spm) for 60 min. Then,

100 µl of PCA/MOPS-I was added and vortexed to neutralize the contents. They were held on ice for 30 min.

3.6.2.c Assay for Carnitine

The NEC, ASAC, and AIAC tubes were centrifuged at 1500 x g at 4 °C for 10 min. A 100 µl aliqout was transferred from each of the fractions to 1.5 ml microfuge tubes separately. Four hundred µl of reagent mixture were added followed by 20 µl of carnitine acetyltransferase (CAT). The tubes were capped, mixed by gentled tapping, incubated in a 37 °C shaking water bath for 30 min. Later, 200 µl of the incubated mixture was transferred onto a mini column, and the elutant was collected into plastic mini scintillation vial (Beckman Instrument, Palo Alto, CA). The mini columns were made by stuffing the area above 5-3/4" Pasteur pipettes (Fisher Scientific Co., Fair Lawn, NJ) with glass wool. Anion Exchange Resin, AG I-X8, 200-400 mesh, Cl-form (Bio-Rad Laboratories, Richmond, CA) was added up to the 9 cm mark measured from the tip of the pipette. When the 100 µl aliqout was fully absorbed, the column was washed with 2 portions of 500 µl GDW, and the washes were also collected into the same respective scintillation vial. Then, 5 ml of scintillation fluid was added to each vial, capped, mixed by swirling, and counted for 10 min/vial in a scintillation counter (LS-3801 Beckman Instruments, Irving, CA).

Dual radioactivity counts were employed to determined the amount and AFB1 conjugation to each of the carnitine fractions (refer to Appendix A-3.1 for the procedures of dual radioactivity counts as described in the Beckman Liquid Scintillation Systems Operating Manual, Beckman Instruments, Irving, CA.) The disintegration per minute (DPM) for the NEC, ASAC,and AIAC, and their standards were adjusted by substracting

the blank's DPM. Standard curves for the carnitine fractions were plotted as shown in Figure 3.1 and 3.2. The calculation for carnitine concentrations were as follows:

Liver : $$\text{DPM} \times \text{I.S.} \times \text{D.F.} = \frac{\mu\text{moles carnitine}}{\text{g tissues}}$$

Blood serum: $$\text{DPM} \times \text{I.S.} \times \text{D.F.} = \frac{\text{nmoles carnitine}}{\text{ml serum}}$$

where: DPM = Disintergration per min

I.S. = Inverse slope

D.F. = Dilution factor (appropriate to bring the amount of carnitine to 1 g or 1 ml)

3.6.3 Total Lipids

Total lipids concentrations of the liver, kidney, and plasma were determined according to the phosphoric acid-vanillin reaction method of Ellenston and Caraway (92). The principle of this method is that unsaturated fatty acids are oxidised to form ketones that react with phosphovanillin to yield a pink-colored product.

3.6.3.a Reagents

1. Sulfuric acid (AR).
2. Phosphoric acid-vanillin reagent: 1 g of vanillin (Sigma Chemical Co., St. Louis, MO) was added to 160 ml of GDW, and brought up to a volume of 500 ml with phosphoric acid.
3. Stock standard : 1 g of olive oil was placed and mixed in a 100 ml volumetric flask containing some chloroform. The volume was brought up to the volume with chloroform, mixed thoroughly, and stored at -20 °C.

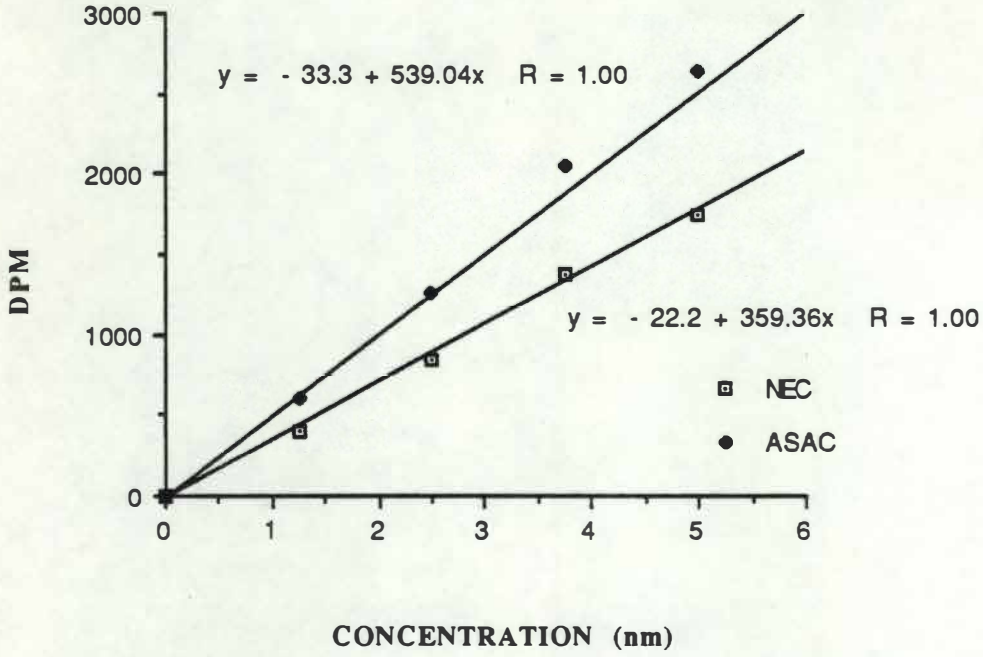


Figure 3.1 Nonesterified (NEC) and Acid Soluble Acylcarnitine (ASAC) Standard Curves.

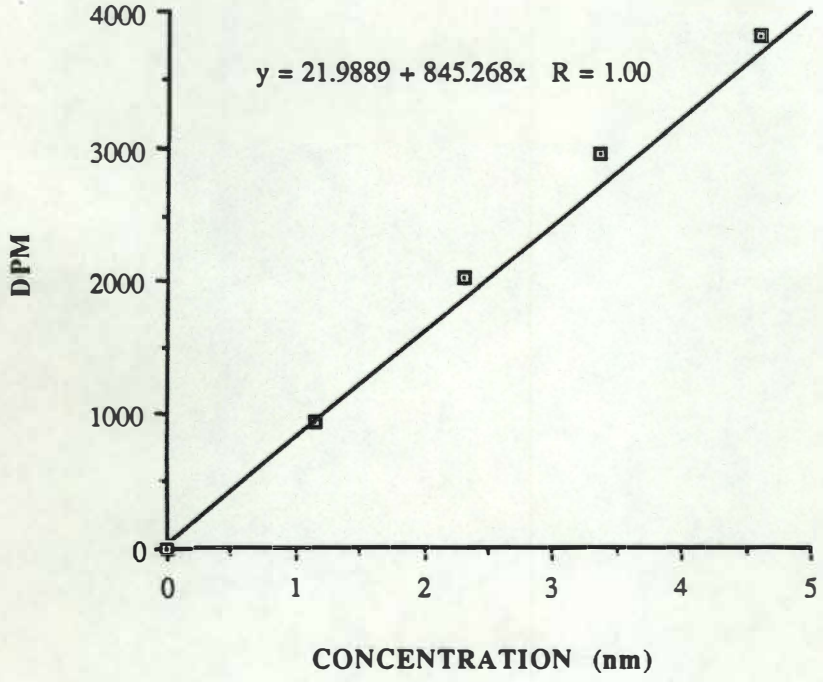


Figure 3.2 Acid Insoluble Acylcarnitine Standard Curve.

4. Working standard: 1 ml of the stock standard was placed in a 10 ml volumetric flask, brought up to the mark with chloroform, and stored at -20 °C.

3.63.b Procedure

Tissue homogenates (1:8 w/v) and blood serum (0.2 ml) were transferred to different 16 x 150 mm glass test tubes, while 0.2 ml GDW was placed for blank. For the standards, 0.05, 0.1, and 0.2 ml of the working standard solution were placed into separate tubes. The chloroform in the standards was evaporated off with a stream of nitrogen, and 0.2 ml GDW was added and 5 ml concentrated sulfuric acid were added to all tubes and vortexed. The tubes were stopped and heated at 100 °C for 10 min in a heating block (Precision, GCA Corp.). The tubes were cooled to room temperature. Two hundred µl of the contents of each tube was transfered to 12 x 75 mm test tubes and 3.0 ml phosphoric acid-vanillin reagent was added and mixed. The tubes were allowed to stand for 60 min in darkness. Absorbances were read at 520 nm in a Beckman spectrophometer. The standard curve is shown in Figure 3.3. The concentrations were calculated as follows:

Total lipids (mg/g tissue or mg/ml serum) = Abs. x Slope x D.F.

where; Abs. = Corrected absorbance of sample

D.F. = Dilution factor

3.6.4 Triglycerides

Triglyceride contents were measured according to the procedure of Geigel et al. (93). The principal of this method is that triglycerides are extracted from the samples by an extraction reagent (nonane). Phospholipids or other interfering components are left

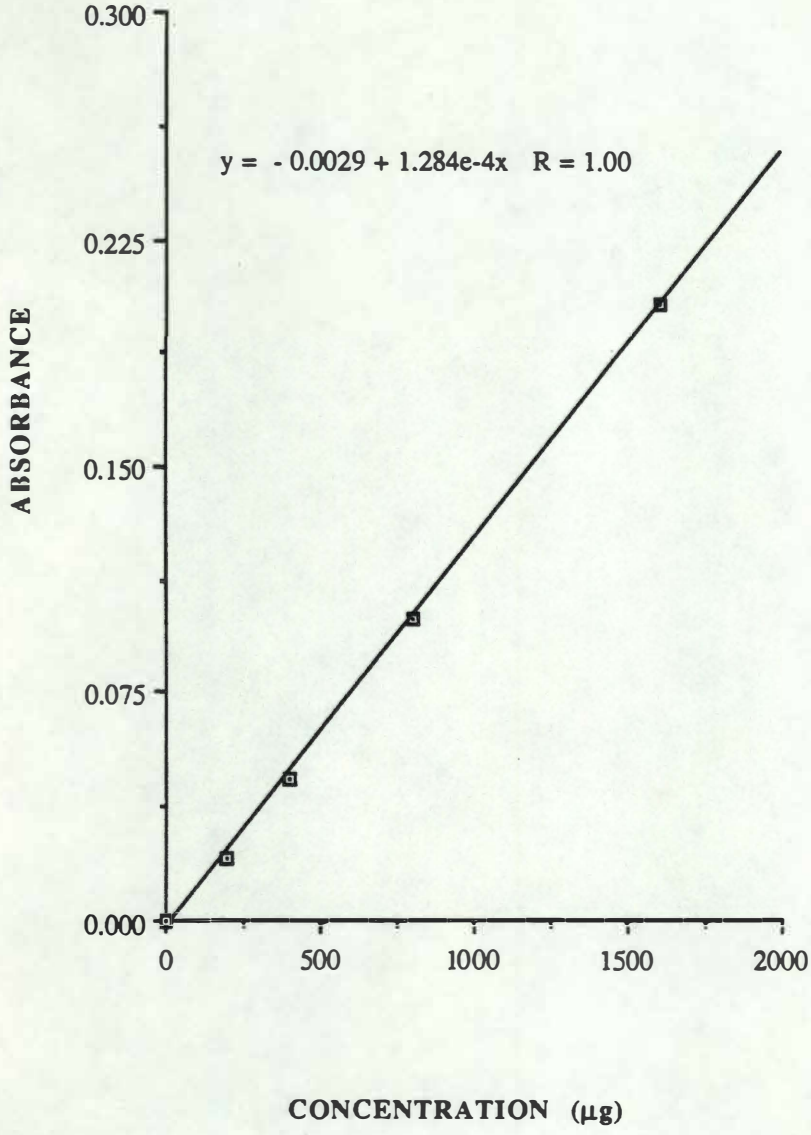


Figure 3.3 Total Lipids Standard Curve.

unextracted. The extracted triglycerides are hydrolyzed by sodium hydroxide and heating. The liberated glycerol is then oxidized by sodium periodate to form formaldehyde which condenses with ammonia and acetylacetone to form yellow diacetyldihydrolutidine which can be measured at 415 nm.

3.6.4.a Reagents

1. Extraction reagent: n-nonane/isoproponal (2.0/3.5, v/v).
2. Triolein standard : 100 mg triolein (Sigma Chemical Co., St. Louis, MO) in 100 ml extraction reagent (1.13 mM).
3. Dilute sulfuric acid, 40 mM.
4. Transesterifying reagent: sodium hydroxide in isopropanol (100mM/l).
5. Oxidizing reagent: sodium periodate (18 mM) in 2 M acetic acid.
6. Color buffer: ammonium acetate (6.0 M, pH 6.0 at 25 °C).
7. Working color reagent: 4.0 ml of acetylacetone mixed with 100 ml of the color buffer, and let stand for more than 15 min before use.

3.6.4.b Procedure

Aliquots of plasma and tissue homogenates (0.2 ml each) were transfered to separate 16 x 100 mm screw-capped test tubes containing 5.0 ml extraction reagent. Then, 1.0 ml dilute sulfuric acid was added. A blank and standards were made by adding 0, 0.1, 0.2, 0.4, and 0.6 ml of triolein standard and diluted to 5.0 ml with extraction reagent. All the tubes were centrifuged at 1500 x g at 4 °C for 10 min. Later, 0.5 ml aliquot of each upper phase was added to 0.5 ml of transesterifying reagent, held at room temperature for 5 min, mixed with 0.5 ml of oxidizing reagent, and held again at room temperature for 5 min. After the two-phase fraction had developed, 6 ml of the working color reagent was added to each tube and incubated at 60 °C for 10 min. The tubes were cooled to room

temperature and the absorbance of the standards and samples was read against the blank at 415 nm. See Figure 3.4 for triglycerides standard curve. The calculations were as follows:

$$\text{Triglycerides (mg/dl)} = \text{Abs.} \times \text{I.S.} \times \text{D.F.}$$

where: Abs. = Absorbance of unknown

I.S. = Inverse slope

D.F. = Dilution factor

3.6.5 Macromolecules and AFB_1-Macromolecule Adducts Quantitation

The extraction and isolation of macromolecules namedly RNA, DNA and protein, were done following the procedure of Glazer et al. (94). Perchloric acid (PCA) was used to precipitate the macromolecules. The pellet was treated with potassium acetate, chloroform:ethanol, and absolute ethanol to remove lipids. The pellet was resuspended with PCA and left overnight. The supernatant contained the RNA fraction. To isolate the DNA and protein, PCA was added to the pellet and heated. The supernatant contained the DNA fraction. Potassium hydroxide was added to the rest of the pellet and heated to get the protein fraction.

3.6.5.a Reagents

1. 0.2N, 0.5N, 1.0N, and 1.5N PCA from 70% PCA.
2. 0.1N potassium acetate (KAc) in absolute ethanol.
3. Chloroform:absolute ethanol (1:1, v/v).
4. 1.0 N potassium hydroxide (KOH).

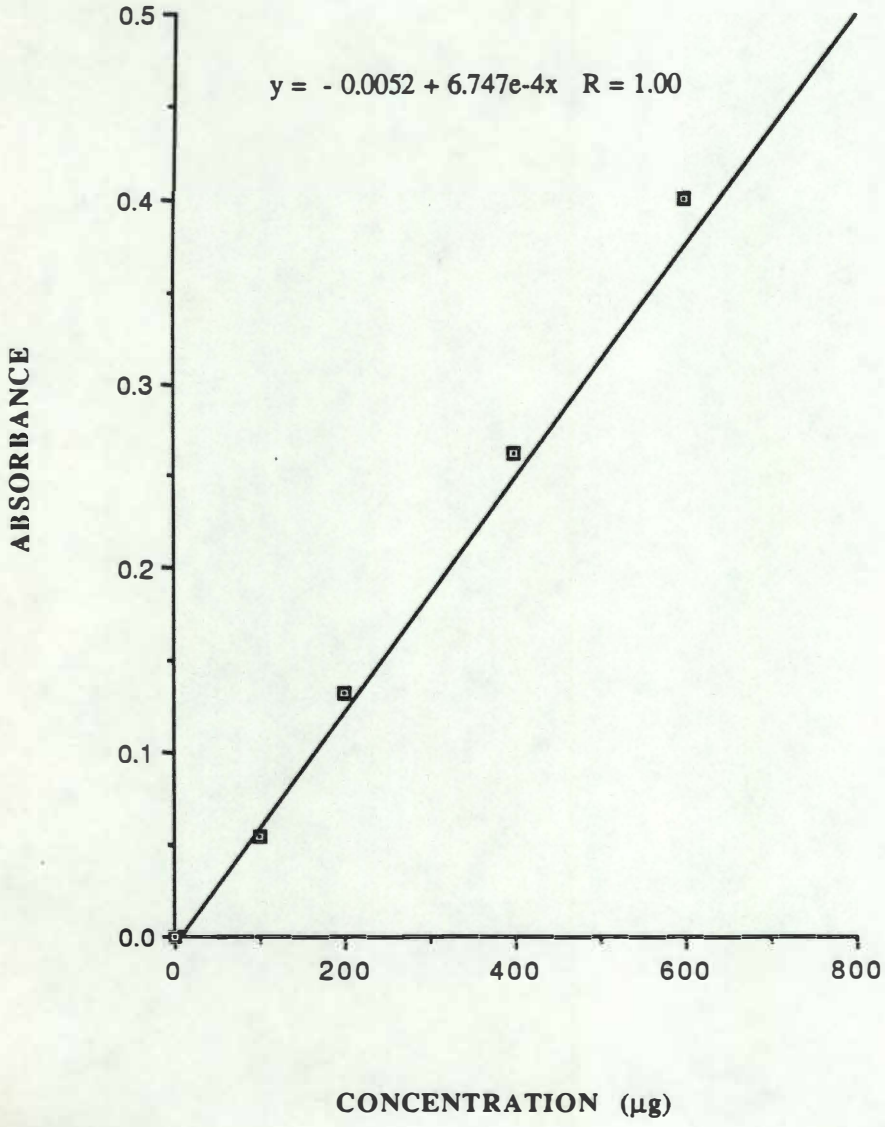


Figure 3.4 Triglycerides Standard Curve.

5. 0.04 % indole.
6. Concentrated HCl.
7. Chloroform.
8. Alkaline Copper Sulfate Solution prepared daily by adding 50 ml of 0.1N NaOH containing 1 g sodium carbonate, 0.5 ml of 2 % (w/v) sodium-potassium tartarate, and 0.5 ml of 1 % (w/v) cupric sulfate.
9. Phenol Reagent (Sigma Chemical Co., St Louis, MO).

3.6.5.b Standard Solutions

The standard solutions were prepared as shown in Table 3.4. Ten ml of calf-thymus DNA (4.0 mg/ml), yeast RNA (8.0 mg/ml) and bovine serum albumin (BSA) for protein (50 mg/ml) were prepared in three different tubes for stock standard solutions. Aliquotes of these stock solutions were dispensed into 4 different tubes such that different concentrations of the DNA, RNA, and protein were mixed together in one tube for each set of working standards. For example, in Standard 1, the tube contained 0.25 mg of DNA, 0.5 mg of RNA, and 10 mg of protein in a total of 5 ml of working standard solution.

3.6.5.c Extraction and Isolation

Aliquotes of O.4 ml of the 1:8 (w/v) liver homogenate (50 mg tissue) and the working standard solutions were added to ice-cold 1.0N PCA, vortexed, and centrifuged at 2700 x g for 15 min at 2 ^{o}C. For blank, 0.4 ml GDW was added to the blank tube. The supernatant was discarded. The pellet was washed and centrifuged 4 times with 1.5 ml 0.2N PCA. The supernatant was removed each time. Lipids were removed by treating the pellet once with 1.5 ml of 0.1N KAc in absolute ethanol, twice with 1.0 ml of

Table 3.4 Preparation of Macromolecule Standard Solutions.

Standard Tubes #	DNA (4 mg/ml)	RNA (8 mg/ml)	Protein (50 mg/ml)	GDW[1]	Total Volume
	----------µl (mg)----------			ml	ml
1	62.5 (0.25)	62.5 (0.5)	200 (10)	4.675	5
2	125 (0.50)	125 (1.0)	400 (20)	4.350	5
3	250 (1.00)	250 (2.0)	800 (40)	3.700	5
4	375 (1.50)	375 (3.0)	1600 (80)	2.650	5

[1]Glass Distilled Water

chloroform:absolute ethanol and once with 1.0 ml of absolute ethanol. During each treatment, the tubes were vortexed, centrifuged, and supernatant was drained completely (especially the last step). The precipitate was resuspended with 1.0 ml of 1.5N PCA, vortexed, and left overnight in the refrigerator (4 ^{o}C for about 18 h). The next day, the tubes were centrifuged and supernatant was collected. The precipitate was washed twice with 0.5 ml of 0.5N PCA, vortexed, centrifuged, and the supernatant was collected each time. The combined supernatant contained the RNA fraction. The pellet was then added to 1.0 ml of 0.5N PCA, vortexed, and incubated in a 70 ^{o}C water bath for 20 min, centrifuged, and the supernatant collected. This was repeated twice and the combined supernatant contained the DNA fraction. The precipitate was dissolved in 1 ml 1.0N KOH, vortexed, and incubated at 70 ^{o}C for 15 min and centrifuged. The supernatant contained the protein fraction. RNA was determined on the same day, while DNA and protein were stored at -70 ^{o}C until analyzed.

3.6.5.d Determination

RNA was measured spectrophometrically against the blank at 260 nm using UV light (94). DNA was determined colorimetrically by the method of Ceriotti (95). One ml of the DNA extract was added with 0.5 ml of 0.04% cold indole and 0.5 ml concentrated HCl. The tubes were vortexed and heated in a 100 ^{o}C water bath for exactly 10 min. The tubes were cooled under running water. The water layer (top layer) was extracted twice with 2 ml chloroform and centrifuged each time. The lower layer (chloroform layer) was removed using a clean Pasteur pipette each time. The samples were read at 490 nm against the blank.

Protein determination was carried out as described by Lowry et al. (96). 0.2 ml of the KOH protein solution was removed and marked-up to 1 ml with GDW in a 10 ml tube.

Six ml of the alkaline copper sulfate solution was added, vortexed, and left to stand for 5 min at room temperature. Then 0.3 ml of phenol reagent was added, vortexed immediately, and left at room temperature for at least 30 min. The tubes were centrifuged for 5 min and the absorbance were read at 650 nm against the blank.

The standard curves were drawn for each of the macromolecules (Figures 3.5, 3.6, and 3.7.) The concentrations were calculated as follows:

DNA/RNA/PROTEIN (mg/g liver) = ABS. x inverse slope x D.F.

Where, ABS. = Corrected absorbance of sample

D.F. = Dilution factor

3.6.5.e [^{3}H]AFB$_1$-Macromolecules Adducts

RNA, DNA, and protein extracts (0.5 ml each) were dispensed into separate 20 ml scintillation vials. Fifteen ml of Aquasol-2 was added to the vials and mixed. For protein, 100 µl of glacial acetic acid was added. The radioactivity was counted in a Beckman liquid scintillation counter for 10 min. The disintergration per minute (DPM) was calculated as follows:

DPM/mg RNA, DNA, or protein = DPM/0.5 ml sample x D.F.

Where, D.F. = Dilution factor

3.7 PRECAUTIONARY AND DECONTAMINATION PROCEDURES

The safety and decontamination procedures followed during handling of AFB$_1$ were as recommended by Stoloff et al. (97), Fischbach et al. (98), and Castegnaro et al. (99). Briefly, when handling the AFB$_1$, disposable rubber gloves, face mask and lab coat were always worn. Dry or concentrated toxins from the manufacturer were handled under the

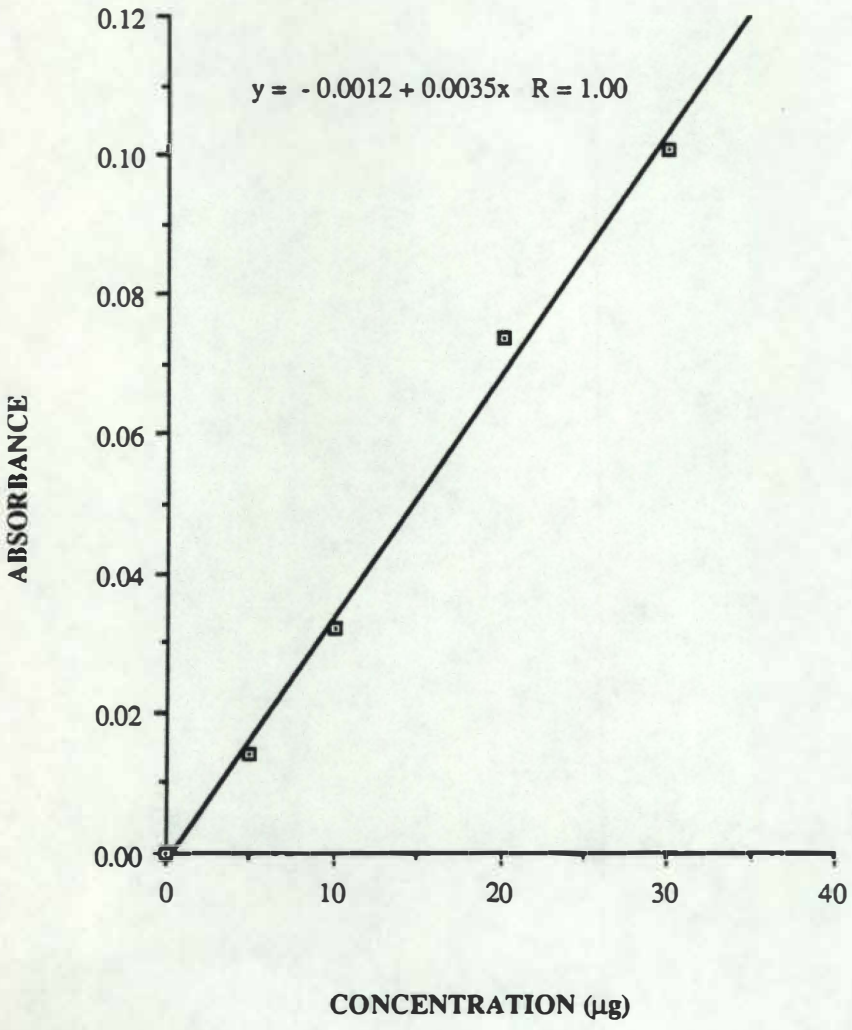


Figure 3.5 DNA Standard Curve.

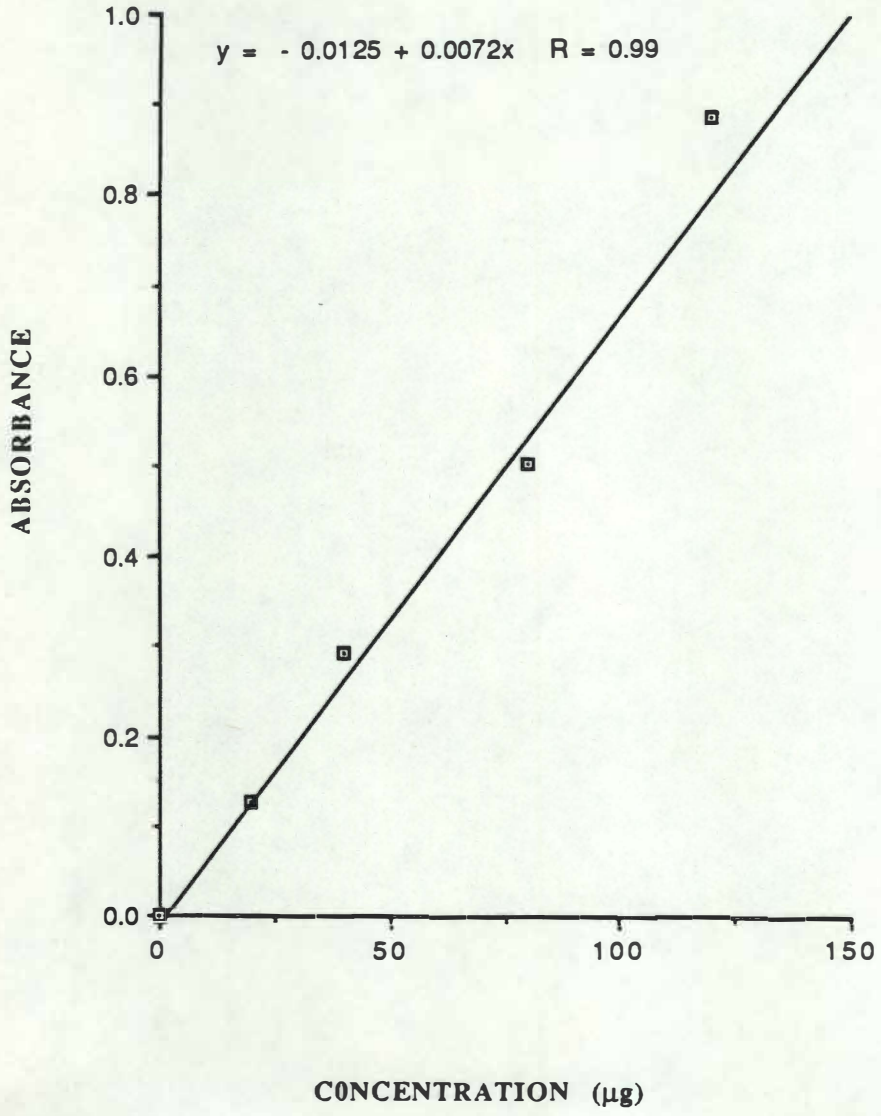


Figure 3.6 RNA Standard Curve.

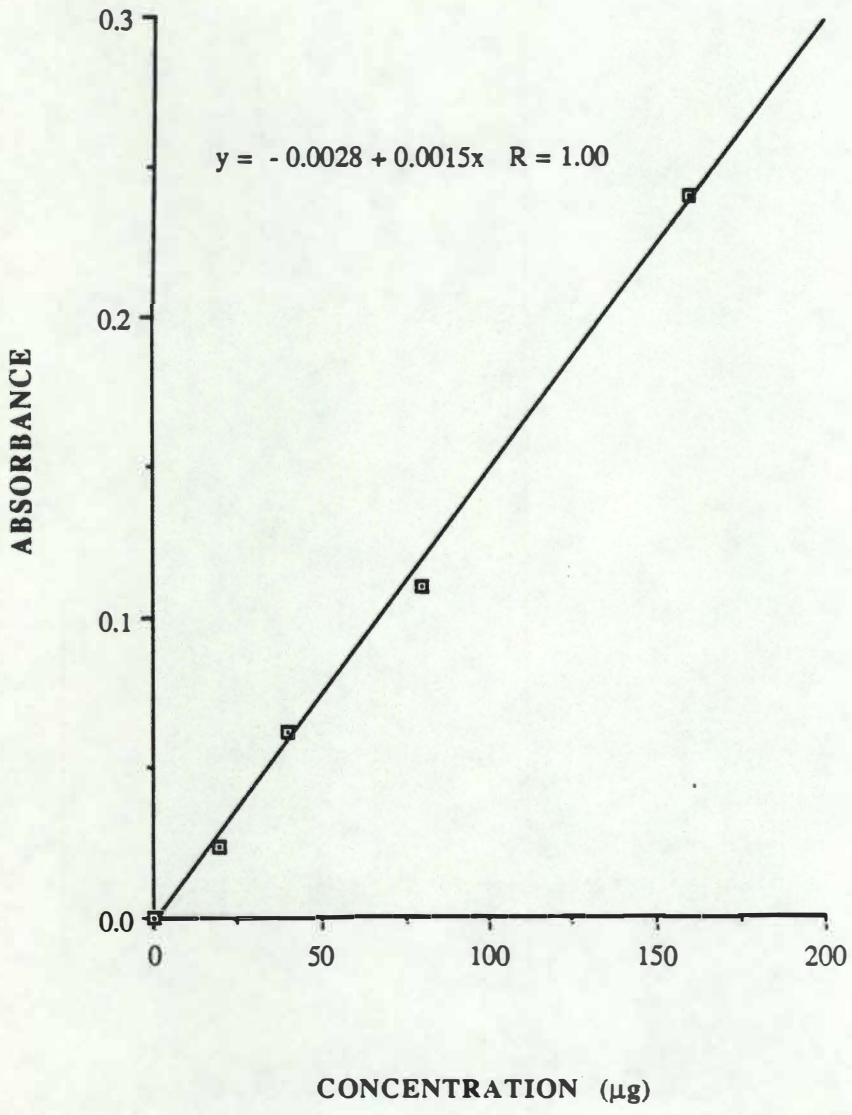


Figure 3.7 Protein Standard Curve.

hood. To decontaminate any spillage, 5% sodium hypochlorite (full strength regular household bleach) was used to wet or soak the contaminated surface for at least 30 sec. The surface was then wiped with paper towels. Before washing contaminated laboratory wares, they were soaked for at least 30 sec with diluted bleach (0.5% NaOCl). Refer to Appendix A-3.2 for the complete details of the procedures.

3.8 STATISTICS

All values are expressed as group mean $\pm$ standard error of mean. Two-way crossed ANOVA were used when data from 4 experimental groups were analyzed and compared, and when significant, Duncan's New Multiple Range Test was used. When analyzing and comparing data from 2 experimental groups, Student's t-test was used. The minimal level of significance accepted was $p \leq 0.05$.

CHAPTER 4

RESULTS

The effect of carnitine supplemented diet and/or AFB_1 treatment on the rats body weight and organ weight are summarized in Tables 4.1 and 4.2. Carnitine supplemention had no significant effect on weight gain or liver and kidney weights. AFB1 treatment too had no effect on organ weights. The 24-hour urinary output and amount of AFB_1 excreted in the urine were not affected by AFB_1 treatment in both the NSA and CSA groups (Table 4.3). Even though it is not significant, the CSA group had slightly larger percentage of AFB_1 dose (58.11%) excreted in the urine than in the NSA group (54.16%).

The results of the effects of carnitine supplemented diet and AFB_1 treatment in Experiment 1 are presented in Table 4.4. In the AFB_1 treated rats, the amount of total lipids in the liver was significantly lower in the carnitine supplemented groups, CSC (59.3 mg/g) and CSA (59.5 mg/g), compared to the NSA group (65.3 mg/g). The liver triglyceride concentrations was lower in the CSC group than the other three groups. Carnitine and AFB_1 treatments did not change the amount of total lipid in the kidney. In the plasma, the diet x AFB_1 interaction significantly affected the total lipid and triglyceride content indicating that in non-supplemented groups, AFB_1 treatment lowers the concentration of the total lipids and triglycerides. In contrast, in the carnitine-supplemented groups, AFB_1 treatment increased the concentrations of the total lipid and triglyceride in the plasma. CSC group had lower amount of total lipids (20% lower) and triglycerides (37.2 % lower) content in the plasma than the NSC group. The percent differences in the total lipid and triglyceride contents are shown in Appendex A-4.1.

Table 4.1 Effects of Carnitine Supplemented Diet on Body, Liver and Kidney Weights of Rats in Experiment 1 (6 h Post-AFB_1).

	Groups[1]			
	NSC	NSA	CSC	CSA
Initial Body Weight (g)	271.0 ± 5.0[2]	270.4 ± 5.9	273.5 ± 4.1	272.3 ± 4.5
Final Body Weight (g)	395.5 ± 13.6	393.0 ± 13.9	398.5 ± 7.4	394.3 ± 8.0
Weight Gain (g)	124.5 ± 8.7	122.6 ± 8.6	124.9 ± 4.6	122.0 ± 11.4
Liver Weight (g)	13.6 ± 0.6	13.2 ± 0.5	12.9 ± 0.3	13.4 ± 0.7
Liver/Final Body Weight (%)	3.4 ± 0.09	3.3 ± 0.08	3.2 ± 0.07	3.4 ± 0.15
Kidney Weight (g)	2.6 ± 0.09	2.5 ± 0.13	2.6 ± 0.05	2.5 ± 0.05
Kidney/Final Body Weight (%)	0.66 ± 0.01	0.63 ± 0.02	0.64 ± 0.01	0.64 ± 0.02

[1]NSC = Nonsupplemented Control; NSA = Nonsupplemented AFB_1; CSC = Carnitine Supplemented; CSA = Carnitine Supplement AFB_1.

[2]The values are mean ± SEM (n = 5). All values are not statistically different ($p \geq 0.05$).

Table 4.2 Effects of Carnitine Supplemented Diet on Body, Liver and Kidney Weights of Rats in Experiment 2 (24 h Post-AFB_1).

	Groups[1]	
	NSA	CSA
Initial Body Weight (g)	338.5 ± 3.7[2]	336.1 ± 3.3
Final Body Weight (g)	403.2 ± 6.3	401.8 ± 2.7
Weight Gain (g)	64.7 ± 4.0	65.7 ± 2.6
Liver Weight (g)	11.6 ± 0.3	12.0 ± 0.2
Liver/Final Body Weight (%)	2.9 ± 0.05	3.0 ± 0.06
Kidney Weight (g)	2.5 ± 0.06	2.4 ± 0.05
Kidney/Final Body Weight (%)	0.61 ± 0.01	0.59 ± 0.01

[1]NSA = Nonsupplemented AFB_1; CSA = Carnitine Supplement AFB_1.

[2]The values are mean ± SEM (n = 5). All values are not statistically different ($p \geq 0.05$).

Table 4.3 Effects of Supplementary Carnitine on AFB_1 Urinary Excretion in Rats of Experiment 2 (24 h Post-AFB_1).

	Groups[1]		
	NSA	CSA	% Different
Amount of Urine (ml/24 h)	21.05 ± 4.05[2a]	18.10 ± 2.02[a]	14.0
Total Amount of [^{3}H]AFB_1 Excreted (μg AFB_1 in 24 h)	223.99 ± 34.85[a]	235.17 ± 15.02[a]	5.0
Dose in Urine (%)	54.16 ± 7.88	58.11 ± 3.74	7.3
Amount of [^{3}H]AFB_1 Excreted/ml Urine (μg AFB_1/ml Urine)	1.40 ± 0.18[a]	1.56 ± 0.18[a]	11.4

[1]NSA = Nonsupplemented AFB_1; CSA = Carnitine Supplemented AFB_1.

[2]The values are mean ± SEM (n = 4). Values with different superscript letter are significant ($p \leq 0.05$).

Table 4.4 Effects of Diet and AFB_1 on Total Lipid and Triglyceride Contents in Rats of Experiment 1 (6 h Post-AFB_1).

	Groups[1]			
	NSC	NSA	CSC	CSA
Liver:				
Total Lipids (mg/g)[2]	60.5 ± 1.6^{abx}	65.3 ± 2.1^{ax}	59.3 ± 4.1^{bx}	59.5 ± 0.5^{bx}
Triglycerides (mg/g)	23.5 ± 1.3^{ax}	27.9 ± 0.5^{ax}	24.4 ± 1.2^{ay}	26.1 ± 0.7^{axy}
Kidney:				
Total Lipids (mg/g)	28.3 ± 2.2^{ax}	29.9 ± 2.5^{ax}	28.1 ± 1.1^{ax}	28.1 ± 0.5^{ax}
Triglycerides (mg/g)	22.3 ± 0.7^{ax}	24.0 ± 0.8^{ax}	22.3 ± 1.0^{ax}	22.6 ± 1.1^{ax}
Plasma:				
Total Lipids (mg/dl)	339.1 ± 10.3^{ax}	280.5 ± 34.3^{ax}	269.1 ± 26.4^{ax}	317.5 ± 25.2^{ax}
Triglycerides (mg/dl)	82.2 ± 4.4^{ax}	57.1 ± 4.9^{ax}	51.6 ± 10.7^{ax}	76.5 ± 13.4^{ax}

[1]NSC = Nonsupplemented Control; NSA = Nonsupplemented AFB_1; CSC = Carnitine Supplemented; CSA = Carnitine Supplement AFB_1.

[2]The values are mean $\pm$ SEM (n = 5 for liver and kidney; n = 4 for plasma). Those values in a line bearing the different superscript letter (a - c) are statistically different ($p \leq 0.05$) due to diet treatment, and those bearing different superscript letters (x - z) are significantly different ($p \leq 0.05$) due to AFB_1 by a two-way crossed ANOVA. Diet x AFB_1 interaction was significant only in plasma's total lipids and triglycerides ($p \leq 0.05$).

Similar results for lipid were observed in Experiment 2 where the animals were sacrificed 24 h after the administration of AFB1 (Table 4.5). Liver total lipid and triglyceride contents were statistically lower in the CSA group than in the NSA group. In the plasma, the reverse was seen, i.e., NSA group had lower while CSA group had higher amount of total lipids and triglycerides. The triglyceride concentration in the CSA group was 15.5% higher than in the NSA group and the difference was highly significant ($p \leq 0.01$). There were no differences in kidney lipids of the two groups.

Tables 4.6 to 4.11 show the effects of carnitine and AFB_1 treatments on liver and plasma carnitine concentrations and the [^{3}H]AFB_1 radioactivities associated with them. Carnitine supplementation did not significantly affect liver carnitine concentrations, although all the carnitine fractions were higher in the CSC group as compared to the NSA group (Table 4.6). However, AFB_1 treatment increased the NEC contents in both NSA and CSA groups when compared to their respective control groups (NSC and CSC). Carnitine and AFB_1 treatments both elevated ASAC concentrations in the NSA and CSA animals as compared to their respective control animals. The amounts of ASAC in the CSA rats were significantly higher (191.25 nmol/g) than in the NSA rats (115.33 nmol/g). The concentrations of AIAC were not significantly affected by the diet and AFB_1 treatments eventhough there was about 50% increase compared to respective controls. The total carnitine contents were higher in both NSA and CSA groups when compared to their control groups. The diet x AFB_1 interactions were not significant in all the groups to affect the concentrations of all the carnitine fractions in the liver.

[^{3}H]AFB_1 radioactivity was only found in the AIAC fraction and is presented in Table 4.7. Though it was not statistically significant, CSA group had 26.2% higher amount of AIAC than the NSA groups per g liver. The amount of AFB_1 dose and

Table 4.5 Effects of Diet and AFB_1 on Total Lipid and Triglyceride Contents in Rats of Experiment 2 (24 h Post-AFB_1).

	Groups[1]		
	NSA	CSA	% Different
Liver:			
Total Lipids[2] (mg/g)	59.8 ± 1.4[a]	55.2 ± 0.5[b]	7.7
Triglycerides (mg/g)	21.7 ± 0.5[a]	19.0 ± 0.4[b]	12.4
Kidney:			
Total Lipids (mg/g)	28.8 ± 0.6[a]	29.0 ± 0.6[a]	0.7
Triglycerides (mg/g)	18.2 ± 0.4[a]	17.9 ± 0.7[a]	1.6
Plasma:			
Total Lipids (mg/dl)	359.5 ± 8.4[a]	396.8 ± 8.2[b]	10.4
Triglycerides (mg/dl)	59.5 ± 3.9[a]	68.7 ± 2.6[c]	15.5

[1]NSA = Nonsupplemented AFB_1; CSA = Carnitine Supplemented AFB_1.

[2]The values are mean ± SEM (n = 5). [b]Significant at $p \leq 0.05$; [c]Significant at $p \leq 0.01$. Values with same superscript are not significant ($p \geq 0.05$).

Table 4.6 Effects of Diet and AFB_1 on Liver Carnitines (nmol/g) in Rats of Experiment 1 (6 h Post -AFB_1).

Carnitine Fractions	Groups[1]			
	NSC	NSA	CSC	CSA
Nonesterified Carnitine (NEC)	224.73 ± 9.53[2ax]	398.19 ± 33.69[ayz]	319.98 ± 41.71[axz]	456.34 ± 55.92[ay]
Acid-Soluble Acylcarnitine (ASAC)	45.88 ± 9.55[ax]	115.33 ± 17.07[aby]	98.04 ± 20.33[abxy]	191.25 ± 31.23[cz]
Acid-Insoluble Acylcarnitine (AIAC)	13.46 ± 3.25[ax]	19.16 ± 2.70[ax]	15.10 ± 3.38[ax]	24.19 ± 6.07[ax]
Total Carnitine	284.08 ± 12.41[ax]	532.69 ± 50.68[abcx]	433.12 ± 59.22[acx]	671.79 ± 9.53[bx]

[1]NSC = Nonsupplemented Control; NSA = Nonsupplemented AFB_1; CSC = Carnitine Supplemented; CSA = Carnitine Supplement AFB_1.

[2]The values are mean ± SEM (n = 5). Those values in a line bearing the different superscript letters a-c are statistically different ($p \leq 0.05$) due to diet treatment, and those values in a line bearing a suprscript letters x-z are statistically different ($p \leq 0.05$) due to AFB_1 treatment by a two-way crossed ANOVA. AFB_1 treatment and AFB_1 x diet interaction was not significant ($p \geq 0.05$).

Table 4.7 [^{3}H]AFB$_1$ Binding to Liver Acid Insoluble Acylcarnitine (AIAC) in Rats of Experiment 1 (6 h Post-[^{3}H]AFB$_1$).

	Groups[1]		
	NSA	CSA	% Different
Amount of AIAC (nmol/g liver)	19.16 ± 2.70[2a]	24.19 ± 6.07[a]	26.25
[^{3}H]AFB$_1$-AIAC adducts/nmol AIAC (nmol AFB$_1$/nmol AIAC)	0.21 ± 0.03[a]	0.22 ± 0.07[a]	4.76
[^{3}H]AFB$_1$ in AIAC/g liver (nmol AFB$_1$/g liver)	3.64 ± 0.27[a]	3.89 ± 0.32[a]	6.87
Amount of dose in AIAC (% Dose/pmol AIAC)	16.60 ± 2.94[a]	17.50 ± 5.77[a]	5.42

[1]NSA = Nonsupplemented AFB$_1$; CSA = Carnitine Supplemented AFB$_1$.

[2]The values are mean ± SEM (n = 5). Values with different superscript letter are significant ($p \leq 0.05$).

Table 4.8 Liver Carnitine Contents and [^{3}H]AFB$_1$-Acid Insoluble Acylcarnitine Binding in Rats of Experiment 2 (24 h Post-[^{3}H]AFB$_1$).

	Groups[1]		
	NSA	CSA	% Different
Nonesterified Carnitine (NEC) (nmol/g liver)	438.22 ± 27.03[2a]	608.42 ± 36.63[b]	38.84
Acid Soluble Acylcarnitine (ASAC) (nmol/g liver)	261.90 ± 35.66[a]	190.96 ± 26.04[a]	27.09
Acid Insoluble Acylcarnitine (AIAC) (nmol/g liver)	18.79 ± 5.61[a]	14.35 ± 3.99[a]	23.63
Total Carnitine (nmol/g liver)	749.46 ± 57.99[a]	834.53 ± 63.19[a]	11.35
[^{3}H]AFB1-AIAC adducts (pmol AFB$_1$/nmol AIAC)	6.47 ± 2.04[a]	4.82 ± 1.24[a]	25.50
[^{3}H]AFB1 in AIAC/g liver (pmol AFB$_1$/g liver)	84.71 ± 10.45[a]	101.22 ± 9.30[a]	19.49
Amount of dose in AIAC (% Dose/pmol AIAC)	0.50 ± 0.14[a]	0.37 ± 0.08[a]	26.00

[1]NSA = Nonsupplemented AFB$_1$; CSA = Carnitine Supplemented AFB$_1$.

[2]The values are mean ± SEM (n = 5). Values with different superscript letter are significant ($p \leq 0.05$).

Table 4.9 Effects of Diet and AFB_1 on Plasma Carnitines (μmol/ml) in Rats of Experiment 1 (6 h Post-AFB_1).

Carnitine Fractions	Groups[1]			
	NSC	NSA	CSC	CSA
Nonesterified Carnitine (NEC)	51.02 ± 0.47[2bcd]	43.82 ±1.49[d]	74.65 ± 7.53[a]	69.98 ± 9.41[ac]
Acid-Soluble Acylcarnitine (ASAC)	11.91 ± 0.33[a]	10.99 ± 0.50[a]	10.72 ± 2.80[a]	11.53 ± 2.58[a]
Acid-Insoluble Acylcarnitine (AIAC)	4.47 ± 0.35[bc]	3.56 ± 0.22[b]	6.33 ± 0.80[a]	5.36 ± 0.61[ac]
Total Carnitine	66.15 ± 1.64[acd]	57.80 ± 2.44[bd]	91.70 ± 10.78[a]	90.80 ± 11.29[ac]

[2]NSC = Nonsupplemented Control; NSA = Nonsupplemented AFB_1; CSC = Carnitine Supplemented; CSA = Carnitine Supplement AFB_1.

[3]The values are mean ± SEM (n = 5). Those values in a line bearing the different superscript letter are statistically different ($p \leq 0.05$) due to diet treatment ($p \leq 0.05$) by a two-way crossed ANOVA. AFB_1 treatment and AFB_1 x diet interaction was not significant ($p \geq 0.05$).

Table 4.10 [^{3}H]AFB$_1$ Binding to Plasma Acid Insoluble Acylcarnitine (AIAC) in Rats of Experiment 1 (6 h Post-[^{3}H]AFB$_1$).

	Groups[1]		
	NSA	CSA	% Different
Amount of AIAC (µmol/ml plasma)	3.56 ± 0.22[2a]	5.36 ± 0.61[b]	50.56
[^{3}H]AFB$_1$-AIAC adducts/µmol AIAC (nmol AFB$_1$/µmol AIAC)	79.90 ± 8.20[a]	57.00 ± 4.70[b]	28.70
[^{3}H]AFB$_1$ in AIAC/ml Plasma (pmol AFB$_1$/ml plasma)	406.93 ± 43.49[a]	420.00 ± 26.77[a]	3.21
Amount of dose with AIAC (% Dose/nmol AIAC)	6.33 ± 0.68[a]	4.50 ± 0.38[b]	28.90

[1]NSA = Nonsupplemented AFB$_1$; CSA = Carnitine Supplemented AFB$_1$.

[2]The values are mean ± SEM (n = 5). Values with different superscript letter are significant ($p \leq 0.05$).

Table 4.11 Plasma Carnitine Contents and [^{3}H]AFB$_1$-Acid Insoluble Acylcarnitine Binding in Rats of Experiment 2 (24 h Post-[^{3}H]AFB$_1$).

	Groups[1]		
	NSA	CSA	% Different
Nonesterified Carnitine (NEC) (μmol/ml)	39.67 ± 1.23[2a]	60.17± 2.51[b]	51.68
Acid Soluble Acylcarnitine (ASAC) (μmol/ml)	8.89 ± 1.07[a]	8.42 ± 0.92[a]	5.29
Acid Insoluble Acylcarnitine (AIAC) (μmol/ml)	13.86 ± 2.59[a]	17.90 ± 5.46[a]	30.85
Total Carnitine (μmol/ml)	62.42 ± 2.64[a]	86.48 ± 3.01[b]	38.54
[^{3}H]AFB$_1$-AIAC adducts (pmol AFB$_1$/μmol AIAC)	15.02 ± 2.58[a]	17.20 ± 5.85[a]	5.81
[^{3}H]AFB$_1$ in AIAC/ml plasma (pmol AFB$_1$/ ml plasma)	181.98 ± 3.40[a]	199.74 ± 18.51[a]	9.76
Amount of dose with AIAC (% Dose/nmol AIAC)	1.16 ± 0.20[a]	1.17 ± 0.46[a]	0.86

[1]NSA = Nonsupplemented AFB$_1$; CSA = Carnitine Supplemented AFB$_1$.

[2]The values are mean ± SEM (n = 5). Values with different superscript letter are significant ($p \leq 0.05$).

[^{3}H]AFB$_1$-AIAC adducts were also not significantly affected by carnitine supplementation.

The amount of NEC in the liver 24 h after the dose of AFB$_1$ was 38.8% ($p \leq 0.05$) higher in the CSA group as compared to the NSA group (Table 4.8). The ASAC, AIAC, and total carnitine were not statistically different between the NSA and CSA groups. The binding of AFB$_1$ and amount of dose in AIAC in both groups were also not significantly different.

Carnitine treatment had significant effect on plasma carnitine concentrations in Experiment 1, but AFB$_1$ treatment did not (Table 4.9). Carnitine supplementation increased NEC concentrations in the CSC group (74.65 μmol/ml) when compared to the NSC group (51.02 μmol/ml). The ASAC fraction was not affected by carnitine supplementation or the AFB$_1$ dose. The AIAC fraction was higher in the CSC rats than in the NSC rats, as well as in the CSA rats when compared with NSA rats. As observed in the liver, the AFB$_1$ was only bound to the AIAC fraction (Table 4.10). The amount of [^{3}H]AFB$_1$-AIAC adducts and the amount of dose in AIAC were significantly higher in the NSA group than in the CSA group. However, since the content of AIAC in the CSA group was higher than in the NSA group, the amount of [^{3}H]AFB$_1$/ml of plasma was not different between the two groups.

Plasma NEC and total carnitine concentrations were significantly higher in the CSA group than in the NSA group 24 h after the [^{3}H]AFB$_1$ administration (Table 4.11). There was no difference in the amounts of ASAC, NEC and [^{3}H]AFB$_1$-AIAC adduct, and the percentage of dose AIAC.

Table 4.12 summarizes the [^{3}H]AFB$_1$ concentrations in the liver, kidney and plasma of rats 6 h after the dose of [^{3}H]AFB$_1$. The amounts of [^{3}H]AFB$_1$ found in the liver, kidney, and plasma were not significantly different. Although the CSA group had 14.7%

Table 4.12 Tissue and Plasma [^{3}H]AFB$_1$ Concentrations in Rats of Experiment 1 (6 h Post-[^{3}H]AFB1).

	Groups[1]		
	NSA	CSA	% Different
Liver:			
DPM (10^{-4})/g	6.77 ± 0.51[2a]	5.77 ± 0.50[a]	14.7
nmole of AFB$_1$/g	3.81 ± 0.28[a]	3.25 ± 0.28[a]	
Kidney:			
DPM (10^{-4})/g	1.21 ± 0.14[a]	1.18 ± 0.13[a]	2.5
nmole of AFB$_1$/g	0.68 ± 0.08[a]	0.66 ± 0.07[a]	
Plasma:			
DPM (10^{-4})/ml	4.38 ± 0.25[a]	5.51 ± 0.73[a]	25.8
nmole of AFB$_1$/ml	2.47 ± 0.14[a]	3.10 ± 0.41[a]	

[1]NSA = Nonsupplemented AFB$_1$; CSA = Carnitine Supplemented AFB$_1$.

[2]The values are mean ± SEM (n = 5).Values with different superscript letter are significant ($p \geq 0.05$).

less nmol of AFB_1 in the liver and 25.8% more [^{3}H]AFB_1 in the plasma than the NSA group, the amounts were not statistically significant due to large standard deviation in the results. The concentrations of [^{3}H]AFB_1 in the liver and kidney 24 h after the dose of [^{3}H]AFB_1 were also not affected by the diet treatment (Table 4.13). However, CSA plasma had 19.8% ($p \leq 0.05$) more [^{3}H]AFB_1 in the plasma than the plasma of the NSA group.

The effects of carnitine on hepatic macromolecules 6 h after the dose of the AFB_1 are shown in Table 4.14 and 4.15. Carnitine had no effect on the RNA, DNA, and protein content in all four groups of animals. However, AFB_1 treatment significantly decreased the liver RNA content in the NSA group as compared to NSC group, but RNA content in the CSC and CSA groups remained relatively unchanged. DNA and protein concentrations were not affected by the AFB_1 treatment. The interaction of diet x AFB_1 was not significant in affecting the macromolecule contents. Although the CSA group had 33.6% less AFB_1-RNA adducts than NSA group, the difference was not significant ($p \leq 0.06$) (Table 4.15). In addition, diet treatment had no effect on the AFB_1-protein adduct concentrations. However, carnitine supplemention markedly decreased ($p \leq 0.01$) the amount of AFB_1-DNA adducts in rat livers as compared to nonsupplemented rats.

The effects of carnitine on the amount of hepatic macromolecules and AFB_1-macromolecules adducts 24 h after the dose of [^{3}H]AFB_1 are shown in Table 4.16. Carnitine supplemented animals had higher amount of RNA, DNA and protein than the NSA group. The quantities of AFB_1 binding to all three of the macromolecules were significantly reduced in the carnitine supplemented group.

Table 4.13 Tissue and Plasma [^{3}H]AFB$_1$ Concentrations in Rats of Experiment 2 (24 h Post-[^{3}H]AFB$_1$).

	Groups[1]		
	NSA	CSA	% Different
Liver:			
DPM (10^{-4})/g	3.74 ± 0.35[2a]	3.55 ± 0.22[a]	5.1
nmole of AFB$_1$/g	2.11 ± 0.20[a]	2.00 ± 0.12[a]	
Kidney:			
DPM (10^{-3})/g	9.95 ± 0.61[a]	9.58 ± 0.22[a]	3.7
nmole of AFB$_1$/g	0.56 ± 0.03[a]	0.54 ± 0.01[a]	
Plasma:			
DPM (10^{-4})/ml	4.20 ± 0.14[a]	5.03 ± 0.34[b]	19.8
nmole of AFB$_1$/ml	2.37 ± 0.08[a]	2.83 ± 0.19[b]	

[1]NSA = Nonsupplemented AFB$_1$; CSA = Carnitine Supplemented AFB$_1$.

[2]The values are mean ± SEM (n = 5). Values with different superscript letter are significant ($p \geq 0.05$).

Table 4.14 Effects of Diet and AFB_1 on Hepatic Macromolecule Contents in Rats of Experiment 1 (6 h Post-AFB_1).

	Groups[1]			
	NSC	NSA	CSC	CSA
RNA (mg/g)	2.08 ± 0.02^{2ax}	1.81 ± 0.09^{ay}	2.02 ± 0.06^{axy}	1.90 ± 0.08^{axy}
DNA (mg/g)	2.90 ± 0.14^{ax}	2.63 ± 0.16^{ax}	2.93 ± 0.24^{ax}	2.78 ± 0.27^{ax}
Protein (mg/g)	140.9 ± 2.88^{ax}	138.1 ± 5.48^{ax}	142.3 ± 4.77^{ax}	135.9 ± 2.69^{ax}

[1]NSC = Nonsupplemented Control; NSA = Nonsupplemented AFB_1; CSC = Carnitine Supplemented; CSA = Carnitine Supplement AFB_1.

[2]The values are mean ± SEM (n = 5). Those values in a line bearing the different superscript letter (a - c) are statistically different ($p \leq 0.05$) due to diet treatment, and those bearing different superscript letters (x - z) are significantly different ($p \leq 0.05$) due to AFB_1 by a two-way crossed ANOVA. Diet x AFB_1 interaction was not significant ($p \geq 0.05$) in all cases.

Table 4.15 Effects of Carnitine Supplemented Diet on Binding of AFB_1 to Liver Macromolecules in Rats of Experiment 1 (6 h Post-AFB_1).

	Groups[1]		
	NSA	CSA	% Different
	(ng AFB_1/mg macromolecules)		
RNA	67.34 ± 8.88[2a]	44.73 ± 9.03[a]	33.6
DNA	21.08 ± 0.98[a]	14.84 ± 1.54[b]	29.6
Protein	3.57 ± 0.28[a]	3.57 ± 0.44[a]	0

[1]NSA = Nonsupplemented AFB_1; CSA = Carnitine Supplemented AFB_1.

[2]The values are mean ± SEM (n = 5). Values with different superscript letter are significant ($p \leq 0.01$).

Table 4.16 Effects of Carnitine Supplemented Diet on Amount of Liver Macromolecules and AFB_1-Macromolecules Binding in Rats of Experiment 2 (24 h Post-AFB_1).

	Groups[1]		
	NSA	CSA	% Different
RNA			
mg RNA/g liver	2.81 ± 0.07[2a]	2.99 ± 0.06[b]	6.4
ng AFB_1/mg RNA	65.96 ± 4.96[a]	53.95 ± 2.26[b]	18.2
DNA			
mg DNA/g liver	2.47 ± 0.07[a]	2.76 ± 0.06[b]	11.7
ng AFB_1/mg DNA	22.29 ± 2.58[a]	16.26 ± 1.36[b]	27.0
Protein			
mg protein/g liver	148.03 ± 4.31[a]	181.18 ± 15.13[b]	22.4
ng AFB_1/mg protein	3.29 ± 0.15[a]	3.04 ± 0.13[b]	7.6

[1]NSA = Nonsupplemented AFB_1; CSA = Carnitine Supplemented AFB_1.

[2]The values are mean ± SEM (n = 5). Values with different superscript letter are significant ($p \leq 0.05$).

CHAPTER 5

DISCUSSION

5.1 CARNITINE AND AFB_1-INDUCED FATTY LIVER

One of the objectives of this study was to examine the effect of carnitine supplement on AFB_1-induced changes in the hepatic and plasma lipids. Carnitine supplement significantly reversed changes in lipid contents of the liver and plasma which are typically induced by AFB_1. Supplementary carnitine significantly inhibited AFB_1-induced increase in hepatic total lipid and triglycerides contents. This effect of carnitine supports preliminary observations (Sachan et al., unpublished) and was analogous to the amelioration of ethanol-induced fatty liver (13,14,15) and fatty liver caused by lysine deficiency (101) further supporting the lipotropic actions of supplementary carnitine. A diet low in lipotropes (i.e. marginally deficient in choline and methionine, and lacking in folacin) has been shown to cause a significant increase in the lipid content of rat liver (52). In poultry, however, choline, inositol, vitamin E and B12 supplementation of an adequate basal diet did not significantly alter the fatty liver caused by aflatoxins (40).

Aflatoxin-induced decrease in plasma total lipid as well as triglyceride concentrations (Table 4.4 and 4.5) is consistent with the studies reported elsewhere (39,41). It has been suggested that the lipid transport from the blood and peripheral tissues is impaired by AFB_1. Carnitine appears to restore the transport system since CSA animals had significantly higher lipid levels in the plasma and lower lipid levels in the liver. This explanation is in accord with the proposed role of carnitine in inter-organ transport of fatty acids and their metabolites (102). Protection of liver from fat accumulation caused by

AFB_1 may also be aided by increased ß-oxidation fo fatty acids caused by carnitine. Actual increase in plasma AIAC of the CSA rats (Table 4.9 and 4.11) supports the argument that exogenous carnitine facilitated exit of fatty acids from the liver. Plasma acylcarnitine were also increased in carnitine supplemented alcoholic rats where exogenous carnitine ameliorated hepatic steatosis (13). The increased excretion of medium-chain dicarboxylic acids in the urine of infants fed carnitine deficient formula (103) as well dicarboxylic aciduria in infant fed carnitine-free formula (104) further lend support to carnitine mediated exit of fatty acid hypothesis.

5.2 CARNITINE AND AFB_1-MACROMOLECULE ADDUCTS FORMATION

In order to address the second objective of this study, examinations of AFB_1-macromolecule adducts were carried out. The data strongly indicate that supplementary carnitine minimized binding of AFB_1 to liver RNA, DNA, and proteins (Table 4.15 and 4.16). This AFB_1-DNA adduct was statistically lowered 6 h as well as 24 h post AFB_1 administration. Decrease in AFB_1-DNA adduct suggest sthat carnitine may modulate carninogenic action of AFB1 since AFB_1-DNA adducts have been typically attributed to cause carcinogenic response in animals (6,23,24,32,33) and humans (1,2,29,32).

A 30% reduction in AFB_1-DNA adduct formation due to carnitine (Table 4.15) is comparable to 9-30% reduction by in vitro addition of riboflavin (46), far better than 9% reduction by 60% food restriction (60), and 18% reduction by marginal deficiency of a mixture of lipotropes (52). Choline deficiency increases (41%) AFB_1-DNA adduct formation (105) which may be related to carnitine since choline deficiency also causes

carnitine deficiency (106). Cruciferous vegetables like Brussel sprouts (61) and chemical compounds in them such as R-goitrin (64) have been shown to reduce AFB_1-DNA adduct 50% and 43%, respectively. Food additives like BHA also reduced AFB_1-DNA adduct by 85% (64). Since many of these studies were done not under conditions identical to carnitine study, a direct comparison cannot be made.

Various mechanisms of action of different nutrients and food additives have been proposed in conjunction with reported studies (52,61,63,64,105,106). Possible mechanisms for interaction of carnitine and AFB_1 may be proposed as follows: 1) carnitine enhances excretion of AFB_1; 2) carnitine retards AFB_1 metabolism; 3) carnitine decreases half-life of AFB_1 binding to albumin; and/or 4) carnitine protects cell membranes.

5.3 CARNITINE AND AFB_1 INTERACTION

It was hypothesized that carnitine by virtue of having an electrophilic nitrogen center and a nucleophilic oxygen at pH 7.4 may may attract AFB_1 and/or its metabolites. AFB_1 was not associated with NEC or ASAC, but AIAC had significant AFB_1 radioactivity associated with it (Table 4.7 and 4.8). However, increases in AFB_1-AIAC complex in CSA animals were not significant. These different may have become significant if one of the group was fed a carnitine deficient diet. It has been reported that AFB_1-albumin was strengthened in the presence of long-chain fatty acids, namely palmitic, stearic, and oleic acids, at the concentration range of 0.5 to 2 moles per mole albumin (57). Carnitine has been found to conjugate with acidic xenobiotics, which are then excreted in the urine

(89,107). Carnitine supplementation, as observed in this study, had no significant effect on the amounts of AFB_1 binding to AIAC.

Carnitine supplementation increased the concentrations of all liver carnitine fractions and total carnitine as compared to the nonsupplemented animals, but the increases were not statistically significant (Table 4.6). However, in the plasma, carnitine supplementation markedly increased the NEC and total carnitine (Table 4.9). These data lead to the suggestion that the liver will take only a certain amount of the exogenous carnitine and the rest of it is goes to the blood and is transported to peripheral tissues or excreted in the urine. It has been reported that the excretion of carnitine in the urine was higher when the animals were fed with carnitine supplemented diet (100).

AFB_1 treatment increased the concentrations of NEC, ASAC and total carnitine in the liver, but had no effect in the plasma. In another words, AFB_1 causes the liver either to synthesize more carnitine or get the carnitine from extrahepatic tissues and/or exogenous sources. Since carnitine supplemented animals had an extra carnitine pool in the blood, the liver may take up this carnitine to accomodate the effect of AFB_1. The NSA group may have to get carnitine supply from the blood. As a result, although the CSA group had lower plasma carnitine than the CSC group, CSA carnitine concentrations were still higher than the NSC and NSA groups (Table 4.9). The higher concentrations of carnitine in the liver may explain the reason for the lower amounts of total lipid and triglyceride in the CSA group as compared to the NSA group. Similar observations were found when the rats were fed with a liquid ethanol diet and carnitine (14).

5.4 CARNITINE AND AFB_1 CONCENTRATION IN TISSUES AND PLASMA

Six hours after AFB_1 dosing, the amounts of AFB_1 in the liver, kidney, and plasma were not statistically different in supplemented or non-supplemented animals. At 24 h after

AFB_1 administration, AFB_1 concentrations were significantly lower in the plasma of the CSA group than in the NSA group, but AFB_1 concentrations in the liver and kidney were not significantly different. Modification of the method, especially in the digestion and decolorization of the tissues, may reduce the deviation among the samples and produce more conclusive results.

5.5 CARNITINE, BODY AND ORGANS WEIGHT AND AFB1 URINARY OUTPUT

This study found no significant changes in the body and organ weights in the rats fed diet with or without supplementary carnitine. These findings are similar to the study done by Berger (100) who fed various levels of carnitine to Sprague-Dawley rats for 10 d. As for the AFB_1 treatment, obviously 6 h or 24 h after a single dose (1 mg/kg) of AFB_1 would not have much affect on the body or organ weights.

The 24-h total urinary output and the concentrations of AFB_1 found in the urine were not significantly different in the NSA and CSA groups. It is important to point out that in this urinary study, the sample size was small (n = 4) due to limited number of metabolic cages. Furthermore, there was too much deviation in the amount of urinary output in the same group of animals (e.g., one of the rats in the NSA group excreted nearly twice as much urine as rest of the rats in its group, and one of the rats in CSA group excreted only 1/3 the amount of urine than the other 3 rats in its group.) Therefore, limited interpretations could be made about these results. However, in a different type of study, carnitine supplementation was found to increase urinary volume in rats treated with methanol and isopropanol but not with ethylene glycol (83). The excretion of methanol and isopropanol was significantly increased by carnitine supplementation. In a food restriction

study, the investigators observed that the 9 h urinary excretion of AFB_1 (as % of dose) was significantly higher in the food restricted than the ad lib. animals (60).

CHAPTER 6

SUMMARY AND RECOMMENDATIONS

6.1 SUMMARY

The effects of carnitine supplementation on AFB1-induced fatty liver and AFB_1-macromolecule adducts formation and effects of AFB_1 on carnitine status were studied in male Sprague-Dawley rats. Supplementary carnitine reduced AFB_1 covalent binding to hepatic macromolecules, namely RNA, DNA and protein. Furthermore, carnitine prevented the reduction of RNA, DNA and protein concentrations caused by AFB_1. Carnitine supplement decreased the total lipid and triglyceride concentrations in the liver caused by AFB_1. In the plasma, supplementary carnitine restored AFB_1-induced decline in total lipid and triglyceride concentrations. AFB1 treatment of rats 6 h prior to sacrifice was observed to cause significant increase in the liver NEC, ASAC, and total carnitine. AFB_1 was found to conjugate with AIAC fraction, but not to NEC or the ASAC fractions. The conjugation, however, was not significantly affected by supplemented carnitine. The concentrations of AFB_1 determined in the liver, kidney and plasma were also not statistically different in the carnitine supplemented and nonsupplemented rats. Urinary excretion of AFB_1 was not affected by carnitine supplement. In conclusion, supplementary carnitine offered protection against AFB_1-induced fatty liver and AFB_1-macromolecules adduct formation. Further studies may reveal anticarcinogenic potential of carnitine.

6.2 RECOMMENDATIONS

The following are some of the recommendations or areas for future studies:

1. The design of the study should include one group of animals that will be fed with carnitine deficient diet. Such a study would be expected to maximize effects of carnitine in cases of aflatoxin toxicity.

2. Determine the effects of AFB_1 on other major lipid fractions such as cholesterol, phospholipids and free fatty acids which may reveal specific lipid fraction(s) affected by carnitine-AFB_1 treatment.

3. Measure the influence of AFB_1 on the urinary excretion of carnitine and determine if the AFB_1 in the urine is conjugated with carnitine.

4. Determine the effects of carnitine on AFB_1 metabolism and MFO enzymes activities. These findings may provide the mechanism on how carnitine inhibits the AFB_1-macromolecule adducts formation.

5. The number of animals should be increased to get more consistent data.

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LITERATURE CITED

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APPENDIX

A-3.1 Dual Radioactivity Count.

DUAL LABEL DPM (LS 1801/3801)

Setting Up A Dual Label DPM Program

While the calculations for Dual Label DPM are much more difficult than for single label, there is very little difference for the operator in the setup of the program. A review of the single label DPM section is recommended before continuing. Figure 10-6 shows the series of questions as they occur on the LS-3801 CRT. LS 1801 questions are identical but appear on the printer. The setup for the Dual Label DPM programs is the same as the single label DPM except that four questions require an answer for isotope 2 as well as isotope 1 in the dual label programs. These questions are: Unknown Normalization Factor, Unknown Units, Half Life, and Standard DPM.

Setting Up The User File

The quench curve you store will determine the accuracy of all the DPM answers you get from this program. It is therefore recomended that you set up rigerous counting conditions for the standards. These can be relaxed for the unknowns as you see fit. Count the samples long enough to reach a 0.5% to 1% 2sigma statistical limit (160,000 to 40,000 total counts). Turn AQC on and take multiple H#'s (4 to 6). Channel 1 must be used for isotope 1, the lower energy isotope and Channel 2 must be used for isotope 2, the higher energy isotope. RCM, if available, should be used.

The channel settings are determined by the two isotopes being counted. Usually, Channel 1 (the lower energy isotope) is set to cover the entire isotope range (0-400 for ^{3}H, for example). Channel 2 (the higher energy isotope) is set so the lower window is the same as the upper window for the lower energy isotope. The Channel 2 upper window is set for the isotope 2 end point (400-670 for ^{14}C in a $^{3}H/^{14}C$ dual label). Spectrum Search can be used to determine window settings for unknown isotopes.

Counting The Standards and Unknowns

For Dual Label DPM programs you must have two sets of standards, one for each isotope to be counted. Place the lower energy isotope standard set in a rack first. Leave a space and put in the higher energy isotope standard set. Isotope 1 is always the lower energy isotope and must be counted first. Isotope 2 is always the higher energy isotope and must be counted second. After placing the Isotope 2 standards in a rack, leave a space and follow that with the unknowns (Figure 10-7). If no unknowns are to be counted immediately, put in a rack with a Halt card. Refer to 10.2 for preparing quench curves.

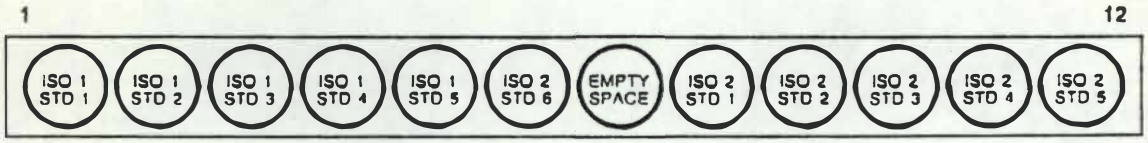
Explanation Of Printouts

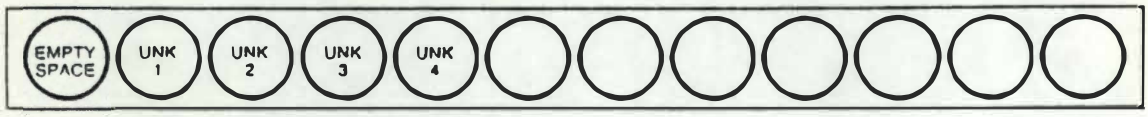
The printout for Dual Label DPM reflects the increased complexity over Single Label DPM. However, the meanings of the printout are the same (Figure 10-8). First, the program summary is printed out. This is followed by the data for Isotope 1 and Isotope 2 standards. Notice here that two efficiencies are calculated for each isotope. One efficiency is the isotope in its own counting channel (isotope 1 counted in channel 1 or isotope 2 in channel 2), and another efficiency is for the spillover of one isotope into the other isotope's counting channel (isotope 1 counted in channel 2 or isotope 2 counted in channel 1). (See Section 1.7 and Chapter 5). This correction for four efficiencies leads to four EFFICIENCY CURVE CORRELATION TABLES and four sets of coefficients. However, this complexity is handled by the instrument so there is little difference to the operator except for the printout.

The printout for the unknowns follows the quench parameters. This printout includes the DPM for the two isotopes and four counting efficiencies for each sample (isotope 1 in channel 1, isotope 1 in channel 2, isotope 2 in channel 1, and isotope 2 in channel 2). The ratio of isotope DPM's is also presented (Isotope 1/Isotope 2 and Isotope 2/Isotope 1) since this is often of interest in dual label experiments.

Source: Beckman LS 1801, 3801and 5801 Series Liquid Scintillation Systems Operating Manual, Beckman Instruments, Irving, CA.

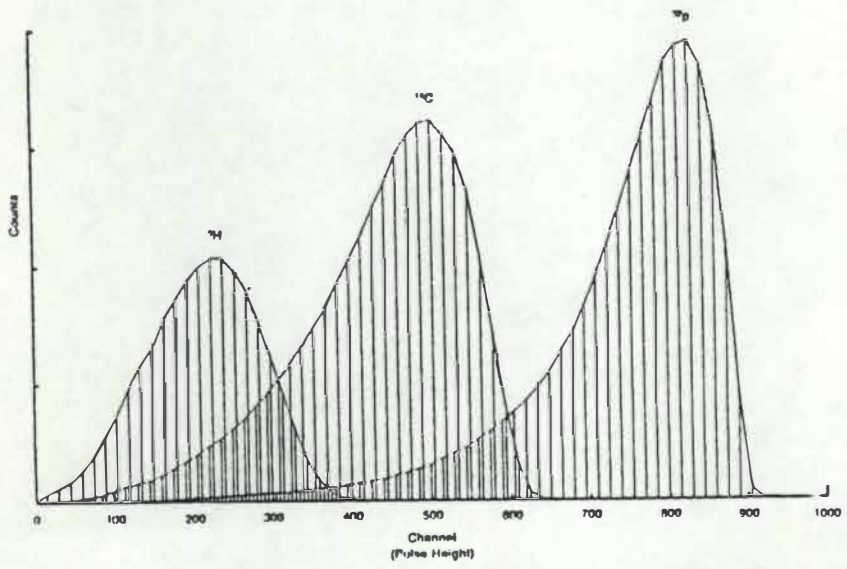
A-3.1 Dual Radioactivity Count (Continued).





ISO 1 & 2 STD = Standards for Quench Curve for Isotopes 1 and 2
EMPTY = No Vial in this Position
UNK = Unknowns

Loading Sequence for DL DPM



Pulse Height Spectra of Three Radioisotopes

A-3.1 Dual Radioactivity Count (Continued).

PAGE: 1

USER: 3 ID:1801 DL DPM PRESET TIME: 5.00 MON 05 NOV 1984 09:44
SAMPLE REPEAT: 1 CYCLE REPEAT: 1 SCR:N RS232:N
H#: 1 AQC:Y QCF:N RCM:N 2 PHASE MONITOR:N
CHANNEL 1-LL: 0 UL: 400 2SIGMA: 1.00 BKG SUB: 0.00 BKG 2SIG: 0.00 LSR: 0
CHANNEL 2-LL:400 UL: 670 2SIGMA: 1.00 BKG SUB: 0.00 BKG 2SIG: 0.00 LSR: 0

DUAL LABEL DPM
UNKNOWN ID:DL DPM TEST UNKNOWN REPLICATES: 1
UNKNOWN NORM FACTOR ISO1:@ 1.00000 ISO2:@ 1.00000
UNKNOWN UNITS ISO1:DPM ISO2:DPM
CALCULATE COEFF:Y HALF LIFE(DAYS) ISO1:N ISO2:N
STANDARD ID:BECKMAN 3H+14C SETS STANDARD DPM ISO1: 357000.0 ISO2: 196500.0

SAM	POS	CH	CPM	2SIG%	TIME	EL TIME	AVG H#	ERR
S1	S1- 1	1	187426.98	0.21	5.00	5.69	34.0	
		2	4069.40	1.40				
					ISO1%EFF CH1: 52.50		CH2: 1.14	
S2	S1- 2	1	144075.78	0.24	5.00	11.50	82.0	
		2	3865.80	1.44				
					ISO1%EFF CH1: 40.36		CH2: 1.08	
S3	S1- 3	1	103466.99	0.28	5.00	17.23	129.0	
		2	3346.00	1.50				
					ISO1%EFF CH1: 28.98		CH2: 0.99	
S4	S1- 4	1	66045.39	0.35	5.00	22.92	180.0	
		2	2659.80	1.48				
					ISO1%EFF CH1: 18.50		CH2: 1.03	
S5	S1- 5	1	44684.00	0.42	5.00	28.59	215.0	
		2	2704.60	1.47				
					ISO1%EFF CH1: 12.52		CH2: 1.04	
S6	S1- 6	1	24296.40	0.57	5.00	34.22	260.0	
		2	1685.60	1.47				
					ISO1%EFF CH1: 6.81		CH2: 1.03	

ISOTOPE 1 CHANNEL 1
QUENCH CURVE COEFFICIENTS
A: 4.123546 B:-0.0045545 C:-0.0000049 D:-0.0000000392

EFFICIENCY CURVE CORRELATION TABLE

STD	H#	MEASURED EFF.	CALCULATED EFF.	PERCENT DIFF.	FLAG
1	34.0	52.50	52.53	0.06	
2	82.0	40.36	40.26	-0.24	
3	129.0	28.98	29.08	0.33	
4	180.0	18.50	18.46	-0.20	
5	215.0	12.52	12.52	0.03	
6	260.0	6.81	6.81	0.01	

ISOTOPE 1 CHANNEL 2
QUENCH CURVE COEFFICIENTS
A: 0.2363036 B:-0.0034566 C:0.00001740 D:-0.0000000272

PAGE: 2

EFFICIENCY CURVE CORRELATION TABLE

STD	H#	MEASURED EFF.	CALCULATED EFF.	PERCENT DIFF.	FLAG
1	34.0	1.14	1.15	0.69	
2	82.0	1.08	1.06	-2.44	
3	129.0	0.99	1.02	2.88	

Printout LS 1801/3801 Dual Label DPM

A-3.1 Dual Radioactivity Count (Continued).

```
  4        180.0          1.03          1.02          -0.54
  5        215.0          1.04          1.03          -0.95
  6        260.0          1.03          1.04           0.45

SAM  POS  CH      CPM     2SIG%  TIME   EL TIME AVG HN                    ERR

S1  S1- 8  1   36419.09   1.00   1.10     36.17  26.0
           2  150981.81   0.49
                                ISO2%EFF CH1: 18.53   CH2: 76.84
S2  S1- 9  1   38194.28   1.00   1.05     37.79 108.0
           2  142339.03   0.52
                                ISO2%EFF CH1: 19.44   CH2: 72.44
S3  S1-10  1   33580.83   1.00   1.20     39.54 192.0
           2  130933.33   0.50
                                ISO2%EFF CH1: 17.09   CH2: 66.63
S4  S1-11  1   25680.62   0.99   1.60     41.72 254.0
           2  118416.88   0.46
                                ISO2%EFF CH1: 13.07   CH2: 60.26
S5  S1-12  1   22492.22   0.99   1.80     44.08 276.0
           2  111004.44   0.45
                                ISO2%EFF CH1: 11.45   CH2: 56.49

ISOTOPE 2 CHANNEL 1
QUENCH CURVE COEFFICIENTS
A: 2.881334      B:0.00161052      C:-0.0000048      D:-0.0000000249

                EFFICIENCY CURVE CORRELATION TABLE

                          MEASURED      CALCULATED      PERCENT
 STD        HN             EFF.           EFF.          DIFF.        FLAG
 ---      -----         --------       ----------      -------       ----
  1        26.0           18.53          18.53          -0.01
  2       108.0           19.44          19.45           0.06
  3       192.0           17.09          17.07          -0.14
  4       254.0           13.07          13.10           0.21
  5       276.0           11.45          11.43          -0.13

ISOTOPE 2 CHANNEL 2
QUENCH CURVE COEFFICIENTS
A:  4.360804      B:-0.0011562      C:0.00000535      D:-0.0000000200

                                                                PAGE: 3
                EFFICIENCY CURVE CORRELATION TABLE

                          MEASURED      CALCULATED      PERCENT
 STD        HN             EFF.           EFF.          DIFF.        FLAG
 ---      -----         --------       ----------      -------       ----
  1        26.0           76.84          76.86           0.03
  2       108.0           72.44          72.33          -0.15
  3       192.0           66.63          66.87           0.35
  4       254.0           60.26          59.92          -0.56
  5       276.0           56.49          56.68           0.33

QUENCH LIMITS    LOW: 34.00     HIGH: 260.0

SAM  POS  CH      CPM     2SIG%  TIME   EL TIME AVG HN                    ERR

 1 11- 2  1   65732.20   0.98   0.65     45.57 247.0
           2  258287.67   0.49
      ISO1%EFF CH1:  8.23 CH2:  1.04 RATIO 1/2:0.1768 ISO1 DPM  :74795.30
      ISO2%EFF CH1: 13.61 CH2: 60.87 RATIO 2/1:5.6564 ISO2 DPM  :423068.5
 2 11- 3  1  101125.00   0.99   0.40     46.51  89.0
           2  310922.50   0.57
      ISO1%EFF CH1: 38.54 CH2:  1.05 RATIO 1/2:0.1141 ISO1 DPM  :48350.08
      ISO2%EFF CH1: 19.47 CH2: 73.28 RATIO 2/1:8.7617 ISO2 DPM  :423630.4
 3 11- 4  1  102155.00   0.99   0.40     47.45  93.0
           2  308762.50   0.57
      ISO1%EFF CH1: 37.56 CH2:  1.04 RATIO 1/2:0.1262 ISO1 DPM  :52221.07
      ISO2%EFF CH1: 19.48 CH2: 73.08 RATIO 2/1:7.9246 ISO2 DPM  :421756.2
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Printout LS 1801/3801 Dual Label DPM (Continued)

A-3.2 Precautionary and Decontamination Procedures During Handling of Aflatoxins.

Aflatoxins have been shown to be extremely toxic and carcinogenic to number of laboratory and domestic animals. Aflatoxin B_1 has been epidemiologically linked to human liver cancer. Therefore, stringent safety measures should be exercised to prevent contact or exposure with these toxins during handling. The following are the safety procedures and recommendations as described by Stoloff et al. (1965), Fischbach et al. (1965), and Castegnaro et al. (1982).

HANDLING OF DRY TOXINS

1. If possible, employ aflatoxins in solution since dry aflatoxins are electrostatic.
2. Always handle dry toxins under hoods that are properly ventilated and filtered.
3. Face masks and disposable rubber gloves should be worn.

DECONTAMINATION

a) Spillage

1. Cover area completly with 5-6% sodium hypochlorite (full strength chlorox, an household bleach) dispensed from a wash-bottle.
2. If the surface cannot be wetted completely, cover the spillage with paper towels, and wet the towels entirely with the bleach.
3. The amount of bleach used should be more than one-tenth of the volume of the spillage.
4. Contact time must be more than 30 seconds.
5. Wipe the surface off with paper towels.
6. Check area with UV lamp to ensure thorough decontamination is attained.

A-3.2 Precautionary and Decontamination Procedures During Handling of Aflatoxins (Continued)

b) Dust and powdered aflatoxins

1. For inaccessible areas and before regular cleaning, vacuum the dust and treat the contents of the vacuum bag with 5% NaOCl before disposing the bag.
2. Same treatment as for spillage if possible.
3. If not, wipe the surface with paper towels soaked in 5% NaOCl.

c) Hoods

1. Wipe the hoods regularly with 5% NaOCl.
2. At the end of the working day, decontaminate the hoods with chlorine gas produced by adding equal volume of 5% NaOCl and 6N HCl.
3. Let the gas disperse for few minutes while the hood's door is shut. Exhaust overnight.

d) Laboratory wares

1. Use 0.5% NaOCl for washing glass and other laboratory wares.
2. Ensure the entire surface of the wares is wetted and soaked for at least 30 seconds.
3. Soaking the wares in detergent + water for 8 hr could be used to substitute for the bleach.
4. Rinse with acetone before drying.
5. Before disposing the solutions used for soaking, add full strength NaOCl (about one-tenth the volume of the soaking solutions).

Personnel

1. Always use rubber gloves and protective masks when handling aflatoxins. Never reused the gloves or masks.
2. Wear lab coat to prevent direct contact of possible spillage and dry toxins with clothes.

A-3.2 Precautionary and Decontamination Procedures During Handling of Aflatoxins (Continued).

3. Rubber gloves provide better protection then vinyl gloves for handling of aflatoxins in dimethyl sulfoxide solution.

4. Aflatoxin in chloroform can penetrate both rubber and vinyl gloves, therefore; immediately change the gloves if the solution spilled onto the gloves.

5. If practical, wear thick or double pair of thin rubber gloves.

6. When skin is contaminated, wash it with 5% NaOCl before washing with soap and water.

7. Sodium perborate with soap can be used if the skin is sensitive to the bleach.

8. If the mouth is contaminated, gaggle a mixed solution of 1% sodium perborate and 1% sodium bicarbonate in water.

9. Contaminated garments should be soaked in 1% NaOCl (for cellulose fabrics) or 5% sodium carbonate solution (for cotton or rayon fabrics) for 1 hr before laundering.

Disposal of Contaminated Materials and Samples

1. Keep all samples and used gloves and masks in tightly closed containers, for examples cans, jars, or plastic bags.

2. Burn the containers.

3. If burning is not possible, soaked the toxic materials in 5% NaOCl for 1/2 hr minimum.

4. Mixing might be necessary to get thorough soaking.

REFERENCES

1. Stoloff, L. & Trager, W. (1965) Recommended decontamination procedures for aflatoxin. J. Assoc. Off. Anal. Chem. 48:681-682.

2. Fischbach, H. & Campbell, A.D. (1965) Note on detoxification of the aflatoxins. J. Assoc. Off. Anal. Chem. 48:28.

3. Castegnaro, M., Van Egmond, H.P., Paulsch, W.E. & Michelon, J. (1982) Limitations in protection afforded by gloves in laboratory handling of aflatoxins. J. Assoc. Off. Anal. Chem. 65:1520-1523.

Table A-4.1 Percent Change in Total Lipid and Triglyceride Content due to Diet and AFB_1 Treatments in Rats of Experiment 1 (6 h Post-AFB_1).

	LIVER		PLASMA	
	Total Lipids (%)	Triglycerides (%)	Total Lipids (%)	Triglycerides (%)
NSC[1] - NSA[2]	7.9	18.7	17.3	30.5
NSC - CSC[3]	2.0	3.8	20.6	37.2
NSC - CSA[4]	1.6	11.1	6.4	6.9
NSA - CSC	9.2	12.5	4.1	9.6
NSA - CSA	8.9	6.4	13.2	34.0
CSC - CSA	0.3	7.0	18.0	48.2

[1]NSC = Nonsupplemented Control

[2]NSA = Nonsupplemented AFB_1;

[3]CSC = Carnitine Supplemented;

[4]CSA = Carnitine Supplement AFB_1.

VITA

Ayub Mohd Yatim was born in Klang, Selangor, Malaysia in June 6, 1959. He received his primary and secondary school education at the Anglo-Chinese Methodist School located in Klang. He entered MARA Institute of Technology, Shah Alam, Selangor on June 1977 and graduated with a Diploma in Food Technology in August 1981. He later worked at Malaysian Agricultural Research and Development Institute at Serdang, Selangor, as an Assistance Research Officer. After working for 4 years, he entered the University of Tennessee, Knoxville, in May 1985 and graduated with a Bachelor of Science degree in Human Ecology, majoring in Nutrition and Food Sciences in March 1988. Since that time, he has been working towards a Master of Science Degree in Nutrition.