CHARACTERIZATION OF A SUBSTANCE FROM CHINESE-STYLE SALTED SHRIMP PASTE THAT CAUSES DNA SINGLE-STRAND BREAKS IN HUMAN CELLS

by

Don Carlos Brunson

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Signature of Author.

Division of Toxicology
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ABSTRACT

Consumption of Chinese-style salted shrimp paste has been correlated with development of nasopharyngeal carcinoma in Northern China. Preliminary experiments demonstrated that extracts of shrimp paste contained biologically-active substances. Solid-phase extraction by polystyrene columns, followed by divalent cation retention chromatography, organic precipitation with chloroform, cation exchange chromatography, inorganic precipitation with base, a second cation exchange chromatography procedure, and anion exchange chromatography yielded a biologically-active fraction, DOW/AG, which is believed to be free of metals. The biologically-active constituent in DOW/AG represents about 200-250 ppm salted shrimp paste has chemical characteristics consistent with a Zwitterion. Cell cycle analysis and analysis of cytotoxicity and DNA damage elicited by DOW/AG led to elucidation of the mechanism of cell death. Exposure of cells to the active constituent in DOW/AG induces DNA SSB and, at much lowers levels, DNA DSB. DNA strand breaks induce G_0-G_1 arrest thereby delaying the progression of G_0-G_1 cells into S phase. DNA single strand breaks elicited do not interrupt the cell cycle of cells in S, G_2, or M phases. Repair or attempted repair of strand breaks signals a checkpoint coupled to the repair event, and the cell cycle checkpoint system increases the probability that release into S phase does not occur until after repair is completed. Cells containing unrepaired DNA are arrested in G_0-G_1, accumulate, and eventually die.

Thesis Supervisor: Dr. Steven R. Tannenbaum

Title: Professor of Toxicology and Chemistry

DEDICATION

To the members of my family.....

Mom

Dad

Joann

Steve

Joshua and Joseph

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This is the place where I get to say my "thank-you's" to all of those people who helped to make me and my research successful. Over the many years (and I do mean many) that have been at MIT, I have interacted with many people who have been a positive influence on me.

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LIST OF ABBREVIATIONS

DSB, double-strand breaks

NPC, nasopharyngeal carcinoma

SSB, single-strand breaks

Chapter 1

INTRODUCTION

Chinese-style salted fish is commonly eaten throughout southeast China, Indonesia, Malaysia. Epidemiological case-control studies have repeatedly linked consumption of this food to the high incidence of nasopharyngeal carcinoma (NPC) in this region as well as in other southern Chinese populations (3-11). The hypothesis that salted fish might be a risk factor for NPC was presented by Ho (4) who noted a higher incidence of NPC among southern Chinese fisherman and their families than among people who lived further inland.

Ning et al. investigated the epidemiology of NPC in Tianjin, a low-risk region for NPC located in northern China (24). In their study, consumption of Chinese-style salted fish was again found to be a major risk factor for NPC, and noted that consumption of Chinese-style salted shrimp paste, another salt-preserved food, was reported to be a major risk factor for NPC. The occurrence of NPC with respect to salted food consumption was influenced by three factors: the age at which consumption began, the frequency of consumption, and the lifetime duration of consumption.

Extracts of salted fish have been found to contain low levels of N-nitroso compounds such as N-nitrosodimethylamine and N-nitrosodiethylamine (35,36), and biological studies have found that exposure of bacterial cells to extracts of salted fish induced both forward and reverse mutations at greater than background levels (37).

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Further studies using rats fed extracts of salted fish found that urine excreted from these animals was also mutagenic to bacteria. Nasal cavity and/or paranasal cavity tumors were developed in the salted fish group (25,26), whereas the development of spontaneous nasal cavity tumors has never been reported in rats (27).

The combination of the high degree of organ specificity associated with NPC, the dose-time dependency associated with the development of the disease, the detection of biological activity in bacteria treated with extracts of salted fish, and the development of nasal cavity tumors in rats fed salted fish strongly suggest that this food contains genotoxic substances which directly or indirectly influence the development of nasopharyngeal carcinomas.

This thesis will investigate the identity of biologically-active material isolated from extracts of Chinese-style salted shrimp paste. It will also investigate the types of biological activities elicited by this material and some possible explanations of how these activities occur. Chapter 2 presents the literature review of the epidemiology of NPC. Chapter 3 presents results of salted fish studies conducted in the laboratory prior to the work in this thesis. Chapter 4 presents chemical and biological evidence justifying the use of salted shrimp paste as a model food for developing a method for purifying genotoxic substances from salted fish. Chapter 5 presents the objective and specific aims of the thesis. Chapter 6 presents further results from investigations toward purifying biologically-active substances from salted shrimp paste. Chapter 7 details the biologically-activities elicited by the purified salted shrimp paste material. and the order

in which these activities occur. Chapter 8 is a summary and conclusion regarding the information presented in this thesis, and Chapter 9 presents suggestions for continuing studies on salted shrimp paste.

Chapter 2

THE EPIDEMIOLOGY OF NASOPHARYNGEAL CARCINOMA

Nasopharyngeal carcinoma (NPC) is a spectrum of diseases. The World Health

organization has classified NPC into three categories: type I, type II, and type III. Type I

NPC is a squamous cell carcinoma which has definite and abundant keratinization. Type

II NPC has no keratinization by light microscopy, yet it is not undifferentiated. Type III

NPC has undifferentiated cells with vesicular nuclei and prominent nuclei (1). Although

other tumors can occur in the nasal cavity, NPC is specifically defined as a carcinoma

which occurs in the nasopharynx (1).

NPC is a rare disease in most parts of the world. The age standardized incidence

for NPC in the world population is less than 1 case per 100,000 person-years (2). In

southeast China, the incidence rate for the disease has been estimated to be 10-20 cases

per 100,000 person-years (3-11), whereas in Northern Chinese provinces, 2-3 cases per

person years have been noted (2-13).

Ebstein-Barr virus (EBV) transformation of epithelial cells was thought to be the

cause of NPC in both northern and southern China because most NPC patients tested

demonstrated a specific immune response to EBV antigens (14-16). This response,

however, occurs mainly for the non-keratinizing form of the disease (1). Subsequent

reports on NPC proved that EBV could not be the major cause of NPC. Zeng et al.(14)

observed similar seropositivity to EBV antigens among populations in both high and low-

risk area for NPC in China and that the primary age of primary infection by EBV was the same in both groups. Other studies have suggested that the height of EBV capsid antigen titers observed among NPC patients is only a reflection of tumor mass (17,18), and attempts to infect cells other than B-lymphocytes *in vitro* with EBV have failed (15). The combined results from these reports suggest that EBV alone cannot be the sole cause of NPC in Chinese populations.

In 1972, Ho suggested that consumption of Chinese-style salted fish might be an important risk factor for NPC in southern Chinese. In a high risk area for NPC in southern China, he noted that the incidence rate for NPC was two times higher among fisherman and their families who were known to consume large quantities of salted fish (4). Salted fish is commonly eaten throughout southeast Asia including southeast China, Indonesia and Maylasia. Yu et. al. (19), in a case-control study conducted in Hong Kong, reported significantly elevated risk factors for NPC associated with occupational exposure to smoke dust and chemical fumes. After considering the additional risk for NPC associated with salted fish consumption, none of the adjusted relative risk factors associated with exposure to the inhalants was significantly different, while the relative risk factor associated with salted fish consumption remained highly significant. This supported the hypothesis of Ho which associates a risk for NPC with exposure to an ingestant rather than an inhalant. Salted fish intake has also been an important risk factor for NPC in both Guangzhou, China (20,21), and Guangxi, China (22), and among Maylasian Chinese (23). Although salted fish is a much less important staple in the diet

of people in northern China, consumption of both salted fish and salted shrimp paste were found to be etiologically important risk factors for NPC in Tianjin, northern China (24).

Salted fish has been shown to have biological activity in whole animals. Huang et al. noted the induction of tumors in the nasal cavities of Wistar rats fed salted fish (25). Twenty inbred rats aged one month were fed steamed salted fish for five days out of a week for six months and then given fish head soup for five days out a week during the remaining time span of the experiment. All animals were killed after two years or when moribund. Four of the twenty rats developed nasal cavity and paranasal cavity carcinomas, but no tumors developed in the six control rats which were fed rat chow.

Nasal cavity tumors were also induced in rats which consumed salted fish quantities in the range of consumption by Cantonese (26). In these investigations, 221 Wistar rats were separated into three equal groups: a high-dose group, a low-dose group, and a control group. The ratios of salted fish:rat chow fed to the high and low-dose groups were 1:3 and 1:5, respectively. After the first six months of salted fish consumption, significant differences in weight were noted among male rats in the high-dose group relative to the control group. Differences in weight were also noted among female rats in the high-dose group six months later (after one year). After eighteen months of salted fish consumption, the two experimental groups were transferred to a rat chow only diet. Two months later, no difference in weight was noted among the three groups of rats. At the termination of the investigations (after three years), three rats from the high-dose group and one from the low-dose group had developed nasal cavity tumors.

No nasal cavity tumors were observed in control rats. The observed rates of tumor

occurrence in the nasal cavities of these animals were similar to the rate of NPC

occurrence in Cantonese. These observations supported the hypothesis that salted fish

consumption was causally linked to NPC development because spontaneous nasal cavity

tumor development has never been reported in rats (27).

Using closely matched NPC patients and control subjects, epidemiological studies

(20-24) have revealed that the following factors strongly influence the occurrence and age

of onset of NPC: 1) the age at which salted fish consumption begins; 2) the frequency of

salted fish consumption; 3) the amount of salted fish consumed; and 4) the duration of

salted fish consumption. These factors demonstrate a dose and time dependence and

suggest that salted fish preparations contain substances which are capable of transforming

epithelial cells in the nasopharynx.

Fong et al. investigated the mutagenicity of salted fish toward bacteria using

salted fish extract and urine from rats fed a mixture of salted fish and rat chow (28).

DMSO extracts of salted fish were tested for mutagenicity toward Salmonella

typhimurium TA98 and TA100 both with and without activation by Arochlor 1254-

induced rat liver PMS and after pre-incubation of bacteria with PMS. DMSO extracts of

salted fish elicited dose-response mutagenicity toward both TA98 and TA100 under all

three conditions, and mutagenicity varied between strains. TA100 was found to be more

sensitive that TA98; and generally, pre-incubation with PMS was more strongly

enhancing than PMS alone which was more strongly enhancing than no activation. After

100-fold concentrations, urine from experimental rats fed a mixture of salted fish and rat chow was mutagenic to TA98 and TA100, although urine from control rats fed rat chow with salt was not. After experimental rats were transferred to a diet consisting of rat chow with salt, mutagenic activity of their urine was greatly diminished compared to the activity during periods of salted fish consumption. These results strengthened the hypothesis that salted fish contains substances which are responsible for the induction of NPC.

Although 90% of all cases of NPC can be attributed to salted fish consumption at an early age (19), the specific role of salted fish in the etiology of NPC remains unclear; furthermore, EBV infection of nasopharyngeal epithelial cells, as a risk factor, cannot be dismissed. Shao et al. investigated the biological activity of a number of foods consumed in high-risk areas for NPC, including southern China, Tunisia, and Greenland (29). Aqueous, hexane, and ethyl acetate extracts were made from each food. All extracts were tested for EBV activation in Raji cells which had been latently infected with the virus. Results from these experiments revealed that aqueous, salted fish extract elicited dose-dependent activation of EBV early antigens in Raji cells. Although two other foods, harissa and qaddid, could activate EBV early antigens in a dose-dependent manner, these foods are not indigenous to the diet of people in southern China. Also, only aqueous food extracts caused EBV early antigen activation. These results suggest that salted fish might have a promotional role in the causation of NPC.

Another hypothesis suggested by E. E. Henderson (30) is that salted fish, as an environmental factor, might act synergistically with EBV and exert an EBV-carcinogen physicochemical effect which would lead to induction. Yu et al. have demonstrated that salted fish is a rat nasal cavity carcinogen (26). Carcinomas that were induced, however, were not NPCs. If, for example, rat nasopharyngeal cells lack either enzymes which are capable of metabolizing nasopharyngeal procarcinogens present in salted fish or DNA sequences which, when disrupted, lead to transformation in these cells, this would explain why only epithelial cells in the rat nasal cavity were transformed. The role of EBV in NPC might, therefore, be as an enhancer of transformation. Evidence for this type of chemical-virus interaction was reported by Henderson and Ribecky (31) who noted that human umbilical cord blood lymphocytes which had been pretreated with N-methyl-N'-nitro-N-nitrosoguanidine prior to EBV infection *in vitro* were 5-10 fold more readily transformed by EBV. Considering the many reports on NPC, it is clear that much more research is needed before the exact role of salted fish in the etiology of NPC will be understood.

In summary, the literature reports reviewed represent a sequence of occurrences which indicate that Chinese-style salted fish contains genotoxic substances. First, a hypothesis linking salted fish to NPC was presented after the EBV-NPC association inadequately explained the prevalence of the disease in southeast China. Next, salted fish consumption was shown to be an important risk factor for developing NPC in high and low-risk areas for NPC in China. Two different studies demonstrated that salted fish

consumption caused nasal cavity tumors in rats. Finally, a hypothesis and evidence

suggesting that salted fish contains biologically active substances were presented.

Chapter 3

CONFIRMATION OF THE EXISTENCE OF BIOLOGICALLY-ACTIVE

SUBSTANCES IN CHINESE-STYLE SALTED FISH

3.1 Introduction

Epidemiological and biological studies concluded that salted fish contained

biologically-active substances. In order to isolate any material, a strategy to extract and

purify such material was required. Preliminary investigations conducted in the

Tannenbaum laboratory yielded results which were crucial to undertaking research in this

thesis. This chapter presents a summary of those investigations, most of which have

never been published.

Biological activity was determined using the forward mutation assay in

Salmonella typhimurium TM677 developed by Skopek et al.(33). The assay was

performed both with and without metabolic activation by Arochlor 1254-induced rat liver

post-mitichondrial supernatant (PMS). Further details regarding this assay are discussed

later in this thesis.

3.2 Selection of Solvent for Optimum Extraction

Research on this project began with investigations toward determining what solvent would be used to achieve optimal recovery of biologically-active material from salted fish.

3.2.1 Methodology

Purification

Salted fish was homogenized in water, centrifuged to remove insoluble material, filtered through glass wool to eliminate fine particles, and subjected to solid phase organic extraction by being passed through a column containing XAD-2 non-ionic polystyrene resin. The column was washed with water, and unbound material and the water wash were combined, concentrated to near dryness and resuspended at a specific concentration. Bound material was eluted from the column with acetone, then methanol, and the two organic washes were combined and concentrated to near-dryness. The residue was resuspended in a minimum volume of water and extracted three times with ethyl acetate (EtOAc) under neutral, acidic, or basic condition. After EtOAc extraction, the residual material was concentrated to near-dryness and resuspended in water prior to testing in the bioassay.

3.2.2 Results and discussion

These experiments showed that most of the mass of water-soluble material after homogenization was not retained by XAD-2 resin. Mutation assay results revealed that aqueous fish extract was mutagenic to TM677 both with and without metabolic activation, although the addition of PMS greatly enhanced mutagenicity. No mutagenicity was induced in TM677 after treatment with any EtOAc fraction of salted fish both with and without metabolic activation. When fractions were frozen and tested days later, previous mutation assay results were reproduced. This study revealed that water was the optimum solvent for extraction of stable, biologically-active material from salted fish.

3.3 Comparison of Mutagenicity of Whole Fish vs. Fish Parts

Investigations were also directed toward understanding whether or not mutagenicity observed was associated with a particular section of the fish anatomy.

3.3.1 Methodology

Salted fish were sectioned as follows

head

lower abdomen

upper abdomen

tail skin

tail flesh

An undissected fish was used as a control.

Purification

Extraction of aqueous, biologically-active material was performed as previously described. Fractions from each portion were resuspended to equal concentrations.

3.3.2 Results and Discussion

Results from these experiments showed that all extracts elicited similar levels of cytotoxicity in TM677. Fish head extract, however, was 15% as mutagenic in TM677 as the other extracts. These experiments showed that, overall, whole fish extraction was a relevant procedure for obtaining biologically-active material from salted fish.

Before further investigation were conducted, the authenticity of biological activity

elicited by salted fish extract was investigated. Mutant TM677 colonies (8-azaguanine

resistant) induced by treatment with fish extract were randomly selected and grown in

medium containing 8-azaguanine. Cells were then treated with salted fish extract to

determine whether or not induced mutant cells were susceptible to cytotoxicity by fish

extract. Lack of susceptibility would imply that mutant colonies were simply selected

and not induced, whereas susceptibility to killing would indicate that mutants were

induced. After treatment by fish extract, mutant TM677 cells were killed at similar levels

as wild-type cells, revealing that they were induced and not selected.

3.4 Biological Effects of Bacterial Enrichment

Chinese-style salted fish is prepared from fresh fish which is gutted, allowed to

autolyze, and is, afterwards, salted. The process is not conducted under controlled

conditions; and therefore, bacterial contamination is a consequence. To determine

whether or not bacterial contamination could influence mutagenicity induced by salted

fish extract treatment, bacterial enrichment of salted fish was attempted.

3.4.1 Methodology

Bacterial Enrichment and Purification

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Salted fish was homogenized in sterile water and incubated overnight at 37 °C.

Purification of the homogenate was performed as previously described.

3.4.2 Results and Discussion

Enrichment of indigenous salted fish bacteria did not significantly enhance

mutagenicity elicited by salted fish extract. This suggested that either sufficient bacteria

to enhance bacteria-mediated reactions associated with salted fish mutagenicity were not

present, insufficient time for such reactions to occur was not allotted, some other factor(s)

needed for such reactions was (were) not present, some combination of the previous

three, all such reactions were already completed, or no such reaction was involved in

mutagenicity elicited by salted fish extract. The presence of a very small number of

bacteria is the most probable cause for the lack of enhancement of activity after bacterial

enrichment. Salting of fish after autolysis (before fish is available in markets) caused

microorganisms present to reside in a highly hypertonic environment. Unless these

organisms were halophilic, their chances of survival were greatly diminished under

salting conditions. Results showing no enhancement of mutagenicity after bacterial

enrichment lead to the conclusion that such investigations were unwarranted because the

addition of other factors, e.g., extended time of incubation, would have created conditions

under which laboratory research (purification) was continued; but salted fish preparations

obtained would not have represented conditions under which the fish were bought,

cooked, or eaten.

3.5 The Effects of Nitrosation on Biological Activity

The high degree of organ specificity associated with NPC along with the detection

on N-nitroso compounds such as N-nitrosodimethylamine and N-nitrosodiethylamine in

salted fish extracts (35,36) raised the question of whether or not mutagenicity of these

extracts is due to this class of compounds.

3.5.1 Methodology

Purification

Salted fish extract was prepared as previously described. An aliquot of fish

extract was further purified by C_{18} reverse-phase chromatography at ambient pressure.

Both aqueous and organic fractions were collected, dried, and resuspended in water prior

to nitrosation.

Nitrosation

Sodium nitrite was added to both fish extract and fish extract fractions, samples were acidified (pH=3), incubated at ambient temperature for 1 hr, and ammonium sulfamate was added to each sample to quench reactions. Samples were neutralized (pH=7) prior to analysis for biological activity.

3.5.2 Results and discussion

Salted fish extract and some extract fractions were mutagenic to TM677 with metabolic activation. After nitrosation, mutagenicity of fish extract was diminished while biological activity in all fish extract fractions was eliminated. The results were reproduced in subsequent experiments and demonstrated, therefore, that the biologically active material were not N-nitroso derivatives.

Chapter 4

BIOLOGICAL ACTIVITY IN SALTED FISH AND SALTED SHRIMP PASTE

4.1 Introduction

Preliminary experiments demonstrated that an aqueous extract of Chinese-style salted fish was biologically active in a bacterial mutation assay both with and without metabolic activation. These results provided biochemical evidence supporting the hypothesis that salted fish consumption is associated with the development of NPC because of the presence of biologically-active substances. Epidemiological studies have also established a positive correlation between consumption of Chinese-style salted shrimp paste and NPC development; but this has only been shown in northern China where salted fish is less available but is an important dietary staple, nevertheless. Access to both foods was easily attainable; so it was decided, therefore, to pursue purification of biologically-active material from preparations of both foods. This chapter presents results and a discussion of experiments directed toward purification of biologically-active substances from extracts of both salted fish and salted shrimp paste as well as characterization of the biological activities.

4.2 Materials and Methods

Water was deionized and distilled prior to use. Organic solvents were HPLC grade. Salted fish (provided by Dr. Mimi Yu of the University of Southern California) was purchased in Hong Kong and made into fish powder as described by Yu et al. (26) prior to shipping to MIT. Shrimp paste was purchased from a local specialty market. Amberlite XAD-2 polystyrene resin was purchased from Aldrich Chemical Company (Milwaukee, WI).

4.2.1 Preparation of Food Extracts

Salted fish powder or salted shrimp paste (250 g) was blended in water (500 ml) and centrifuged at 3000 rpm for 30 minutes. The supernatant was filtered through glass wool, centrifuged again, and passed through a 1.5 cm by 36 cm column of XAD-2 equilibrated in water. Adsorbed material was eluted with 1 L of 1:1 acetone : methanol, stirred, and evaporated to near-dryness. Water was added to dilute the residue to a final volume of 50 ml.

The resuspended residue was diluted with water (250 ml) and filtered through an Amicon YM-2 ultrafiltration membrane (molecular weight exclusion = 1000 daltons) at 4 °C under N_2 pressure. After 150 ml of permeate was collected, 150 ml of water was added to the retentate. This was repeated twice so that the total volume of permeate

collected was 450 ml. The permeate was concentrated to near-dryness and resuspended in a minimum volume of water.

Acetone was added to the permeate (2:5 by volume acetone : permeate), the mixture was vortexed, and the resulting precipitate was removed by centrifugation. The supernatant was evaporated to near-dryness, resuspended in a minimum volume of water, frozen at -80 °C, thawed, and filtered through a 0.22 μm filter.

4.2.2 Biological activity of food extracts

Cytotoxicity in Human Cells

TK6 human lymphoblasts were treated with various doses of salted fish extract for 16 hours. After treatment, cells were centrifuged, resuspended in saline and stained with Nigrosin dye. The number of Nigrosin-excluding cells was recorded for each sample and used to calculate the percentage of surviving cells.

$$\% \text{ survival} = \frac{\text{\# of Nigrosin excluding cells in treated sample}}{\text{\# of Nigrosin-excluding cells in untreated sample}}$$

DNA-Damaging Activity in Human Cells

A modified version of the DNA precipitation assay described by Olive (32), was used to detect DNA strand-breaks in human cells.

4.3 Results

To isolate biologically-active substances from salted fish and salted shrimp paste, a methods was developed to extract large amounts of material. The procedure used to prepare both extracts is summarized in Figure 4.1. Aliquots of both food extracts were lyophilized and weighed. The mass of material recovered after acetone precipitation and concentration was typically 12-13 g from the original 250 g. Salted fish and salted shrimp paste extracts, therefore, contained about 5 % of the mass of fish powder or salted shrimp paste.

Salted shrimp paste and salted fish extracts were cytotoxic and mutagenic to *Salmonella typhimurium* TM 677 in forward mutation assays both with and without metabolic activation by Arochlor 1254-induced rat liver PMS (Figures 4.2a-b and 4.3a-b). Cytotoxicity and mutagenicity were dose-dependent for both extracts. TM 677 cells respond (cytotoxicity and mutagenicity) to a variety of chemical substances (37), and it was difficult, therefore, to determine the type of DNA damage which led to either death or mutations in this cell line. Typically, the equivalent of one gram of fish powder increased the mutant fraction by 16-20 fold above background levels with metabolic activation and 6-10 fold without activation.

Fish Powder or salted shrimp paste

↓

Homogenize, Centrifuge

↓

Filter, Centrifuge

↓

**XAD-2 Column Chromatography
(elute with 1:1 acetone:methanol)**

↓

Mix Eluant, Centrifuge

↓

Remove solvents, Resuspend in Water

↓

Ultrafiltration (< 1000 daltons)

↓

Concentrate Permeate

↓

Acetone Precipitate

↓

**Remove Supernatant, Concentrate
(food extract)**
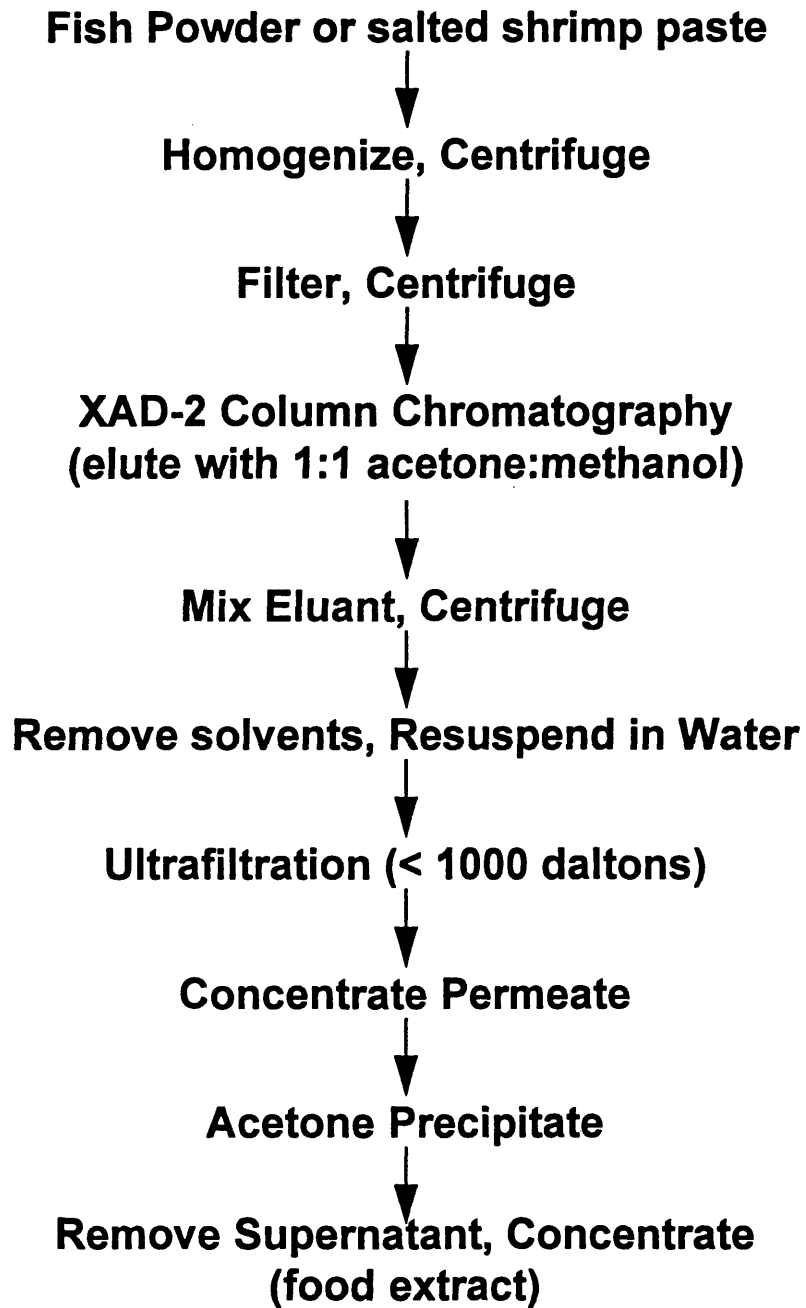
Figure 4.1 Preparation of Salted Fish and Salted Shrimp Paste Extracts
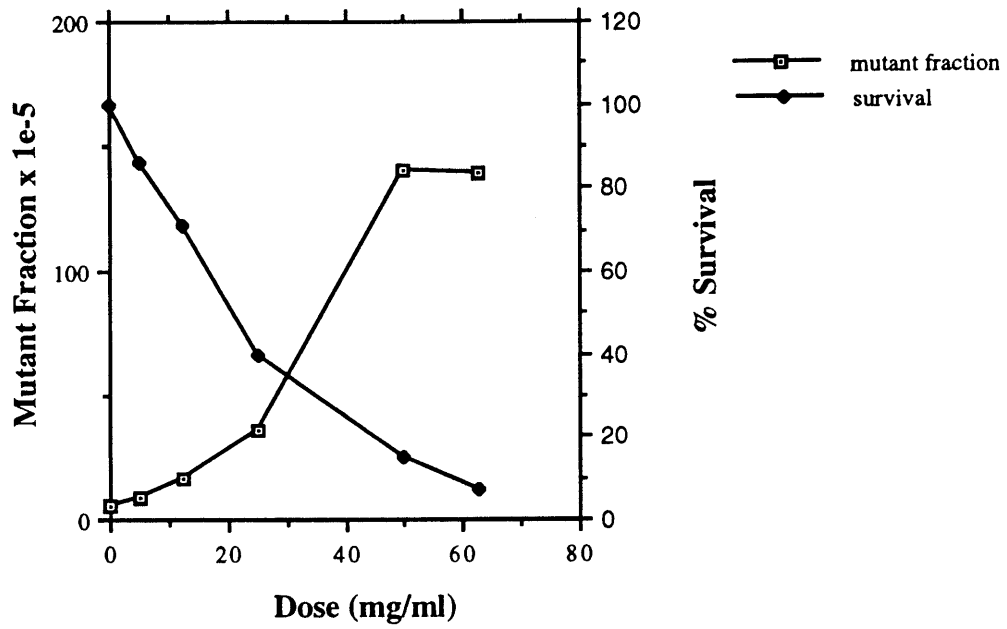
Figure 4.2a Mutagenicity of Salted Fish Extract in TM 677 with Metabolic Activation
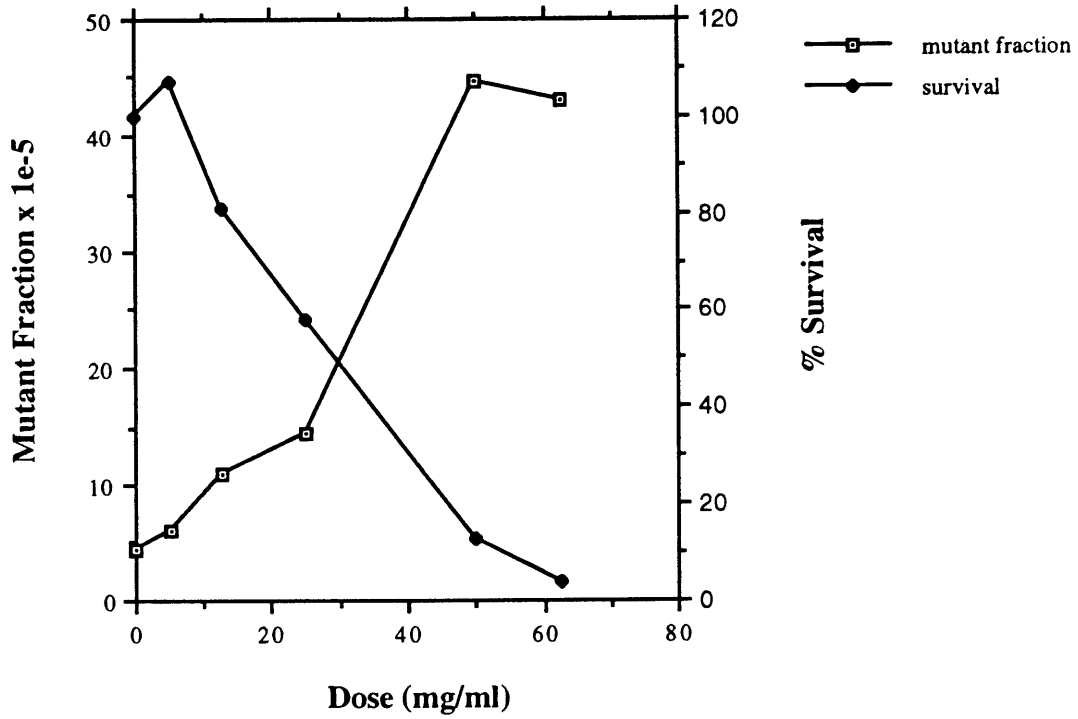
Figure 4.2b Mutagenicity of Salted Fish Extract in TM677 without Metabolic Activation
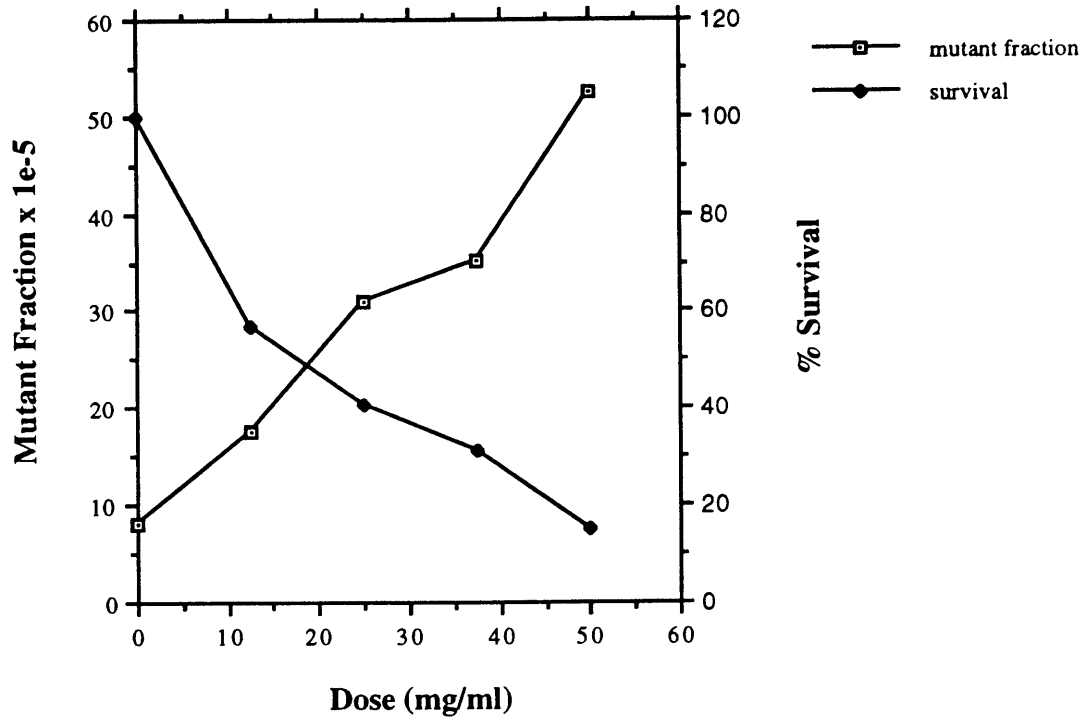
Figure 4.3a Mutagenicity of Salted Shrimp Paste in TM677 with Metabolic Activation
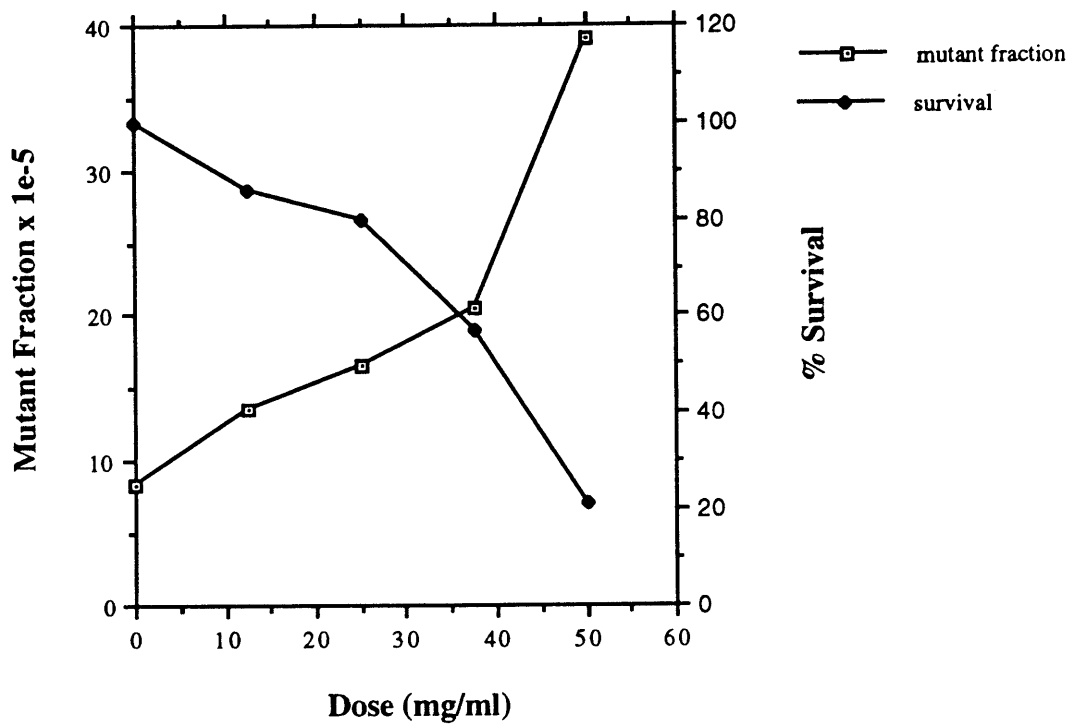
Figure 4.3b Mutagenicity of Salted Shrimp Paste in TM 677 without Metabolic

Activation

The mutagenicity of salted fish was investigated using other types of rat liver PMS to determine whether maximum levels of metabolic activation had been achieved. Arochlor 1254 induced , acetone induced , and uninduced PMS were tested for their enhancing effects on the mutant fraction in bacteria after treatment with salted fish extract. Arochlor 1254-induced PMS was the most strongly enhancing, being 50% more potent than uninduced PMS and twice as potent as acetone induced PMS (Table 4.1).

To determine the source of activation of salted fish extract by PMS, Arochlor 1254-induced PMS was centrifuged by at 10^5 x g to isolate microsomes from cytosol. TM 677 cells were treated with salted fish extract in the presence of unfractionated PMS + cofactors (glucose, NADP, Mg^{2+}, and glucose-6-phosphate-dehydrogenase), cytosol + cofactors, microsomes + cofactors, cofactors only, or in the absence of all of the above (no activation). No difference in levels of mutagenicity elicited was observed between cells treated with cytosol or whole PMS, although both treatments were three times more potent than microsomes (Table 4.2).

Salted fish extract was tested for mutagenicity and cytotoxicity toward TK6 human lymphoblast cells both with and without metabolic activation. Dose dependent mutations occurred in TK6 cells at the hypoxanthine-guanine phosphoribosyl transferase and thymidine kinase loci under both conditions (Figure 4.4a -b). With activation, mutagenicity increased with dose, and cytotoxicity was dose-dependent both with and without metabolic activation.

Table 4.1 Comparison of Different PMS Fraction on Activation of Substances in Salted

Fish Extract

PMS Type	Sample	Mutant Fraction $(x\,10^{-5})$	% Survival
Arochlor 1254	-control	6.8	100
	salted fish extract	133	17
Acetone	-control	7.5	100
	salted fish extract	86.1	22
Uninduced	-control	4.8	100
	salted fish extract	68.5	23

Table 4.2 Comparison of Mutagenicity Enhancement by Cytosol and Microsomes

Sample	Mutant Fraction x 10^{-5}	% Survival
backgound (water + PMS + cofactors)	8.0	100
benzo[a]pyrene + PMS + cofactors	75.2	58
salted fish extract + PMS + cofactors	70.4	21
salted fish extract + PMS - cofactors	32.9	24
salted fish extract + boiled PMS + cofactors	28.3	26
salted fish extract + boiled PMS - cofactors	14.5	40
bezo[a]pyrene + cofactors + cytosol	8.1	78
benzo[a]pyrene + cofactors + microsomes	120	19
salted fish extract + cofactors + cytosol	74.5	13
salted fish extract + cofactors + microsomes	24.5	19
salted fish extract alone	18.7	48
salted fish extract + cofactors	17.8	42
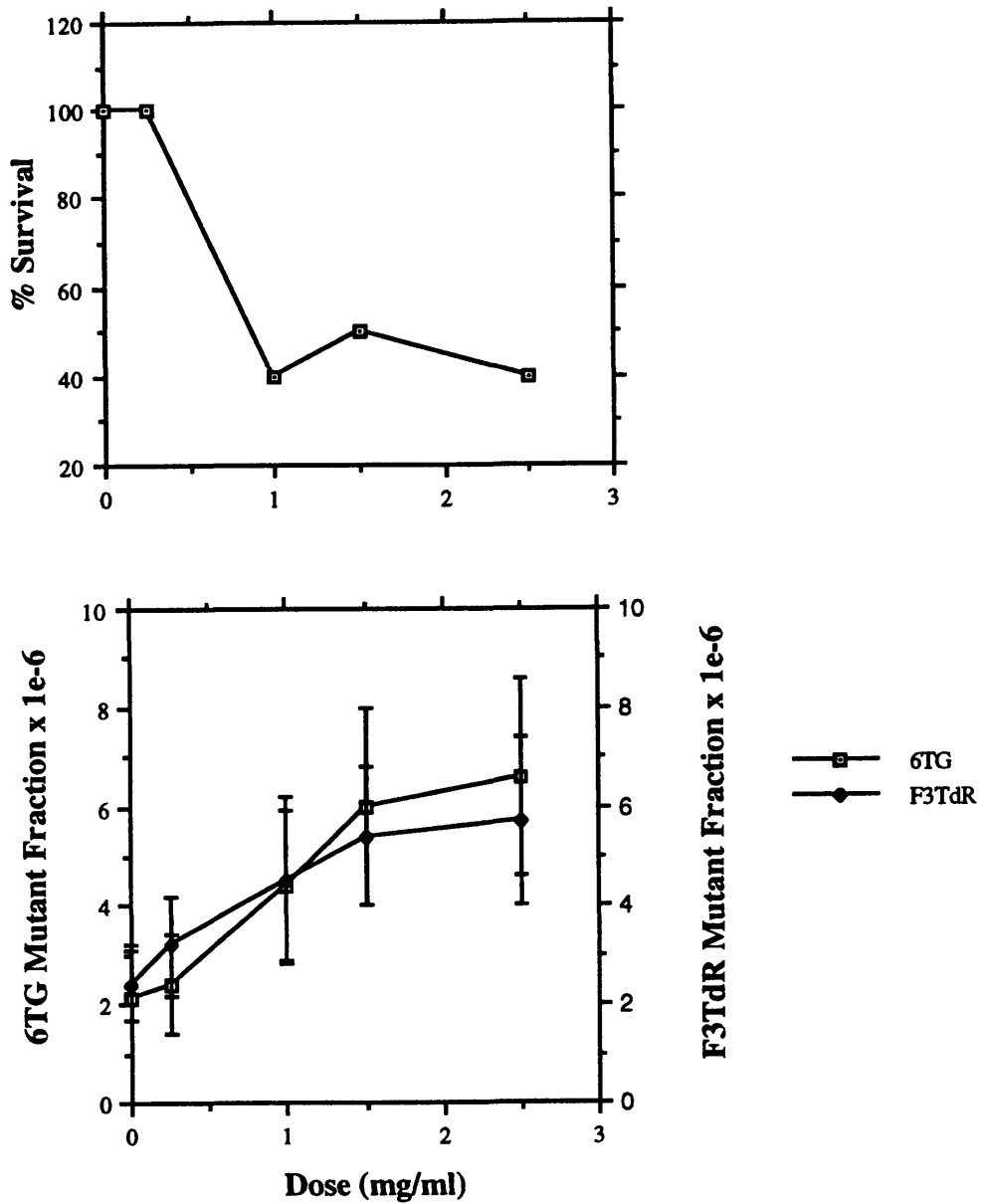
Figure 4.4a Mutagenicity of Salted Fish Extract toward TK6 Human Lymphoblasts with Metabolic Activation
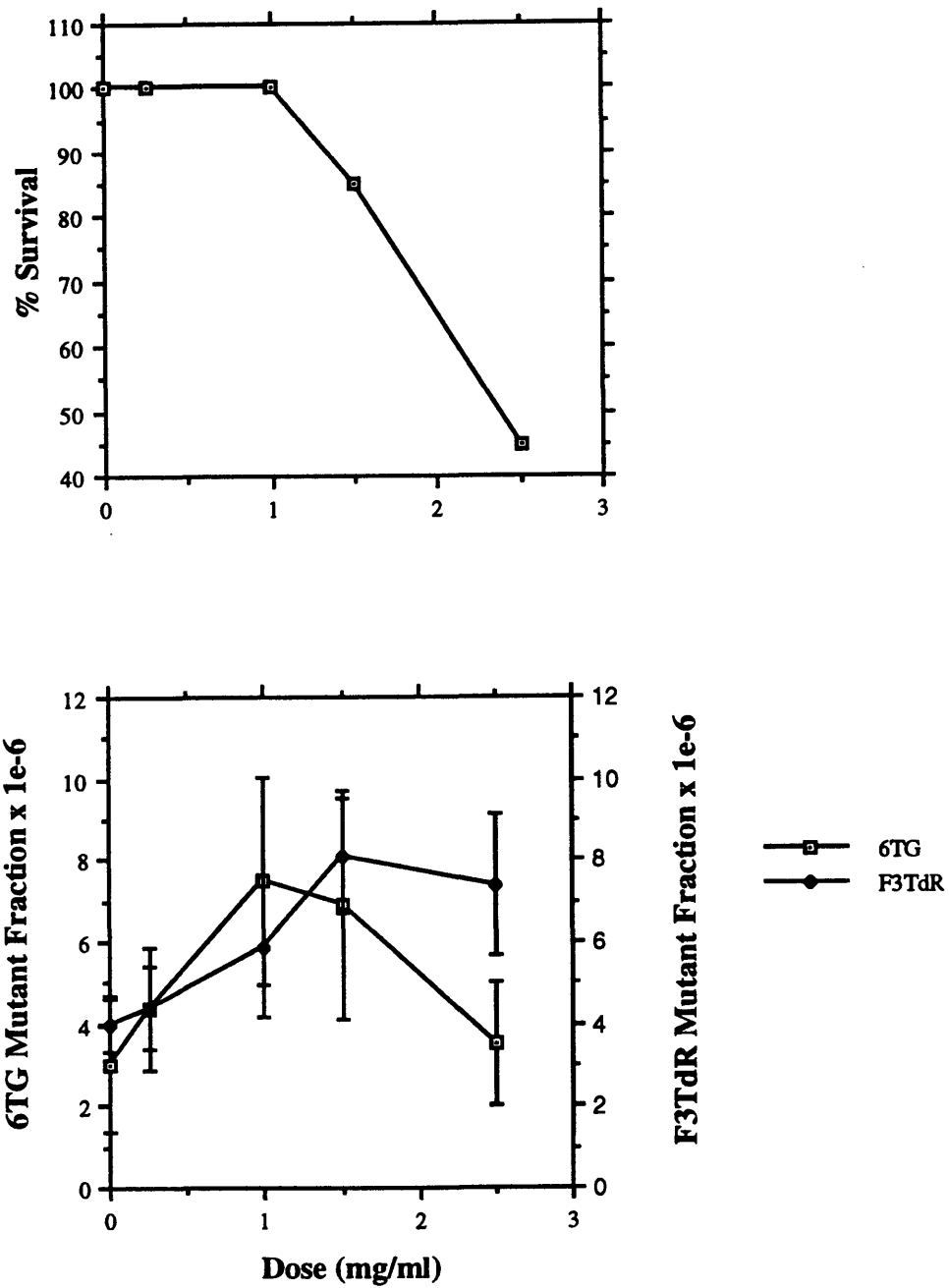
Figure 4.4b Mutagenicity of Salted Fish Extract toward TK6 Human Lymphoblasts without Metabolic Activation

Extracts of both foods were also cytotoxic toward human cells. TK6 cells were treated with salted fish or shrimp paste extract for 16 hrs, and and cell survival was determined by staining with Nigrosin dye and counting the number of Nigrosin-excluding cells. Both salted fish and salted fish extracts caused dose-response cytotoxicity in TK6 cells (Figure 4.5).

Both salted fish and salted shrimp paste extracts caused DNA single-strand breaks in TK6 cells both with and without metabolic activation by Arcochlor 1254-induced PMS. The dose-response relationship was complex (Figures 4.6a-b); low doses of extracts (<100 mg/ml and 50 mg/ml for salted fish extract and salted shrimp paste extract, respectively) elicited DNA single-strand breaks at or near background levels while increasing the dose caused increases in DNA single-strand breakage to a maximum level, followed by a decline.

To further purify genotoxic substances from both food extracts, ambient pressure C_{18} reverse-phase column chromatography was used. Fractionation of both extracts on C_{18} with water as the eluant yielded two major fractions with UV absorbance at 250 nm. Chromatograms are shown in Figures 4.7a-b. Although, 2.75 g of material were loaded onto the columns, only 2.50g and 2.60 g of salted fish extract and salted shrimp paste extract, respectively, were recovered.

The first fraction from each extract contained about 90% of the mass of material recovered from the column, and this material was cytotoxic to TM 677 both with and without metabolic activation (data not shown). No mutagenicity was elicited by material
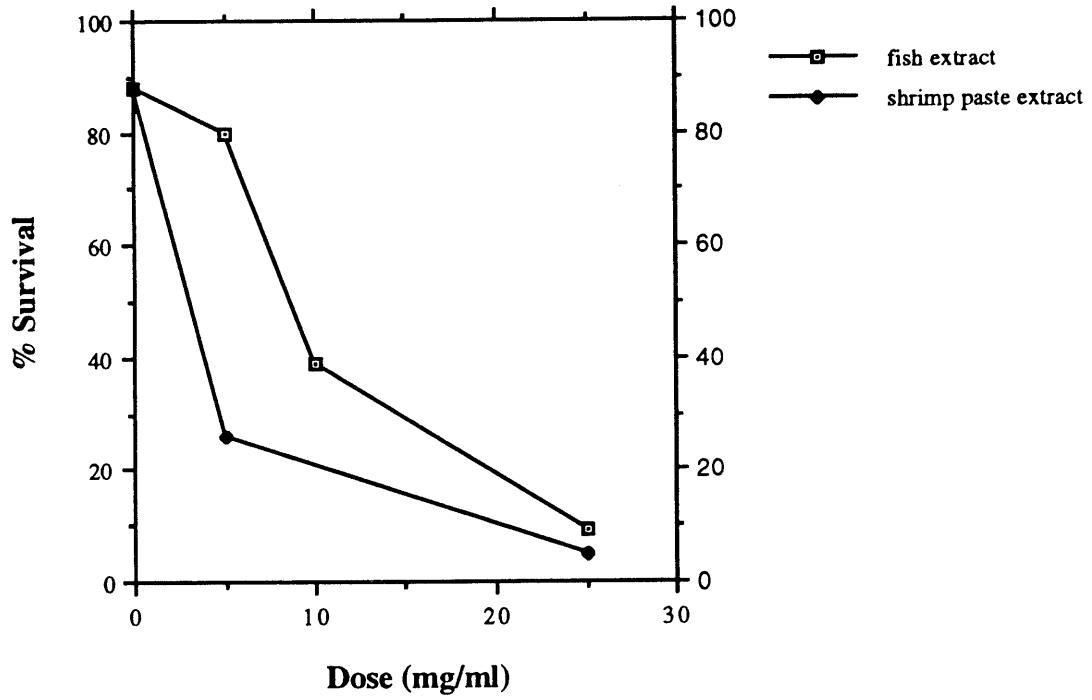
Figure 4.5 Cytotoxicity of Salted Fish and Salted Shrimp Paste Extracts in TK6 Cells
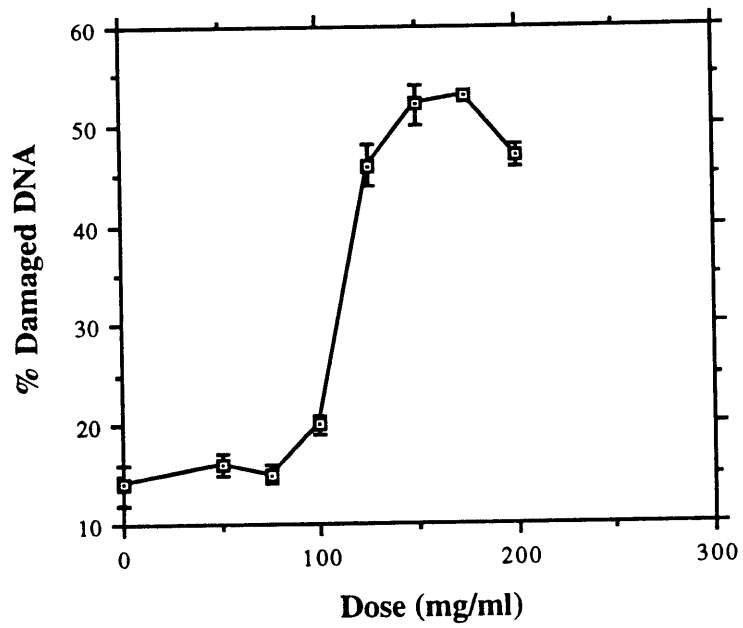
Figure 4.6a DNA Single Strand Breaks Elicited in TK6 Cells by Treatment With Salted
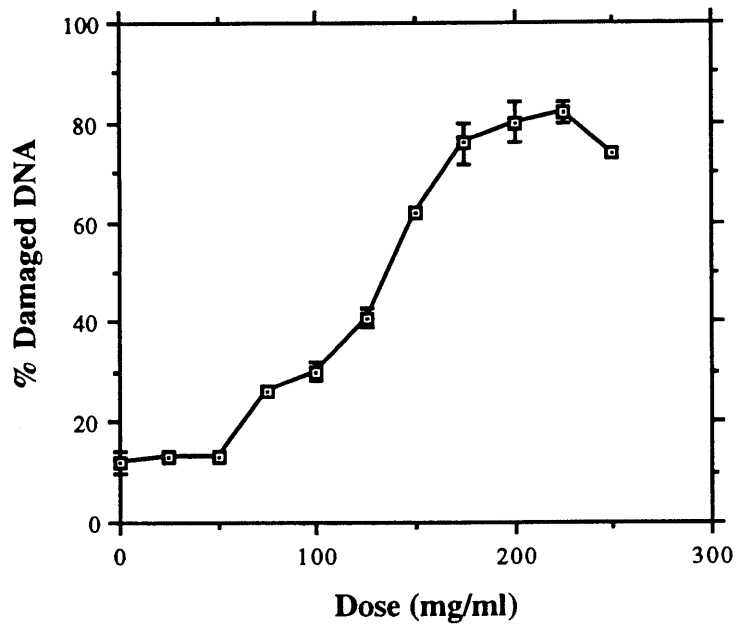
Fish Extract

Figure 4.6b DNA Single Strand Breaks Elicited in TK6 Cells by Treatment With Salted

Shrimp Paste Extract
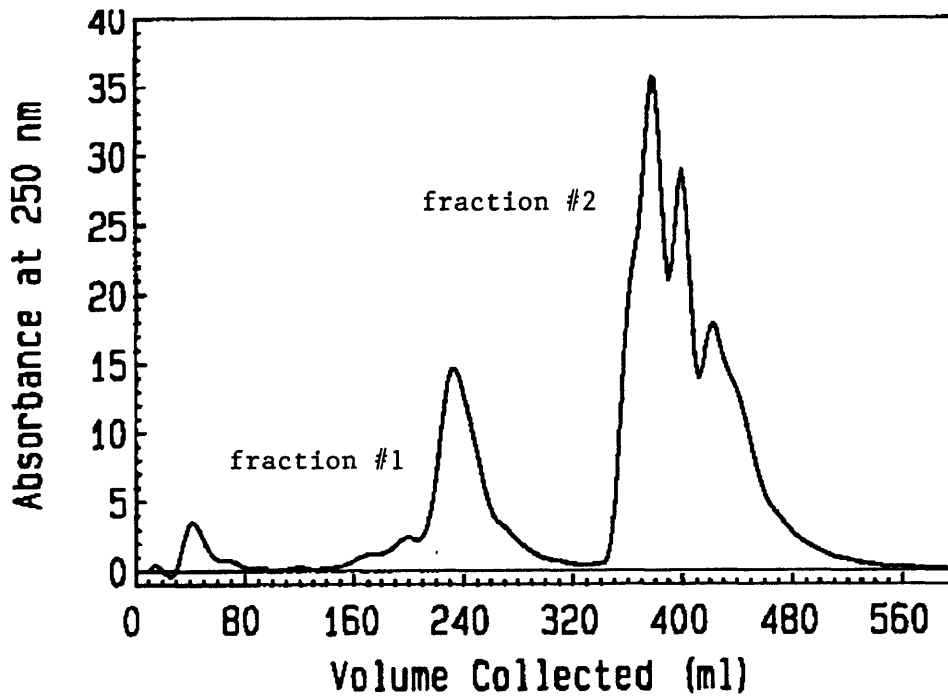
Figure 4.7a Chromatogram Reconstruction: Fractionation of Salted Fish Extract by C_{18}

Reverse-Phase Chromatography
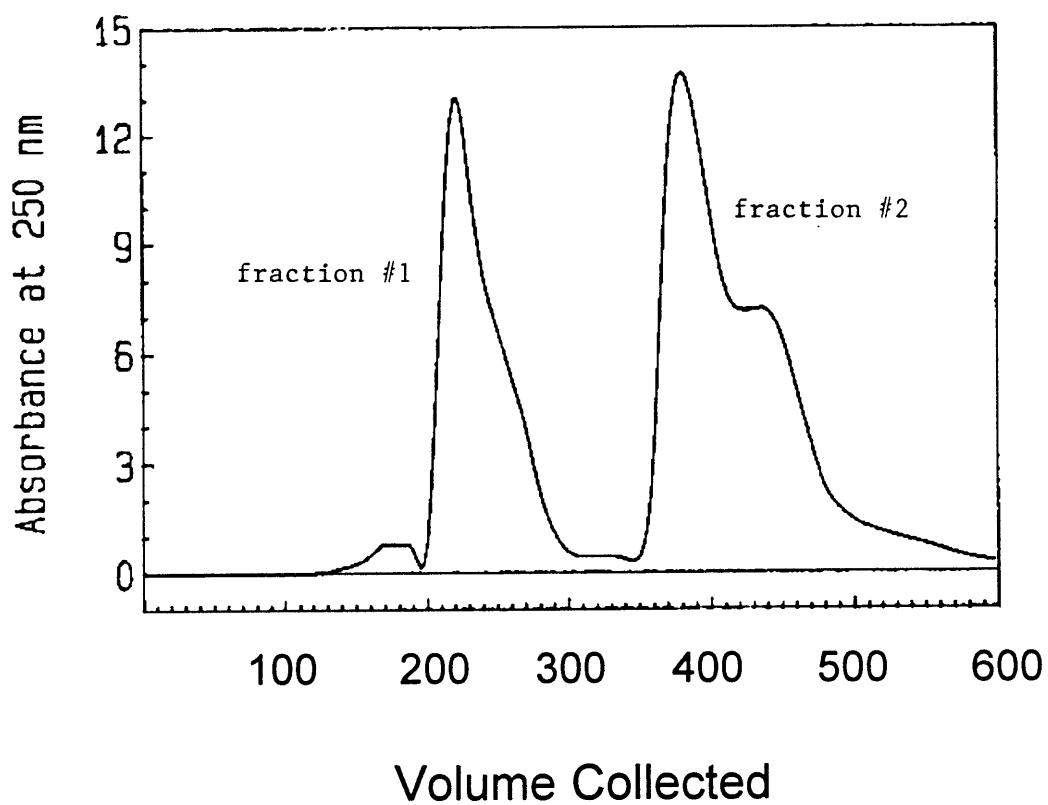
Figure 4.7b Chromatogram Reconstruction: Fractionation of Salted Shrimp Paste

Extract by C_{18} Reverse-Phase Chromatography

in either fraction 1. DNA single-strand breaks were noted in TK6 after treatment with fraction 1 from each extract. Treatment of TK6 cells with this fraction from salted fish extract caused DNA single-strand breaks at higher levels than other salted fish extract fractions, but fraction 1 was slightly less potent than unfractionated salted fish extract.

The second fraction from each extract contained about 5% of the mass of recovered material. Material contained in this fraction was mutagenic but generally, not cytotoxic to TM 677 with metabolic activation. Without metabolic activation, only the salted fish fraction was mutagenic to TM 677. Fraction 2 from neither salted fish extract nor salted shrimp paste extract was cytotoxic to TM 677 or caused DNA single-strand breaks in TK6 cells.

The elution washes in 25 % methanol (30 ml) and 100 methanol (500 ml) were combined and tested for genotoxicity. This fraction from each extract was slightly cytotoxic but not mutagenic to bacteria both with and without metabolic activation, and elicited DNA single-strand breaks in TK6 cells,

An aqueous, low-molecular weight extract of canned tuna was prepared and fractionated in the same manner as the salted fish and salted shrimp paste extracts. C_{18} reverse-phase chromatography on tuna extract yielded three major fractions with UV absorbance at 250 nm. (Figure 4.8). A fourth fraction was obtained by combining a 25% methanol and 100 % methanol elution as previously described. No significant mutagenicity was noted in TM 677 after treatment with tuna extract or fractions of tuna extract, although the combined methanol fraction was cytotoxic in these cells with
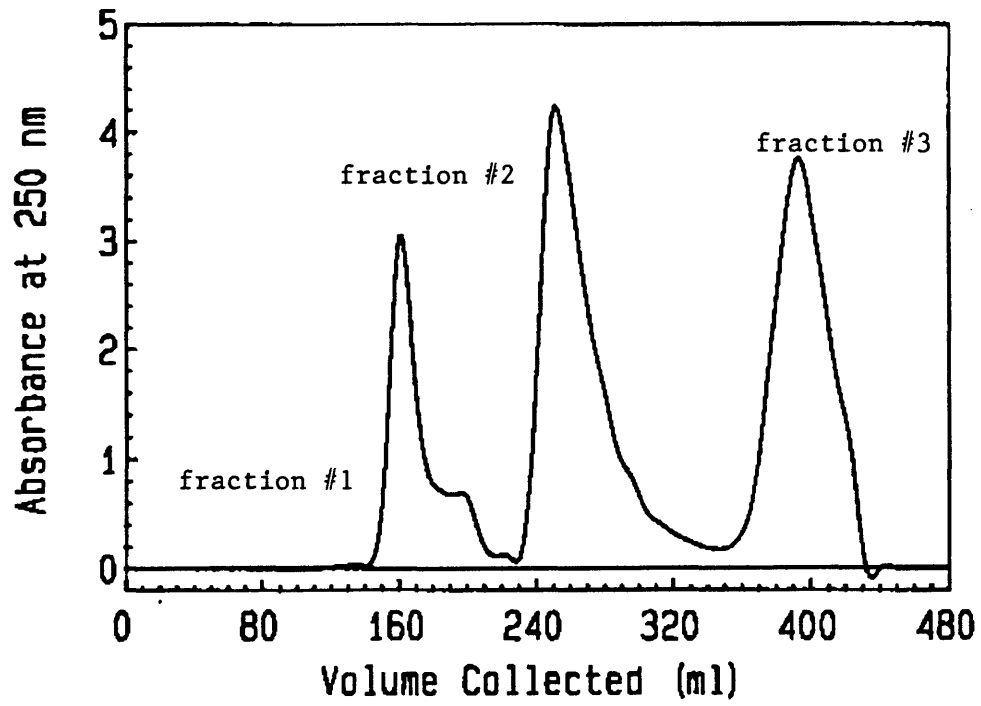
Figure 4.8 Chromatogram Reconstruction: Fractionation of Tuna Extract by C_{18}

Reverse-Phase Chromatography

metabolic activation (Table 4.3). Neither tuna extract nor any fraction of tuna extract elicited any DNA damage in TK6 cells after treatment. The absence of mutagenic activity and DNA-damaging activity proved that biological-activity in salted fish and salted shrimp paste extracts did not result from the isolation procedure.

4.4 Discussion

The initial objective of the research was to investigate a possible biochemical role for salted fish in the etiology of NPC. The investigations were extended to include salted shrimp paste after it was learned that other preserved foods, including salted shrimp paste, were etiologically linked to NPC among Chinese patients (24).

The original goal of this research was to identify a mutagen from extracts of salted fish and salted shrimp paste and to determine whether or not this mutagen was associated with NPC. Results presented in this chapter have demonstrated that aqueous extracts from both foods were genotoxic to bacterial cells both with and without metabolic activation. Mutagenic activity of salted fish extract toward bacteria was enhanced by cytosolic enzymes from Arochlor 1254-induced PMS. Others characteristics of the particular enzymes involved in activation, e.g. their molecular weights, were not determined.

Arochlor1254-induced PMS was the most effective enhancer of salted fish extract mutagenicity in bacteria. Untreated PMS was more potent than boiled PMS indicating

Table 4.3 Biological Activity of Tuna Extract and Tuna Extract Fractions in TK6 Cells

both with and without Metabolic Activation

| Sample | with activation | | without activation | |
	Mutant Fraction x 10^{-5}	% Survival	Mutant Fraction x 10^{-5}	% Survival
background	6.4	100	5.8	100
tuna extract	6.5	94	5.8	103
fraction #1	3.9	74	4.8	101
fraction #2	5.3	85	5.4	94
fraction #3	5.1	94	5.9	106
fraction #4	12.1	24	8.3	75

that proteins were responsible for enhancement. The observation that both cytosol and cofactors were also required suggested that the proteins were cytosolic enzymes. Treatment by cofactors alone did not influence the mutant fraction observed.

The bacterial mutagens are low molecular weight substances associated with the second chromatographic peak from the C_{18} fractionation of either salted fish extract and shrimp paste extract. This fraction elicited low levels of cytotoxicity in bacterial cells, which suggests that both extracts contain low molecular weight substances which are capable of mutating bacterial cells without killing them. It is unknown whether the two foods contain the same mutagen. In addition to these results in forward assays in *S. typhimurium* TM 677, salted fish extract has also been shown to be mutagenic in reversion assays in *S. typhimurium* TA 98 and TA 100 both with and without activation (28). More importantly, isolation of this fraction demonstrated that both extracts contained material which could mutate bacterial cells while causing low levels of cell death. If these mutagens elicited a similar activity in human cells, then this could at least partially explain the role of these foods in NPC etiology.

An extract of canned tuna prepared identically as salted fish and salted shrimp paste extracts was not mutagenic to TM 677 either with or without metabolic activation, This demonstrated that biological activity elicited by salted fish and salted shrimp paste extracts was authentic rather than artifacts resulting from purification.

Salted fish extract was also cytotoxic and mutagenic to TK6 human lymphoblasts both with and without metabolic activation and at two different loci (HPRT and TK).

This further substantiated that salted fish consumption could affect biology associated development of NPCs. Isolation of mutagenic material, therefore, became the primary objective of this thesis. That objective was changed, however, after it was noted that mutagenic material (fractions) were unstable to purification. In numerous experiments, mutagenic fractions isolated from salted fish extract were found to lose potency after as little as two days of storage at -70 °C following purification. The bacterial mutation assay in TM 677 requires 2 days to obtain results, whereas the TK6 human cell mutation assay requires a minimum of 19 days. Purification of labile material would have been exceedingly difficult to complete. Other assays, e.g., the *umu* assay in bacteria (38), were also attempted to screen for biological activity, but these assays were abandoned because of problems with reproducibility, sensitivity, or both. It was decided, therefore, to pursue purification of material which elicited DNA SSB. This material was much more stable than the mutagenic material, the assay required less time than either the TM 677 or TK6 mutation assay, and the semi-quantitative results obtained were sufficient for achieving the desired research goals. In concurrent experiments, salted shrimp paste extract was found to be more potent than salted fish extract in the DNA damage assay. Isolation of biologically-active material from salted shrimp paste that causes DNA SSB in human cells became the objective of this thesis.

Chapter 5

OBJECTIVE AND SPECIFIC AIMS

5.1 Objective

To identify and characterize a substance from Chinese-style salted shrimp paste which is toxic and/or genotoxic to human cells.

5.2 Specific Aims

The first aim of this thesis is to isolate biologically-active material from salted shrimp paste. Epidemiological data have strongly suggested that biologically-active material is contained in this food, and isolation of such material will provide tangible evidence of its existence. The second aim of this thesis is to determine mechanism of the biological activity elicited by the isolated material. This knowledge could prove valuable in determining whether or not and how such material plays a role in the epidemiology of NPC associated with salted shrimp paste consumption.

Chapter 6

ISOLATION OF BIOLOGICALLY-ACTIVE MATERIAL FROM CHINESE-STYLE SALTED SHRIMP PASTE

6.1 Introduction

Purification procedures previously described were developed for preparing aqueous extracts containing low molecular weight substances from salted fish and salted shrimp paste. Research objective changes required the development of a method to isolate very polar material which causes DNA SSB in human cells. This material is among the most polar substances present in salted shrimp paste extract. Further purification of biologically-active fractions was necessary to elucidate the structure of the material of interest. This chapter presents details regarding the isolation and purification of material from salted shrimp paste which causes DNA SSB noted.

6.2 Materials and Methods

Solvents used for purification were HPLC grade. Salted fish powder and salted shrimp paste were obtained as previously described.

6.2.1 Preparation of Shrimp Paste Extract

Shrimp paste (325 g) was blended in water (2 ml/g shrimp paste), centrifuged at 1500 x g for 25 minutes, and the supernatant was filtered through a Fisherbrand G6 Glass Filter Circle (G6 filter). The resulting solution was passed through a 6.0 cm x 36 cm column of Amberlite XAD-2 equilibrated in water. The column was then washed with 1 L of water, and all unbound material (XAD/U) was combined. An aliquot of the material was dried and weighed to determine the amount of material recovered.

6.2.2 Divalent Cation Retention Chromatography

For divalent cation retention chromatography (DCRC), XAD/U was diluted 1:4 with water and loaded onto a 6.0 cm x 36 cm column containing Bio Rad Gel Chelex 100 with H^+ as the counterion. The column was washed with 2.5 L of water to remove any unbound material, and bound material was eluted with 600 ml of 1.2 N HCl and concentrated to dryness. After three 325 g equivalent volumes of shrimp paste extract were processed by DCRC, the bound fractions were combined, concentrated to dryness, and resuspended in a minimum amount of water (Chelex/R). An aliquot of this fraction was dried and weighed to determine the mass of material recovered.

6.2.3 Organic Extraction and Organic Precipitation

Chelex/R was concentrated to dryness and extracted twice in methanol (12 ml/g).

The supernatant was removed and concentrated to one extraction volume. The

supernatant was constantly stirred while chloroform was added in aliquots of 0.1

extraction volumes to a final concentration of 3:1 chloroform:methanol. The solution

was then filtered through a G6 filter, concentrated to dryness, and lyophilized to

determine the mass of material recovered. This material was referred to as the $CHCl_3$-

sup.

6.2.4 Cation Exchange Chromatography I

$CHCl_3$-sup was resuspended in water to a final concentration of 70 mg/ml, and

loaded onto a 6.0 cm x 36 cm column containing Bio Rad Gel AG 50W-X8 (Dowex).

Bound material was eluted from the column with 3 volumes of 1.25 N HCl, the column

was washed with 2 volumes of water, and the water wash and acid eluant were combined

(frac 1.25), concentrated to dryness and lyophilized to determine the mass of material

recovered. Frac 1.25 was resuspended in water to a final concentration (350 - 400 mg/ml)

and kept frozen at -4° C.

6.2.5 Inorganic Precipitation

Frac 1.25 (1 g) was added to 10 ml of concentrated ammonium hydroxide and allowed to precipitate for 3 hr. The sample was centrifuged at 3500 x g for 5 minutes, the supernatant was removed, and the pellet was washed twice with cold water. The supernatant and water washes were combined (frac 1.25-OH), concentrated to dryness and lyophilized to determine the mass of material recovered.

6.2.6 Cation Exchange Chromatography II

For chromatographic purposes, frac 1.25-OH was assumed to be composed entirely of NH_4Cl. The sample was resuspended in 0.2 N HCl to a final concentration of 70 mg/ml and loaded onto a 2.5 cm diameter column containing Dowex resin (10 meq / meq frac 1.25-OH). Unbound material was removed from the column with 5 volumes of water (Dowex-unbound). Moderately bound material was eluted from the column by washing with 2 volumes of 3 M NH_4OH followed by three volumes of water (Dowex-NH_4); and tightly bound material was eluted from the column with 3 volumes of 1.2 N HCl followed by 2 volumes of water (Dowex-bound). Dowex-unbound and Dowex-bound were concentrated to dryness, lyophilized, and weighed to determine the mass of material recovered.

6.2.7 Anion Exchange Chromatography

Dowex-bound was reconstituted in water to a concentration of 60 mg/ml and loaded onto a 5 cm x 30 cm column containing Bio Rad Gel AG 1 - X8 (OH⁻ form). Unbound material was removed from the column by washing with 5 volumes (2 L) of water (AG-unbound); and bound material was eluted with 3 volumes (1200 ml) of 1.2 HCl (AG-bound).

6.3 Results and Discussion

The procedure for isolating biologically-active (DNA damaging) material from salted shrimp paste is summarized in Figure 6.1. The aqueous extract of shrimp paste (SE) was typically a solution with a pH of 6.2 - 6.5 containing both organic and inorganic substances. Centrifugation removed undigested tissue. Other insoluble products were removed by filtration through a G6 glass filter.

SE was passed through a column containing Amberlite XAD-2 polystyrene resin which has a high affinity for nonionic and non-polar organic molecules. The unbound fraction (XAD/U) which was collected contained very polar and/or ionic, organic and inorganic, hydrophilic substances with NaCl as a major constituent. The amount of material recovered after this procedure was typically between 25 - 35% of the mass of shrimp paste.

Chelex 100 chelating resin was used to eliminate NaCl from the biologically-active fraction, XAD/U. This resin contains iminodiacetic acid active sites which are

Salted Shrimp Paste

↓

aqueous extraction (removes solid material)

↓

solid phase extraction (removes organic material)

↓

divalent cation retention chromatography
(removes NaCl)

↓

chloroform precipitation (further salt removal)

↓

cation exchange chromatography I (greatly reduces amino acid conc.)

↓

hydroxide precipitation (removes divalent metal ions)

↓

cation exchange chromatography II (further removal of amino acids)

↓

anion exchange chromatography (removes excess ammonium ions)

Figure 6.1 Purification of Salted Shrimp Paste Extract

highly specific for divalent cations; however, other cations may be retained when present at concentrations greater than 1 M. To reduce binding by Na^+, the sample was treated as if it were composed entirely of NaCl and diluted to a concentration equivalent to 0.2 N. Chromatography using Chelex 100 yielded two fractions. The unbound fraction which was collected contained mostly NaCl as well as nonionic, monovalent, trivalent, and anionic substances. The biologically-active material (Chelex/R) bound Chelex 100 tightly and was removed by eluting with two volumes of 1 N HCl. Among substances present in this fraction were divalent salts, and salts of organic amines. The use of HCl for the elution caused all of the salts present in the sample to be in a chloride or hydrochloride form.

To convert Chelex 100 resin from the Na^+ form which was purchased to the H^+ form which was required in the purification procedure, 1 N HCl (10 volumes) was passed through the resin. At low pH's (pH < 4), the resin will have positively charged imino sites in addition to its desired negatively charged acetate sites. Consequently, Chelex 100 will act, to a limited degree, as both a cation exchanger and an anion exchanger when in the H^+ form. This, therefore, would allow for binding of some negatively charged ions to the resin, and these anionic substances would be present in the bound biologically-active Chelex/R fraction.

Chelex/R was dried, extracted three times with methanol, centrifuged, and the supernatant was removed. Chloroform was added to the supernatant to a final solvent

composition of 75% to induce precipitation. The biologically-inactive material precipitated as a white powder, and was believed to be predominately inorganic salts which were not soluble in large volumes of nonaqueous solvent, i.e., 3:1 chloroform:MeOH. Biologically-active material remained soluble in chloroform:MeOH.

Previous experiments using GC/MS to analyze a derivatized biologically-active fraction revealed that a large percentage of the mass of material soluble in chloroform:MeOH was composed of lysine hydrochloride (LDH) and putrescine dihydrochloride (PDH). To remove contaminating LDH and PDH, cation exchange chromatography using Bio Rad Gel AG 50W-X8 (Dowex) was performed. Dowex has been shown to be useful for eliminating amino acids and diamines from biological samples (38). Stepwise elution using 1.25 N HCl (5 vols.), and 6 N HCl (2 vols.) successfully separated the biologically-active material from the biogenic amines. The biologically-active material was recovered in the 1.25 N HCl fraction (frac 1.25), while higher acid concentrations were needed to remove LDH and PDH from the resin.

Elemental analysis by secondary-ion mass spectrometry and inductively coupled plasma analysis revealed that Mg^{2+} and Cl^- were the predominate substances present in frac 1.25. In previous experiments, Mg^{2+} (in the form of $MgCl_2$) did not elicit DNA SSB in TK6 cells and was, therefore, considered to be a contaminant. To eliminate Mg^{2+} from the sample, concentrated NH_4OH was added to form insoluble $Mg(OH)_2$, and precipitation was allowed to occur for 3 hr. After precipitation, the sample was centrifuged, the

supernatant was removed and saved, and the pellet was washed twice with water to recover as much soluble material as possible. The supernatant (frac 1.25-OH) was concentrated to dryness with a rotary evaporator to remove excess NH_3 from the sample. Water was added to the sample, and both the neutral pH of the sample and lack of strong odor indicated that excess NH_3 had been removed. No biologically-active material was detected in the pellet.

Addition of NH_4OH to solutions containing Cl^- will cause the formation of non-volatile NH_4Cl. This suggested that the following reaction occurred in frac 1.25 after addition of NH_4OH

$$2\ NH_4^+(OH)^- + Mg^{2+} + 2\ Cl^- \longrightarrow Mg(OH)_2\ (i) + 2\ NH_4^+Cl^-.$$
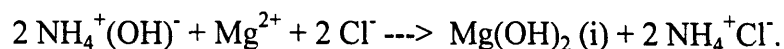
Cation exchange chromatography previously performed on the sample eliminated most of the biogenic amines. Previous results from analyses by HPLC after precolumn derivatization by phenylisothiocyanate revealed that some amino acids remained as constituents of frac 1.25. Precipitation of the sample by NH_4OH as was performed would not have removed any base-soluble amino acids. Partial elimination of amino acids can be accomplished using Dowex and eluting with 3 M NH_4OH (38). For chromatographic purposes, frac 1.25-OH was considered to be composed entirely of NH_4Cl. The sample was dried and weighed to determine its mass, resuspended in 0.2 N HCl to ensure all

possible substances were protenated, and loaded onto a 2.5 cm diameter Dowex column

having a maximum capacity equal to 10x the number of equivalents of NH_4Cl. Three

fractions were collected from this column: an unbound fraction (Dowex-unbound)

containing neutral and anionic material, an amino acid fraction (Dowex-NH_4: collected

by eluting with 3 M NH_4OH), and a fraction containing tightly bound material (Dowex-

bound: collected by eluting with 1.2 N HCl). Most of the mass of material recovered

was present in the bound fraction. Material present in the unbound (Dowex-unbound)

and bound fractions (Dowex-bound) were found to be biologically-active.

Upon elution with 3 M NH_4OH, much of the H^+ counterion on the Dowex resin

was exchanged with NH_4^+. The composition of the Dowex-bound fraction (which was

eluted from the column with HCl) was almost entirely NH_4Cl, and screening for

biologically-active material from this fraction was nearly impossible due to masking by

the large excess of biologically-inactive NH_4Cl. A procedure was sought, therefore, to

eliminate NH_4Cl by converting the contaminating salt to more volatile compounds. Trial

experiments using other forms of ammonium salts, e.g., $NH_4^+CH_3COO^-$, revealed that

many NH_4^+ salts are not sufficiently volatile under normal conditions of vacuum and/or

heating to be efficiently eliminated. Conversion of NH_4Cl to aqueous ammonia was

considered to be an ideal method to eliminate excess NH_4^+. Addition of NaOH to

Dowex-bound, for example, would have successfully produced volatile NH_4OH;

however, this procedure would have yielded NaCl. The net reaction using NaOH or any

other OH^- salt would have resulted in substituting NH_4Cl with some other unwanted salt.

The objective for this step in the procedure was to convert NH_4Cl to volatile NH_4OH while creating a minimal amount of contaminating by-products.

Bio Rad Gel AG 1-X8 (AG1) is a strong anion exchange resin which is highly selective toward Cl^-. Relative to OH^- as the starting counterion (relative selectivity = 1.0), Cl^- is greater than $20x$ more selectively retained (relative selectivity = 22.0) by AG1. This resin was selected, therefore, to produce the following reaction because it could facilitate the removal of NH_4Cl by acting as a solid phase stationary cation which removed Cl^- while releasing OH^- which would act as a counterion for NH_4^+, thus yielding volatile NH_4OH.

$$AG1(OH^- \text{ form}) + NH_4^+Cl^- \longrightarrow AG1(Cl^- \text{ form}) + NH_4^+OH^- \longrightarrow AG1(Cl^- \text{ form}) + NH_3(g) + H_2O$$
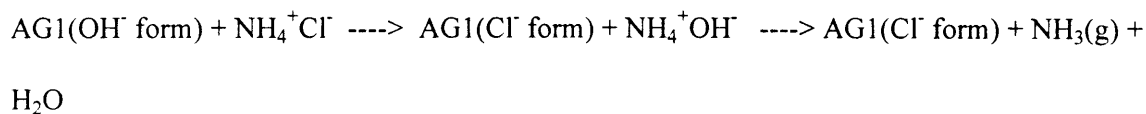
Dowex-bound was diluted and loaded onto a 5.0 cm x 30 cm column containing AG1 and allowed to flow through the column very slowly to allow for maximal conversion of the resin from a OH^- form to a Cl^- form. The column was then washed with 5 volumes (2 l) of water to remove all unbound material (AG-unbound), particularly, NH_3 / NH_4^+. During the elution, a strong odor of ammonia was noted suggesting that the conversion was successful. Bound material (AG-bound) was removed from the column by eluting with three volumes (1200 ml) of 1.2 N HCl. Both fractions were concentrated

to dryness, lyophilized, and weighed. Only material in the bound fraction was found to be biologically-active suggesting that the material of interest had anionic characteristics. This result showing anionic characteristics associated with the biologically-active fraction which earlier had been tightly bound to a cation exchanger, Dowex, was consistent with results from chromatography results using Chelex 100 in a H^+ form which can also bind anionic substances.

The physical appearance of the biologically-active Dowex-unbound fraction and the biologically-active AG-bound fraction were identical. The fact that material from a biologically-active fraction (frac 1.25) could bind to both a strong cation exchanger (Dowex) and a strong anion exchanger (AG1) suggested that either two different substances elicited DNA SSB (one present in the Dowex-unbound fraction; the other present in the Dowex-bound fraction) or that a Zwitterion was the biologically-active substance in the fractions of interest. Considering the chemistry associated with the biologically-active fraction from the divalent cation retention chromatography using Chelex 100 and the physical appearance of the Dowex-unbound and Dowex-bound material, it is most probable that the biologically active substance is a Zwitterion. It was suspected that these two fractions contained the same biologically-active material; so, they were, therefore, combined and referred to as fraction DOW/AG. This mass of material present in the combined fraction represented about 700-1000 ppm salted shrimp paste.

The characteristics of material of interest present in DOW/AG are as follows

low-molecular weight

hydrophilic

ionic

acid/base soluble

slightly soluble in alcohol.

Identification of the biologically-active constituent was exceedingly difficult. Substances

eluted from DOW/AG have no characteristic UV, IR, Raman, or NMR spectra and have

yielded little interpretable data during analyses by ESI/MS. This, therefore, precluded

using the most valuable analytical techniques available to aid in identification.

Moreover, the limited solubility and high polarity of the material of interest precluded

using methods such as reverse-phase chromatography and normal phase chromatography

as results from earlier experiments revealed that the biologically-active substances either

did not bind these resins or could not be detected following chromatography. Low or no

solubility in organic solvents associated with DOW/AG also eliminated the possibility of

derivatizing the biologically-active component to form a substance which was either

more suited for purification by either HPLC or GC or was more easily detected by

standard analytical techniques. When obtained, analytical data usually provided

information about the identity of or toward identifying substances which were

contaminants (e.g., PDH, LDH, tyramine) in a biologically- active fraction.

Unsuccessful attempts using NMR and UV to obtain useful information about the composition of DOW/AG and knowledge of the presence of ionic substances in the fraction led to analyses of this material by ESI/MS. Data from these procedures identified the major constituent in this fraction as a substances having $m/z = 103$ in positive ion mode. Further analyses by ESI/MS/MS and comparison using manufactured material led to identifying the substance with $m/z = 103$ as choline. Semi-quantitatively, choline appears to constitute at least 75% of the mass of DOW/AG. Choline did not elicit DNA SSB in the DNA single-strand breakage assay, and negative ion ESI/MS yielded spectra having no major parent ions. This, therefore, meant that the actual quantity of biologically active material recovered in DOW/AG comprised a maximum of 50-250 ppm of salted shrimp paste. Elemental analyses (e.g., by inductively coupled plasma analysis) would most likely have yielded little information regarding the identity of the active constituent due to masking by the large excess of contaminating choline.

The purification procedure developed was directed toward isolating an unknown substance by using the DNA damage assay to screen for biological activity. Each step in the procedure was equally or more greatly directed toward eliminating an unwanted contaminant as it was toward retaining biologically-active material. To this end, it is likely that material of interest was lost during purification, and the amount of lost material could not be quantitated. Identification of the substance which elicited DNA SSB in TK6 cells could not be completed due to the characteristics of the constituents present in the active fraction (DOW/AG), the technology available to isolate/separate

limitedly soluble, polar compounds, and the lack of spectroscopic characteristics

available to permit detection of any isolated material. Characterization of the biological

activity of DOW/AG was undertaken to complete research in this thesis.

Chapter 7

BIOLOGICAL ACTIVITY ELICITED BY CHINESE-STYLE SALTED SHRIMP

PASTE

7.1 Introduction

Treatment of TK6 cells with extracts of salted shrimp paste has been shown to cause cytotoxicity and DNA single-strand breaks in these cells. Chromatographic fractions isolated from shrimp paste extract elicited the same biological activity as the starting material. The order of the biological effects and the mechanism by which DNA SSB and cytotoxicity occurred were not known. Experiments discussed in this chapter were undertaken to elucidate how the purest material isolated from salted shrimp paste elicited biological activity in TK6 cells.

7.2 Materials and Methods

7.2.1 Chemicals used in Biological Assays

[^{3}H]Thymidine (2 Ci/mmol) was purchased from NEN Research

Products. Liquiscint scintillation fluid (National Diagnostics, Manville, NJ) was used for

scintillation counting.

7.2.2 Analysis of Cytotoxicity by Incorporation of Radioactivity during DNA Synthesis

TK6 cells (46 µl; 5 - 6 x 10^5 cells/ml) in RPMI 1640 medium supplemented with

10% horse or calf serum (medium may have contained penicillin [18 units/ml] /

streptomycin [18 µg/ml]) were added to samples (20 µl) in sterile, 0.5 ml microcentrifuge

tubes (final cell concentration = 3.4 - 4.2 x 10^5 cells/ml). Tubes were capped, and 3 - 22

gauge holes or 1- 18 gauge hole was made in the cap of each tube to allow gas exchange.

Tubes were placed in a humidified 37 °C incubator with a 5 % CO_2 atmosphere during

treatment. After treatment, sample were mixed and added to 50 ml centrifuge tubes

containing 30 ml of supplemented medium. Aliquots(1 ml) of each treatment were plated

in quadruplicate in the wells of 5 or 6 - 24-well or 48-well tissue culture plates, and plates

were placed in a humidified 37 °C incubator in a 5 % CO_2 atmosphere .

Each day, one set of quadruplicate aliquots from each treatment was removed

from the incubator and labelled with 0.1 µCi/ml ^{3}H-thymidine. Plates containing the cell-

^{3}H-thymidine mixture were returned to the incubator for 24 hr to allow incorporation of

the radioactive nucleotide into cellular DNA. After the incorporation period, each aliquot

was transferred to a 1.5 ml centrifuge tube and spun at 16,000 x g for 15 sec to separate

cells from the radioactive medium, and the medium was removed and discarded. PBS (1 ml) was added to each tube, cells were centrifuged at 16,000 x g for 15 sec, and the supernatants were removed and discarded. PBS washing was repeated once after which 0.5 ml or 0.1 ml lysis buffer (10 mM tris, 10 mM EDTA [pH=8.4], 2% SDS), was added to each sample for at least 1 min. Cell lysates were transferred to 20 ml scintillation vials containing 5 ml of scintillation liquid, and PBS or water (1 ml) was used to wash each centrifuge tube to ensure complete transference of all radioactivity. Samples were counted for 1 min, and incorporation of ^{3}H-thymidine was charted for 5 or 6 consecutive days following treatment. Cell survival was determined by dividing the amount of radioactivity incorporated by treated cells by the amount of radioactivity incorporated by untreated cells.

% survival = (radioactivity of treated sample / radioactivity of untreated sample) x 100

7.2.3 Analysis of DNA Damage by SDS Precipitation

A modified version of the DNA Precipitation Assay described by Olive (32) was used to detect DNA strand breaks in mammalian cells. Cells in media supplemented with 5% or 10% serum were radiolabelled for 20 hr with 0.02 µCi/ml [14C] thymidine in a 5% CO_2 atmosphere at 37 °C followed by incubation for 30 min, 2 hr, or 24 hr in fresh

medium. Cells were then incubated with samples for 30 min and resuspended at a density of 4-6 x 10^5 cells/ml.

Cells (100 µl) were lysed in plastic tubes at 0 °C for one minute in 500 µl of solution containing 2% SDS, 10 mM EDTA, and 10 mM Tris. NaOH (250 µl; 0.1 M) was added just prior to lysis. After lysis, KCl (0.5 ml, 0.12 M) was gently added, and the tubes were capped and placed in a water bath at 65 °C for 10 min, cooled on ice for 5 min, and centrifuged for 10 min at 3500 x g. The supernatant was decanted into a liquid scintillation vial containing one ml of 0.5 M HCl. One ml of warm water (65 °C) was added to the pellet, the sample was resuspended by pipetting up-and- down using a 1000 µl pipet, and the sample was deposited into a scintillation vial. A second ml of warm water was added to the sample tube, mixing was repeated, and the solution was deposited into the same scintillation vial as the previous wash to assure complete removal of all DNA. Radioactivity was counted using a Beckman scintillation counter and liquid scintillation fluid (5 ml). The percentage of damaged DNA was calculated by dividing the radioactivity in the supernatant (R_s) by the radioactivity in the supernatant plus pellet ($R_s + R_p$) and multiplying by 100. All analyses were done in triplicate.

$$\% \text{ damaged DNA} = (R_s / (R_s + R_p)) \times 100$$

7.2.4 Analysis of DNA Damage by Agarose Gel Electrophoresis

TK6 cells (3 - 4 x 10^5 cells/ml) were incubated with samples in microcentrifuge tubes for 24 hr at 37 °C. After treatment, cells were centrifuged at 16,000 x g for 15 sec, resuspended in extraction buffer (10 mM Tris-Cl [pH=8.0], 100 mM EDTA [pH=8.0], 0.5% SDS, 20 mg/ml pancreatic RNAse), and incubated in a water bath for 1 hr at 37 °C. Proteinase K was added to each tube to a final concentration of 100 mg/ml, and samples were returned to the water bath for 1 hr.

An equal volume of 1:1 buffered phenol (pH=8.0):CHCl$_3$ was added to each sample. Samples were briefly vortexed and centrifuged at 16,000 x g, and the organic layer was removed. Phenol:CHCl$_3$ extraction was repeated at least twice more, and one volume of 24:1 CHCl$_3$:isoamyl alcohol was added to the remaining solution containing the DNA. Each sample was briefly vortexed and centrifuged at 16,000 x g, the organic layer was removed, and two volumes of 100% ethanol was added to each tube. Samples were briefly vortexed, set on ice for 5 min, centrifuged for 15 min at 16,000 x g, and the supernatants were removed and discarded. Pellets were washed with one volume of 70% ethanol, briefly vortexed and centrifuged, and the supernatants were removed. Pellets were washed once more with 95% ethanol, vortexed, and centrifuged at 16,000 x g. The supernatants were removed and discarded, and the pellets were dried under vacuum.

For alkaline electrophoresis, agarose was dissolved in water, melted, and the solution was made 50 mM NaOH and 1 mM EDTA after cooling to 60 °C. The gel

solution was poured and allowed to set, after which it was equilibrated twice in alkaline

electrophoresis buffer (30 mM NaOH, 1 mM EDTA) for 30 min with shaking. Dried

DNA pellets were resuspended in electrophoresis buffer, and 6x alkaline loading dye (300

mM NaOH, 18% Ficoll [type 400; Pharmacia] in water, 0.15% bromocresol green, 0.25%

xylene cyanol FF) was added prior to loading samples into wells of the gel.

Electrophoresis was performed at 1.2 volts/cm for 8 - 12 hours. Thirty minutes after the

start of electrophoresis, a glass plate was placed over the gel to prevent dye diffusion into

the electrophoresis buffer and to hold the gel in place. After electrophoresis, the gel was

neutralized twice for 30 min in neutralizing buffer (1 M Tris-Cl [pH=7.6], 1.5 M NaOH)

with shaking and stained for 30 min in a 2 μg/ml ethidium bromide solution or a $1/10^5$

dilution of SYBR green II in TBE.

7.2.5 Analysis of Cell Cycle by Fluorescence Activated Cell Sorting

TK6 cells (460 μl; 5 - 6 x 10^5 cells/ml) in RPMI 1640 medium supplemented with

10% horse or calf serum (medium may have contained penicillin [18 units/ml] /

streptomycin [18 μg/ml]) were added to samples (200 μl) in sterile, 0.5 ml

microcentrifuge tubes (final cell concentration = 3.4 - 4.2 x 10^5 cells/ml). Tubes were

capped, and 3 - 22 gauge holes or 1- 18 guage hole was made in the cap of each tube to

allow gas exchange. Tubes were placed in a humidified 37 °C incubator with a 5 % CO_2

atmosphere during treatment. After treatment, cells were centrifuged at 16,000 x g for 10

sec, the medium was removed, and cells were resuspended in 250 µl staining solution. (50 µg/ml propidium iodide, 180 units/ml RNAse, 0.2% Triton X, 0.1% sodium acetate) and incubated at 37 °C for 20 min. Afterwards, an equal volume of hyperosmotic solution (50 µg/ml PI, 0.2% Triton X, 0 36 M sodium chloride) was added and samples were left at 4 °C for at least 16 hr prior to analysis by fluorescence activated cell sorting (FACS).

7.2.6 Analysis for Interference with Catalytic activity of Topoisomerase I

DOW/AG was incubated with plasmid DNA (pUC19; 1 µg) and wheat germ topoisomerase I (4 units) for 1 hour at 37 °C to determine whether biologically-active material in this fraction could interfere with the catalytic activity of topoisomerase I. After treatment, the reaction was stopped by the addition of SDS to a final concentration of 1% (w/v). Samples were phenol:chloroform extracted and ethanol precipitated, dried under vacuum, and resuspended in TBE buffer prior to analysis by electrophoresis on a 1% agarose gel. Electrophoresis was performed for 4 hr at 2 v/cm, after which the gel, stained with ethidium bromide and photographed.

7.3 Results and Discussion

Results from preliminary experiments showed that salted shrimp paste extract was cytotoxic to TK6 cells. Cells were treated for 2 hr and 24 hr with 0, 50, 100, 250, 500 and 1000 µg/ml DOW/AG to determine the level of cytotoxicity induced by that fraction. After treatment for 2 hr, cytotoxicity was noted in cells treated with 1000 µg/ml DOW/AG only. No other treatment dose elicited cell death above background levels (Figure 7.1). After 24 hr treatment, dose-response cytotoxicity was noted in TK6 cells (Figure 7.2). These results indicated that cell death was a function of time.

Earlier biologically-active fractions contained Mg^{2+} and Ca^{2+} which are naturally occurring metal ions in salted shrimp paste. In earlier experiments, DNA damage and cytotoxicity appeared to be enhanced in the presence of Mg^{2+}. Ca^{2+} also appeared to enhance cytotoxicity of DOW/AG. Subsequent experiments proved that Mg^{2+}, in fact, did not enhance levels of cytotoxicity noted. When TK6 cells were treated with 250 µg/ml DOW/AG and Mg^{2+} concentrations were varied from 2.5 - 25 mM, no enhancement of cytotoxicity elicited by DOW/AG was noted except at the highest metal concentration (25 mM). This concentration of Mg^{2+} caused about 50% cell death in TK6 cells. Calcium ion concentrations ranged between 0.5 - 2.5 mM and did not significantly increase cytotoxicity in the presence or absence of DOW/AG (data not shown). Previous metal enhanced toxicity noted was due to the solvent used to dissolve metal salts ($MgCl_2$ 6 H_2O and $CaCl_2$). Phosphate buffered saline, the solvent used to dissolve metal solutions, reduces toxicity of Mg^{2+} and increases toxicity of Ca^{2+}. After treatment of cells
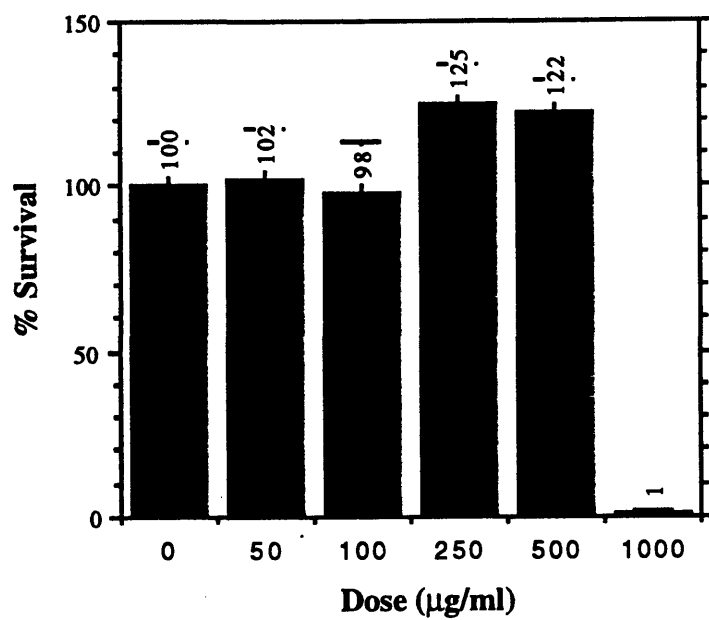
Figure 7.1 Survival of TK6 Cells after 2 hr Exposure to DOW/AG
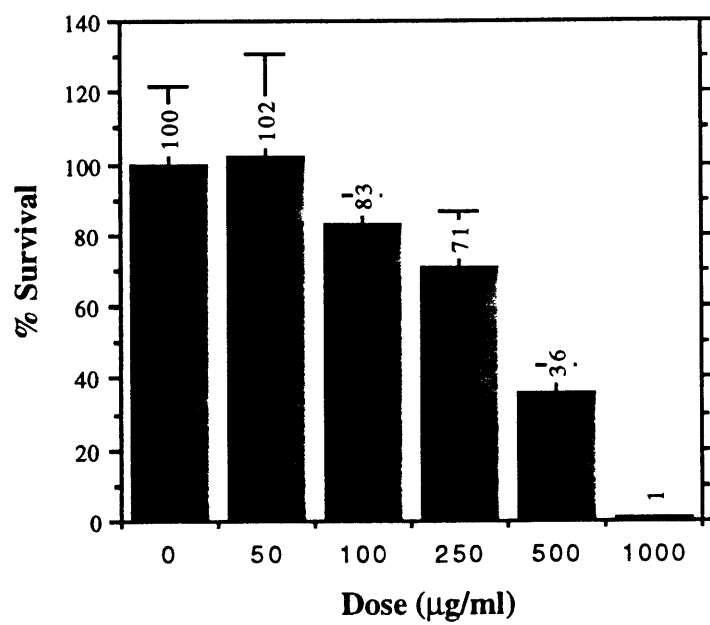
Figure 7.2 Survival of TK6 Cells after 24 hr Exposure to DOW/AG

with metal solutions in water, no enhancement of cytotoxicity was attributed to the presence of either metal.

To understand the relationship between cell death and time, TK6 cells were treated with 250 µg/ml DOW/AG for 4, 6, 12, and 24 hr; and cell survival was determined for each treatment time. Low levels of cytotoxicity were noted in TK6 cells during treatments of 6 hr or less. Cells treated for 12 hr or more exhibited much higher levels of cell death (Figure 7.3). These results revealed that events leading to cell death could be noted 6-12 hr after treatment.

The primary method used to assess biological activity was the ability of a fraction to elicit DNA SSB in TK6 cells. DOW/AG (0, 50, 100, 250, 500, and 1000 µg/ml) was incubated with TK6 cells for 2 hr and 24 hr. After incubation for 2 hr, no DNA damage above background level was noted in TK6 cells at any treatment dose (Figure 7.4). After incubation for 24 hr, cells treated with 1000 µg/ml showed significantly higher levels of DNA damage relative to background levels (Figure 7.5).

DOW/AG was incubated with TK6 cells in the presence of Mg^{2+} to determine whether this metal enhanced levels of DNA damage noted. Prior to treatment, DOW/AG was incubated with 50 mM Mg^{2+} at room temperature for 90 min, and the DNA damage assay was performed as described. Mg^{2+} only enhanced levels of DNA damage elicited by very high doses (1500 µg/ml) of DOW/AG after 30 min of treatment (Figure 7.6). This suggested that Mg^{2+} was not an important factor in eliciting DNA damage observed.
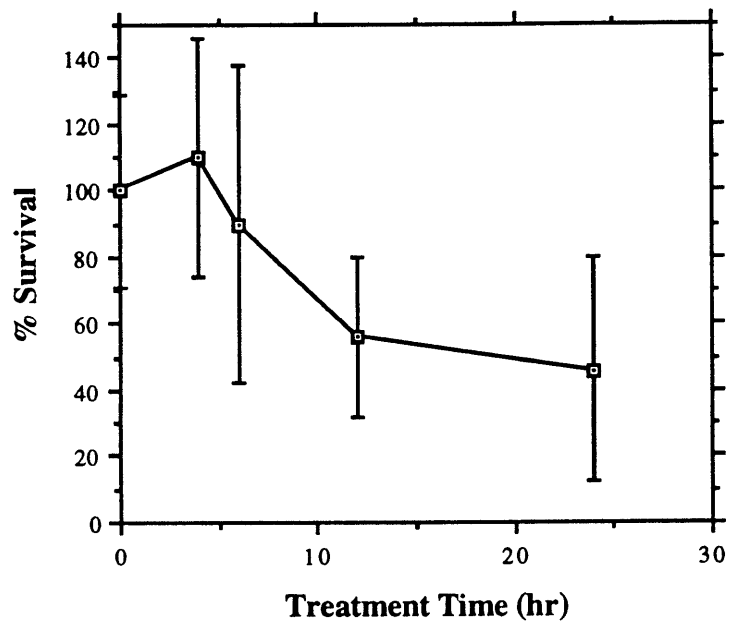
Figure 7.3 Dose- and Time- Response Cytotoxicity of DOW/AG in TK6 Cells
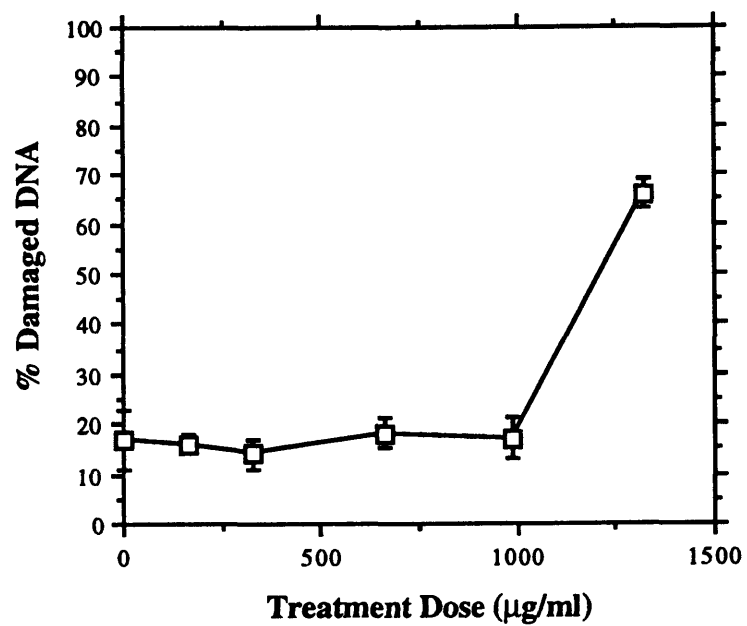
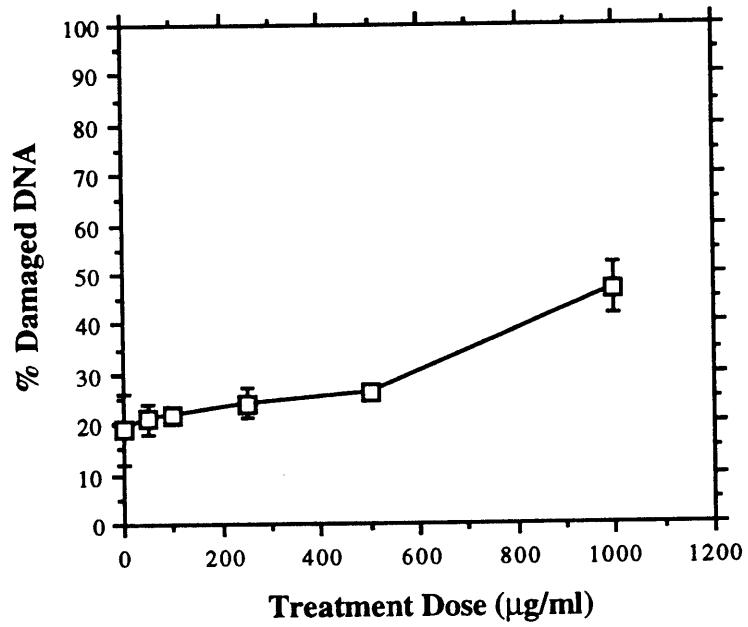Figure 7.4 DNA Damage Elicited by DOW/AG in TK6 Cells after 2 hr Exposure

Figure 7.5 DNA Damage Elicited by DOW/AG in TK6 Cells after 24 hr Exposure
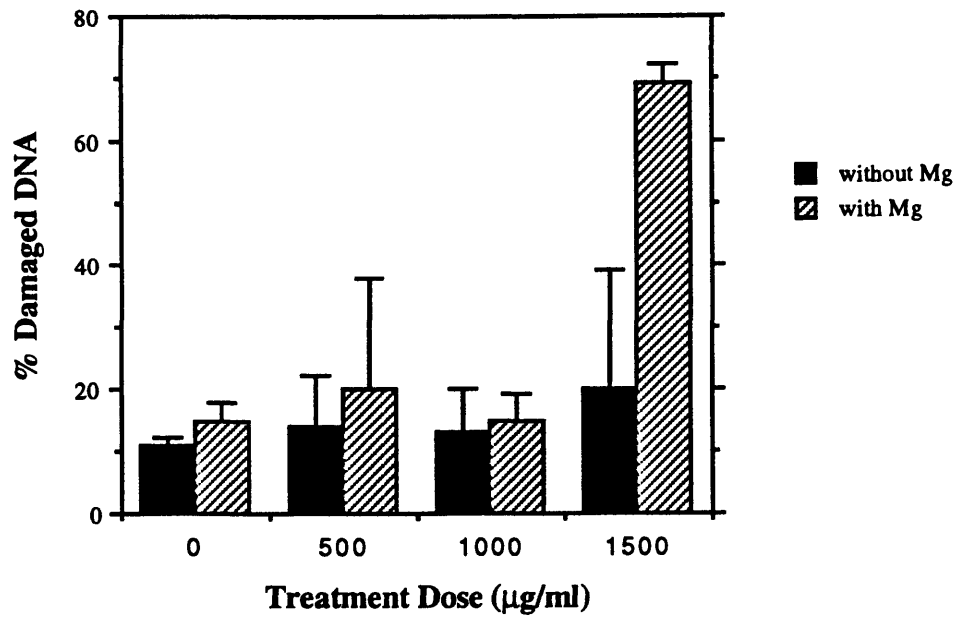
Figure 7.6 Effects of Mg^{2+} on DNA Damage Elicited by DOW/AG

TK6 cells were also treated with different doses of DOW/AG for 2, 4, 6, 12 and

24 hr (Figure 7.7). Results from this experiment show that the relationship of dose and

time relative to DNA damage was complex. The lowest treatment dose (250 μg/ml)

elicited background levels of DNA damage between 2-12 hr. After incubation for 24 hr,

cells treated with this dose showed slightly elevated levels of DNA damage. At 4x this

dose (1000 μg/ml), DNA damage was slightly higher than background levels at treatment

times between 2 and 12 hr. After 24 hr treatment, DNA damage significantly increased

in cells treated with this dose. In cells treated with 1250 μg/ml DOW/AG, levels of DNA

damage noted were slightly greater than background levels during the first 4 hours of

treatment. After 6 hr treatment with this dose, DNA damage peaked at about 66%

followed by a declined in DNA damage after 12 hr to slightly greater than background

levels, and a further decline after 24 hr to less than background levels.

The DNA damage assay used is a semi-quantitative method to evaluate DNA

SSB in mammalian cells. This assay has been shown to be useful for evaluating DNA

damage (SSB or DSB) caused by agents which damage DNA by different mechanisms of

action (32). This assay, however, does not provide information regarding the size of

DNA fragments generated by the damaging agent. To determine the size of DNA

fragments resulting from treatment, TK6 cells were incubated with frac 1.25 (a

biologically-active, less pure fraction than DOW/AG) at doses which induced DNA

damage at levels similar to (0, 100, 250, 500 μg/ml) DOW/AG and DNA damage was

observed using alkaline agarose gel electrophoresis. Preliminary
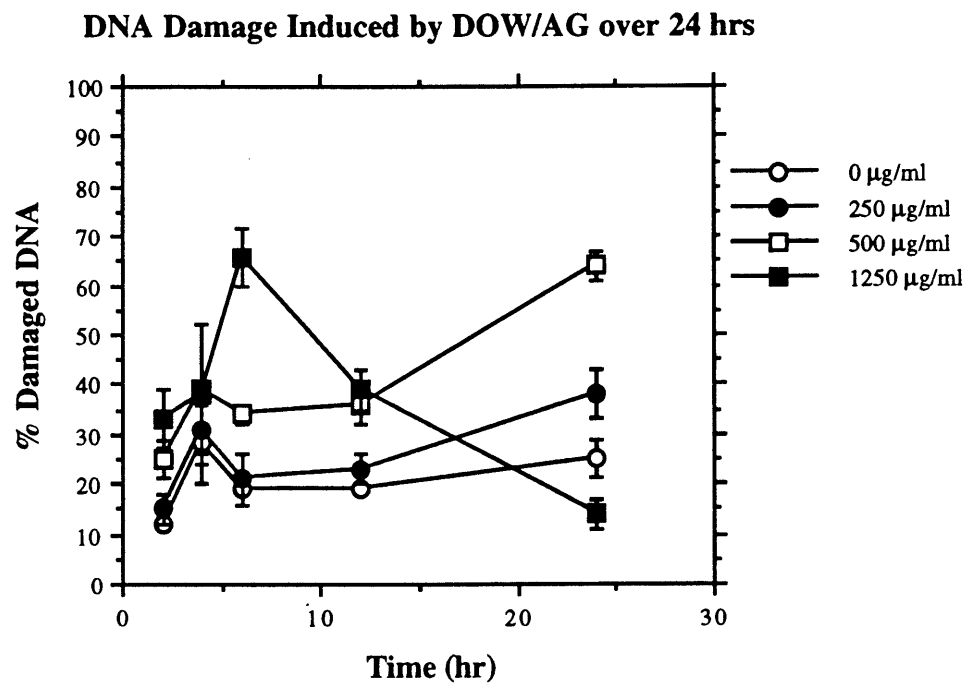
DNA Damage Induced by DOW/AG over 24 hrs

Figure 7.7 Dose- and Time- Response DNA Damage Elicited by DOW/AG in TK6 Cells

experiments analyzing DNA from DOW/AG treated cells on 0.7 % and 1 % alkaline

agarose gels showed that DNA fragments never exited the wells of the gel (data not

shown); and therefore, were probably greater than 10 kb in length (the limit of resolution

on a 0.7 % agarose gel). Other experiments analyzing DNA from cells treated with

DOW/AG demonstrated that DNA fragments generated were large; however, poor

staining or insufficient quantities of marker DNA precluded determining the size of the

fragments. Analysis of DNA SSB by electrophoresis produced results in agreement with

the DNA precipitation assay. Treatment doses which elicited DNA SSB in the

precipitation assay also yielded positive results in the electrophoresis assay; doses

yielding negative results in one assay also yielded negative results in the other. Using

lambda Hind III digest as DNA markers, the major distributions of both undamaged and

damaged DNA from all treatments were larger than 23 kb in length; however, the

distribution of DNA fragments from cells treated with a high dose of DOW/AG migrated

further down the gel than DNA from cells treated with lower doses (Figure 7.8).

The size of damaged DNA fragments generated after treatment, the fact that

previous experiments demonstrated that cellular components were required to elicit DNA

SSB, and the fact that no evidence that DSB were made raised the question of whether or

not the biologically-active material from DOW/AG interfered with the biological activity

of topoisomerase I (topo I). Interference with topo I activity can occur in two ways.

Firstly, substances which bind topo I or DNA can prevent the enzyme from completing

its catalytic activity of relaxing supercoiled DNA. The other manner by which a
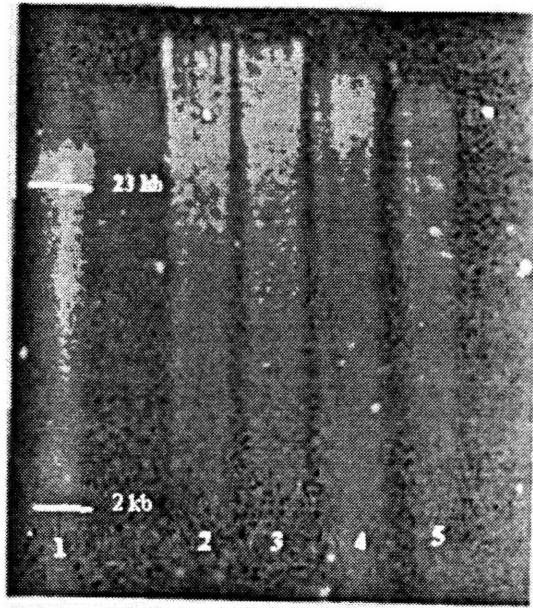
Lane 1 Lambda Hind III DNA markers

Lane 2 DNA from untreated cells

Lane 3 DNA from cells treated with 100 µg/ml DOW/AG

Lane 4 DNA from cells treated with 250 µg/ml DOW/AG

Lane 5 DNA from cells treated with 500 µg/ml DOW/AG

Figure 7.8 Analysis of DNA Damage in TK6 Cells by Alkaline Agarose Gel
Electrophoresis

substance can interfere with the enzyme by forming the cleavable complex. When this occurs, topo I will bind and knick DNA but will remain bound to DNA rather than dissociate to DNA and enzyme.

Plasmid DNA (pUC19) was incubated for 60 min with wheat germ topo I and various concentrations (250, 500, and 1000 μg/ml) of DOW/AG to determine whether the active fraction could inhibit the catalytic activity of the enzyme. Results from electrophoresis using a 1% agarose gel showed slight inhibition at the highest level (1000 μg/ml) of DOW/AG treatment (Figure 7.9; lane 6) suggesting that DOW/AG could only slightly inhibit topo I catalytic activity. An unexpected observation in this experiment was evidence that DOW/AG treatment alone (Figure 7.9, lane 2) elicited DNA DSB in plasmid DNA at 1000 μg/ml. DNA DSB had never before been noted in DNA after treatment *in vitro* or *in vivo*.

Analysis of the cell cycle in TK6 after treatment was performed in order to determine the extent to which treatment with DOW/AG exerted biological effects in cells. Cells were treated for 24 hr with 250 μg/ml DOW/AG and consequently stained with propidium iodide (PI). After PI staining, cells were counted using a fluorescence activated cell sorter (FACS) to determine the distribution of diploid cells in each phase of the cell cycle. After 24 hr treatment with 250 μg/ml DOW/AG, 50% more cells were present in the G_0 - G_1 phase relative to S phase in the treated sample compared to the untreated sample (i.e., G_0-G_1/S for treated = 1.5 x G_0-G_1/S untreated). Compared to cells at the start of the experiment, G_0-G_1/S for treated cells was nearly 4x greater after 24 hr
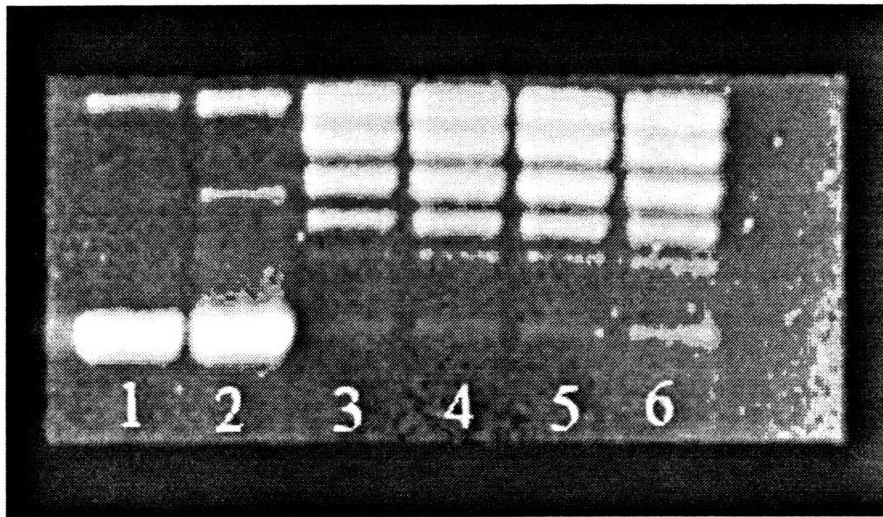
Lane 1 DNA only

Lane 2 DNA + 1000 μg/ml DOW/AG

Lane 3 DNA + enzyme

Lane 4 DNA + enzyme + 250 μg/ml DOW/AG

Lane 5 DNA + enzyme + 500 μg/ml DOW/AG

Lane 6 DNA + enzyme + 1000 μg/ml DOW/AG

Figure 7.9 Effect of DOW/AG on Catalytic Activity of Topoisomerase I

compared to a 2.5x increase in untreated cells (Table 7.1). This demonstrated that cell

cycle arrest occurred in G_0-G_1, that is, cells were prevented from continuing in their cycle

from the first gap phase into DNA synthesis.

To elucidate how quickly cell cycle interruption occurred, cells were treated with

250 µg/ml DOW/AG for 6, 12, and 24 hr. After treatment, cells were again strained with

PI and analyzed by FACS. Between 6 and 12 hr of treatment, 30% more untreated cells

were arrested in G_0-G_1, that is, G_0-G_1/S is 30% greater after 12 hr compared to the ratio

after 6 hr. In treated cells, no difference in the ratio of cells in G_0-G_1/S was noted after 12

hr compared to the ratio at 6 hr (Table 7.2). Between 12 and 24 hr, G_0-G_1/S increased by

90% in untreated cells compared to a 400% increase in G_0-G_1/S in treated cells . These

data showed that events or consequence of events that caused cell cycle arrest at the G_1/S

checkpoint occurred between 12 and 24 hr.

The combined data demonstrating cytotoxicity, DNA damage, and cell cycle

arrest raised questions regarding the mechanism by which these events occurred. At

lower doses (250 µg/ml DOW/AG), cytotoxicity appears to occur in TK6 cells after 6 hr

of treatment. DNA damage and cell cycle arrest occur between 12 and 24 hr of treatment.

The cytotoxicity assay requires a small number of cells and measures the total amount of

radioactivity incorporated by replicating cells, an event which occurs continuously. The

DNA damage assay, however, requires a much greater number of cells and measures

levels of radioactivity of labeled DNA fragments based on fragment solubility after

Table 7.1 Effects of DOW/AG on Cell Cycle of TK6 Cells after 24 hr Exposure

	Time (hr)	(G0-G1)/S	S/(G2-M)	(G2-M)/(G0-G1)
untreated	0	0.63	4.7	0.34
untreated	24	1.6	4.3	0.15
treated	24	2.4	2.0	0.21

Table 7.2 Time-Reponse Effects of DOW/AG on Cell Cycle of TK6 Cells

	Time (hr)	(G0-G1)/S	S/(G2-M)	(G2-M)/(G0-G1)
	0	0.63	4.7	0.34
untreated	6	0.68	6.7	0.22
	12	0.90	5.0	0.22
	24	1.7	3.4	0.18
treated	6	0.47	4.9	0.43
	12	0.49	3.5	0.59
	24	2.4	1.9	0.20

precipitation. Data from the three types of experiments suggest the following mechanism for cytotoxicity.

Exposure of cells to the active constituent in DOW/AG induces DNA SSB and, at much lowers levels, DNA DSB. DNA strand breaks induce G_0-G_1 arrest (39) thereby delaying the progression of G_0-G_1 cells into S phase. DNA single strand breaks elicited do not interrupt the cell cycle of cells in S, G_2, or M phases. Repair or attempted repair of strand breaks signals a checkpoint coupled to the repair event (40), and the cell cycle checkpoint system increases the probability that release into S phase does not occur until after repair is completed. Cells containing unrepaired DNA are arrested in G_0-G_1, accumulate, and eventually die.

The cytotoxicity assay shows a lack of ^{3}H incorporation in cells which are dead or in cells which are cytostatic and eventually die. The stage of the cell cycle at which a cell is present during exposure and the ability to repair DNA damage appear to be the major factors regarding cell survival. As more cells acquire unrepaired DNA damage and become arrested in G_0-G_1, cell cycle analysis by FACS and the DNA damage assay detect biological consequences of exposure. A role for topoisomerase I in this mechanism has yet to be shown.

Chapter 8

SUMMARY AND CONCLUSIONS

Consumption of Chinese-style salted fish has been repeatedly shown to be a major

risk factor for developing NPC. Epidemiological investigations involving cases of all

ages from a number of places have shown that the correlation is both time and dose

(quantity) dependent. Consumption of salted shrimp paste has also been shown to be a

major risk factor for developing NPC in Northern China.

Aqueous extracts of both foods contain compounds with similar biological

activities. These substances have low molecular weights (<1000 daltons) and can be

classified into three different categories based on C_{18} reverse-phase chromatography on

the aqueous extracts.

Class I   hydrophilic, cytotoxic, genotoxic

Class II   hydrophilic, mutagenic

Class III hydrophobic, genotoxic, cytotoxic

Exposure of bacterial and human cells in forward mutation assays to extracts of

salted fish caused dose-response cytotoxicity and mutagenicity. Salted shrimp paste

extract demonstrated similar biological activity in bacteria, but was not investigated in

human cells. It would not be surprising to find that salted shrimp paste extract exposure

elicited mutagenicity at levels similar to those of salted fish extract. The major difference

between the two extracts was that shrimp paste extract was more stable upon purification.

Using a semi-quantitative, biological assay which screens for the ability of a substance to elicit DNA SSB in mammalian cells, purification of the class I material from salted shrimp paste extract was attempted. A purification procedure was developed and used to isolate a biologically-active fraction which apparently contains an acidic zwitterion as its active constituent. The fraction, DOW/AG, is highly contaminated with choline (which does not elicit DNA SSB), and the active constituent appears to be present in salted shrimp paste at levels of 200-250 ppm or less. The identity of this substance was not completed. DOW/AG has little UV absorption associated with it (mainly end absorption), little NMR activity (very small peaks that appear to be unrelated), no IR activity, and ESI/MS data revealed the presence of choline only, which is masking the true active constituent.

The biologically active fraction elicits DNA SSB breaks in human cells, and these strand breaks appear to lead to cell cycle arrest at G_1/S due to attempted DNA repair, and subsequently, cell death due to the inability of the cell to repair DNA damage at the treatment levels used. Lower levels of treatment, however, were less cytotoxic, presumably because of successful DNA repair which would preclude G_1/S cell cycle arrest.

The research in this thesis demonstrate that Chinese-style salted shrimp paste contains direct- and indirectly acting genotoxic substances. DOW/AG, in particular, elicits both activities, but it may be the attempted repair of DNA damage by the cell may be more important. Low level exposure of cells to DOW/AG appears to cause DNA

damage which is repaired, and, at some low frequency, misrepaired. Non-lethal misrepair, furthermore, could give rise to non-lethal mutations. These possibilities along with contributions from non-cytotoxic, indirectly-acting mutagenic class II material, and perhaps an additional effect from class III material would suggest that exposure to Chinese-style salted shrimp paste could have serious biological consequences.

Chapter 9

RECOMMENDATIONS FOR FUTURE RESEARCH

Research in this thesis demonstrated that Chinese-style salted shrimp paste contains direct-acting biologically active material. Further investigations involving salted shrimp past extracts should include the following.

(1) Investigations on whether DOW/AG exposure in the presence of topo I could cause formation of the cleavable complex. This is a relatively quick and simple experiment which could demonstrate how DNA damage (DNA SSB) occurs and would further correlate the relationship of cytotoxicity and DNA Damage.

(2) Purification of the putative genotoxic agent. choline has been shown to be the major contaminant in DOW/AG. Removal/separation of choline from the active constituent in DOW/AG could be accomplished by ion exchange HPLC. The limitations of this technique remain to be that the identity of active material is unknown; and therefore, screening for biological activity is still the only method to detect the material. A more sensitive biological assay than the DNA damage could facilitate this endeavor.

(3) Isolation of the class II (mutagenic) material from salted shrimp paste extract. This material is very interesting because it causes mutations and elicits low levels of

cytotoxicity. Identification of this material could possibly explain why/how salted

shrimp paste consumption could lead to the development of NPC.

(4) Isolating biologically active material from extracts of Chinese-style salted

fish. The procedures developed for isolating toxic/genotoxic material from salted shrimp

paste could be applied to salted fish. Application of a method which has been shown to

isolate apparently similar material from a similar matrix would greatly reduce

complications associated with loss of biological activity as was experienced during

attempts to isolate biological activity from salted fish extract.

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APPENDIX

During the oral defense of this dissertation, Professor J.M. Essigmann revealed that a published scientific paper[1] reported that choline was a suspected agent which could methylate DNA bases ultimately leading to the formation of DNA damage in the form of AP sites. Results from experiments in this thesis showed that neither choline nor choline + Mg^{2+} significantly enhanced background levels of DNA damage noted in TK6 cells or levels of DNA damage noted after DOW/AG treatment. In light of the journal paper, one possible explanation for these observations could be that choline might actually enhance DNA damage elicited by the active constituent in DOW/AG; however, at the naturally-occurring high levels present in DOW/AG, any enhancement may have reached saturation. Therefore, any further addition of choline had no notable effect on DNA damage. The scientific value of this report is appreciated in the context of the work in this thesis.

[1] Burcham, P.C. and Marnett, L. J. Endogenous DNA adducts: potential and paradox. Chem Res Toxic 6: 771-85, 1993.