CHAPTER IV

RESULTS AND DISCUSSION

Much attention has recently been brought to the fact that many natural components of the diet are mutagenic and/or carcinogenic (Zeiger, 1993). Many chemicals, chemical mixtures, and plant extracts are allowed as direct food additives by the Thai FDA (Food and Drug Association). Some chemicals found in the body are natural components of foods. However, synthetic chemicals such as nitrite is one of the currently used as food additives that involving *in vivo* mutagen formation. Various drugs have been shown to react with nitrite to produce mutagenic compounds (Andrew *et al.*, 1980). Reaction products of some tranquilizers were shown to be mutagenic (Takeda and Kanaya, 1981).

Mutagenic modification of drug with nitrite

It is interesting to evaluate whether some drugs (which are generally suggested to be taken after meal or with meal for a long time) can react with nitrite to produce mutagens in acid condition. In this study twenty-seven preparations were selected to be tested for their mutagenicity using microbial assay developed by Ames *et al.*(1975). This method is currently used to detect a high proportion of those chemical in our environment which are potentailly hazardous as mutagens, carcinogens, or (as in more usual) both. The basic method involves incorporating the test chemical in an agar overlay containing a small inoculum of one of several carefully chosen auxotrophic

mutant strains of the bacteria *S. typhimurium*, and then scoring the numbers of mutants to prototrophy which appear on the appropiate selective medium (Macphee and Podger, 1977). This procedure could be useful to screen out the drug forming mutagens by drug-nitrite interaction from numerous pharmaceutical preparations.

A rapid screening method of Ames test (spot test) was used firstly to evaluate whether these drugs could react with nitrite to produce some direct mutagen. Eleven out of twenty-seven drugs (Table 4) show their mutagenicity after being treated with excess sodium nitrite in dilute acidic condition. Only the positive samples were selected for further preincubation method.

The number of His^+ revertants of eleven nitrite treated drugs of plate incorporation test are shown in Table 6. Only five samples are positive in both strains. The explanation why these samples were positive only in the spot test but negative in the preincubation method is the dilution effect. The concentrated samples were applied on a limited area of the paper discs in spot test, however, in plate incorporation method the samples were mixed with the top agar and spreaded onto the agar plate.

Nitrite treated chlordiazepoxide and nitrite treated phenytoin found positive in the present spot test were reported to produce nitrosamines at very low yield i.e. 0.28 % and 0.12 % respectively (Gillatt et al.,1983). This may suggested that the two samples could produce some direct non-nitroso mutagens after interacting with nitrite. On the other hand, Gillatt et al. (1983) found that the nitrosamine yield of nitrite

Table 4 Mutagenicity of nitrite treated drugs by spot test

Chemical	Mutagenicity on *S. typhimurium*	
	TA 98	TA 100
Amitriptyline	–	–
Atenolol	–	–
Bromazepam	+	+
Carbamazepine	–	–
Chlordiazepoxide	+	+
Chlorpromazine	+	+
Cimetidine	+	+
Clobazepam	–	–
Diazepam	+	+
Diltiazem	–	–
Chlorazepate	–	–
Enalapril	–	–
Griseofulvin	+	+
Isoniazid	+	+
Isosorbide dinitrate	+	+
Ketoconazole	–	–
Metformin	–	–
Mexilitine	–	–
Nifedipine	+	+
Nortriptyline	–	–
Phenytoin	+	+
Prazepam	–	–
Pyrazinamide	–	–
Ranitidine	+	+
Rifampicin	–	–
Thioridazine	–	–
Trihexyphenidyl	–	–

\+ postive test for Ames test.
– negative test for Ames test.

treated nortriptyline in acid condition was 9.8 %, but negative result of this drug was obtained from the Ames test in the present study.

Although it is shown that the interaction between any drug and nitrite in the acid condition produce some direct non-nitroso mutagens, it is not wise to assume that if no mutagenicity is detected in the present study the drug is considered safe. Because most nitrosamines are indirect mutagens and it is presumed that the target organ is liver. Therefore the information on the mutagenicity of the nitrite treated drug and the formation of nitroso compound in the present experiment should be both aware that it concern only in the gastric like condition not for the whole body of the patient.

The results shown in Table 5 revealed that five preparations were easily modified to be mutagens by interacting with nitrite in acidic condition which was a mild condition. The present results show that when three preparations tested (such as bromazepam, chlordiazepoxide and isoniazid) were incubated with nitrite, they became capable of causing both base-pair substitution and frameshift mutation while cimetidine and ranitidine became capable of causing only base-pair substitution mutation in *S. typhimurium*. The mutagen products occurred were probably nitroso- or nitro-compounds because all drugs were nitrogen-containing compounds. Chlorpromazine was negative in both strains in Ames test but it has been recently reported that it was positive in SMART (somatic mutation and recombination test) (Vanschaik and Graf, 1993). This showed that nitrite treated chlopromazine produced indirect mutagen.

Table 5 Mutagenicity of nitrite treated drugs in acidic condition (pH 3-3.5) on *S. typhimurium* TA98 and TA100. Bold number indicate positive mutagenic response.

Chemicals	Amount (μg/plate)	No. of His^+ revertants/plate			
		TA98		TA100	
		without $NaNO_2$	with $NaNO_2$	without $NaNO_2$	with $NaNO_2$
Bromazepam	0	12 ± 2	12 ± 2	104 ± 6	104 ± 6
	200	15 ± 2	**120 ± 15**	121 ± 8	175 ± 7
	400	11 ± 2	**230 ± 2**	132 ± 7	**320 ± 16**
	800	11 ± 1	**406 ± 36**	114 ± 6	**750 ± 12**
Chlordiazepoxide	0	10 ± 1	10 ± 1	140 ± 13	140 ± 13
	200	18 ± 2	**44 ± 5**	108 ± 6	128 ± 6
	400	31 ± 3	**56 ± 6**	123 ± 15	**293 ± 15**
	800	32 ± 2	**196 ± 16**	115 ± 8	**403 ± 8**
Chlorpromazine	0	19 ± 1	19 ± 1	134 ± 24	134 ± 24
	200	15 ± 1	36 ± 6	87 ± 5	120 ± 7
	400	15 ± 3	58 ± 3	87 ± 8	130 ± 12
	800	16 ± 3	85 ± 9	84 ± 6	180 ± 27
Cimetidine	0	19 ± 1	19 ± 1	134 ± 24	134 ± 24
	200	18 ± 2	24 ± 6	94 ± 5	**225 ± 20**
	400	19 ± 3	26 ± 6	97 ± 15	**746 ± 4**
	800	17 ± 3	25 ± 6	97 ± 5	**1636 ± 59**
Diazepam	0	10 ± 1	10 ± 1	140 ± 13	140 ± 13
	200	21 ± 2	29 ± 6	139 ± 4	121 ± 12
	400	17 ± 3	66 ± 6	123 ± 12	155 ± 5
	800	39 ± 4	137 ± 6	124 ± 9	180 ± 8

Table 5 (Cont.) Mutagenicity of nitrite treated drugs in acidic condition (pH 3-3.5) on *S. typhimurium* TA98 and TA100. Bold number indicate positive mutagenic response.

Chemicals	Amount (μg/plate)	No. of His^+ revertants/plate			
		TA98		TA100	
		without $NaNO_2$	with $NaNO_2$	without $NaNO_2$	with $NaNO_2$
Nifedipine	0	12 ± 2	12 ± 2	104 ± 13	104 ± 13
	200	18 ± 2	44 ± 5	100 ± 5	124 ± 3
	400	10 ± 2	29 ± 5	96 ± 4	108 ± 4
	800	19 ± 1	51 ± 7	147 ± 2	192 ± 2
Griseofulvin	0	19 ± 1	19 ± 1	182 ± 5	182 ± 5
	200	36 ± 6	27 ± 2	132 ± 3	123 ± 4
	400	20 ± 1	35 ± 2	160 ± 4	156 ± 4
	800	28 ± 2	47 ± 3	141 ± 5	190 ± 2
Isoniazid	0	19 ± 1	19 ± 1	134 ± 16	134 ± 16
	200	16 ± 4	**548 ± 10**	150 ± 5	**326 ± 4**
	400	31 ± 3	**1286 ± 8**	143 ± 3	**474 ± 4**
	800	33 ± 3	**2430 ± 9**	169 ± 3	**1094 ± 5**
Isosorbide dinitrate	0	22 ± 1	22 ± 1	133 ± 3	133 ± 3
	200	19 ± 2	22 ± 1	103 ± 4	116 ± 2
	400	20 ± 1	30 ± 1	127 ± 4	118 ± 4
	800	26 ± 1	23 ± 1	118 ± 3	159 ± 3
Phenytoin	0	19 ± 1	19 ± 1	182 ± 5	182 ± 5
	200	35 ± 4	35 ± 4	149 ± 3	123 ± 4
	400	19 ± 2	41 ± 2	134 ± 5	120 ± 3
	800	24 ± 1	25 ± 1	134 ± 5	170 ± 3

Table 5 (Cont.) Mutagenicity of nitrite treated drugs in acidic condition (pH 3-3.5) on *S. typhimurium* TA98 and TA100. Bold number indicate positive mutagenic response.

Chemicals	Amount (μg/plate)	No. of His^- revertants/plate			
		TA98		TA100	
		without $NaNO_2$	with $NaNO_2$	without $NaNO_2$	with $NaNO_2$
Ranitidine	0	16 ± 4	16 ± 4	109 ± 6	109 ± 6
	200	12 ± 1	20 ± 2	131 ± 2	161 ± 6
	400	16 ± 3	52 ± 3	154 ± 4	**261 ± 5**
	800	23 ± 2	60 ± 5	148 ± 5	**426 ± 5**

Results show in means ± SD (n=5). Aminopyrene is positive control and DMSO is negative control for Ames Test.

Antimutagen formation of ivygourd fiber or BSA on drug treated with nitrite

It was reported that some plant fibers protected the consumers against a number of diseases (Yamaguhi, 1992). One of the main mechanisms involved including the absorption of toxic substances as well as the precursors. Epidemiological data showed a possible correlation between the intake of nitrates and/or nitrites and human gastric cancer suggesting that nitrosation was its possible cause (Armijo and Coulson 1975 ; Cuello *et al.*, 1976).

Ivygourd was selected to evaluate whether its fiber could prevent the mutagenicity of drug-nitrite interaction, according to the suggestion of Laohavechvanich (1994). Five drug samples, namely bromazepam, chlordiazepoxide, isoniazid, cimetidine, and ranitidine were treated with nitrite in the present of fiber

under acidic condition mixture. Using the procedure described in chapter III, ivygourd fiber inhibited the formation of mutagens in drug-nitrite interactions. The results are summarized in Table 6. Results of antimutagen formation of fiber demonstrated that fiber prepared in this experiment was efficient nitrite and/or mutagen scavenger under acidic condition. The data presented here confirm such an idea that the precursors of toxic substance can be adsorbed by fiber, hence, minimize the hazard to human being. Drug formulation in future may require the addition of fiber in order to scaverge nitrite if such formulation is to be taken in the presence of hydrochloric acid during food digestion.

Interaction of drug and nitrite does not occur alone in the gastrointestinal tract. Some proteins found in the digestive tract effectively scavenged nitrite and thus inhibited the formation of mutagens from nitrosation (Kato and Kikugawa, 1992). In this study bovine serum albumin was used to study the effect of protein on the formation of mutagen. The results (Table 8) show that all nitrite treated drugs are negative in Ames test in the present of imitated gastric condition mixture (containing bovine serum albumin, sodium chloride and sodium thiocyanate). It was found that high concentrations of protein similar to those found in the digestive tract effectively scavenged nitrite and thus inhibited the formation of mutagen due to nitrite (Kato and Kikugawa, 1992). Kato and Kikugawa stated that some amino acids could also convert nitrite into nitrogen gas; proline was converted into non-mutagenic nitrosoproline, thiocysteine to S-nitrosocysteine, tryptophan to weakly mutagenic nitrosotryptophan and tyrosine to non-mutagenic diazotyrosine.

Table 6 Mutagenicity of nitrite treated drugs in acidic condition (pH 3-3.5) on *S. typhimurium* TA98 and TA100 compared with drug samples treated with nitrite and fiber.

Chemicals	Amount (μg/plate)	No. of His^+ revertants/plate			
		TA98		TA100	
		without fiber	with fiber	without fiber	with fiber
Bromazepam + $NaNO_2$	0	13 ± 1	13 ± 1	165 ± 6	165 ± 6
	200	**135 ± 6**	42 ± 6	**320 ± 15**	105 ± 8
	400	**280 ± 23**	51 ± 3	**750 ± 7**	150 ± 9
	800	**450 ± 20**	96 ± 7	**1290 ± 53**	192 ± 15
Chlordiazepoxide + $NaNO_2$	0	13 ± 1	13 ± 1	165 ± 6	165 ± 6
	200	**37 ± 1**	22 ± 3	114 ± 5	92 ± 4
	400	**60 ± 4**	20 ± 1	**230 ± 7**	95 ± 4
	800	**244 ± 5**	35 ± 3	**447 ± 4**	100 ± 3
Cimetidine + $NaNO_2$	0	ND	ND	117 ± 8	117 ± 8
	200	ND	ND	**250 ± 10**	120 ± 3
	400	ND	ND	**846 ± 11**	256 ± 6
	800	ND	ND	**1670 ± 41**	404 ± 7
Isoniazid + $NaNO_2$	0	19 ± 1	19 ± 1	117 ± 8	117 ± 8
	200	**560 ± 9**	243 ± 7	**440 ± 7**	190 ± 5
	400	**1120 ± 21**	540 ± 7	**760 ± 8**	205 ± 10
	800	**2405 ± 13**	920 ± 14	**1104 ± 14**	293 ± 4
Ranitidine + $NaNO_2$	0	ND	ND	140 ± 5	140 ± 5
	200	ND	ND	**270 ± 4**	158 ± 13
	400	ND	ND	**372 ± 5**	155 ± 7
	800	ND	ND	**504 ± 6**	290 ± 30

Results show in means ± SD (n=3). Aminopyrene is positive control and DMSO is negative control for Ames test.

Relationships between chemical structure and mutagenic properties

On the basis of well-known structure activity relationship relating to carcinogenicity in the nitrosamine series (Williem and Louis, 1975), the carcinogenic properties of the nitroso derivatives of the amine drugs could be predicted. To our knowlegde, no experiment evidence in support of such a view is available at present. It seem wise to consider the above mentioned amine drugs (and all other nitrosatable drugs prescribed over longer periods. Moreover, *in vivo* conversion of such new drugs into carcinogenic nitrosamines and specially designed animal experiments should be included to test this possibility. Many drugs containing a secondary amino or amido group potentially capable of reacting with nitrous acid to form a relatively complex N-nitroso derivatives (Gillatt *et al.*, 1983). Some other drugs are tertiary amines with either a dialkylamino substituent or an alkyl or other group attached to a ring tertiary nitorgen atom. Bromazepam has the highest specific mutagenicity (Table 8). It is suggested that bromazepam is easily react with nitrite and produce mutagens. Gillatt and his colleages (1983) stated that such amine structure was hardly nitrosated : this lack of susceptibility may reflect the delocalization of the lone pair electrons as a result of proximity or benzyl group. Therefore, the mutagenicity found in this experiment may belong to other non-nitroso compounds. They may probably be nitro-compounds which some of them are direct mutagen and can be detected in the Ames test without metabolic activation. Manoonpol (1994) suggested that PAHs were easily modified to be direct mutagen after interacting with nitrite in acid condition. It was suggested that the new substances mostly exhibited direct acting mutagenic activity toward both TA

Table 7 Mutagenicity of nitrite treated drugs in acidic condition on *S. typhimurium* TA 98 and TA 100 compared with drug samples treated with nitrite in gastric condition.

Chemical	Amount	No. of His^+ revertants/plate acidic condition		Amount	No. of His^+ revertants/plate gastric condition (1)	
	(μg/plate)	TA98	TA100	(μg/plate)	TA98	TA100
Bromazepam +$NaNO_2$	0	13 ± 1	165 ± 6	0	13 ± 2	130 ± 5
	200	**135 ± 6**	**320 ± 15**	183	20 ± 3	135 ± 3
	400	**280 ± 23**	**750 ± 7**	366	26 ± 2	193 ± 5
	800	**450 ± 20**	**1290 ± 53**	732	30 ± 3	240 ± 5
Cimetidine +$NaNO_2$	0	ND	117 ± 8	0	ND	130 ± 5
	200	ND	**250 ± 10**	188	ND	121 ± 2
	400	ND	**846 ± 11**	375	ND	162 ± 2
	800	ND	**1670 ± 41**	750	ND	198 ± 2
Chlordiazepoxide +$NaNO_2$	0	13 ± 1	165 ± 6	0	13 ± 2	130 ± 5
	200	**37 ± 1**	114 ± 5	179	16 ± 2	119 ± 3
	400	**60 ± 5**	**230 ± 7**	357	11 ± 2	168 ± 3
	800	**244± 5**	**447 ± 4**	714	29 ± 1	209 ± 2

Results show in means ± SD (n=3). Aminopyrene is positive control and DMSO is negative control for Ames test.

(1) gastric condition mixture concists of 300 mg/l BSA, 0.3 g/l of sodium thiocyanate and 2g/l of sodium chloride

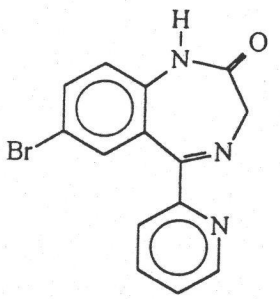
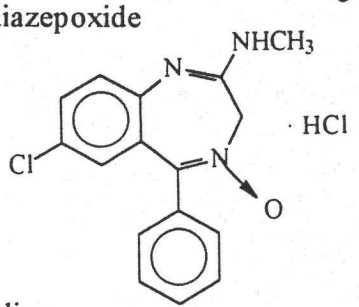
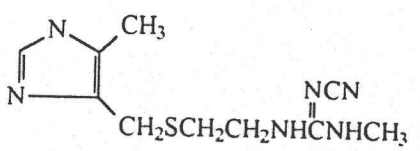
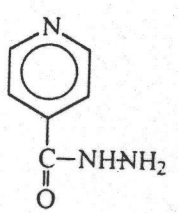
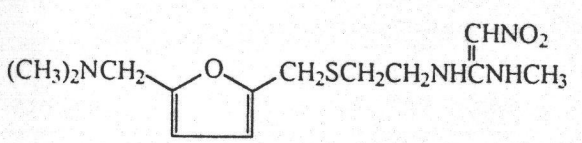
Table 7 (cont.) Mutagenicity of nitrite treated drugs in acidic condition on *S. typhimurium* TA 98 and TA 100 compared with drug samples treated with nitrite in gastric condition.

Chemical	Amount	No. fo His^+ revertants/plate acidic condition		Amount	No. of His^+ revertants/plate gastric condition (1)	
	(μg/plate)	TA98	TA100	(μg/plate)	TA98	TA100
Isoniazid	0	19 ± 1	117 ± 8	0	13 ± 2	130 ± 5
	200	**560 ± 9**	**440 ± 7**	183	16 ± 2	109 ± 3
	400	**1120 ± 21**	**760 ± 8**	366	11 ± 2	168 ± 3
	800	**2405 ± 13**	**1104 ± 14**	732	29 ± 1	231 ± 2
ranitidine	0	ND	109± **6**	0	ND	130 ± 5
	200	ND	141± 6	188	ND	109 ± 3
	400	ND	**261± 5**	375	ND	168 ± 3
	800	ND	**426 ±5**	750	ND	231 ± 2

Results show in means ± SD (n=3). Aminopyrene is positive control and DMSO is negative control for Ames test.

(1) gastric condition mixture concists of 300 mg/l BSA, 0.3 g/l of sodium thiocyanate and 2g/l of sodium chloride

Table 8 Specific mutagenicity of positive samples.

Chemical	Specific mutagenicity	
	TA 98	TA 100
Bromazepam 	1765	3260
Chlordiazepoxide 	784	1612
Cimetidine 	ND	56
Isoniazid 	45	21
Ranitidine 	ND	28

98 and TA 100. The products were proved not to be N-nitroso compounds since the information of mutagenic N-nitroso compounds required nitrosable substances such as amines and amides but studied PAHs did not contain nitrogen atom; therfore, the mutagenicity of the products after nitrite treatment must be other compounds not related to N-nitroso ones.

Although no information on the chemical structure or properties of the reaction products is available. The results of the screening procedure described above afford useful information as to whether or not a drug or chemical form any hazardous products in reaction with nitrite. In a report of a WHO meeting held in Geneva I 1978, Coulston (1980) stated that the development of a systemic approach to the investigation of potential hazard of nitrosatable drugs must be based on two considerations, namely the reactive speed at which drugs undergo N-nitrosation under standardized condition *in vitro* and the carcinogenicity of the more strongly-relating compounds in suitable animal models. Further criteria upon which the selection of drugs for evaluation should be made are those of the doses and periods of use, particularly when children are involved. Overall, therefore, it is apparent that many drugs containing nitrogen atoms are capable of forming mutagen but under standardized conditions their susceptibilities to attack by nitrous acid vary widely. It is proposed to undertake further studies simulating as closely as possible the conditions within the human stomach.

In conclusion, the results presented in this paper should be interpreted solely as indicating that some drugs can induce mutagens when react with nitrite in foods. It ought to be careful on using such drugs with meal or after meal which

composed of nitrite containing food for long term treatment. The consumption of fruits and vegetables will provide a good source of nitrite scarvenger and can protect the consumer from the mutagens derived from nitrite treated drugs during the stomach digestion. In addition modification on drug formulation by the addition of suitable dietary fiber is a choice to prevent mutagens from drug-nitrite interaction.