FURTHER STUDIES ON THE INFLUENCE OF THIAMINE DEFICIENCY ON DMN-DEMETHYLASE SYSTEM IN THE RATS

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A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE
(TOXICOLOGY)

IN
FACULTY OF GRADUATE STUDIES
MAHIDOL UNIVERSITY
1988

Thesis

entitled

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was submitted to the Faculty of Graduate Studies, Mahidol University

for the degree of Master of Science (Toxicology)

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ACKNOWLEDGEMENTS

I would like to express my sincere appreciation and gratitude to Dr. Mathuros Ruchirawat for her valuable supervision and encouragement throughout the course of this thesis.

Sincere appreciation is expressed to the Supervisory Committee, Dr. Voranant Suphiphat and Archan Auratai Aramphongphan, for their valuable comments and suggestion of this work.

I am thankful to Miss Dusadee Kittikool for her assistance and helpful technical quidance.

Much appreciation is expressed to Miss Prisana Chaichoo, Miss Suwanit Suwannawong, Mr. Satid Phranomsinth for their help in doing experiment, statistically analysis and typing. Without all of them, this thesis could not be accomplished.

Finally, special thanks are also given to my parents for their encouragement and understanding throughout my study.

Chuthaporn Akathos

ชื่อวิทยานิพนธ์ การศึกษาระบบเอ็นไซม์ DMN-demethylase ในตับไตและอิทธิพลของการขาดวิตามินบี 1 ในระบบนี้

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บทคัดย่อ

ระบบเอ็นไซม์ DMN-demethylases ใน microsome ของไตของหนูขาว (Fischer F-344) ประกอบด้วย isozymes อย่างน้อย 2 isozymes เช่นเดียวกับระบบเอ็นไซม์ในตับ Isozymes นี้ สามารถจำแนกเป็น DMN-demethylase I และ DMN-demethylase II ซึ่งจะมีปฏิกิริยาที่มีความเข้มข้นต่ำ (4-50 mM) และที่ความเข้มข้นสูงของ DMN (มากกว่า 50 mM) ตามลำดับ ความเข้มข้นที่เหมาะสมสำหรับ DMN-demethylase I คือ 4 mM และ DMN-demethylase II คือ 200 mM ระบบ DMN-demethylase ในไตนี้มี Activity น้อยมาก เมื่อเทียบกับเอ็นไซม์ในตับ Activity ของ DMN-demethylase I และ DMN-demethylase II ในไตมีเพียง 11 และ 16 เปอร์เซ็นต์ของตับ ตามลำดับ

ในการทดลองที่ใช้ Phenobarbital เป็นตัวกระตุ้นเอ็นไซม์พบว่าความสามารถในการทำปฏิกิริยาของ DMN-demethylase I และ II ไม่เกิดการเปลี่ยนแปลง แต่เมื่อใช้ 3-methylcholanthrene เป็นตัวกระตุ้นเอ็นไซม์ ปรากฏว่า มีผลในการเพิ่มความสามารถในการทำปฏิกิริยาของ DMN-demethylase I สูงขึ้น 37 เปอร์เซ็นต์ ส่วน DMN-demethylase II นั้นเพิ่มขึ้น 30 เปอร์เซ็นต์

ผลของวิตามินบี 1 ที่ทำให้ความสามารถในการทำปฏิกิริยาของ DMN-demethylase I และ II สูงขึ้นอย่างมีนัยสำคัญ และปรากฏการณ์นี้มีความแตกต่างจากระบบ DMN-demethylase ในตับ ซึ่งการขาดวิตามินบี 1 มีผลแค่ในเฉพาะ DMN-demethylase II ในขณะที่ไม่ทำให้เกิดการเปลี่ยนแปลงของความสามารถของ DMN-demethylase I นอกจากนี้ Phenobarbital ไม่ทำให้เกิดการเปลี่ยนแปลงของระบบเอ็นไซม์ DMN-demethylase ในหนูที่ขาดวิตามินบี 1 แต่ 3-methycholanthrene ทำให้เกิดการลดลงของปฏิกิริยาของระบบเอ็นไซม์มี 22เปอร์เซ็นต์ในไต การให้ Thiamine hydrochloride ในภาวะการขาดวิตามินบี 1 สามารถทำให้การเปลี่ยนแปลงของระบบเอ็นไซม์ DMN-demethylase ในไตกลับสู่ภาวะปกติได้

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ABSTRACT

The DMN-demethylase system in rat kidney microsomes consisted of at least two isozymes designated as DMN-demethylase I and DMN-demethylase II which operated at low concentration (4-50 mM) and high concentration of DMN (above 50 mM) respectively. The optimum substrate concentrations determined were 4 mM for DMN-demethylase I and 200 mM for DMN-demethylase II. The activity of DMN-demethylase I and DMN-demethylase II of kidney microsomes were only 11% and 16% of the liver enzymes, respectively. In the kidney enzyme system, phenobarbital pretreatment had no effect on DMN-demethylase I and DMN-demethylase II activities. While 3-MC pretreatment incresed both DMN-demethylase I (37%) and DMN-demethylase II (30%).

Thiamine deficiency enhanced both the kidney DMN-demethylase I and DMN-demethylase II activities. This effect is different from that of the liver system in which thiamine deficiency markedly enhanced the activity of DMN-demethylase II without a significant effect on DMN-demethylase I. Phenobarbital pretreatment in thiamine deficient rats produced no effects on kidney DMN-demethylase activity as also observed in the controls. For 3-MC pretreatment, only DMN-demethylase II was 22% decreased.

Thiamine hydrochloride supplementation could reverse the enhanced effect of thiamine deficiency and this effect was not mediated through the level of TPP in the microsomal enzyme system.

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LIST OF ABBREVIATIONS

B_1 DEF	Thiamine deficiency
B.W.	Body weight
°C	Degree Celcius
DMN	Dimethylnitrosamine
DMN-d	DMN-demethylase enzyme
DNA	Deoxyribonucleic acid
fig	Figure
gm	Gram
HCHO	Formaldehyde
hr	Hour
hrs	Hours
i.p.	Intraperitoneally
kcal	Kilocalories
kg	Kilogram
LD 50	Lethal dose-fifty
M	Molarity
7-MeG	7-Methylguanine
MFO	Mixed function oxidase
mg	Milligram
min	Minute
ml	Millilitre
mM	Millimolar
m-RNA	Messenger ribonucleic acid
NAD	Nicotinamide adenine dinucleotide
NADP	Nicotinamine adenine dinucletide phosphate (Oxidized form)

NADPH	Nicotinamide adenine dinucleotide phosphate (Reduced form)
O^6-MeG	O-6-Methylguanine
RNA	Ribonucleic acid
rpm	Revolution per minute
S.C.	Subcutaneously
S.D.	Standard deviation
S.E.	Standard error
SGPT	Serum glutamic pyruric transaminase
SKF 525A	2-Diethylaminoethyl-2, 2-diphenylvalerate HCL
TPP	Thiamine pyrophosphate
w/v	Weight/volume

CHAPTER I

INTRODUCTION

Dimethylnitrosamine [DMN], one of the most widely occurring carcinogenic compound in the environment, requires metabolic activation for its hepatotoxic and carcinogenic action. The metabolic breakdown of DMN by the mixed function oxidase (MFO) system gives rise to an electrophilic intermediate, a carbonium ion, which can alkylate DNA, RNA and protein. The highest MFO activities are in mammalian liver, but other organs such as kidney can also metabolize DMN but to a less extent and kidney tumors developed under certain conditions.

DMN metabolism can be affected by many factors, including the nutritional status. One of the interesting factors is vitamin B deficiency. In this study, thiamine deficiency is used as a modulator of DMN metabolism primarily in kidney. In earlier works, thiamine deficiency showed an increased in DMN metabolism in liver. It is of interested to investigate the response of the kidney enzyme system under this condition since kidney could play a significant role in the metabolism of DMN during thiamine deficiency.

Objectives :

1. To investigate and characterize the DMN-demethylase system in the kidney and to compare the kidney enzyme system with that of the liver, by means of the enzyme inducers (PB, 3-MC)

2. To study the effect of thiamine deficiency on hepatic and renal DMN metabolism.
3. To investigate the effect of some enzyme inducers and inhibitors on DMN metabolism in normal and thiamine deficient rats.

CHAPTER II

LITERATURE REVIEWS

Part I Occurrence of Dimethylnitrosamine

Dimethylnitrosamine [N-Nitrosomethylamine, DMN] is a potent hepatotoxic and carcinogenic agent (1). It has been strongly implicated as a major environmental carcinogen and also atmospheric pollutant and food contaminant. DMN has been found in nitrite-preserved meat, bacon (2), cigarettes (3) and food stuffs such as fruit, cheese, mushrooms, vegetables, wine, beer and some drugs. It can be formed in food products such as salt-dried fish (4) due to reduction of nitrate in curing salt to nitrite by S. aureus which is responsible for DMN formation. In marine fish which contain high concentration of trimethylamine oxide, DMN can be formed by the reaction of nitrite with appropriate amines within the gastrointestinal tract (5). The susceptible amine substrates studied include drugs such as aminopyrine (analgesic), disulfiram [antialcoholic], tolazamide [oral hypoglycemic], oxytetracycline [antibiotic], methadone [narcotic], quinacrine (antimalarial) and chlorpromazine (tranquilizers), some pesticides such as dimefox (22%), thiram (9%), chloroxuran (3%). All of the naturally occurring compounds present in food can be converted to DMN (6,7,8,9). DMN can be synthesized by the reaction of dimethylamine and nitrous acid to yield yellow oily liquid which is readily miscible with water. It is stable in alkali but decomposes in acid solution.

Absorption and uptake of DMN

DMN is absorbed relatively slowly from the stomach with a half life of more than 1 hour (10,11). Uptake of this carcinogen is very rapid from the intestine and particularly in the duodenum, where the half-life is about three minutes. DMN is distributed uniformly throughout the body water within a short period of exposure and pass readily through cell membrane, therefore it is found in about the same concentration in most of the body tissues (12).

Metabolism of DMN

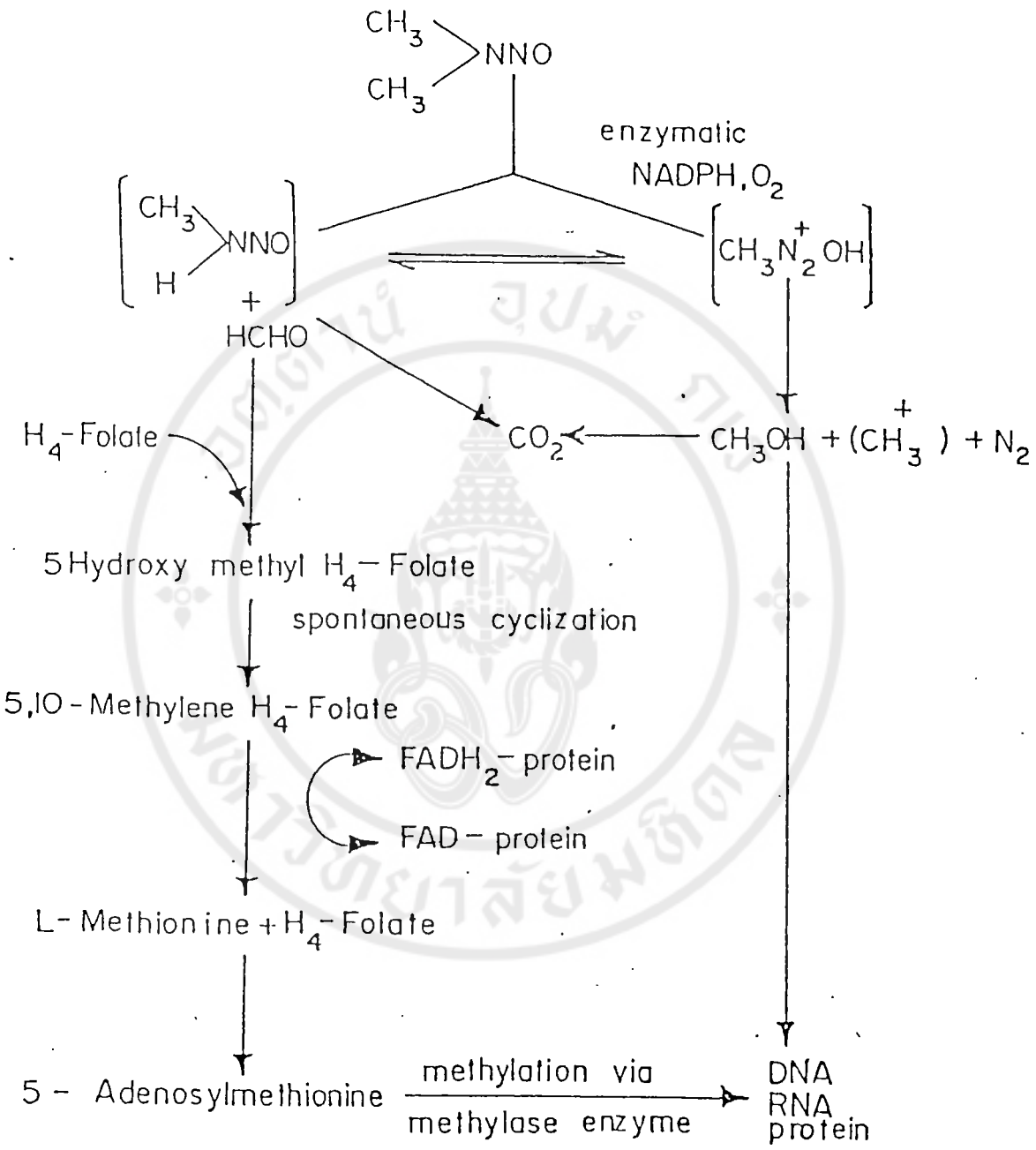
It is generally recognized that dimethylnitrosamine [DMN], like most chemical carcinogens, must be converted by metabolic processes to chemically reactive species to exert its hepatotoxic and carcinogenic effects in a number of mammalian species including rat (13). Metabolism of DMN takes place mainly in the liver, with lesser activity in the kidney, lung and other organ (13,14,15). The enzyme system responsible for metabolizing DMN is active at birth, though the activity is quite low in one-day old rat liver but it reaches the highest level between the 5^{th} and 21^{st} day of age and then decreases (16). This result agreed with the studies of Davies et al (17), about the age-dependence of hepatic DMN demethylase activity in rat that reached a maximum value on the 29^{th} day and decreased rapidly towards the 59^{th} day, thereafter, enzyme activity remained essentially stable up to at least the 110^{th} day.

The metabolism, distribution and excretion of DMN were studied in vivo in rats and mice by Magee and Barnes (18). DMN is rapidly and completely metabolized, therefore, there is very little excretion of unchanged DMN. Only 30% of the dose given was still recoverable at 6 hours and none at all at 24 hours. Dutton and Health (19) also studied disappearance of DMN by using DMN-C^{14} in rat and mouse and found that in mouse 65% of injected C^{14} was recovered in the expired carbondioxide 6 hours after injection of DMN (50 mg/kg body weight). In rat metabolism was slower, about 40% of C^{14} -DMN was recovered in 8 hours.

DMN metabolism proceeded through an oxidative demethylation to give monomethylnitrosamine which was unstable and followed by non-enzymatic cleavage of hydroxylated methyl group giving rise to formaldehyde (13,20). Further oxidation of formaldehyde yielded carbondioxide. Decomposition of monomethylnitrosamine proceeded via the formation of an alkylating agent which could be diazomethane or a carbonium ion (21,22,23). The work of Lijinsky et al (24) seemed to favor the formation of carbonium ion in preference to diazomethane, the proximate agent capable of methylating nucleic acids and proteins. This process may be responsible for the mutagenic, carcinogenic and toxic action of DMN.

The supporting evidence came from the experiment in which rats were treated with fully deuterium-labelled DMN and recovered 7-methylguanine from nucleic acid of liver. The alkylated nucleic acid contained methyl group that retained all three deuterium atom [CD_3] instead of two's [$-CD_2H$], so the alkylated agent should be the carbonium ion [CH_3^+]. Another route for

SCHEME I



The proposed metabolic pathways of DMN
(Ruchirawat, unpublished)

alkylation of DNA besides the carbonium ion pathway was demonstrated by Ruchirawat (25) by means of labelled C^{14}-methionine and non-labelled DMN. The result of 7-methyl-C^{14}-guanine indicated that methionine was also the methyl donor and methylated to base in the nucleic acids.

DNA Alkylation by DMN

From previous studies, DMN biotransformation yields the carbonium ion that can alkylated nucleic acids. DNA alkylation in one or several positions will alter the base pairing properties and may give rise to an incorrect nucleotide insertion during replication. Misreading of DNA template is thought to be the basis of mutagenic and carcinogenic effects. For DMN, the major DNA adducts was 7-methylguanine (7-MeG) which was thought to be associated with carcinogenic response. However, its significance as a critical target in carcinogenesis was reduced due to inability to correlate the presence of 7-MeG in target tissue, its rapid loss from DNA and tumor induction.

In addition to 7-MeG, many studies have demonstrated the methylation of DNA at the sixth position of oxygen atom, O^6-methylguanine [O^6-MeG] that are thought to be more closely correlated with carcinogenesis. Magison and O'Connor (26) proposed the possibilities of mutation by 3-alkyladenine. Lawley (27) also found that the ratio of O^6-MeG to N^7-MeG formation was one tenth in the methylation of DNA by DMN.

It also appeared that tumor production was not only attributed to DNA alkylation, but the capacity to remove and

repair such an unusual base from DNA was also a significant factor. Pegg and Hui (28) showed that methylated adenines were rapidly lost from DNA, presumably by specific enzymic removal. Klaude and Decken (29) demonstrated that deficiency of amino acid methionine and cysteine decreased the repairing enzymes at O^6-MeG, O^6-methyguanine transferase, therefore leading to an increase in the ratio of O^6-MeG to N^7-MeG in liver. The final result is the difference in the methylation pattern of guanine in such condition.

The extent of DNA alkylation was highest in liver followed by lung and kidney (30). According to the study of Skipper et al, (31), the level of alkylation of DNA in the kidney is 10% of that in the liver and the rate of metabolism by the kidneys is only one-fortieth of the rate by the liver. The highest susceptibility for DNA methylation and DNA alkyltransferase activity confined to parenchymal liver cells (32).

The in vitro metabolism of DMN

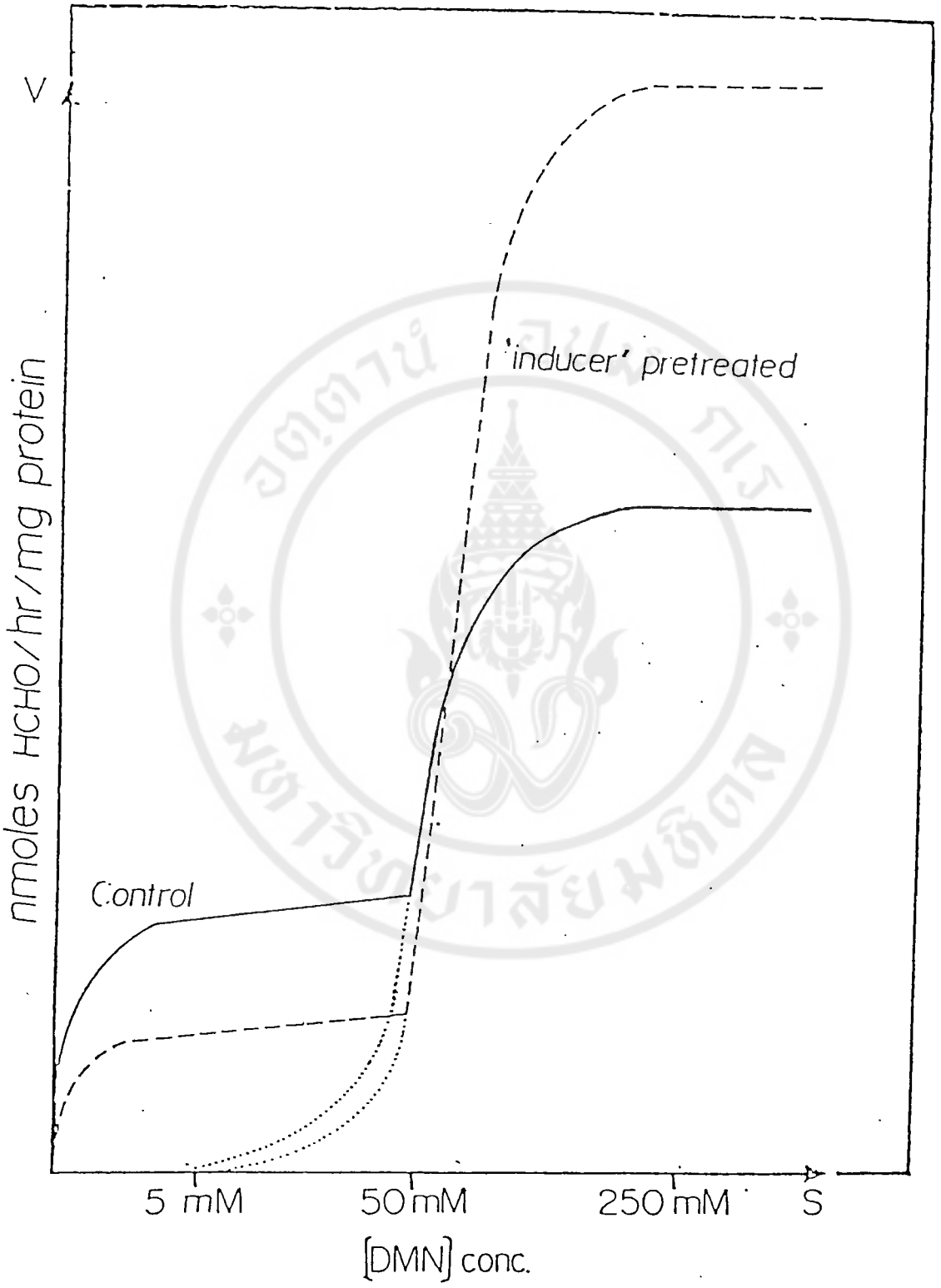
Metabolism of dimethylnitrosamine in vitro was first reported by Magee and Vandekar (12) by measuring the production of carbondioxide or formaldehyde by liver slices or liver homogenated, respectively in the presence of oxygen. However, the measurement of activity of DMN demethylase enzyme in terms of formaldehyde production should reflect the metabolism of DMN more accurately than does the carbondioxide excretion rate. They also found that among the tissue tested; i.e. liver kidney, lung, esophagus, spleen and brain slices: liver was the most active

organ in metabolizing DMN, but the others were inactive with the possible exception of kidney. The conclusion is supported by evidence from hepatectomized rat (33). It was shown that rat liver homogenates was slow and less effective than rabbit liver. The ability to metabolize DMN was found only in the microsome plus cell-sap fraction of the liver. Neither microsome nor cell-sap was appreciably active alone but activity was restored on recombination (12,13). Similar results have been achieved by using isolated microsomes and a system for generating NADPH (34). And activity was removed from tissues suspension by dialysis and could be partially restored by addition of NADP or NAD; the former was more effective.

Arcos et al (35) have reported the existence of two kinetically distinct DMN demethylase and theirs response in opposite way towards the pretreatment with enzyme inducers in vivo.

It is conceivable that DMN demethylase I was defined as the isoenzyme active in the substrate range of 0-5 mM [low Km] while DMN demethylase II was the isoenzyme responsible for DMN metabolism at higher concentration of 50-200 mM [high Km]. These two DMN-demethylases respond differently to enzyme inducers such as pretreatment with phenoparbital or 3-MC.

Analysis of the kinetic suggests that Vmax of DMN demethylase I is reached at the substrate concentration of approximately 4 mM, then shows a plateau but with a slightly upward slope until the concentration of 50 mM where there is rapid rise of Vmax, that is the beginning of the onset of DMN demethylase II. This upward slope represents the initial phase of kinetic response of DMN demethylase II measuring by subtract the



The Michaelis - Menten curves of DMN-demethylase I and II in control and inducer-pretreated rats and mice. (Arcos et al.(1977)).

reaction velocity between 4 mM and 50 mM, Vmax of DMN-demethylase I. Substraction yields the dotted line segment of the curves. Since, the apparent total kinetic response of DMN-demethylase II is that of an allosteric enzyme.

In addition, Arcos et al (35) also suggested that DMN-demethylase II may arise from DMN-demethylase I by a conformational modification, like in apparent induction due to a much greater sensitivity to conformational change of microsome, that lead to a higher activity of this enzyme. Besides this, microsome lost its activity at DMN concentration above 300 mM due to denaturating effect of DMN on protein at very high concentration (36).

Factors affecting DMN Metabolism

Effect of Nutritional status on DMN Metabolism

1. Protein deficiency

Protein depleted rats are more resistant to the acute toxic and lethal effects of DMN (37). In such rats, degree of liver damage was decreased due to the reduction in rate of DMN metabolism in liver (38) and increased renal tumor formation (29). Mclean & Day (37) and Czygan et al, (39) also found the cytochrome P-450 content of liver homogenate reduced to one half compare to control group and disappearence of DMN from plasma in protein-deficient rats was lower than the control. This effect can be reversed by fasting or phenobarbitone pretreatment and can not be affected by benzo-a-pyrene, another enzyme-inducer.

Therefore, a protein-free-diet can decrease hepatotoxicity of DMN and also increase LD 50 nearly 2 folds (40,41).

Additionally, Klaude & Decken (29) reported the effect of methionine-cysteine deficiency that increased methylation of DMN at O^6 position in liver. On the contrary, this condition decreased methylation in kidney of subadult but increased in adult mice.

2. Fasting

Fasting enhances hepatic dimethylnitrosamine demethylase activity and this elevation of enzyme activity can be blocked by Actinomycin D administration (42). This report showed an increase in formaldehyde production and reduction in the amount of plasma DMN when compare to a control (37). From the experiment of Venkatesan et al, (43), it was suggested that the increase enzyme activity is initiated at the transcription process. Besides increase in demethylase activity, Lorr et al, (44) also observed the potentiation of hepatotoxicity by elevated glutamate-pyruvate transaminase (GPT) level.

3. Carbohydrate and amino acids

Venkatesan et al, (42) reported the repression of DMN demethylase activity in glucose fed rats. This result may be due to increase enzyme degradation especially at the level of translation process. Contrary to the effects produced by glucose feeding, DMN demethylase activity was markedly stimulated in high casein diet. Therefore, in starvation when glycogen and protein stores are break down, the observed effect represented the net

effect of interplay between inhibition by carbohydrate and stimulation by amino acid.

4. Vitamins

Thiamine [Vitamin B_1] exerts an effect on DMN metabolism. Ruchirawat et al (45) showed that thiamine deficiency increased metabolism and rate of disappearance of DMN. It also caused the lowering of lethal dose of DMN.

For vitamin B_2, its deficiency selectively stimulated DMN-demthylase system since it enhanced demethylase I while demethylase II was unaffected. Ruchirawat and Saengchan (46) reported that the metabolism of DMN was dependent on the severity of riboflavin deficiency. Mild deficiency increase DMN-demethylase I activity whereas DMN-demethylase II was enhanced by severe deficiency. In contrast to the liver system, riboflavin deficiency mainly enhanced the kidney DMN-demethylase II activity while no significant increase in DMN-demethylase I activity (47).

Fong and Ton, (48) found that ascorbate [Vitamin C] deficiency decreased rate of metabolism and clearance from plasma of DMN. As opposed to low vitamin C intake, DMN metabolism was marginaly enhanced by ascorbate megadose.

From the study of Dashman and Kamm (49), it was found that high dose of vitamin E [55 mg/kg/day i.m. for 3 days] can inhibit hepatic metabolism of DMN to its active intermediate. There was a decrease cytochrome P-450 levels owing to binding of vitamin E to the terminal oxidase, reduction of DMN clearance rate and decreased hepatotoxicity of DMN. This is in accordance with the finding of Skaare and Nafstad, (50) that vitamin E

decreased the acute hepatotoxicity of DMN. The inhibitory effect is reversed in about 3 days following cessation of vitamin E intake.

5. Fat-supplement diet

Agrelo et al (51) have shown that the presence of fat retards rate of absorption and metabolism of DMN. This condition may increase DMN clearance rate by liver and lead to a higher incidence of renal tumors and a lower incidence of liver tumors than rat on normal diet (52). However, Wade et al (53) observed an elevation of cytochrome P-450 in hepatic microsome in high fat diet.

6. Choline

Repeated administration of choline can enhance hepatic necrosis caused by DMN due to an increase in activity of DMN demethylase in female rats. Choline causes an increase activity of microsomal enzyme and increase smooth endoplasmic reticulum in liver (54).

Effect of various chemical on DMN metabolism

The biotransformation of DMN may be modified by previous or concurrent exposure to other agents, such as an enzyme inducers e.g. Phenobarbital and 3-Methylcholanthrene.

Phenobarbital treatment increased liver DMN-demethylation. Moreover, there is an increase in average liver weight and yield of microsomal protein concentration per gm liver (55,56).

According to Mostafa and Weisburger (57), DMN demethylase I was markedly decreased but the activity of DMN demethylase II was increased after phenobarbital pretreatment. Earlier results described inhibition of DMN demethylase activity by PB although it brought about an increase of cytochrome P-450 content: Venkatesan et al, (43) studied the inhibition action by pretreatment of rat with 3-methylcholanthrene [3-MC] and suggested the decrease of activity was due to a reduction in the amount of enzyme in microsome especially DMN demethylase I in the step of lower synthesis of m-RNA specific for this enzyme. In addition, Phillips et al (56) found that 3-MC increased the susceptibility to the acute toxicity of DMN measured by lowering of the LD 50 values than in the control group. As opposed to these results, Frantz and Malling (55) suggested an increase DMN metabolism and hepatic tumors in rat after 3-MC induction.

Several compounds have been found to inhibit the metabolism of DMN, such as acetoaminonitrile, pregnenolone-16- α carbonitrile thereby lowering its toxic effect in animal (41,55). Pretreatment of rats with SKF 525A, and carbonmonoxide, known inhibitors of the mixed function oxidase system, inhibited DMN demethylase activity (55,58,59,60). Additionally, Frantz and Malling, (55) reported the complete inhibition of SKF 525A at 1 mM, and exposure of microsomes to 100% CO for 10 min resulted in a 93.7% decrease in DMN demethylase activity (60). Pyrazole and 3-amino -1,2,4-triazole [3-AT], known inhibitors of alcohol metabolism, profoundly inhibited the metabolism of DMN and led to elevate blood level of DMN. They also reduced clearance rate and increased LD 50 of DMN (59).

Acetone and isopropanol pretreatment enhance the rate of N-demethylation of DMN and produced no significant changes in the levels of microsomal protein or cytochrome P-450 (61). This potentiation of metabolism and hepatotoxicity of DMN were further studied by Lorr et al, (44), who suggested that enhancement of DMN demethylase was due to the induction of specific cytochrome P-450 isozymes. The hepatotoxicity was demonstrated by a significant increase in plasma GPT activity. Additionally, there were marked centrilobular necrosis, hemorrhage and vascular congestion. In addition to being inducers, acetone and isopropanol are also competitive inhibitors of DMN demethylase when they are present during metabolism of DMN (62).

Ruchirawat, et al, (63) studied the protective effect of CCl_4 against DMN hepatotoxicity and found that CCl_4 resulted in a marked decrease in the activities of hepatic microsomal DMN demethylase I and II and a decrease in microsomal protein. The inhibitory action was dose-dependent, the activities of DMN demethylase decreased with increasing doses of CCl_4, and it appeared that CCl_4 had a more pronounced effect on DMN demethylase I. A decreased level of O_6-methylguanine was also observed by Pound & Lowson, (64).

Toxicity of DMN

Toxicity of DMN was originally studied because it produced acute and subacute poisoning in man exposed to it in industries and in laboratories. DMN is metabolized mainly in liver followed by kidney and lung. Induction of liver toxicity is more common

than other tissues. A large dose of DMN [50 mg/kg] produced acute haemorrhagic centrilobular necrosis in several mammalian species (65). It can also produce malignant tumors of the liver in the rat after prolonged adminstration (18). After giving nearly LD 50 (30 mg/kg BW, i.p) of DMN, Mclean et al, (66) observed treated rat in ten days and found occurence of reno-occlusive lesions in the liver. At 6 hours loss of cytoplasmic basophilia in the centrilobular zones occurs and a reduction in the level of liver RNA, not DNA was observed (58). After DMN administration, acute changes have been mainly cytoplasmic with detachment of ribosome and dilation of the cisternae of the endoplasmic reticulum (13). Svoboda et al, (67), also observed changes in the nucleus after 24 hours injection of DMN mainly in nucleolus described as microaggregation with partial separation of the fibrillar and granular components. As the liver lesion developed, there was an increase in accumulation of lipid and marked loss of liver glycogen (68). However, loss of liver glycogen could be very largely prevented by prior treatment with cysteine (69).

Inhibition of hepatic protein synthesis by DMN in vivo was studied and the breakdown of liver ribosomal aggregation was also observed. The extent of breakdown was proportional to the degree of inhibition of protein synthesis (58), which was supported by the reduction in incorporation of labelled amino acid into liver protein about 50% by 3 hours after a necrotizing dose of DMN and damage to microsome structures. This effect was not significant in the kidney. It is also suggested that defect in hepatic protein synthesis due to reduction in amino acid incorporation after DMN intoxication occurred in the stage of assembly on m-RNA

which is not normally methylated and make it unsuitable template (70). This correlated with smaller proportion of polysomes and increase in free single 80-S ribosome. This is analogous to the disaggregation and disorganization of rough endoplasmic reticulum seen in DMN poisoning (71,72). Additionally, Somogyi et al, (41) and Kleinhues et al, (73) studied the protective effect of pregnenolone-16- α-carbonitrile against acute toxic effects of DMN but this compound could not inhibit protein synthesis and polyribosomes disaggregation caused by DMN.

Besides this finding, incorporation of P^{32} into phosphorus-containing fraction of rat liver was studied during the period 4-6 hours after the necrotizing dose of DMN and found that the P^{32} incorporation into partially purified ribonucleic acid was significantly reduced (58).

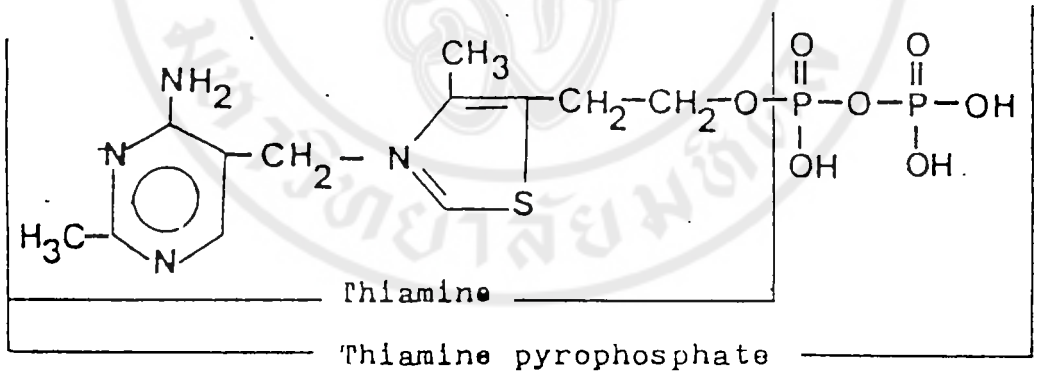
Bailie and Christie, (74) found that aerobic oxidation of pyruvate, octanoate, L-malate, citrate, L-glutamate, α -oxoglutarate and β -hydroxybutyrate progressively decreased after 12 hours following injection of DMN. However, anaerobic glycolysis was unaffected. In addition, Rees and Shotlander (75) reported production of fatty liver in DMN treated rats after 22 hours as a result of accumulation of triglyceride due to inhibition of protein synthesis. Reynolds (76) indicated that protein synthesis was the most general and sensitive indicator of injury. DMN suppresses protein synthesis similar to those of halomethane such as CCl_4 by increase lipid peroxidation through biologically active methylating agent and this destroys the composition and functional of endoplasmic reticulum.

The works of McLean and Day, (37) suggested that rate of DMN metabolism was not related to its toxicity whether its rapidly metabolism induced by enzyme inducers, like phenobarbitone or slowly metabolism repressed by some factors such as low protein diet. They also stated that DMN has no alternative rate, all have to be metabolized either slowly or rapidly. Furthermore, toxicity of DMN involved in repair process of that alkylated cell components.

Part II Thiamine

Chemistry Source and daily intake

Thiamine [Vitamin B_1 or Aneurine], one of the water soluble vitamins, was the first member of vitamin B complex to be identified chemically. It is proved as an antiberiberi factor. Thiamine was first isolated from rice pericarp. It is a complex organic molecule containing a pyrimidine ring linked through a methylene bridge to a thiazole nucleus. Its structure was established by R.R. William (77).



(Goodman and Gilman's: The Pharmacological Basis of Therapeutics 6th ed.,1980)

Thiamine is stable in strong acid solution, but in alkali medium, it is destroyed rapidly by oxidizing and reducing agents.

Thiamine occurs in all living cells and it is also present in rice husk, wheat cereals, eggs, meat, beans, peas, bread and

yeast. There is an evidence indicating that this vitamin may be synthesized by intestinal flora (78).

The requirement of thiamine depends upon the metabolic rate. In man, the minimum requirement is 0.33 mg/1000 kcal. To provide a margin of safety, the Food and Nutrition Board of the National Research Council recommends daily thiamine intake of 0.5 mg per 1000 kcal (79). There is a close relationship between thiamine requirements and total caloric intake. The thiamine content of the human body is about 20-30 mg (78). The minimum need of thiamine requirement for maximum growth of rat and mouse are 2 and 3 mg/kg diet respectively (80).

Thiamine Absorption, Fate and Excretion

Thiamine is absorbed from the small intestine. Under normal conditions, thiamine transport in human and animal is biphasic. At low or physiological thiamine concentrations, transport is a saturable, carrier-mediate, active transport, but at higher concentration, the transport of thiamine is predominantly passive (81). Thiamine is distributed in all tissues. The higher concentration presents in liver, brain, kidney and heart. Substantially smaller amounts are found in spleen, lung, adrenal and muscle. Subcellular distribution of thiamine as detected by radiolabelled techniques reveals 60-70% in the mitochondria with about 20% of its total thiamine in microsomes (82). Intramuscular administration of thiamine results in a rapid and complete absorption. On the contrary, absorption from gastrointestinal tract, small intestine and the duodenum is

limitted.

Thiamine is stored in the body and existed in 3 forms; 80% is in its active form, thiamine pyrophosphate [TPP], or cocarboxylase; 10% is thiamine triphosphate and the rest are in the forms of thiamine monophosphate and free thiamine (78). In the presence of ATP; thiamine undergoes phosphorylation to form thiamine pyrophosphate [TPP] (83,84).

$$\text{Thiamine} + \text{ATP} \xrightleftharpoons[\text{Thiamine pyrophosphokinase}]{Mg^{++}} \text{TPP} + \text{AMP}$$

When thiamine intake is at low level, little or no thiamine is excreted in the urine. But in excess thiamine intake and the tissue storage of thiamine is saturated, it will be excreted in urine in the form of major metabolite, 2-methyl-4-amino-5-pyrimidine carboxylic acid. If the intake of thiamine is further increased, it is excreted in unchanged form.

Impairment of thiamine absorption has been demonstrated in malnourished alcoholic with and without liver disease (85), folate deficiency (86). Somogyi and Wageli, (87) reported interference with thiamine absorption by coffee. It has been demonstrated that tea leaves (88), betel nuts and fermented fish (89) interfere with bioavailability of thiamine, even in adequate dietary intake.

Physiological and Biochemical functions

It is well recognized that thiamine pyrophosphate [TPP], the physiologically active form of thiamine, is a vital cofactor

for the enzymes involve in pyruvate and α-ketoglutarate dehydrogenase systems, the key enzyme of the tricarboxylic acid cycle. Therefore thiamine plays an important function in the metabolism of carbohydrate. One of these important reactions is the conversion of pyruvate to acetyl coenzyme A. It has also been established about the relationship of thiamine requirement and metabolic rate. If energy source is from carbohydrate, requirement of thiamine is greatest. In case of thiamine deficiency, pyruvate can not be metabolized, so it accumulates and blocks ATP production. In addition, thiamine pyrophosphate is also an essential cofactor for transketolation reaction, such as transketolase in the pentose phosphate pathway; the keto group is transferred from xylulose-5-phosphate to ribose-5-phosphate to produce glyceraldehyde-3-phosphate and sedoheptulose-7-phosphate (90).

Decrease in the transketolase activity due to thiamine deficiency has an indirect effect on the synthesis of RNA (92) and fatty acid (84).

Besides its function as a coenzyme, thiamine also plays a direct role in the excitability of neurons (93,94). Itokawa et al (94) studied the specific function of thiamine in membrane transport in nerves. It was demonstrated that in addition to electrical stimulation, neuroactive drugs such as acetylcholine and tetrodotoxin can release the thiamine from nerve preparation. It was also showed that thiamine played a role in the initiation phase of the action potential by increasing the permeability of the excitable membrane to sodium due to the mechanism of thiamine phosphorylation. Therefore, interference

with this system could lead to abnormal function and tissue damage. Moreover, Volpe and Marasa, (95) reported rate of fatty acid synthesis in thiamine deficiency glia was reduced to approximately 15% and about 60% reduction in cholesterol biosynthesis. This disturbed the cellular integrity and cell growth.

Thiamine deficiency

Sign & symtom of deficiency

The symtoms and signs of mild thiamine deficiency are remarkably nonspecific. But in severe thiamine deficiency, it leads to the condition known as beriberi, the first deficiency disease to be recognized. Classically, there are two major types-dry beriberi in which the features of peripheral neuropathy are predominant and wet beriberi where the signs and symtoms of high output cardiac failure dominate. In reality clinical beriberi is usually a mixed deficiency syndrome. The major symptoms are related to the nervous system and cardiovascular system. In man, thiamine deficiency has produced anorexia, irritability and weight loss. Electrocardio graphic alteration has been produced (96). In the early state of cardiac beriberi, tachycardia, wide pulse pressure, sweating and warm skin are usual finding. In the acute form there is severe hypotension and lactic acidosis (97). Late in the course, heart failure appears, cutaneous vasoconstriction occurs to maintain systemic blood pressure and the extremities become cold and cyanotic (98). A

recent review paper reported an abnormality of animal behavior and the impairment in the metabolism of neurotransmitter including acetylcholine, serotonin and amino acids in thiamine deficiency (99).

The central nervous system is also affected by thiamine deficiency described as a Wernicke Korsakoff syndrome. Its characterization is ataxia, ophthalmoplegia and global confusion. Ocular abnormality are usually corrected within minutes after I.V. thiamine and the response is much lower in cirrhosis and is greatly delayed in protein or nucleic acid deficiency (100). Mental changes occur both acutely and chronically. Besides this, disorientation especially to time and place, postural hypotension and impaired vestribular functions are common (101,102). A characteristic defect is an amnestic syndrome with profound defects in new learning and memory for recent events. All of these symptoms occur specifically with a deficiency of thiamine, mainly but not exclusively in alcoholics and may occur singly or in combinaticns. Now it is clear that features of Wernicke's Korsakoff descriptions are extremely prevalent in alcoholics or in thiamine deficiency without alcoholism.

The involvement of peripheral nervous system due to thiamine deficiency is termed "poly neuritis" that involves both sensory and motor nerves (103). Weakness in the extremities and paresthesia of the legs are common finding and more severely than the arms (104,105). Tenderness of the muscles on pressure is common and the deep tendon weakness. According to Victor et al, (106) finding, thiamine deficiency may stimulate senile dementia. And the clinical lesions due to thiamine deficiency are more

complicated if other deficiency states is involved.

Thiamine deficiency may result from :

1. inadequate intake of thiamine

2. malabsorption of thiamine associated with abnormal condition such as in liver disease, alcoholism (107) or resection of small intestine. Chronic alcoholism can cause vitamin deficiency including thiamine. The cause of deficiency may be due to (81)

   1. insufficient food intake
   2. impairment of thiamine absorption secondary to other states of nutritional deficiency
   3. reduction in hepatic storage such as in patients with fat metamorphosis.

3. Consumption of food containing thiaminase enzyme or antithiamine factor [ATF]

Antithiamine factors are classified into two classes

1. natural occuring ATF, which is structure-altering compound, is divided into 2 groups
   a. thermolabile as in raw fish and sea food
   b. thermostable in tea, betel nuts (89), caffeic acid, blueberries, bracken fern, etc.

2. synthetic compounds which is structurally similar compounds that can compete with thiamine in TPP reactions

(108,109) such as pyrithiamine, oxythiamine and amprolium.

Effect of thiamine on Drug Metabolism

Thiamine is one of the essential nutrients that has an effect on the rate of drug metabolism by liver. Effect of thiamine on drug metabolizing enzymes has first been studied by Wade et al (110) because of its importants role involving carbohydrate metabolism. They found that in vitro high thiamine intake depressed heptachlor metabolism and also observed the microsomal metabolism of aniline which is increasingly depressed and reaches the maximum peak when thiamine level is 100 ug/day or greater. The studies of Grosse and Wade, (111) indicated the significant reduction in metabolic rate of aniline, zoxazolamine in both sexes, in vitro, in rats fed on high thiamine diet, whereas no significant change in hexobarbital metabolism in either sex. Aminopyrine metabolism was depressed only in male but not in female rats. In vivo, hexobarbital sleeping time was increased only in female whereas zoxazolamine paralysis time was increased in both sexes. High thiamine diet reduced the level of hepatic microsomal cytochrome P-450, cytochrome b_5 and NADPH cytochrome C reductase without reduction in microsomal protein content (111,112). In such condition quantity of enzyme [Vmax] responsible for aniline hydroxylase was decreased but the binding affinity [Km] was unaltered when compared to pair-feeding rats. It may be assumed that either of these component of mixed function oxygenase system are rate limitting of aniline hydroxylase but for hexobarbital oxidase neither Vmax nor Km is

altered. In addition, high thiamine increases activity of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase. However, the depression in aniline hydroxylase, ethylmorphine demethylase activities may not result only from lower level of these cytochrome but other factors such as high sucrose consumption. Wade et al, (113) showed that cytochrome P-450 content was not significantly depressed in both sexes when rats were fed on high level of thiamine in starch-based diet. This suggested that sucrose, not thiamine was responsible for depressing cytochrom P-450 etc. In addition, oxythiamine [thiamine antagonists] appeared to produce further depression of aniline hydroxylation in large doses of thiamine, (112,114).

Thiamine deficiency enhances aniline, aminopyrine, and zoxazolamine metabolism (111), with a decrease in glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase activities. There was an evidence showing that hexobarbital sleeping time was shorter only in female rats, whereas zoxazolamine paralysis time was shorter in both sexes. In low dietary thiamine, microsomal cytochrome b_5, cytochrome P-450 and NADPH cytochrome C reductase level were significantly increased. The alteration in cytochrome P-450 amount was demonstrated by altered ethyl isocyanide difference spectra (112). It appeared that thiamine might induce the synthesis of a new species of cytochrome P-450 designated as cytochrome P_1-450. Thiamine might act in a manner analogous to that produced by the administration of enzyme inducer, 3-methylcholanthrene, (115). This suggestion was by an increase in absorption spectrum of aniline binding from microsome of thiamine deficiency rats. Therefore increase level

of cytochrome P-450 might result in increased metabolism of aniline, zoxazolamine and aminopyrine. Goldhar and Pawar (116) studied drug metabolizing enzyme involving lipid peroxidation and found that NADPH linked and ascorbate induced lipid peroxidation in both sexes was also increased. They also suggested that an increase in microsomal fraction might be due to increased amount of cofactors or decrease repressors or inhibitors. Additionally, they also reported that phenobarbital treatment induced drug metabolizing enzyme in male more than in female. However, to evaluate the result, several factor such as amount diet consumption, age or stress should be considered.

Determination of thiamine status

Thiamine state of tissue has been measured by assay of thiamine-dependent enzyme, pyruvate decarboxylase, α-ketoglutarate decarboxylase and transketolase. The most practical method for determining thiamine status is the assay of transketolase in erythrocyte (117,118). The principle reaction used to assay transketolase is shown in Scheme III.

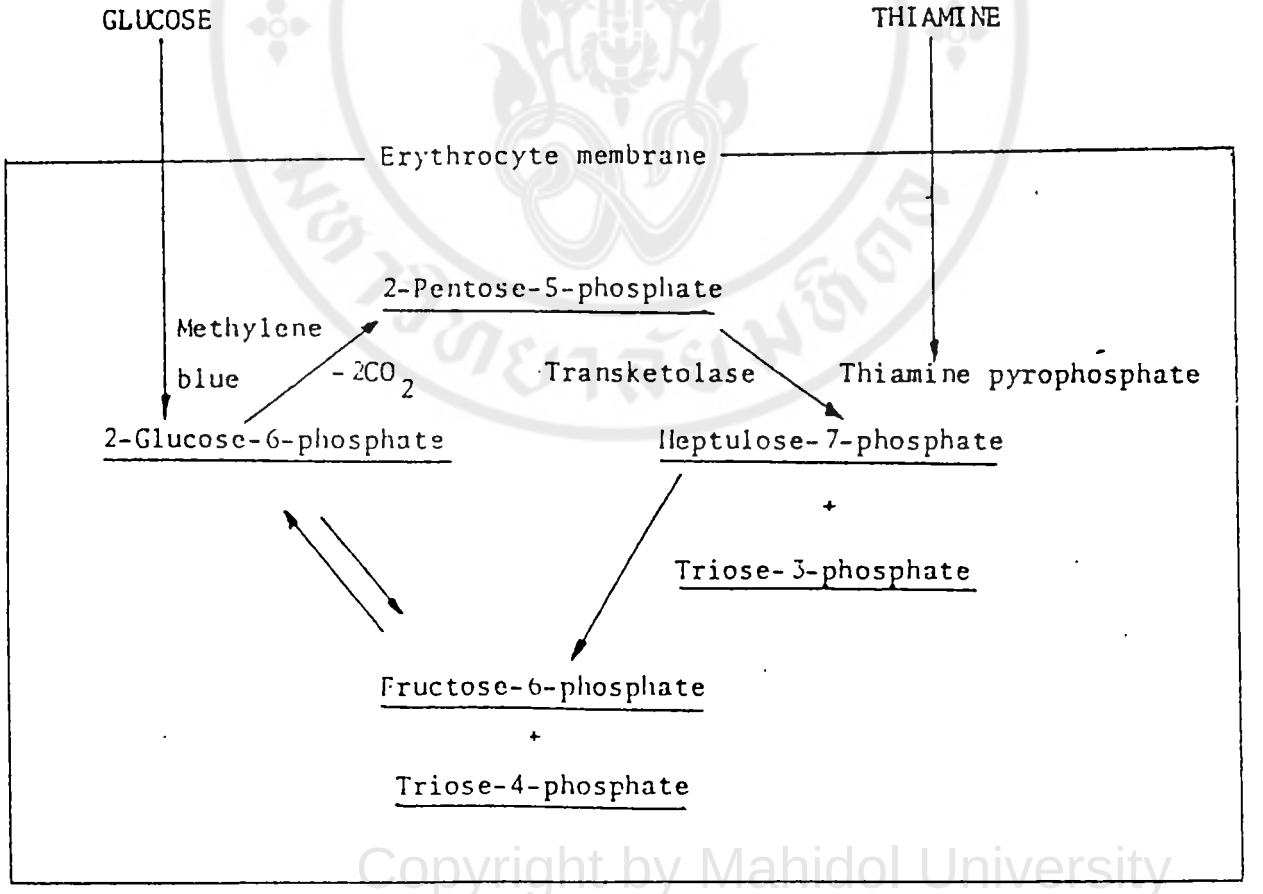
The decrease in activity of this enzyme in red blood cell by thiamine deficiency was due to an insufficient amount of TPP coenzyme to bind with the apotransketolase. The addition of TPP *in vitro* [TPP effect] would restore the transketolase activity toward normal, the results are expressed as percentage stimulation of enzyme activity (107,118,119).

The erythrocyte transketolase activity is considered quite a specific and sensitive measure of vitamin B_1 status (120,121).

The TPP effect correlates well with the degree of deficiency and demonstrates a biochemical defect before other adverse clinical sign are noted in the rats. But in severe active cirrhosis, RBC transketolase activity may show no relationship to blood thiamine in patients (107).

It was also reported that after 2 weeks on thiamine deficiency there was an actual weight gain but RBC transketolase is depressed 49%. At 3 weeks, a slightly weight loss was observed but the enzyme activity was depressed by 82% compared to the control groups. Additionally, Brin et al (122) reported that the TPP stimulation effect from 0-14% was considered to be normal and marginally deficiency from 15-24% TPP effect and if over 25%, it was considered to be severely deficient.

Scheme III A diagramatic representation of the principal reactions involved in the glucose oxidative pathway used in the assayed of transketolase as related to thiamine deficiency. Glucose and thiamine are phosphorylated when utilized as metabolic mediators within the cell (118).



Part III Xenobiotic Metabolism by the Kidney Metabolizing Enzymes and their requirements

Although it is well established that liver is the primary organ in metabolism and biotransformation of xenobiotics, a number of studies have shown that the kidney is metabolically very active in the biotransformation of a variety of chemicals and drugs and, in some cases, surpasses the liver. In those case, kidney plays a significant role in determining the pharmacological activity of a drug, especially when chemical metabolism by liver is low or when the liver is damaged or its metabolism activity is inhibited (123). The metabolism in kidney is also mediated by the mixed-function oxidase system involving cytochrome P-450. The presence of the mixed-function oxidase [MFO] system in kidney microsomes of various animal species has been described by many investigators (124,125). Kidney microsomal cytochrome P-450 was first demonstrated by Kato [1966] who found that it is about 4-7 times less than that in liver microsomes (126). This result is in accordance with those of Muller et al (127), who found a small amount of cytochrome P-450 and cytochrome b_5 in kidney about 10-15 percent of the concentration in liver. Additionally, comparative studies between hepatic and renal tissues have suggested the existence of multiple forms of cytochrome P-450 that differ from organ to organ and species to species (128). Zenser et al (129), studies the mixed-function oxidase activities in renal cortex, outer and inner medulla in rabbit and found the presence of cytochrome P-450 in renal cortex but not in outer and inner medulla. But

the lauric acid hydroxylation in inner medulla can be inhibited by known inhibitors of cytochrome P-450 mediated oxidations, therefore it is possible that lauric acid hydroxylation by the inner medulla is cytochrome P-450 mediated though there was no spectral evidence for cytochrome P-450 in inner medulla. Fowler et al (130), suggested the presence of distinct mixed-function oxidase system within the kidney. This is supported by the observation that NADPH cytochrome C reductase activity in the renal outer medulla is approximately 8-fold greater than the inner medulla from 3-methylcholanthrene treated rabbits. By contrast, Lauric acid hydroxylase activity was 3-fold higher in inner medulla compared to medulla while cytochrome P-450 content, aryl hydrocarbon hydroxylase and aminopyrine demethylase activities were observed in outer medulla but not in inner medulla. In contrast to the liver, mono-oxygenase, cytochrome P-450 in rats kidney cortex appears to have a higher substrate specificity and catalyzes predominantly fatty acid hydroxylation in the ω and ω-1 position of lauric acid, which is the most commonly studies fatty acid substrate because of its high affinity and rapid rate of hydroxylation (126). These reactions require molecular oxygen, NADPH and cytochrome P-450 which is designated as P-450k owing to spectral and catalytic characteristics. The finding of low or absent hydroxylation activity with various drugs or steroid hormone as a substrate proved the highly substrate specific in kidney cortex microsome such as slow rate of oxidation of aniline, acetanilide or nortriptyline and unmeasurable rate of hydroxylation of cyclohexane and testosterone.

The renal enzyme system was similar to that found in the liver in that carbonmonoxide inhibited the reaction and that laurate produced a type I spectral change with oxidized microsomal preparation. In contrast to liver hydroxylation of lauric acid catalyzed by different enzymes (126), it appeared to be involved in a single enzyme in renal fatty acid hydroxylation. Although the renal metabolic rates were low, activity was present in a variety of species, including man. Jakobsson and Cinti (131) reported the specific rates of ω and ω -1 hydroxylation of laurate in man to be approximately one-half of those observed in rats. Besides laurate, aminopyrine was also metabolized at the same rate, suggesting a lower substrate specificity for human kidney cortex P-450 than in rats.

Litterst et al (132), studied the effect of various factors known to affect hepatic drug metabolism that might be involved in drug metabolism in kidney:

The sex difference which were marked in the liver, such as aminopyrine N-demethylase and cytochrome P-450 level, were not observed in the kidney.

Starvation was found to increase both renal cytochrome P-450 levels and biphenyl 4-hydroxylase activity while in the liver, it showed bidirectional changes, depending on the specific parameter study and the duration of starvation.

Carbon tetrachloride produced dramatic changes in the hepatic drug metabolism without influencing in kidney such as reduce the levels of cytochrome P-450 and NADPH cytochrome c reductase activity in liver but less effect in kidney, However, CCl_4 administration significantly reduced biphenyl hydroxylase

activity in liver and kidney. It was also noted by Litterst et al (132), that CCl_4 produced a marked increase in the specific activity of UDP-glucuronyl transferase in liver but to a lesser degree than in kidney.

SKF 525A, a known inhibitor of mono-xygenase activity, inhibited aminopyrine and biphenyl metabolism in both liver and kidney but failed to inhibit ethylmorphine demethylation in the kidney microsomes.

In addition to cytochrome P-450 dependent oxidation, kidney also processed cytochrome P-450 dependent reduction. Sugiura et al (133), found the reduction of tertiary amine N-oxide in kidney microsome but in low activity when compared to liver. Besides, benzo(a)pyrene 4,5-oxide were also reduced in slow rates by renal cytochrome P-450 dependent reductase.

Kidney Enzyme Induction

The ability of the rat kidney cortex monooxygenase to be induced by a variety of chemicals is much more limited than the liver system. Muller et al (127), found no significant induction in rat kidney by phenobarbitone of cytochrome P-450 on any age or the rate of hydroxylation of fatty acid (134). This agrees with the work of Uehleke and Greim (135), who found no induction by phenobarbital in the kidneys of adult rats. Moreover, Feuer et al (136), could not observe the stimulation of enzymes in any extrahepatic tissues. Zenser et al (129), showed that the rabbit renal mixed function oxidase system was more sensitive to induction by 3-methylcholanthrene than the liver. 3-

Methylcholanthrene induced renal cortical and outer medullary cytochrome P-450 content and aryl hydrocarbon hydroxylase activity. In addition, 3-MC increased both renal cortical and outer medullary aminopyrine demethylase activity. But for reductase activity such as microsomal NADPH-cytochrome C reductase, a functional unit of mixed-function oxidase system, was not increased by 3-MC treatment. Lake et al (123), reported that 3-MC treatment in rats did not induce chloromethyl-aniline N-demethylase [CMA-N-demethylase], aniline 4-hydroxylase and biphenyl 4-hydroxylase in kidney. Additionally, 3-MC also failed to enhance the severity of renal necrosis initiated by paracetamol owing to the inability of 3-MC to induce the renal microsomal system for activating paracetamol to toxic metabolites (137). However, 3-MC treatment caused a significant increase in microsomal cytochrome P-450 content in rat kidney cortex (132) similar to the study of Zenser et al (129), in rabbit kidney cortex. 3-MC pretreatment also stimulates benzpyrene hydroxylase activity in rat kidney (123), which correlated with the report of Gelboin and Blackburn (138), who found a 5-15 fold increase in benzpyrene hydroxylase in rat kidney after 3-MC treatment. In addition, Nebert et al (139), also reported the 80-fold induction of aryl hydroxylase activity [AHH] in rat kidney microsomes following 3-MC administration in rats, B_6 mice, hamsters and rabbits.

Pretreatment with benzo(a)pyrene, Ellin and Orrenius (134) caused no enhancement of fatty acid hydroxylation while it significantly increased the kidney benzpyrene hydroxylase activity (138). Additionally, Grundin et al (140), observed an

increase in different species of P-450 in benzyprene-treated rats compared to control group. Swann et al (141), revealed that a higher kidney tumor incidence due to pretreatment with benzpyrene corresponded well with the increase in dialkylation of kidney DNA induced by nitrosamine.

Fowler et al (130), reported the enhancement of renal biphenyl-4-hydroxylase activity in rats induced by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). This may be due to TCDD induce the proliferation of smooth endoplasmic reticulum localized in S_3 proximal tubule cell. This localized induction may explain the small changes in microsomal yields reported in TCDD treated animal and normally low microsomal enzyme activity found in control kidney.

Variety of Xenobiotics Metabolized by Kidney Microsomes

From the report of Orrenius et al (126), it was shown that many good substrates for liver monooxygenases were not metabolized by kidney cortex microsome. However, several xenobiotics were potentially metabolized in the kidney (140). Weekes (142), studied the metabolism of dimethylnitrosamine [DMN] by kidney microsomal enzymes from C_3H/He and Ha/ICR mice and found that these two strains could generate mutagenic metabolites from DMN and produced mutagenic effect like the kenetics exhibited by hepatic microsomal enzymes. The rate of metabolism correlated with the relative susceptibility of these strains to induction of carcinoma by DMN. Furthermore, testosterone administration [2 mg/day, 10 days] increased DMN N-demethylase

activity in the kidney and had also increased the ability to convert DMN to mutagenic metabolites in S-9 enzyme preparation from kidney (143).

Litterst et al (144), demonstrated low activities of aniline 4-hydroxylase and biphenyl 4-hydroxylase and a higher activity of P-chloro-N-methyl-aniline [CMA]-N-demethylase in rat kidney. In addition, the renal toxicity of paracetamol may be due to an active metabolite generated by kidney microsomes (137), arylates kidney macromolecules and the enzyme-mediated covalent binding of radiolabelled material to microsomal protein requires NADPH and molecular oxygen. Cobaltous chloride and piperonyl butoxide can decrease both renal and hepatic necrosis initiated by paracetamol due to inhibition in the synthesis of cytochrome P-450 in liver and kidney.

Human foetal kidney can metabolite certain xenobiotics, including chlorpromazine (145) and benzpyrene (146) by mixed-oxidase system, although the activities are lower than in foetal liver. Furthermore, there was a report of drug metabolizing enzyme, O-demethylation of p-nitroanisole, in neonatal and adult swine kidney (147). By using suspensions of uninduced rat kidney tubule fragments, Fry et al (148) have shown that the substrate 7-ethoxycoumarin was metabolized at a significant rate by kidney cells, even the maximal capacity for O-deethylation of 7-ethoxycoumarin was only 3% of that of liver cells. Additionally, Zilting et al (149), found that i.p. injection of Chinese hamsters with acrylonitrile (30 mg/kg) decreased deethylation activity of 7-ethoxycoumarin.

Recently, Bekersky & Popick (150) reported the metabolism of a potent diuretic, Bumetamide by rat kidney microsome. This drug was metabolized to an inactive form, aminobutyric acid and hydroxybutylamino rapidly in rat. Thus, when microsomal drug metabolism was inhibited, bumetamide produced a diuretic response correlated well with an increased urinary excretion rate of unchanged bumetamide.

Smith & Hook (151), studied the kidney metabolism of chlorofrom [$CHCl_3$] in vitro and indicated that chloroform could be metabolized by renal cortex to a nephrotoxic intermediate possibly by a cytochrome P-450 dependent mechanism similar to that occurring in the liver. The optimum metabolism of chloroform to carbondioxide required more concentration of oxygen and NADPH generating system only, not NADH. This suggested the different forms of cytochrome P-450 between renal and hepatic microsome. In addition, renal microsome showed no consistent pattern of dose-dependence produced by an inhibitor, SKF 525A and metyrapone or an enzyme inducer, phenobarbitone in contrast to liver. Chloroform metabolism significantly occurred in male mouse but little or no metabolism in female renal microsome Smith & Hook, (152,153) also showed relationship between renal toxicity and metabolism of chloroform.

CHAPTER III

MATERIAL & METHODS

1. Materials

1.1 Chemicals and Drugs

All drugs and chemicals were analytical grade and were used without further purification.

DMN was obtained from Aldrich Chemical Co. Inc. Milwaukee, Wis. U.S.A.

All dietary ingredients were obtained from ICN Pharmaceuticals Inc. Life Science Group. Cleveland, Ohio, U.S.A.

1.2 Animals and Diet

Young male Fischer 344 rats, weanling, aged 21 days were used throughout all experiment, they were housed individually, food and water were provided ad libitum. Semipurified agar-gel diet was used throughout the study. The control diet contained all vitamins and minerals required by rats. In thiamine deficient diet, thiamine was omitted from the vitamin mixture. The composition of the diet was given in appendix I. All animals were fed with control diet until 30 days old, they were then divided into two groups and fed with either control or thiamine deficient diet for 3 weeks.

1.3 Animal Treatment

All rats were fasted overnight and were killed by decapitation without anesthesia between 7.00-9.00 a.m. to avoid diurnal variation in drug metabolizing enzyme activity. Livers and kidneys were quickly removed and placed in ice-cold normal saline (0.9% NaCl). All injections were done intraperitoneally with the volume ranged from 0.05-0.25 ml.

1.4 Enzyme inducer pretreatments

Phenobarbital pretreatment

Phenobarbital was administered in 0.9% NaCl at a daily dose of 75 mg/kg body weight. Pretreatment was done by intraperitoneal injection for 4 consecutive days. In control group, animals were given 0.9% NaCl solution with the schedule similar to the treated group.

3-Methylcholanthrene (3-MC) pretreatment

3-MC was administered as a single intraperitoneal injection in corn oil at the dose level of 40 mg/kg body weight, 24 hours prior to the experiment. The control animals received an equal volume of corn oil, i.p., as in the treated group.

Cobaltous Chloride pretreatment

$CoCl_2$ was dissolved in normal saline (0.9% NaCl) and administered to animals at the dose of 60 mg/kg body weight. Pretreatment was done by a single subcutaneous injection, 24 hours prior to the experiment. Control animals received only the vehicle.

1.5 In vivo Thiamine supplement

Thiamine hydrochloride was dissolved in distilled water and was injected i.p. as a single dose of 650 µg/rat/day (24 hours) prior to the experiments.

2. Methods

2.1 Preparation of liver microsomes

2.0 gm of liver was homogenized at 4°C in 3 volume (W/V) of 1.15% KCl in 0.01 M potassium phosphate buffer pH 7.4. Homogenization was performed for 10 strokes. The homogenate was centrifuged at 9,000 g (11,000 rpm) for 25 min. The supernatant was centrifuged at 105,000 g (40,000 rpm) for 60 min. producing the microsomal pellet which was resuspended in 3 volume of ice-cold 0.1 M potassium phosphate buffer pH 7.4.

2.2 Preparation of kidney microsomes

Pooled kidneys (at least 8.0 gm) were homogenized at 4°C in 3 volume (W/V) of 1.15% KCl in 0.01 M potassium phosphate buffer pH 7.4. Homogenization was performed for 15 strokes and centrifuged for 25 min at 9,000 g (11,000 rpm). The supernatant fraction was centrifuged at 105,000 g for 60 min, producing the microsomal pellet which was suspended in 0.25 volume (W/V of the original, tissue) of ice-cold 0.1 M potassium phosphate buffer pH 7.4.

2.3 DMN-demethylase assay

Microsomal conversion of DMN to formaldehyde was assayed in a standard incubation mixture containing :

MgCl	10 µmole in DW 0.025 ml
Niacin	20 µmole in DW 0.1 ml
Semicarbazide	37.5 µmole in DW 0.025 ml
NADP	2 µmole in DW 0.025 ml
G-6-P	20 µmole in DW 0.025 ml
G-6-PD	2 µunits in DW 0.025 ml
phosphate buffer, pH 7.4	0.5 M 1.4 ml
KCl	1.15% W/V 0.325 ml
Microsomal preparation	0.5 ml
DMN (4 or 200 mM)	0.05 ml

DMN was omitted in the blank tube

(DW = Distilled Water)

The total volume of incubation mixture was 2.5 ml. One millilitre of the reaction mixture was taken out as the initial point of reaction immediately after DMN was added and the reaction was stopped by addition of $ZnSO_4$ [15% W/V, 0.5 ml] and $Ba(OH)_2$ [saturated solution, 0.5 ml]. After 10 min equilibration at 37°C, the remaining incubation mixture was further incubated in a Dubnoff metabolic shaker for 30 minutes. Then 1 ml of this mixture was taken out, and the reaction was stopped by $ZnSO_4$ and $Ba(OH)_2$ as previously mentioned. The amount of formaldehyde formed was determined by the methods of Nash (154). After substracting the initial readout values from those of the final

readout, the demethylase specific activity was expressed as nmole of formaldehyde formed per hour per mg microsomal protein.

2.4 Determination of formaldehyde by the methods of Nash (1953)

After the enzymatic reaction was stopped by $ZnSo_4$ and precipitated with $Ba(OH)_2$, the reaction mixtures obtained were centrifuged at 3,000 rpm for 10 min. Then 0.75 ml of supernatant was taken and mixed with 0.3 ml of double strength Nash reagent [7.5 gm ammonium acetate + 0.1 ml acetyl acetone diluted to 25 ml with ice-cold distilled water] and incubated at 60^0C in Dubnoff metabolic shaker for 30 minutes. The reaction mixtures were then cooled and centrifuged 10 min at 3,000 rpm. The amount of formaldehyde formed was estimated by measuring the absorbance at 415 nm.

The calibration curve were prepared by using various concentration of formaldehyde standard solutions and assayed by the same procedure.

2.5 Determination of protein by the method of Bradford (1976) using the Biorad reagent

Protein in liver and kidney microsomes were determined according to the method of Bradford using the Biorad reagent. The Biorad reagent was diluted 1:5 with distilled water. 1 ml of the diluted reagent was mixed with 2 µl of diluted liver or kidney microsomal preparation [1:10]. The absorbance was measured at 600 nm within half an hour.

The calibration curve was prepared by using a series of bovine serum albumin (BSA) standard solutions. The absorbances were plotted against the protein concentration [µg/ml] of BSA standard.

2.6 Analysis of Data

All the results records in the tables and graphs were expressed in mean ± standard error. The significance of difference between two means were determined by the student's t-test or analysis of variance.

CHAPTER IV

EXPERIMENTAL PROTOCOL

Part I Studies of the microsomal DMN-demethylase system in the kidney.

Study I Characterization of DMN-demethylase system of the kidney.

Experiment 1 : Determination of the kinetic properties of DMN-demethylase

Renal microsomes were prepared from kidney pooled from 8 rats. The microsomes, equivalent to approximately 6-10 mg of protein, were incubated in the incubation mixture containing various concentrations of DMN (0.05, 0.5, 1, 2, 4, 25, 50, 100, 200 mM) and NADPH generating system at 37^{0}C for 30 min. Formaldehyde production in the system was determined by the method of Nash by taking an aliquot of 1 ml at time zero and time 30 min. DMN-demethylase activity was calculated from the amount HCHO produced at time 30 minus time 0 and expressed as n mole HCHO formed per mg microsomal protein per hour. Michaelis-Menten constant [Km] and maximum velocity (Vmax) of the enzyme were determined from the Lineweaver-Burke plot of 1/V and 1/S.

Experiment 2 : Effects of the microsomal enzyme inducers: phenobarbital [PB] and 3-methylcholanthrene [3-MC] on DMN-demethylase system in the kidney.

Rats were pretreated with PB [daily injection, 75 mg/kg, i.p. for 4 consecutive days] or 3-MC (a single dose of 40 mg/kg, i.p. 24, hours prior to experiment). Microsomes prepared from kidneys pooled from the control or inducer-pretreated rats were incubated in the reaction mixture containing DMN [4 mM or 200 mM] and NADPH generating system for 30 min at 37°C. DMN-demethylase activity was determined. Duplicated samples were run for each determination.

Study II Comparative studies of hepatic and renal DMN-demethylase system.

Experiment 3 : Comparison of the kinetic properties of DMN-demethylase from liver and kidney

The microsomes were prepared from livers pooled from 3 rats, or kidneys pooled from 8 rats and were incubated in the reaction mixture containing various concentrations of DMN [4, 25, 50, 100, 200 mM], essential cofactors including NADPH generating system at 37°C for 30 min. DMN-demethylase activity was determined by formaldehyde production, measured as described earlier.

Experiment 4 : Effect of phenobarbital [PB] and 3-methyl-cholanthrene [3-MC] on DMN-demethylase system in liver and kidney.

Rats were pretreated with PB and 3-MC as described earlier. After the pretreatment, microsomal preparations from the individual livers or microsomal preparations from pooled kidneys were assayed for DMN-demethylase activity with DMN (4 mM and 200 mM) in standard condition.

Part II Studies on the influence of thiamine deficiency on the hepatic and renal microsomal DMN-demethylase systems.

Study III Effects of thiamine deficiency on the DMN-demethylases.

Experiment 5 : Effects of thiamine deficiency on the kinetic properties of hepatic and renal DMN-demethylases.

Microsomes were prepared from kidneys or livers pooled from 8 or 3 rats, respectively. These rats were fed with the control or the thiamine deficient diets for 3 weeks. DMN-demethylase assay was carried out by incubating the microsomes with various concentrations of DMN (0.05, 0.5, 1, 2, 4, 25, 50, 100, 200 mM) and NADPH generating system at 37°C for 30 minutes. DMN-demethylase activity was determined as described earlier. Duplicate samples were run for each determination.

Kinetic parameters, Km and Vmax, were determined from the double-reciprocal plots of substrate concentration and velocity.

Experiment 6 : Counteracting the effects of thiamine deficiency by *in vivo* supplementation of thiamine hydrochloride

The effect of *in vivo* thiamine supplementation was carried out by giving a single dose of thiamine hydrochloride (650 µg/rat, i.p.) to the thiamine deficient group. These animals were killed 24 hours after thiamine supplementation. Microsome were prepared from kidney pooled from 8 rats/group. Microsomes were incubated with DMN (4 mM and 200 mM) and NADPH generating system at 37°C for 30 minutes. Formaldehyde determination was done by the method of Nash. Duplicate samples were run for each determination.

Experiment 7 : *In vitro* effects of thiamine hydrochloride and thiamine pyrophosphate [TPP] on hepatic DMN-demethylase of the normal and thiamine deficient rats.

To investigate whether the alteration on DMN-demethylase activities was the result of direct action of active form of thiamine, thiamine pyrophosphate [TPP] on the DMN-demethylase enzyme, TPP or thiamine hydrochloride was added into the *in vitro* microsomal assay for DMN-demethylase prior to the addition of DMN. In this experiment, hepatic microsomes were prepared from 3 rats/group, fed with either normal or thiamine deficient diet. Three different concentrations of thiamine pyrophosphate (3.70 mM, 18.51 mM and 36.36 mM) dissolved in 0.5 M phosphate buffer were added in the assay system with NADPH generating system prior to DMN (4mM or 200 mM) administration and incubate at 37°C for 30 min, DMN-demethylase activity was determined in duplicate samples.

Part III Studies on the effects of microsomal enzyme inducers and inhibitors on DMN metabolism in the normal and thiamine deficient rats.

Study IV Effects of the microsomal enzyme inducers on hepatic and renal DMN-demethylase system.

Experiment 8 : Effects of phenobarbital [PB] pretreatment on the hepatic and renal DMN-demethylase systems in the normal and thiamine deficient rats.

Groups of normal and thiamine deficient rats were injected with PB [75 mg/kg B.W., i.p.] for 4 consecutive days prior to the experiment. The animals were decapitated 24 hours after the last dose of PB. Pooled hepatic or renal microsomes (prepared from groups of normal or thiamine deficient rats) were incubated with DMN [4 mM and 200 mM] together with NADPH generating system. Formaldehyde determination was done by the method of Nash. Duplicate samples were run for each determination.

Experiment 9 : Effect of 3-methylcholanthrene [3-MC] on the hepatic and renal DMN-demethylase system in the normal and thiamine deficient rats.

Pretreatment of 3-methylcholanthrene was done by administration of a single dose (40 mg/kg B.W. in corn oil, i.p.) 24 hours prior to the experiment. Microsomes were prepared from

livers and kidneys pooled from pretreated rats fed either normal diet or thiamine deficient diet for 21 days. Microsomes were incubated in the reaction mixture containing DMN (4 mM or 200 mM) in the presence of NADPH generating system at 37°C for 30 min. DMN-demethylases activity was determined in duplicate samples.

Study V Effect of enzyme inhibitor on hepatic DMN-demethylation in normal and thiamine deficient rats.

Experiment 10 : Effect of Cobaltous Chloride [$CoCl_2$] pretreatment on hepatic DMN-demethylase system in normal and thiamine deficient rats.

Animals were pretreated with $CoCl_2$ [60 mg/kg, S.C., 24 hours prior to the experiment]. After the pretreatment, the microsomes obtained from livers from normal or thiamine deficient rats were assayed for DMN-demethylase activity with DMN (4 mM and 200 mM) in standard condition.

CHAPTER V

RESULTS

Part I Studies of the microsomal DMN-demethylase system in the kidney.

Normally liver is the main organ that metabolize foreign substances including DMN. Kidney can also metabolize DMN but to a lesser extent. The liver enzyme system has been well characterized and studied but little is known about the kidney enzyme system. Under certain condition such as that when rate of metabolism is low in liver or when the liver is damaged, kidney will play a significant role in metabolizing DMN, consequently, kidney tumor usually develops. In the present study kidney enzyme system involved in DMN metabolism was studied in details and compare to that in the liver system.

Study I Characterization of DMN-demethylase system of the kidney

Experiment 1 : Determination of the kinetic properties of DMN-demethylase in the kidney

This experiment was designed to investigate the kinetics of DMN-demethylation by the kidney microsome. The results demonstrated the pattern of enzyme activity which increased with increasing substrate concentrations up to 4 mM and gradually reached a plateau between 4 to 50 mM then rapidly increased and

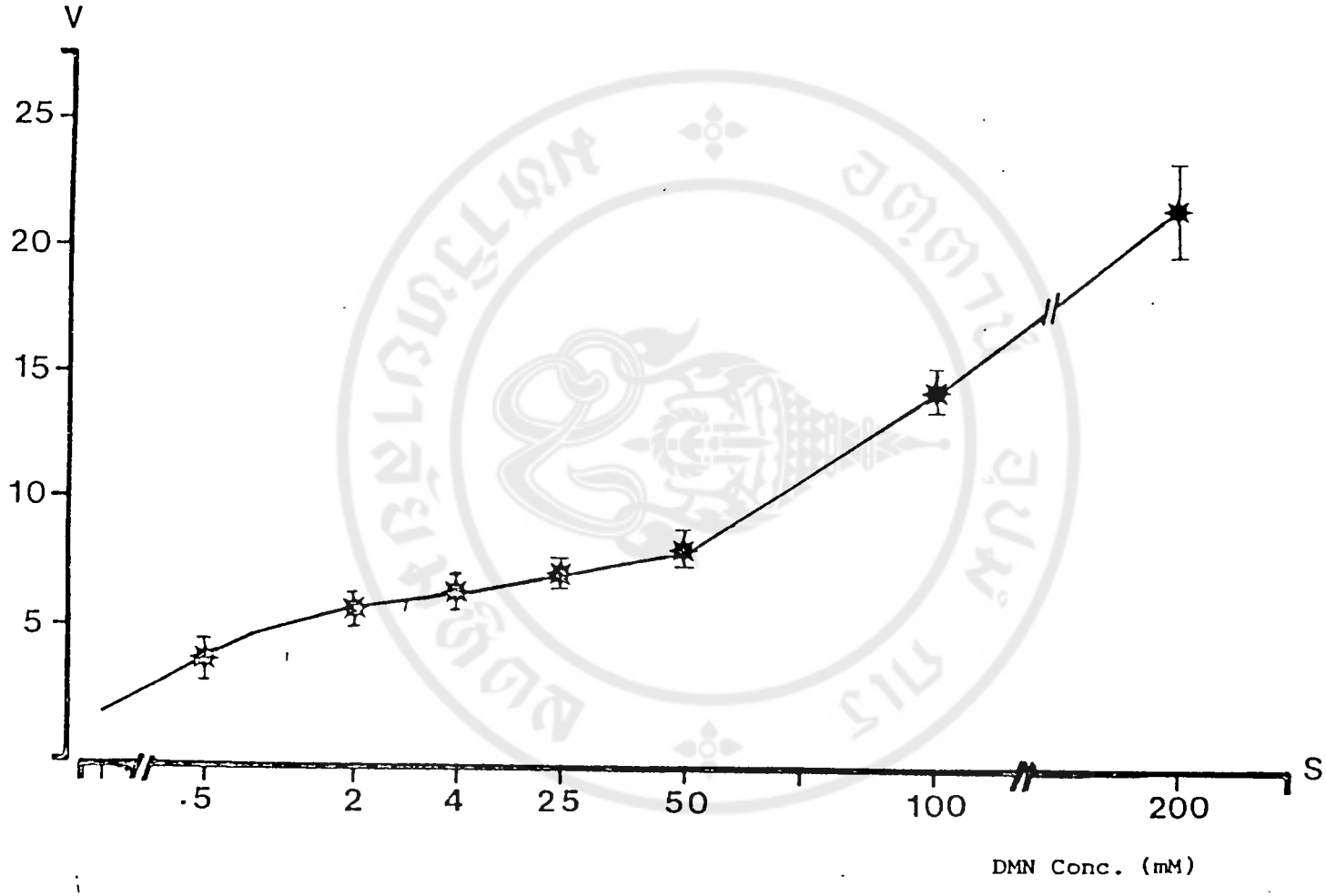
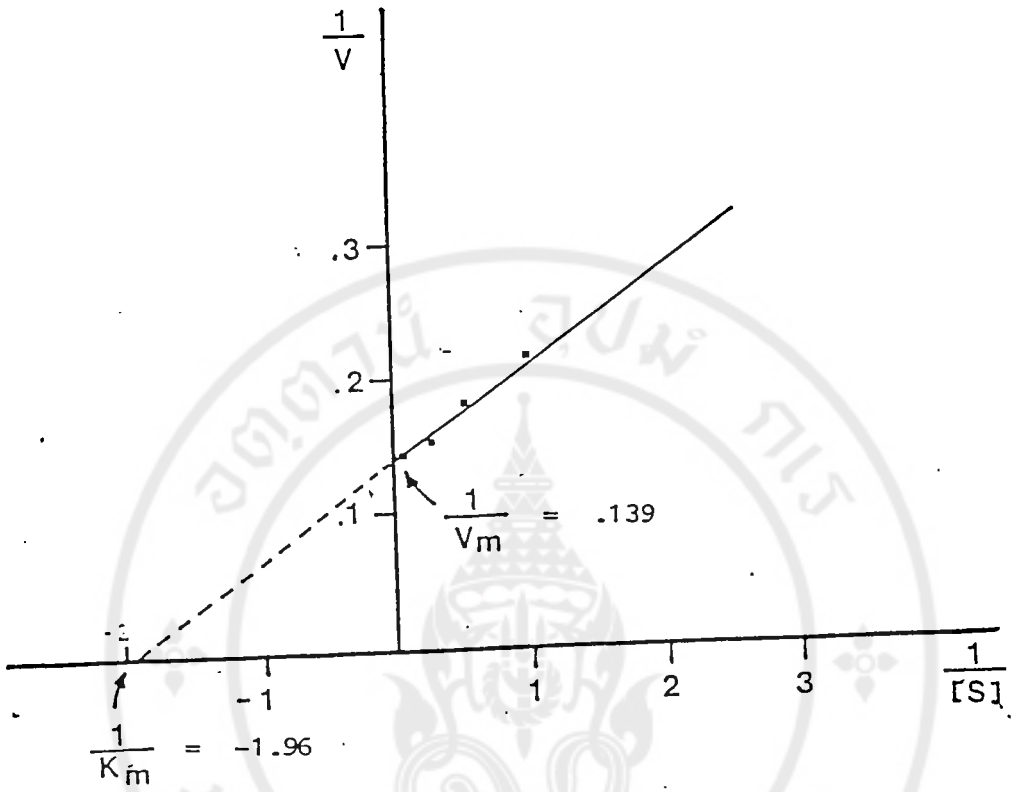
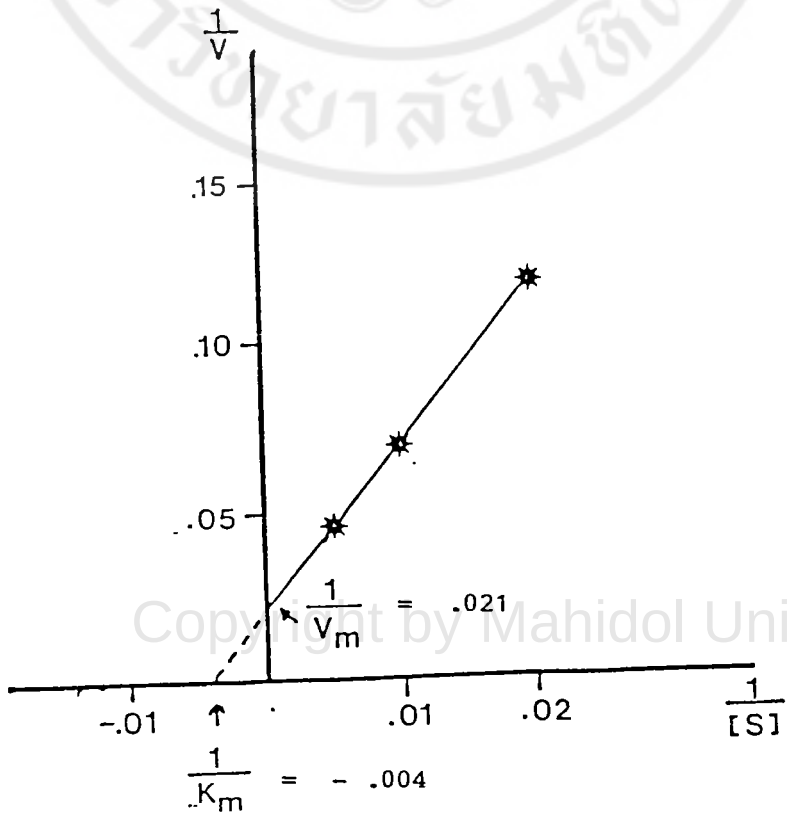


Fig. 1 V. Versus S Plot of Kinetics of DMN-Demethylation. Each Data Point Represents the Means of a Triplicated Assay.

Fig. 2 The Double Reciprocal Plots of DMN-Demethylation by Kidney Microsomes.



A. Low Substrate Conc.



B. High Substrate Conc.

Table 1 Kinetic Study of DMN-demethylase System in Kidney

Microsomal prot.	DMN-d I activity +	DMN-d II activity ++	DMN-demethylase I		DMN-demethylase II	
mg/g tissue	[nmole HCHO/hr/(mg microsomal prot)]	[nmole HCHO/hr/(mg microsomal prot)]	Km	Vmax	Km	Vmax
8.5 ± 0.49	6.44 ± 0.46	22.01 ± 1.83	0.51	7.19	232.55	47.62

Value are expressed as the mean ± S.E. from 3 pooled samples

\+ 4 mM DMN was used

++ 200 mM DMN was used

did not reach the maximum even around to 200 mM [Fig. 1]. This sharply rise after 50 mM indicated that another isozyme may play a role in DMN metabolism. The Lineweaver-Burke plot of DMN-demethylation demonstrated at least 2 Km values for the enzyme [Fig. 2]. The apparent Kms determined were 0.51 and 232.55 mM with the corresponding Vmax values of 7.19 and 47.62 nmol formaldehyde formed per hour per mg protein [Table 1]. Therefore, it is concluded that the DMN-demethylase system of the kidney microsomes in normal rats appeared to involved at least two isozymes of low and high Kms like those of the liver. From their apparent Kms, these isozymes can be classified as DMN-demethylase I and DMN-demethylase II which operated at the low concentration (4-50 mM) and high concentration of DMN (above 50 mM), respectively. The optimum substrate concentrations selected for the subsequent experiments were 4 mM for DMN-demethylase I and 200 mM for DMN-demethylase II as those of the livers.

Experiment 2 : Effects of microsomal enzyme inducers: phenobarbital [PB] and 3-methylcholanthrene [3-MC] on DMN-demethylase system in the kidney.

DMN-demethylase system is recognized as a cytochrome P-450 dependent monooxygenase system of multiple Km values (155) which may result from the presence of different P-450 isozymes in the microsomes with different affinity for the substrate. These characteristics are usually manifested as differential responses after pretreatment with various microsomal enzyme inducers such as phenobarbital [PB], 3-methylcholanthrene (3-MC) etc.

Table 2 Effects of PB and 3-MC Pretreatment on DMN-demethylase System in Kidney

Pretreatment	Microsomal protein (mg/gm tissue)		DMN-d I activity+ [nmole HCHO/hr/(mg microsomal prot)]		DMN-d II activity++ [nmole HCHO/hr/(mg microsomal prot)]	
	Control	exptl	Control	exptl	Control	exptl
PB	6.97 ± 0.37	7.81 ± 0.35	4.01 ± 0.22	4.10 ± 0.27	12.85 ± 0.85	14.39 ± 1.75
	(12.05% increase)		No effect		(11.98% increase)	
	NS		NS		NS	
3-MC	7.41 ± 0.28	7.96 ± 0.27	5.55 ± 0.41	7.65 ± 0.22	15.51 ± 4.18	20.23 ± 5.77
	No effect		(37.83% increase)		(30.43% increase)	
	NS		P < .05		P < .05	

Values are expressed as the mean ± S.E. from 3 pooled samples.
+ 4 mM DMN was used
++ 200 mM DMN was used

This study showed that PB had no effect on DMN-demethylase I, while it caused a slight increase (11.98%) but not statistically significant in DMN-demethylase II activity. There was no changed in microsomal protein content under this condition. This result is in agreement with that from the investigations of Uehleke and Greim (135) who found no induction by phenobarbital in the kidney of adult rats. It can be seen that the kidney enzyme system was much less affected by PB pretreatment.

3-MC pretreatment on the other hand, caused a significant increase (37% and 30% in the activity of DMN-demethylase I and II respectively), while it caused no changed in microsomal protein [Table 2]. This result is in accordance with the finding of Kluwe et al (156) who found that 3-MC increased renal microsomal enzyme activity such as aryl hydrocarbon hydroxylase.

Study II Comparative studies of hepatic and renal DMN-demethylase system.

This study was designed to compare DMN-demethylase system of the liver and kidney by using the kinetic properties of the enzyme the response to the pretreatment with the well-known microsomal enzyme inducers, PB and 3-MC, as a tool to characerize the system.

Experiment 3 : Comparison of the kinetic properties of DMN-demethylase from liver and kidney

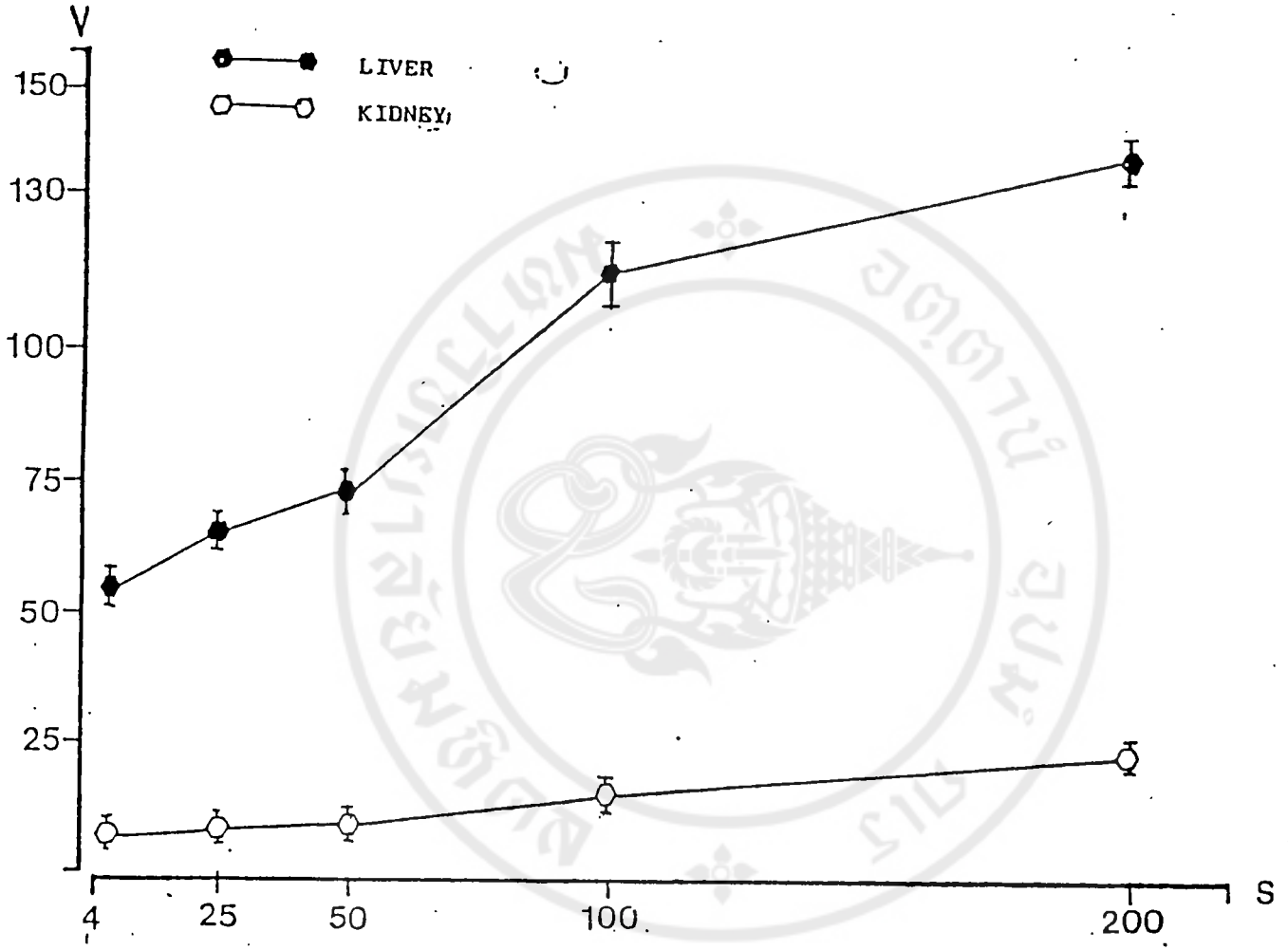


Fig. 3 The Michaelis-Menten Plots of DMN-Demethylation by Liver and Kidney Microsomes. Each Data Represented the Means of a Triplicated Assay.

Table 3 The Comparative Studies of DMN-demthylation by Liver and Kidney of Normal Rats.

Organ	DMN-d I activity + [nmol HCHO/hr/(mg prot)]	Activity, % of liver	DMN-d II activity ++ [nmol HCHO/hr/(mg prot)]	Activity, % of liver	DMN-demethylase I Km	DMN-demethylase I Vmax	DMN-demethylase II Km	DMN-demethylase II Vmax	Microsomal prot mg/gm tissue
Liver	54.18 ± 1.44	100	135.02 ± 5.28	100	1.81	71.42	147.05	285.71	23.85 ± 0.81
Kidney	6.44 ± 0.46	11.9	22.01 ± 1.83	16.3	0.51	7.19	232.55	47.62	8.5 ± 0.49

Values are expressed as the mean ± S.E.

\+ 4 mM DMN was used

++ 200 mM DMN was used

The kinetic properties of DMN-demethylation by the kidney and liver microsomes are compared in Fig. 3 where DMN-demethylation was examined over substrate concentrations ranged from 4-200 mM. The plots demonstrated that isozymes of low and high Km were present in both organs. The velocity of DMN-demethylation increased with increasing DMN concentration to 4 mM, after which the plateau was gradually reached at approximately 50 mM. In the liver, after 50 mM the velocity increased again with increasing concentrations until another plateau was reached when DMN concentration is around 200 mM. In kidney, the maximum activity is not attained even at 200 mM.

Two Km values determined from kidney and liver microsomes were 0.51 and 232.55 mM; and 1.81 and 147.05 mM for kidney and liver enzymes, respectively. These two sets of enzymes operated at the optimal substrate concentrations of 4 and 200 mM, respectively. The results also showed that the activities of kidney DMN-demethylase were much lower than those of the liver. The activities of DMN-demethylase I and DMN-demethylase II of kidney were only 11.9% and 16.3% of the liver enzymes, respectively [Table 3]. The microsomal protein content of kidney was also much lower than that of the liver, being 8.5 ± 0.49 mg/gm kidney as opposed to 23.85 ± 0.81 mg/gm liver.

Experiment 4 : Effect of phenobarbital [PB] and 3-methyl-cholanthrene [3-MC] on DMN-demethylase system in liver and kidney.

Table 4 The Comparative Studies on the Effect of PB and 3-MC on DMN-demethylase System in Liver and Kidney.

Organ	Pretreatment	Microsomal protein mg/g tissue		DMN-d I activity+ [nmol HCHO/hr/(mgprot)]		DMN-d II activity++ [nmole HCHO/hr/(mg prot)]	
		control	exptl	control	exptl	control	exptl
Liver	PB	20.11 ± 0.87	27.36 ± 0.74	85.29 ± 6.95 (n = 13)	43.04 ± 4.17 (n = 17)	160.16 ± 14.93 (n = 13)	252.80 ± 20.68 (n = 12)
		(36.05% increase) P < .05		(49.53% decrease) P < .005		(57.84% increase) P < .05	
	3-MC	20.70 ± 2.72	23.09 ± 2.23	60.08 ± 2.36 (n = 8)	41.56 ± 2.03 (n = 9)	136.70 ± 2.57 (n = 6)	150.45 ± 4.46 (n = 5)
		(11.54% increase) NS		(30.82% decrease) NS		(10.06% increase) NS	
Kidney	PB	6.97 ± 0.37	7.81 ± 0.35	4.01 ± 0.22	4.10 ± 0.27	12.85 ± 0.85	14.39 ± 1.75
		(12.05% increase) NS		No effect NS		(11.98% increase) NS	
	3-MC	7.41 ± 0.28	7.96 ± 0.27	5.55 ± 0.41	7.65 ± 0.22	15.51 ± 4.18	20.23 ± 5.77
		No effect NS		(37.85% increase) P < .05		(30.43% increase) P < .05	

Values are expressed as the mean ± S.E. from 3 pooled samples in kidney
\+ 4 mM DMN was used
++ 200 mM DMN was used
Table of effect of 3-MC on hepatic DMN-demethylation was taken from Thesis of Sornprasit A, 1982.

Results obtained from the PB and 3-MC pretreated experiments were summarized in Table 4. In the liver system, PB pretreatment decreased the activity of DMN-demethylase I by 49% but increased the activity of DMN-demethylase II (57%). There was also an increase (36%) in microsomal protein content. This finding is in agreement with the study of Mostafa et al (1981). In addition, PB pretreatment is known to induce a high Km DMN-demethylase. Kidney DMN-demethylases responded differently to PB pretreatment since there were no changes in the activities of DMN-demethylase I and DMN-demethylase II and microsomal protein content.

3-MC pretreatment caused a decrease (30%) in the activity of DMN-demethylase I in the liver, with no change in the activity of DMN-demethylase II and microsomal protein. However, kidney enzymes responded differently to 3-MC. It significantly increased the activity of DMN-demethylase I and demethylase II by 37% and 30%, respectively with no effect on the microsomal protein content.

Part II Studies on the influence of the thiamine deficiency on the hepatic and renal microsomal DMN-demethylase systems.

Study III Effect of thiamine deficiency on the DMN-demethylases.

Experiment 5 : Effects of thiamine deficiency on the kinetic properties of hepatic and renal DMN-demethylases.

The effect of thiamine deficiency on DMN-demethylase system was studied in the liver and kidney by measuring kinetics

of DMN-demethylation using various substrate concentrations ranged from 4 mM to 200 mM. From figure 4, it can be seen that in liver, thiamine deficiency cause a slight increase but not significant difference in DMN-demethylase I activity. However, the increase in the activity is more pronounced as the concentration increased. At 200 mM, the activity of DMN-demethylase II was significantly enhanced in thiamine deprivation. This result was in agreement with the earlier report of Sornprasit (157).

The Km and Vmax of hepatic DMN-demethylase determined from Figure 4 by using the Lineweaver-Burke plots were shown in Table 5. DMN-demethylase I had the apparent Km values of 1.54 and 4.34 mM in the control and thiamine deficient rats, respectively, with the corresponding maximal velocities (Vmax) of 75.75 and 133.33 nmole formaldehyde formed/hr/mg microsomal protein. The apparent Michaelis constants of DMN-demethylase II were 147.06 and 29.41 mM in the control and thiamine deficient microsomes, respectively, with the corresponding Vmax of 303.03 and 217.39 nmole formaldehyde formed/hr/mg protein.

In kidney, thiamine deficiency apparently increased the activity of DMN-demethylase at all concentrations but more pronounced in the range of DMN-demethylase II (Figure 5). The apparent Michaelis constants of DMN-demethylase I and DMN-demethylase II of the kidney were 0.51 and 232.55 mM, respectively, for the control rats and 0.34 and 181.81 mM for those of the thiamine deficient rats. The corresponding maximal velocity of DMN-demethylation was 7.19 and 47.62 nmole formaldehyde formed/hr/mg microsomal protein for the controls and

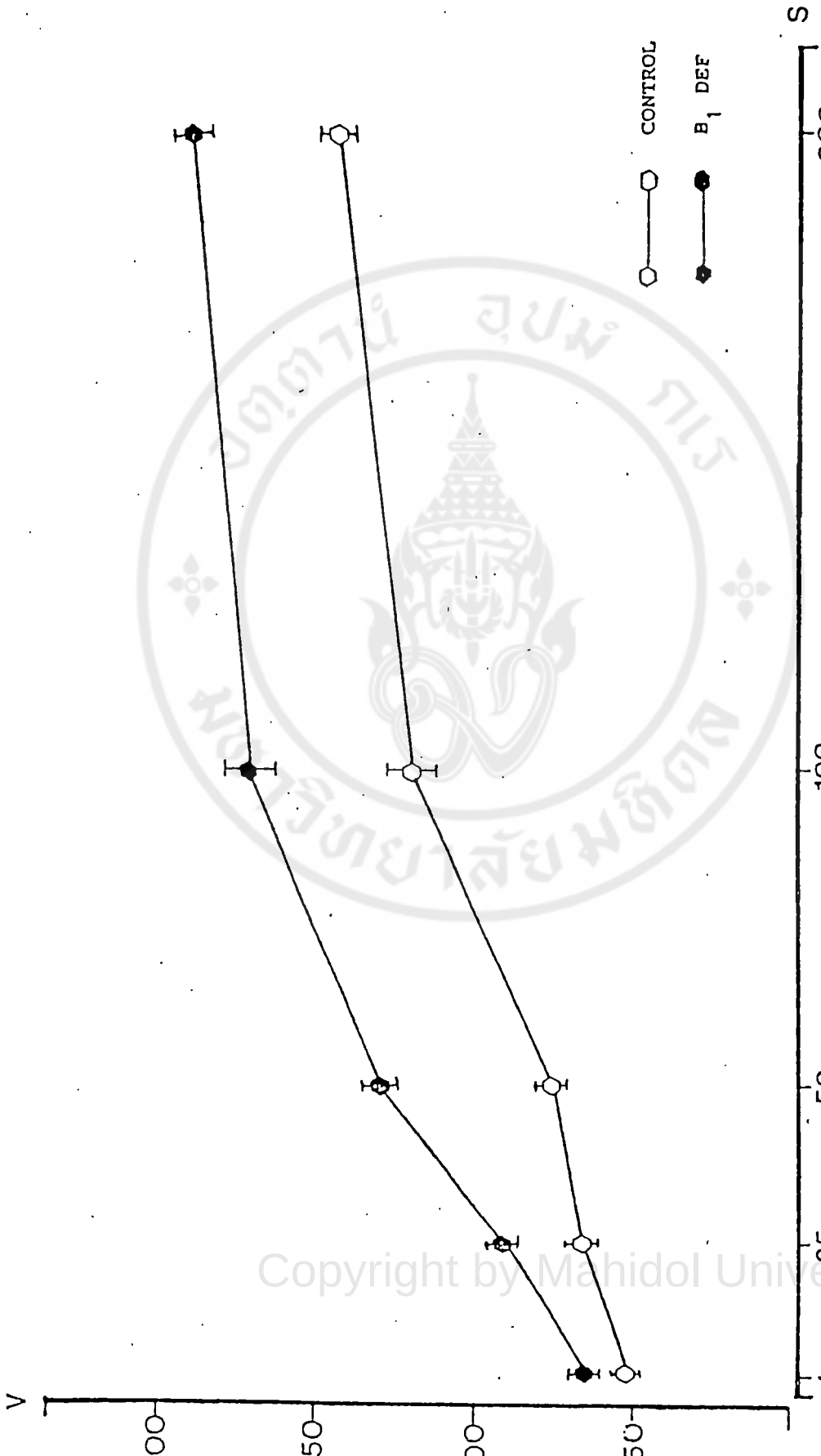


Fig. 4 V. Versus, S Plot of Kinetics of DMN-Demethylation by Pooled Liver Microsomes from Control and Thiamine Deficient Rats. Each Data Point Represents the Means Pooled of a Triplicated Assay.

Table 5 Effects of Thiamine Deficiency on DMN-demethylase System in Liver

Group	Microsomal protein (mg/gm liver)	DMN-demethylase Activity		DMN-demethylase Kinetic Properties			
		DMN-demethylase^{+} I	DMN-demethylase^{++} II	DMN-demethylase I		DMN-demethylase II	
				Km	Vmax	Km	Vmax
Control	24.81 ± 0.75	54.86 ± 2.39	144.06 ± 1.45	1.54	75.75	147.06	303.03
B_1-def	17.01 ± 0.20 [a]	61.85 ± 1.69 [b]	192.54 ± 0.97 [a]	4.34	133.33	29.41	217.39

DMN-demethylase activity was expressed as nmole HCHO/hr/mg microsomal protein
Values are expressed as the mean ± S.E. from 3 pooled samples.
+ 4 mM DMN was used
++ 200 mM DMN was used
a significant different from its control value at $P < .005$
b not statistically significant

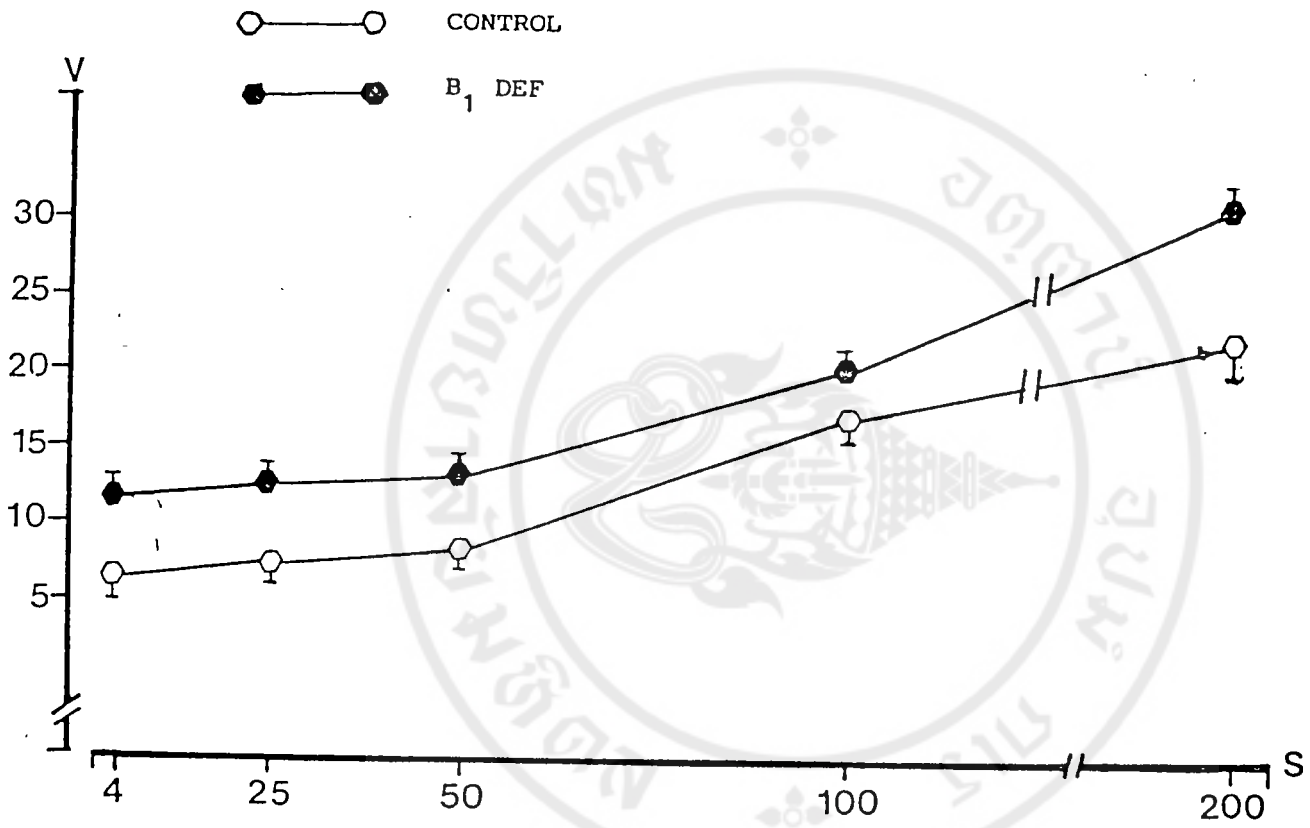


Fig. 5 V. Versus S Plot of DMN-Demethylation by Kidney Microsomes from Control and Thiamine Deficient Rats. Each Data Point Represented the Means of a Triplicated Assay.

Table 6 Effects of Thiamine Deficiency on DMN-demethylase System in Kidney

Group	Microsomal prot. (mg/gm kidney)	DMN-demethylase Activity		DMN-demethylase Kinetic Parameters			
		DMN-demethylase[+] I	DMN-demethylase[++] II	DMN-demethylase I[a]		DMN-demethylase II[b]	
				Km	Vmax	Km	Vmax
Control	8.51 ± 0.55	6.44 ± 0.46	22.01 ± 1.83	0.51	7.19	232.55	47.62
B_1-def	8.65 ± 0.28[b]	11.79 ± 0.06[a]	31.72 ± 0.77[a]	0.34	12.5	181.81	62.5

DMN-demethylase activity was expressed as nmole HCHO/hr/mg microsomal protein.
Values are expressed as the means ± S.E. from 3 pooled samples.
+ 4 mM DMN was used
++ 200 mM DMN was used
a significant different from control group $P < .005$
b not significant statistically

12.5 and 62.5 nmole formaldehyde formed/hr/mg protein for the thiamine deficient kidney, respectively (Table 6). This result indicated that thiamine deficiency brought about differential responses of the enzymes from different organs.

Experiment 6 : Counteracting the effects of thiamine deficiency by *in vivo* supplementation of thiamine hydrochloride.

This experiment was designed to examine whether the alteration on DMN-demethylase activity during thiamine deficiency was solely due to the effect of the vitamin deficiency, not by deprivation of other nutrients which may occur secondary to anorexia. The effect of supplementation of thiamine hydrocholride (650 µg/rat, i.p.) to the deficient rats was shown in (Table 7). Previously, it had been shown that *in vitro* supplementation of thiamine over the concentrations range up to 100 uM could not reverse the effect of dietary thiamine deficiency on hepatic microsomal DMN demethylase I and II (Table 8). *In vivo* supplementation, however, could counteract the effect of dietary thiamine deficiency. The activity of DMN-demethylase I and II was decreased to normal level 24 hours after thiamine supplementation to the deficient rats.

Microsomal protein content was unaffected by either thiamine deficiency or supplementation to the rats.

Experiment 7 : *In vitro* effects of thiamine hydrochloride and thiamine pyrophosphate (TPP) on hepatic DMN-

demethylase of the normal and thiamine deficient rats.

This experiment was designed to investigate the mechanism underlying changes in DMN-demethylation during thiamine deficiency.

Earlier studies in this laboratory (157) had revealed that *in vitro* addition of thiamine hydrochloride to the reaction could not counteract the effect of dietary thiamine deficiency on the microsomal enzyme system (Table 8). However, it was not clear whether the active form of thiamine, i.e., thiamine pyrophosphate (TPP) was the prerequisite for the enzyme interaction. In this experiment the effect of TPP was tested in the *in vitro* microsomal DMN-demethylase assay. Table 9 demonstrated that TPP concentrations from 3.7-36.4 mM progressively enhanced the activity of DMN-demethylase I in the control rats. In the thiamine deficient rats, this effect was not observed at low concentrations of TPP. At highest TPP concentration tested (36.36 mM), DMN-demethylase I activity in the deficient rats was significantly increased.

For DMN-demethylase II, TPP could enhance its activity only at the high concentrations in the control animals. In the deficient animals, the effect was not significant.

The results demonstrated that the increase in the activity of DMN-demethylase during dietary thiamine deficiency was not due to removal or low level of TPP which may be inhibitory to the enzymes.

Table 7 Effects of thiamine supplementation on the activity of DMN-demethylase I and II in kidney.

Group	DMN-demethylase I activity [nmole HCHO/hr/(mg prot)]	DMN-demethylase II activity [nmole HCHO/hr/(mg prot)]	Microsomal Prot. mg/gm kidney
Control	5.61 ± 0.28 (n = 4)	12.02 ± 0.50 (n = 4)	9.55 ± 0.73
Deficient	7.63 ± 0.70 [a] (n = 3)	14.95 ± 0.49 [b] (n = 3)	9.11 ± 1.01
Deficient Supplemented	5.96 ± 0.29 [c] (n = 4)	10.64 ± 0.76 [c] (n = 4)	9.19 ± 0.72

Supplemented rats were given thiamine hydrochloride (650 µg/rat, i.p.) 24 hours prior to sacrifice, DMN-demethylase activity was determined and expressed as nmole formaldehyde formed per hour per mg microsomal protein.

Values are expressed as the mean ± S.E. from 3 or 4 pooled samples

a significant different from the control value $P < .1$

b significant different from the control value $P < .01$

c not significantly statistic

Table 8 The *in vitro* effect of thiamine supplement on hepatic DMN-demethylase system in normal and deficient rats.

Enzyme	Group	Thiamine concentration (μM)								
		0	10	20	30	40	50	60	80	100
DMN-d I+	normal	63.52 ± 2.21	63.47 ± 1.68	63.06 ± 3.11	62.41 ± 1.99	62.11 ± 4.50	61.78 ± 2.86	63.31 ± 2.58	62.73 ± 1.92	61.24 ± 2.23
	B_1 def	61.88 ± 4.25	61.19 ± 3.24	62.96 ± 2.49	60.99 ± 2.23	63.68 ± 3.19	60.70 ± 2.32	63.46 ± 2.36	61.55 ± 2.84	63.62 ± 1.77
DMN-d II++	normal	100.63 ± 1.24	105.04 ± 3.18	101.82 ± 1.49	101.21 ± 1.74	101.14 ± 0.34	100.78 ± 1.06	100.80 ± 1.31	99.65 ± 1.13	101.23 ± 0.70
	B_1 def	172.60 ± 1.45	177.50 ± 2.28	174.17 ± 2.26	171.30 ± 0.75	173.24 ± 1.41	169.35 ± 2.05	171.49 ± 1.63	171.94 ± 2.42	171.43 ± 0.65

Values are expressed in mean ± SE
DMN-demethylase activity was expressed as nmole HCHO/hr/mg protein
\+ 4 mM DMN was used
++ 200 mM DMN was used

This table is taken from Thesis of Sornprasit A, 1982.

Part III Studies on the effect of some enzyme inducer [PB and 3-MC] and inhibitor [$CoCl_2$] on DMN-demethylase system in normal and thiamine deficiency.

Experiment 8 : Effect of PB pretreatment on DMN-demethylase system in liver and kidney of normal and thiamine deficient rats.

Rats were pretreated with PB as described in the experimental protocol and the results were summarized in Table 10. In the liver system, PB pretreatment markedly decreased the activity of DMN-demethylase I in control and thiamine deficient groups by 49% and 63%, respectively. On the other hand, DMN-demethylase II responded differently. After PB pretreatment, the activity of DMN-demethylase II activity in control rats was markedly increased (58%) while that in the deficient group was decreased (30%). However, in both groups the microsomal protein content was greatly enhanced. This results was in agreement with the previous study of Sornprasit (157).

In kidney, PB pretreatment caused no significant change in neither DMN-demethylase I, DMN-demethylase II nor the microsomal protein content both in normal and thiamine deficient rats.

Experiment 9 : Effect of 3-MC pretreatment on the hepatic and renal DMN-demethylase system in the normal and thiamine deficient rats.

In liver, the effect of 3-MC had been previously studied by Sornprasit (157). DMN-demethylase I was decreased both in

Table 9 Effect of Thiamine Pyrophosphate [TPP] in vitro on Hepatic DMN-demethylase System in Normal and Thiamine Deficient Rats.

TPP conc. (mM)	DMN-d I activity+ [n mol HCHO/hr/(mg protein)]		DMN-d II activity++ [n mol HCHO/hr/(mg protein)]	
	control	deficient	control	deficient
0	25.42 ± 3.08	37.04 ± 0.95	54.07 ± 1.27	136.25 ± 5.26
3.70	34.16 ± 1.41[a]	35.24 ± 2.01	55.22 ± 1.52	137.46 ± 3.75
18.51	58.41 ± 1.61[a]	34.50 ± 1.35	66.68 ± 8.58[a]	144.45 ± 4.89[c]
36.36	96.27 ± 4.84[a]	112.29 ± 3.80[a]	93.48 ± 1.81[a]	145.84 ± 2.56[b]

The values are expressed in mean ± S.E. from 3 pooled samples
+ 4 mM DMN was used
++ 200 mM DMN was used
a significant different from the corresponding control value $P < .005$
b significant different from the corresponding control value $P < .01$
c significant different from the corresponding control value $P < .025$

Table 10 Effect of PB Pretreatment on Hepatic and Renal DMN-demethylase System in the Normal and Thiamine Deficient Rats.

Organ	Group	microsomal protein (mg/g tissue)		DMN-d I activity+ [n mol HCHO/hr(mg prot)]		DMN-d II activity++ [n mol HCHO/hr/(mg prot)]	
		control	exptl	control	exptl	control	exptl
Liver	Control	20.11 ± 0.87	27.36 ± 0.74 (36.05% increase) P < .05	85.29 ± 6.95	43.04 ± 4.17 (49.54% decrease) P < .005	160.16 ± 14.93	252.80 ± 20.68 (57.84% increase) P < .05
	B_1 -def	18.27 ± 1.25	28.70 ± 1.22 (57.08% increase) P < .05	117.28 ± 11.17	42.42 ± 3.90 (63.83% decrease) P < .005	303.07 ± 26.28	211.53 ± 26.69 (30.20% decrease) P < .05
Kidney	Control	6.97 ± 0.37	7.81 ± 0.35 (12.05% increase) NS	4.01 ± 0.22	4.10 ± 0.27 No effect NS	12.85 ± 0.85	14.39 ± 1.75 (11.98% increase) NS
	B_1 -def	7.86 ± 0.13	8.91 ± 0.47 (13.35% increase) NS	4.27 ± 0.43	4.48 ± 0.35 No effect NS	15.57 ± 1.53	15.20 ± 1.26 No effect NS

Values are expressed in mean ± S.E.
\+ 4 mM DMN was used
++ 200 mM DMN was used

Table 11 Effect of 3-MC Pretreatment on Hepatic and Renal DMN-demethylase System in the Normal and Thiamine Deficient Rats.

Organ	Group	microsomal protein (mg/g tissue)		DMN-d I activity+ [n mol HCHO/hr(mg prot)]		DMN-d II activity++ [n mol HCHO/hr/(mg prot)]	
		control	exptl	control	exptl	control	exptl
Liver*	Normal	20.70 ± 2.27	23.09 ± 2.23	60.08 ± 2.36	41.56 ± 2.03	136.70 ± 2.57	150.45 ± 4.46
		(11.54% increase)		(30.82% decrease)		(10.06% increase)	
		NS		NS		NS	
	B_1 -def	34.32 ± 1.93	37.32 ± 1.66	55.18 ± 2.12	38.30 ± 1.93	215.63 ± 12.14	224.43 ± 11.38
		(8.7% increase)		(30.59% decrease)		No effect	
		NS		NS		NS	
Kidney	Normal	7.41 ± 0.28	7.96 ± 0.27	5.55 ± 0.41	7.65 ± 0.22**	15.51 ± 4.18	20.23 ± 5.77
		(7.42% increase)		(37.84% increase)		(30.43% increase)	
		NS		P < .05		P < .05	
	B_1-def	8.18 ± 0.30	8.44 ± 0.26	7.95 ± 0.50	7.92 ± 0.68**	24.03 ± 3.02	18.61 ± 0.64
		No effect		No effect		(22.55% decrease)	
		NS		NS		NS	

Values are expressed as the means ± S.E.
+ 4 mM DMN was used
++ 200 mM DMN was used
NS = non significant
* The effect of 3-MC on hepatic DMN-demethylase system was taken from thesis of Sornprasit A, 1982.
** There was an interaction effect between 3-MC and diet treatment with P < 0.05 in Renal DMN-d I activity.

control and thiamine deficient rats to the same extent. However, the microsomal protein content in both group was not changed. On the contrary, 3-MC pretreatment caused no significant change in DMN-demethylase II activity in both groups. The results in Table 11 demonstrated the effect of 3-MC pretreatment on the kidney enzymes. It caused an increase in the activity of DMN-demethylase I and DMN-demethylase II in control rats by 37% and 30%, respectively. In the deficient group, it had no effect on DMN-demethylase I while it caused a slight decrease (22%) in DMN-demethylase II activity. However, there was an interaction between 3-MC treatment and dietary group on the DMN-demethylase I activity whereas no interaction was shown on the DMN-demethylase II activity.

Experiment 10 : Effect of Cobaltous Chloride on hepatic DMN-demethylase system

Cobaltous chloride is a well known inhibitor of the microsomal mixed function oxidase. Pretreatment of rats with $CoCl_2$ showed an inhibitory effect on DMN-demethylase I and DMN-demethylase II activities in both the control and thiamine deficient group (Table 12). However, the effect is more pronounced in control rats. This result was in accordance with many other studies and especially that of Stanley et al (158) that $CoCl_2$ induced protection against acetaminophen hepatotoxicity by depressing its metabolism and hepatic cytochrome P-450 levels.

Table 12 Effect of $CoCl_2$ Pretreatment on Liver DMN-demethylase System in Normal and Thiamine Deficient Rats.

Treatment	Group	Microsomal protein mg/gm Liver		DMN d-I activity+ [n mol HCHO/hr/(mg prot)]		DMN d-II activity++ [n mole HCHO/hr/(mg prot)]	
		control	exptl	control	exptl	control	exptl
$CoCl_2$	Normal	17.52 ± 2.09	12.02 ± 0.63	93.16 ± 5.85 (n = 5)	36.69 ± 0.77 (n = 7)	165.43 ± 6.61 (n = 5)	65.29 ± 1.18 (n = 7)
			P < .005	(60.61% decrease) P < .005		(60.5% decrease) P < .005	
	B_1 -def	13.11 ± 1.17	13.09 ± 1.26	125.62 ± 5.27 (n = 6)	83.52 ± 8.26 (n = 4)	289.92 ± 18.92 (n = 6)	133.59 ± 7.47 (n = 4)
			No effect	(33.51% decrease) P < .005		(53.92% decrease) P < .005	

The values are expressed as mean ± S.E. from individual rat
Explt = rat pretreated with $CoCl_2$ (60 mg/kg, S.C.) 24 hour
Contl = rat injected with an equal volume of NSS
+ = 4 mM DMN was used
++ = 200 mM DMN was used
n = number of rats

CHAPTER VI

DISCUSSION

Part I Studies of the microsomal DMN-demethylase system in the kidney.

DMN, a precarcinogen, must be activated mainly by hepatic microsomal enzyme to elicit its carcinogenic effect. Although liver is the major site of metabolism of DMN, but in certain circumstance such as the rate of metabolism of DMN by liver is decreased or when the liver is damaged or its metabolic activity is inhibited, kidney can play a significant role in metabolism and thus be coming a target organ for DMN. In many reports it has been shown that kidney is active in metabolizing DMN although to a less extent than the liver. In this study, the kidney DMN demethylase system has been characterized in detail.

Kinetic studies of DMN-demethylase system in kidney showed a pattern similar to that of the liver. The presence of at least 2 Km values in kidney showed the different forms of cytochrome P-450 isozyme designated as DMN-demethylase I and DMN-demethylase II and these two enzymes operated with a similar ranges of the optimum substrate concentrations (4 mM and 200 mM) as in liver although the maximum velocities of the kidney enzymes were 7-10 times lower than those of the liver enzymes. This may be due to lower concentration of cytochrome P-450 in renal cell which according to Kluwe et al (156) was about 1/3 to 1/4 of the concentration in liver. The N-demethylation of other substrates

such as aminopyrine and ethylmorphine in kidney was also reported to be much lower than in the liver (126). These isozymes may have different affinity for DMN. The multiple Km values of DMN-demethylase of the kidney could, therefore, be due to the presence of various forms of cytochrome P-450 in kidney which was reported by Ogita et al (159).

Classical microsomal enzyme inducers such as PB and 3-MC, which have differential effects on specific forms of cytochrome P-450, were employed to further characterize the kidney enzyme system. Kluwe et al, (156) showed that 3-MC increased aryl hydrocarbon hydroxylase and p-chloro-N-methylaniline-N-demethylase activities in rat kidney. Ogita et al, (160) reported that 3-MC pretreatment resulted in a striking increase of cytochrome P-448 and benzo(a)pyrene hydroxylase activity in rabbit. However, Mitchell et al, (137) demonstrated that 3-MC pretreatment could not induce biphenyl-4-hydroxylase and p-chloro-N-methylaniline-N-demethylase in kidney of the rat. In our system, 3-MC exerted no effect on DMN-demethylase II from the liver but had opposite effect on the kidney enzyme (30% increase). In liver, it caused a 30% decrease in DMN-demethylase I activity while in kidney, it increased the activity by 38%. The result in liver was in accordance with the earlier studies that exhibited the repressive effect of 3-MC on DMN-demethylase activity (43) due to the reduction of the amount of enzyme in microsome. From the available data, it is not possible to postulate any mechanism underlying such phenomenon. Detailed studies on different forms of cytochrome P-450 in the kidney and their responses to 3-MC induction will be necessary for the explanation.

Various studies on the effect of PB pretreatment on renal microsomal MFO activity demonstrated that this agent did not have any influence on such system (156,159). Kuo et al (161) also found that PB produce little effect on rat renal MFO system. In this study; PB has no effect on both forms of DMN-demethylases in the kidney while increasing only the activity of DMN-demethylase II in liver.

Part II Studies on the influence of thiamine deficiency on the hepatic and renal microsomal DMN-demethylase systems.

The effect of dietary thiamine deficiency on DMN-demethylase system in kidney differed from that of in the liver as shown in previous study in this laboratory. In kidney, thiamine deficiency caused an increase both in DMN-demethylase I and DMN-demethylase II while in the liver only DMN-demethylase II was affected. This suggested that demethylation occurring at the low and high DMN concentrations is mediated by different cytochrome P-450 isozymes which have different sensitivity to the effect of thiamine deficiency. A markedly decrease in Km value of DMN-demethylase I and II in thiamine deficiency might reflect the alteration in the interaction between cytochrome P-450 and its substrate DMN. This low Km value may be due to a slight different in the active sites of enzyme between control and thiamine deficient rat or may be a different or may be a different in the environment adjacent to P-450 in the microsomal membrane, like cytochrome b_5. Thiamine deficiency caused an increase in maximum velocity of DMN-demethylase enzyme. This stimulatory effect at

first was thought to be due to lowering concentration of thiamine or thiamine pyrophosphate which might be inhibitory to the enzyme system since no detectable increase in the cytochrome P-450 content [Ruchirawat, unpublished]. However, the in vitro effect of the active form of thiamine in the body, TPP, which resulted in stimulation of DMN-demethylase activity indicated that it was not the lowering of TPP during deficiency that enhanced DMN-demethylase activity. Thiamine deficiency should act indirectly via an unidentified mechanism.

Supplementation of thiamine hydrochloride by single dose i.p. injection to the deficient rat could reverse the effect on the DMN-demethylase activity suggesting that the increased effect is the true action of thiamine deficiency, not by other nutrient deficiencies which may result secondary to anorexia induced by thiamine deficiency.

Part III Studies of the effects of microsomal enzyme inducers and inhibitors on DMN-metabolism in the normal and thiamine deficient rats.

Pretreatment with the known microsomal enzyme inducers such as PB and 3-MC demonstrated that PB has no effect on the kidney DMN-demethylase system both in normal and thiamine deficiency rats. This is in agreement with the observation of Lake et al (123) that PB did not appear to stimulate enzyme chloromethyl aniline N-demethylase and aniline 4-hydroxylase in kidney. Uehleke and Greim (135) also found no induction on renal MFO by PB in the kidney of adult rats. This result may be due to

a small quantity of cytochrome P-450 present in kidney. Therefore, induction by PB was not apparent. In liver, PB cause a markedly decrease in DMN-demethylase I activity both in normal and thiamine deficient rats but for DMN-demethylase II, PB caused an increase in activity in normal rat but depressed in thiamine deficient group. This observation is in agreement with a previous result in this laboratory Sornprasit (157). Though PB are known to bring about an increase of P-450 content in liver microsomes but it repress DMN-demethylase I activity in our result as in the earlier work of Venkatesan et al (43) and Phillips et al (56). Possible mechanisms are that this DMN-demethylase I is not directly mediated by the cytochrome P-450 inducible by PB. This result was supported by Tu and Yang (162) who found that microsomal DMN-demethylase activity is usually not induced by PB and 3-MC when assayed at 4 mM DMN but is induced when assayed at 200 mM DMN.

Effect of 3-MC pretreatment on renal microsomal enzyme showed that 3-MC caused an increase in DMN-demethylase I and DMN-demethylase II activity in control rat, whereas in thiamine deficient group, 3-MC exerted no effect on DMN-demethylase I but slightly decreased in DMN-demethylase II. It is possible that the effect of diet alone is not pronounced enough but the combine effect of 3-MC and thiamine deficient diet can be further extended and showed statistically significant difference with $P < 0.05$ in the DMN demethylase I activity but no interaction was shown in DMN-demethylase II activity. From the previous study 3-MC caused an induction in cytochrome P-450 content (132), the form which may be responsible for the increased activity of DMN-

demethylase I and DMN-demethylase II. Other reports suggested inductive effect of 3-MC on Aryl hydrocarbon hydroxylase activity in rat kidney microsome (139) and induction of 3-MMAB by 3-MC as demonstrated by Sladex and Mannering (115). However 3-MC caused no inductive of chloromethyl-aniline-N-demethylase or biphenyl-2-hydroxylase in kidney (123).

Pretreatment with $CoCl_2$, a well known inhibitor of cytochrome P-450 resulted in significantly decrease in the DMN-demethylase activities of the normal and thiamine deficient rats. The decreased effect was more pronounced in control rats. It is possible that thiamine deficiency had already increased the amount of cytochrome P-450 isozyme (112), thus inhibition by $CoCl_2$ could not lowering the level of activity as much as control. This suggests that demethylation of DMN is a cytochrome P-450 dependent reaction. Although several forms of cytochrome P-450 may participate in the metabolism of DMN, and under certain condition, some forms may be predominant, however only some cytochrome P-450 isozymes are inhibited by $CoCl_2$ pretreatment. This inhibitory effect of $CoCl_2$ was supported by the study of Honnavar et al (163) who demonstrated that administration of cobatous chloride leaded to a suppression of phenobarbitone-mediated increase in total cytochrome P-450. It was due to a decrease in the rate of synthesis of protein in the step of translation of cytochrome P-450 RNA without affecting total protein synthesis. Additionally, Stanley et al (158) found that $CoCl_2$ decreased hepatic cytochrome P-450 level by acceleration of heme degradation, therefore decrease in oxidative metabolism of acetaminophen and toxicity.

CHAPTER VII

SUMMARY

The experimental results have demonstrated the following:

1. Kidney DMN-demethylase consists of at least two isozymes which operate in the same concentration range as DMN-demethylase I and DMN-demethylase II of the liver.

2. The activity of kidney DMN-demethylase is much less than that in liver and it shows different type of the responses to the microsomal enzyme inducers (phenobarbital and 3-MC) from those of the hepatic DMN-demethylases.

3. Thiamine deficiency enhances both the renal DMN-demethylase I and DMN-demethylase II

4. The effect of thiamine deficiency in kidney can be counteracted by a single injection of thiamine hydrochloride i.p. (650 µg/rat) 24 hours prior to the experiment.

5. The enhanced effect of thiamine deficiency in liver can not be reversed by in vitro supplementation of thiamine hydrochloride or thiamine pyrophosphate.

6. Coboltous chloride, a known inhibitor of cytochrome P-450 synthesis, markedly decreases DMN-demethylase activities in the control and thiamine deficient rats.

7. Variable responses of DMN-devethylases to the pretreatment with PB and 3-MC were observed in the control and thiamine deficient rats.

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APPENDIX I

Composition of Control Agar-Gel Diet

Ingredient	Amount (gm)	% in diet
	1 kg	
Vitamin Free Casein	220	22
Dextrose	200	20
Sucrose	160	16
Dextrin	200	20
Roger-Harper's Salt Mix	50	5
Vitamin mix No. 13	20	2
Mazola oil	150	15
Choline (20%)*	15#	
Vitamin B (0.1%)*	5#	

Can be made up in a 3% agar soluting. # Volume used in ml

* Choline solution (20%); Chloline chloride 200.0 gm, water up to 1000 ml.

Vitamin B_{12} solution ; Vitamin B_{12} triturate (0.1%) 10.0 gm, water up to 1000 ml.

Vitamin mix No. 13

Ingredient	Total mixture 2 kg
Thiamine HCl	0.8 gm
Menadione	0.1 gm
Riboflavin	0.5 gm

Ingredient	Total mixture 2 kg
Pyridoxine	0.8 gm
Calcium Pantothenate	2.0 gm
Folic Acid	1.0 gm
Nicotinic acid	5.0 gm
Inositol	25.0 gm
Vitamin A & D mix (500/50)	6.0 gm
DL-Alpha Tocopheral Acetate	22.50 gm
Biotin	0.05 gm
Sucrose	1936.20 gm

Salt-Mixture (Roger-Harper)

Ingredient	Amount in percent
$CaCo_3$	29.29
$CaHPO_4.2H_2O$	0.43
KH_2PO_4	34.31
NaCl	25.06
$MgSO_4.7H_2O$	9.98
$Fe(C_6H_5O_7).6H_2O$	0.623
$CuSO_4$	0.156
$MnSO_4.H_2O$	0.121
$ZnCl_2$	0.020
KI	0.0015
$(NH_4)_6Mo_7O_{24}$	0.0025
$Na_2SeO_3.5H_2O$	0.0015

The salts at 5% of the diet provided in percent of element: Ca, 0.592; P, 0.394; k, 0.493; Na, 0.493; Cl, 0.760; Mg, 0.049; Fe, 0.0048; Cu, 0.0019; Mn, 0.00195; Zn, 0.004; I, 0.000019; Mo, 0.000005; Se, 0.0000025.