การทดสอบความเป็นพิษต่อยีนของยางวัลคาไนซ์และสารเคมีตกค้างที่ใช้ในกระบวนการทำให้ยางคงรูปโดยการก่อกลายพันธุ์กลับคืนของ *Salmonella typhimurium*

นางสาวปัทมาวดี แสนเขื่อนแก้ว

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาชีวเคมี ภาควิชาชีวเคมี
คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2547

ISBN 974-53-1726-8

GENOTOXICITY TEST OF VULCANIZED RUBBER AND RESIDUAL CHEMICALS USED IN CURING PROCESSES USING REVERSE MUTATION ASSAY OF *Salmonella typhimurium*

Miss Pattamawadee Sankheangaew

A Thesis Submitted in Partial Fulfilment of the Requirements
For the Degree of Master of Science in Biochemistry

Department of Biochemistry
Faculty of Science
Chulalongkorn University

Academic Year 2004
ISBN 974-531726-8

Thesis Title	Genotoxicity test of vulcanized rubber and residual chemicals used in curing processes using *Salmonella typhimurium*
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นางสาวปัทมาวดี แสนเขื่อนแก้ว : การทดสอบความเป็นพิษต่อยีนของยางวัลคาไนซ์และสารเคมีตกค้างที่ใช้ในกระบวนการทำให้ยางคงรูปโดยการก่อกลายพันธ์กลับคืนของ *Salmonella typhimurium* (GENOTOXICITY TEST OF VULCANIZED RUBBER AND RESIDUAL CHEMICALS USED IN CURING PROCESSES USING REVERSE MUTATION ASSAY OF *Salmonella typhimurium)* อ.ที่ปรึกษา รศ.ดร.จริยา บุญญวัฒน์
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วิธีก่อกลายพันธุ์กลับคืนของ *Salmonella typhimurium* 2 สายพันธุ์ คือ TA 98 และ TA 100 ถูกนำมาใช้ในการตวจสอบความเป็นพิษต่อยีนของสารเคมีที่ใช้ในอุตสาหกรรมยางและแผ่นยางคงรูป งานวิจัยนี้ตรวจสอบความเป็นพิษต่อยีนของสารเคมี13 ชนิด ในกลุ่ม สารตัวเร่ง สารกระตุ้น สารต้านทานความเสื่อม สารตัวเติม และสารวัลคาไนซ์ พบสารเคมี 3 ชนิดในกลุ่มสารตัวเร่ง ได้แก่ zinc dibenzyl dithiocarbamate (ZBEC) zinc dibuthyl dithiocarbamate (ZDBC) และ mercapto benzothiazole (MBTS) เป็นพิษต่อยีนของสายพันธุ์ TA98 ที่ความเข้มข้น 100 ไมโครกรัม/1จานเพาะเลี้ยง ZDBC เป็นพิษต่อยีนในภาวะที่ผ่านการกระตุ้นโดยเอ็นไซม์จากตับหนู แต่ ZBEC และ MBTS เป็นพิษต่อยีนโดยตรง ดังนั้นศักยภาพความเป็นพิษต่อยีนจากมาก ไปหาน้อยคือ ZBEC> MBTS> ZDBC>DPTT>ZMBT ตามลำดับสำหรับกลุ่มสารตัวเร่ง นอกจากนั้นพบว่า บิวทิลไฮดรอกซีโทลูอีน (BHT) ซึ่งเป็นสารต้านทานความเสื่อม และ ซัลเฟอ ร์เป็นพิษต่อเซลล์แบคทีเรีย แต่เมื่อทำให้ยางคงรูปโดยระบบซัลเฟอร์เปอร์ออกไซด์และฉายรังสีรวม 6 ตำรับแล้วใช้โทลูอีนและคลอโรฟอร์มต่อเมธานอลในอัตราส่วน1:1 สกัดสารเคมีตกค้างจากยางคงรูป ไม่พบความเป็นพิษต่อยีน จำนวนโคโลนีกลายพันธุ์ เมื่อสกัดด้วย คลอโรฟอร์มต่อเมธานอล (1:1) มากกว่าโทลูอีน แต่จำนวนโคโลนีกลายพันธุ์น้อยกว่าสองเท่าเมื่อเทียบกับตัวควบคุมผล ผลการทดลองบ่งชี้ว่าระบบวัลคาไนซ์โดยใช้ซัลเฟอร์ไม่เหมาะที่จะนำมาใช้ในการทำผลิตภัณฑ์ยางทางการแพทย์ เพราะอาจมีสารเคมีตกค้างที่เป็นพิษต่อยีนและต่อเซลล์ จึงมีความเสี่ยงมากกว่าการวัลคาไนซ์โดยใช้เปอร์ออกไซด์ และ การฉายรังสี

ภาควิชา ชีวเคมี ลายมือชื่อนิสิต.....
สาขาวิชา ชีวเคมี ลายมืออาจารย์ที่ปรึกษา.....
ปีการศึกษา 2547 ลายมืออาจารย์ที่ปรึกษาร่วม.....

4372482023 : MAJOR BIOCHEMISTRY

KEY WORD: GENOTOXICITY / VULCANIZATION / RESIDUAL CHEMICALS / REVERSE MUTATION ASSAY

PATAMAWADEE SANKHEANGAEW : GENOTOXICITY TEST OF VULCANIZED RUBBER AND RESIDUAL CHEMICALS USED IN CURING PROCESSES USING REVERSE MUTATION ASSAY OF *Salmonella thyphimirium.* THESIS ADVISOR: ASSOC. PROF. JARIYA BOONJAWAT, Ph. D., THESIS COADVISOR : MISS ANONG TEPSUWAN, pp ISBN 974-53-1726-8

Reverse mutation assay of *Salmonella typhimurium* strain TA98 and TA100 was used for determination of genotoxic effect of 13 chemicals used in rubber industry and vulcanization as accellerator, activator, antioxidant, filler and vulcanizing agent . Three chemicals in the accellerator group were found genotoxic for strain TA98 namely zinc dibutyl dithiocarbamate (ZBEC), zinc dibuthyldithiocarbamate (ZDBC), mercapto benzothiazole (MBTS) at the concentration 100 microgram/plate . ZDBC exhibited indirect genotoxic effect in the presence of metabolic activation by enzyme from rat liver, but ZBEC and MBTS exhibited the direct genotoxic, therefore the genotoxic potential of the chemical use as accelerator were ranked from high to low as the following . : ZBEC>MBTS>ZDBC>DPTT>ZMBT. Besides, the result indicate that butyl hydroxytoluene (BHT) used as antioxidant and sulfur are cytotoxic to bacterial cells. How ever when vulcanizates were prepared from 6 formulations by using sulfur, peroxide and radiation vulcanization system and extracting residual chemicals by toluene and chloroform:methanol (1:1), none of the rubber vulcanizate show genotoxic effect under this testing condition. The number of mutant colonies when extract by chloroform:methanol(1:1) were more than toluene but less than double of negative control. The result indicate that sulfur curing system was not suitable to produce medical device, because many chemicals were added that may be genotoxic and cytotoxic and therefore more risk than peroxide and rediation curing systems.

Department Biochemistry Student signature……

Field of study Biochemistry Advisor's signature……

Academic year 2004 Co- Advisor's signature……..

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude and deep appreciation to my advisor, Assoc. Prof Dr. Jariya Boonjawat, for her guidance, invaluable advice, helpfulness and encouragement throughout this study. I would like to acknowledge Miss Anong Tepsuwan, my thesis co-advisor for her advice and laboratory facilities throughout this research. I would like to acknowledge my thesis committee Assoc.Prof Aran Incharoensakdi. Assoc. Prof. Chyagrit Siri-Upathum, Dr. Alisa Vangnai for serving as thesis committee and for comments.

I would like to extend my sincere thanks to SK. Polymer Co.,Ltd. for supplying chemicals and helping to produce vulcanized rubber.

All staff members in Ban Pan Biotechnology Co.,Ltd. for their help, supplying Latex materials and laboratory equipment.

I wish to thank all staff members and friends of Biochemistry Program, especially in room 604 for their assistance and friendship. Finally I would like to extend my deepest gratitude to my parents, Mrs. Pranom and Mr. Saney, my younger brother Mr. Katanyoo and my uncle Mr. Sanong for their love, understanding and encouragement

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ABBREVIATIONS

AF-2	2-Aminofluorene
ASTM	American Society for Testing and Materials
BHT	Butyl hydroxytoluene
BP	Benzo[a]pyrene
DCP	Dicumyl peroxide
DMSO	Dimethylsulfoxide
DRC	Dry rubber content
DPTT	Dipentamethylene thiuram tetrasulfide
IR	Isoprene rubber
ISO	International Standard Organization
KDa	Kilodalton
LPS	Lipopolysaccharide
mm	millimeter
MPa	Mega pascal
MBTS	Mercapto benzothiazole
NR	Natural rubber
NRL	Natural rubber latex
Phr	part per hundred rubber
PV-NR	Peroxide vulcanized natural rubber
RVNRL	Radiation vulcanized natural rubber latex
SV-NR	Sulfur vulcanized natural rubber
SV-IR	Sulfur vulcanizrd isoprene rubber
WEP	Water extractable polymer
ZBEC	Zinc dibenzyl dithiocarbamate
ZDBC	Zinc dibutyl dithiocarbamate
ZDEC	Zinc ethylene bis dithiocarbamate
ZDMC	Zinc dimethyl dithiocarbarmate
ZEPC	Zinc ethylphenyl dithiocarbamate
ZMBT	Zinc mercapto benzothiasole
μg	microgram

CHAPTER I

INTRODUCTION

1.1 Natural rubber latex (NRL)

Natural rubber latex (NRL) is obtained from milky secretion of various plants, but the most important commercial source of is the tree *Hevea brasiliensis.* The latex is cloudy white liquid, similar to cow milk (Archer *et al.,* 1963). It is collecting by cutting of the inner bark of the rubber tree, that process called " tapping ". Natural rubber latex is natural polymer that is built up of many units into very long polymer chains. Each unit of polymer is called "monomer". The monomer of natural rubber is *Cis*-1,4-polyisoprene. (Campos and Angulo, 1976). The chemical structure of Cis-1,4-poly isoprene is shown in Figure 1.1.

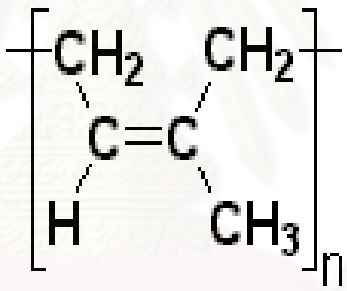


Figure 1.1 Basic structure of *Cis*-1,4-poly isoprene (Nyburg, 1954)

1.1.1 Composition of Natural rubber latex

The latex is a milky white or slightly yellowish opaque liquid. Natural rubber latex is a cytoplasmic fraction which normally contains about 30-45 % rubber hydrocarbon (*Cis*-1,4-poly isoprene) and about 3-5 % w/w non rubber component, of which 1-1.5 % w/w is made up of protein. The composition of the fresh latex is rather complex due to its origin and proportion of certain protein and minerals (Habibah and lim, 1986). The variation is due to many factors such as clone, season and tapping process. A typical composition of fresh field latex is shown in Table 1.1.

Table 1.1 Composition of fresh fied latex (Fong 1992 and Keven1999)

Constituents	Percent (w/v)
Total Solids Content	25.00 – 45.00
Dry Rubber Content	23.00 – 42.00
Amino Acids and N-bases	0.30 – 0.40
Neutral lipids	0.40 – 1.00
Proteins	1.00 – 1.80
Phospholipids	0.50 – 0.60
Inositols Carbohydrates	1.0 – 2.00
Salt (mainly K , P and Mg)	0.40 – 0.60
Water	48.50– 71.80

When the latex is centrifuged, it is separated into three fractions. The top layer contains the cream of rubber particles. The middle layer was yellow which contains the free Wyssling particles and serum. The bottom layer containing predominantly lutoid (Dickenson, 1969). These three layers are shown in Figure 1.2.

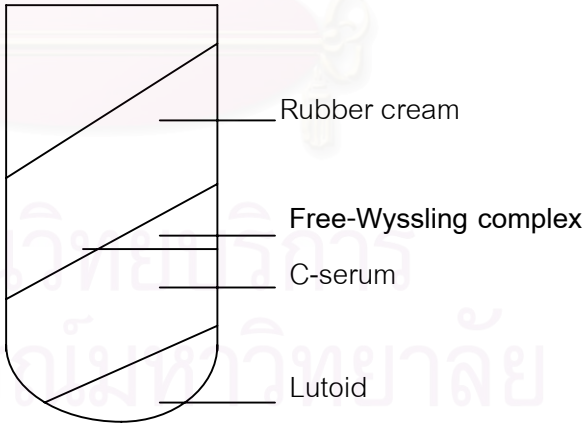


Figure 1.2 Separation of latex by ultracentrifugation at 54,000xg , 40 min (Gomez and Moir, 1979)

1.1.2 Rubber particles in Hevea Latex

Rubber hydrocarbon is the major component of Hevea Latex . The dry rubber content varying from 25 % to 45 % of latex . The average molecular weight ranges from 200 to 600 KDa .

The rubber molecules are found as particle in the latex. These consisting mainly of rubber (90 %) association with lipophilic molecules (mainly lipids and proteins) forming the film that encloses the rubber particle (Ho *et al* , 1979). This film carries negative charges and is responsible for the stability of rubber particle when suspended in aqueous system .

The size of the particles range from 0.01 μm to 5 μm. (Pendle and Swinyard, 1991). The particles are numerous. The common size observed in electron microscopy is about 0.1 μm (Gomez and Moir, 1979).Each particle contain several hundred rubber molecules. The structure of rubber particle by electron microscope is shown in Figure 1.3

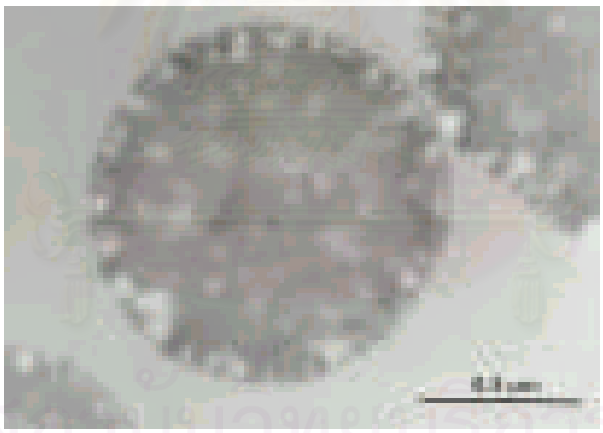


Figure 1.3 Electron micrograph of a rubber particle (Jacob *et al*., 1993)

Rubber particles are surrounded by lipid and protein (Arnold and Evans, 1991). These lipids are similar to the common lipids in our body, and so would not be expected to be recognized as foreign substances by our immune system. However the rubber particle proteins are quite different from protein in our bodies and hence will be recognized as ' foreign ' by our immune system , this is where health problem being (Alennius *et al*., 1994b).

1.1.3 Rubber latex protein

The total protein content of fresh field latex is about 1.0-1.8 %w/v. Proteins in fresh field latex are distributed in three phases, the rubber phase (27%) the C-serum (48%) and within the lutoid or the B-serum (25%). The proteins in the rubber fraction are mainly associated with the interfacial layer surrounding the rubber particles. They are mostly water insoluble. The major proportion of water soluble proteins in fresh latex is derived from B and C-sera. They consist of variety of both anionic and cationic proteins with PI ranging from PH 3.5-9.5 (Dennis *et al*., 1989) The majorities are anionic proteins with molecular weights between 14.0 to 66.0 KDa. A few low molecular proteins (<14 KDa) are also observed. Hevein , a 5.0 KDa (PI 4.7) and B-serum protein could be one of them. Protein do not confer any significant advantages to the properties of NRL (Slater and Chahabra, 1989). Tanaka, 1984 reported that protein should be covalently boundto rubber molecule on the alpha end.

1.1.4 First step of natural rubber production

By analyzing latex freshly tapped from the rubber tree, many small rubber particles less than a thousandth of millimeter were observed (Ho *et al* , 1979). Around the rubber particle is layer of protein, which prevent the rubber from forming a lump. If the protein layer is broken, the rubber particles will coagulate. A subsequent investigation of the coagulated dried rubber showed that the material can change to become elastic. The elastic property of rubber can be enhance by curing process.

1.1.5 Vulcanizing system

Rubber industry has been initiated by the Amazon indian in the 18th century from which rubber shoes were made directly from fresh field latex. The sulfur vulcanizing system was patented in the 1920s and has been developed continously to improve the vulcanizate properties. In dry rubber industry heavy machinery and motors are used to masticate rubber and mix the compounding ingradients by passing between rollers.The rubber compounding ingradients can be added (sekhar, 1960). The long polymer chains must be partially broken by mastication, mechanical shearing force applied by passing the rubber between rollers. Thus , for most purpose , the rubber is ground and dissloved in a suitable solvent and compounded with other ingredients e.g.

accelerators, filler, activator and vulcanizing agents. The compounded rubber is sheeted, extruded in special shapes, applied as molded and then vulcanized.

A crosslinked rubber (vulcanizate) shows the following differences when compared with an uncrosslinked rubber :

- The vulcanizate undergoes deformation upon stretching and when released can recover almost completely its original dimension over time
- The vulcanizate does not dissolve in a good solvent for uncrosslinked rubber but shows swelling
- The properties of a vulcanizate are less sensitive to temperature.

Several cure systems are used in the rubber industry , and each cure system has its own advantages. Selection of a cure system is based upon several considerations such as functional performance desired of the vulcanizate or article and processing and safety risks of the curing process (Kadir, 1994).

1.1.5.1 Sulfur vulcanization

Elemental sulfur is predominant vulcanizing agent for general-purpose rubbers. It is used in combination with one or more accelerators and an activator system such as zinc oxide and a fatty acid (normally stearic acid). The most popular accelerators are delayed action sulphenamides, thiazole, thiuram sulphide , dithiocarbamate and guanidines part of all of the sulfur replaced by sulfur donor , The accelerator determines the rate of vulcanization whereas the accelerator to sulfur ratio dictate the efficiency of vulcanization. In natural rubber an accelerator to sulfer ratio typically of 1-5 is called a conventional vulcanizing system. Most of the crosslinks are poly sulphidic and a high proportion of the sulfur is in the form of cyclic sulphide main chain modifications.

This combination provides good mechanical properties and excellent low temperature resistance.

An accelerator to sulfur ratio of 5:1 is typical of an efficient vulcanizing (EV) system. An intermediate accelerator to sulphur ratio of 1:1 is typical of a semi-effieient vulcanizing (semi-EV) system and provide properties between those of conventional and EV systems (Freakley, 1985 and Franho, 1989). The presumed reaction is shown in Figure 1.4

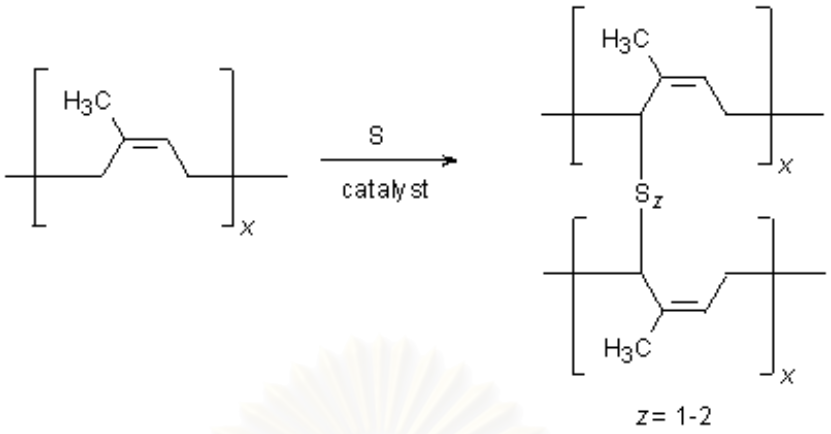


Figure 1.4 Sulfur vulcanization

1.1.5.2 Peroxide vulcanization

Organic peroxide are used to crosslink rubber having no main chain insaturations and to cross link unsaturated rubber vulcanization.

A peroxide initiate cross linking through a free radical reaction. In pratice the reaction is more complicated because coagents are often used with the peroxide to enable a reduction in peroxide levels and improve processing safety. The principal classes of peroxide cross linking agents are dialkyl peroxide , peroxyketals and peroxyesters. Peroxide vulcanization has only a few applications in natural rubber, SBR and other general purpose rubber because the mechanical and dynamic properties are not as good as sulfur vulcanizates (Brydson, 1988). The 3 steps of peroxide vulcanization reaction is shown in Fig 1.5

1.Homolytic breakdown

$$R-O-O-R \xrightarrow{\Delta} 2RO\bullet$$

dialkyl peroxide coagent

2. Abstraction of hydrogen from polymer

$$P-H + R-O\bullet \longrightarrow P\bullet + ROH$$

3. Combination

$$P\bullet + P\bullet \longrightarrow P-P$$

Crosslinked polymer

Fig 1.5 Peroxide vulcanization

For crosslinking of rubber , peroxides provide many advantages e.g. Superior heat and ageing stability, excellent compression set at higher temperatures, nitrosamine free system, good compound shelf life etc.

1.1.5.3 Radiation vulcanization

Radiation vulcanization using electron beams(EB), X-rays, gamma rays is very effective for improving properties of various polymers (Makuuchi, 2003). The reactions of cross-linking, degradation and grafting on polymers initiated by radiation have found many useful application in plastic and rubber materials. Important properties of polymer materials, such as mechanical properties , thermal stability , chemical resistance, melt flow, procressability and surface properties can be significantly improved by radiation processing (Hien *et al*., 1990)

Radiation vulcanization of natural polymers has been resarched and developed for many years. A well-known example is the radiation vulcanization of natural rubber latex for high purity medical product (e.g. glove and condom). The chemical reaction of radiation vulcanization is shown in Figure 1.6

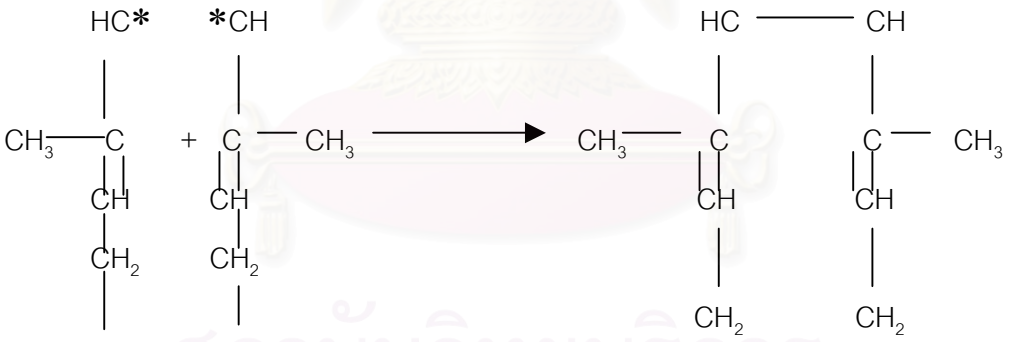


Figure 1.6 Radiation vulcanization

1.1.6 Natural rubber latex product

The Natural rubber consumption of many countries is increasing. The rubber plantation areas is growing up. NRL is most common source material for production of surgical , pharmacuetical including dental product (Mooibroek and Cornish, 2000). Several other household goods were produced from NRL e.g. rubber

band, carpet backing, rubber thread for underwear and eraser etc. (Loos, 1998). The Table 1.2 shows the wide spread use of NRL for medical and household product

Table 1.2 Some medical and household products made from NR

Medical	Household
Gloves	Rubber band
Urinary catheter	Condom
Face mask	Balloon
Wound drain	Eraser
Injection port	Toy
Electrode pad	Sport equipment
Rubber syringe stopper	Shoe soles
Dental Device	Household latex glove
Medication vial	Computer mouse pads
Pressure cuff and tubing	Button on electronic equipment

1.1.7 Allergic reaction to NRL

Rubber particles contain also lipids and proteins . These lipid are similar to the common lipid in our body , and so would not be expected to be recognized as foreign substances by our immune system (Doom and Goossens , 1988). However the rubber particle proteins are quite different from proteins in our bodies and hence will be recognized as ' foreign ' by our immune system , this is where health problem occure . Latex contains low – Molecular – weight soluble proteins, which are the cause of , IgE – mediated allergic reaction . At least 10 different protein have been reported (Slater and Chhabra, 1992). Accellerators and antioxidants added in the vulcanizing system may also be significant mediators of type IV or allergic contact dermatitis . Allergy can probably occur to any of these chemicals , rubber accelerators seem to cause the greatest problems.

Rubber accelerators are chemicals used to speed up the manufacturing process of rubber (Vulcanisation) . This process makes untreated natural rubber

latex suitable for use in the manufacture of many rubber products. There are many types of rubber accelerators in use e.g. Dithiocarbamates, Thiurams, Hexamethylenetetramine that may cause type IV allergy (Brehler and Kutting, 2001)

1.1.7.1 Irritant dermatitis

This is a skin reaction that does not involve the immune system , therefore it is not considered an allergic response. Causes of irritant dermatitis are frequent hand washing, strong detergents, inadequate drying, climatic irritation , or emotional stress. This type of reaction can cause breaks in the skin, which may later lead to latex allergy.

1.1.7.2 Immediate hypersensitivity (Type I)

It is systemic allergic reaction caused by circulating IgE antibodies to the proteins of natural latex. Symptoms include hives , rhinitis con junctivitis , asthma and in severe cases anaphylaxis and hypotension. Symptom occur after exposure to latex within about 5-30 minutes. The symtom are characterized by local or generalized urticaria and edema (McFadden, 2002).However, it can involve more severe symptoms. including asthma marked by difficult breathing, coughing spells, and wheezing, cardiovascular and gastrointestinal ailments and in rare cases, anaphylaxis and death.

Direct contact with the medical product is not needed for sensitisation to latex. Allergenic latex proteins are also adsorbed on the glove powder; when latex gloves are snapped on and off, these become airborne and can be directly inhaled. Direct latex exposure at mucosal or serosal surfaces also occurs by repeated use of rubber catheters or gloves used intraoperatively during abdominal or urological surgery.

1.1.7.3 Delayed hypersensitivity (type IV)

Type IV hypersensitivity can result from exposure to accelerators such as mercaptobenzothiazole, carbamate and ρ-phenylenediamine and other chemicals used in the manufacture of rubber gloves (Brehler and Kutting, 2001) also known as allergic contact dermatitis. The reaction is local and limited to the skin . The skin may become leathery and express papules or blisters.The reaction is delayed occurring several hours after contact, reaching maximum after 24-48 hours and then subsides (Hamilton, 1997). Repeated exposure to rubber latex may cause the skin condition to extend beyond the area of contact with the gloves or other medical device.

1.1.8 Cytotoxic and genotoxic effect of chemicals in Latex manufacturing

Natural rubber latex (NRL) products have been widespreadly used for over a century. Reports of immediate and delayed hypersensitivity to latex have increased dramatically since the first case was reported in 1979 in England (Nutter, 1979). In 1991 U.S. Food and Drug Administration (FDA) (Gelfand , 1991) has warned of the risk of a life - threatening type I allergy associated with NRL products. Ten to 11 percent of health care workers have already become sensitized, and over 2 percent have occupational asthma as a result of latex exposure (Turjanmaa K, 1987) Dithiocarbamates are widely used in the agriculture and rubber industry (Franekie-J *et.al*.,1994) e.g. Ziram (zinc dimethyldithio carbamate), thiuram (tetramethyl thiuram disulphide) and Zinc-ethylene bisdithiocarbamate. Shirasu *et. al*.(1976) reported that zinc dimethyldithiocarbamate (ZDMC) can be mutagenic in *Salmonella typhimurium* strains TA 1530, TA, 1534 and TA 100 without metabolic activation, but non mutagenic in strain TA 1531, TA 1538. This chemical was mutagenic in the rec assay (Kada *et al*., 1974) but Moriya et. al, 1983 got negative results in the *Escherichia coli* reversion assay.

There are relatively few data in the literature on mutagenic activity of zinc-ethylene bis dithiocarbamate (ZBEC). It was negative in the Ames test and cultured mamalian cells.

Zenzen *et al*. (2001) studied the mutagenic and cytotoxic effectiveness of the vulcanization accelerators zinc dimethyldithiocarbamate (ZDMC) and zinc disononyl dithio carbamate (ZDINDC) by lymphocyte culture without metabolic activation ZDMC cause more genotoxic effect than ZDINDC which has been developed recently as a potentially less toxic analogue of ZDMC.

Mohanan *et.al*. (2000) studied the genotixic potency of zinc mercapto benzothiazole (ZMBT) by *in vivo* chromosomal aberrations using 5 group of Swiss albino

mice. The first three groups received ZMBT at 1920, 960, and 480 μg/20 g animal. The remaining two groups received the vehicle (Cotton seed oil) and positive (methyl methane sulfonate) controls. All animals were sacrificed at the end of 36 hours. Bone marrow preparation were made, stained with Giemsa and examined for chromosomal abnormalities. The results showed that no incidence of chromosomal abnormalities. The results indicated a lack of incidence of chromosomal abnormalities in the test and control groups.

Medical Devices Agency (MDA) (Sumana, 1998) has investigated potential human health hazard arising from a presence of dithiocarbamate vulcanization accelerators in latex products (mainly gloves). After collection of manufacture's data on usage and residues of these accelerators, an independent investigation of solvent extractable residue and dithiocarbamate migration into aqueous stimulants were commissioned, to complement equivalent "in-house" test data from two major manufactureres. The presence of extractable accelerator residues in commercial products was confirmed. Potential human health hazards associated with Zinc dimethyl dithiocarbarmate, Zinc ethylene bis dithiocarbamate and Zinc dibutyl dithiocarbamate (ZDMC, ZDEC and ZDBC) include genotoxicity and possible carcinogenicity. ZDMC must be considered a genotoxin (and probable carcinogen) so residue of this substance in latex medical devices should be minimized. ZDEC was proved genotoxic *in vitro* but was not cleary genotoxic *in vivo*, and may have activity intermediate between that of ZDMC and ZDBC by chromosome aberration test.

Dillon *et. al.* (1998) studied the effectiveness of *Salmonella* strains TA 100, TA 102 and TA 104 for detecting mutagenicity of some aldehyde and peroxide. Several aldehyde and peroxide were tested for mutagenicity using *Salmonella typhimurium* tester strains TA100 and TA102 in the presence and absence of S9 mix. The result shown that acetaldehyde and dicumyl peroxide gave no mutagenic effect in Salmonella typhimurium strain TA100 and TA 102 with S9. Formaldehyde and glutaraldehyde were

mutagenic in TA100 and TA102 with S9. All chemicals were mutagenic in strains TA102 and also mutagenic in TA100.

Hageman *et. al*. (1988) studied the mutagenicity of butylated hydroxyanisole, butylated hydroxytoluene and tert - butylhydroguinone using Salmonella tester strains TA97, TA102, TA104 and TA100. None of the phenolic antioxidants showed mutagenic activity, either with or without metablolic activation. At doses of 100 μg/plate and higher all 3 phenolic antioxidants exhibited toxic effects. Mckee and Tometsko (1979) studied Inhibition of promutagen activation by antioxidants butylated hydroxya nisole and butylated hydroxy toluene using Salmonella typhimurium reversion test. The result showed that BHA and BHT reduced reversion induced by chemicals requiring metabolic activation for effectiveness. However, they did not affect reversion induced by direct-acting motagens. These result suggested that BHA and BHT may inhibit the metabolic activation process.

Kaniwa *et. al*. (1994) reported zinc ethylphenyldithio carbamate (ZEPC), a dithiocarbarmate - type accelerator (DTC) was causative in a case of allergic contact dermatitis from worker using natural gloves. Subsequently, they have clarified that DTCs such as zinc dimethyldithiocarbamate (ZDMC), zinc diethyldithiocarbamate (ZDBC) and amines such as dimethylamine (DMA), diethylamine (DEA) and piperidine (PIP) were also causative in cases from surgical rubber gloves.

Knudsen *et.al*. (2000) suggested that to prevent contact with specific rubber accelerators, sensitized patient have to know in which glove brands these accelerators are present. ZDEC, ZDBC and ZMBT were the most frequently detected residual chemicals in gloves.

Soloneski *et al.* (2002) studied the genotoxicity of zinc-ethylene bis dithiocarbamate by using Chinese hamster overy (CHO) cell by the analysis of the sister chromatid exchange (SCE) and single cell gel electrophoresis (SCGE) assays. This chemical was tested at dose ranging from 0.1 to 100 μg/ml. Concentrations of 0.1 - 25.0 μg/ml chemical induced the increase of SCE frequency and doses higher than 50.0 μg/ml were cytotoxic. SCGE assay shown that dose 25 - 100 μg/ml of zinc ethylene bis dithiocarbamate increased cells damage.

1.2 Genotoxicity and description of genotoxin

Natural rubber latex was used to make various medical devices such as catheter, surgical gloves, anesthesia tubing and bag, endotracheal tube, injection port and tubing etc. In case of using medical device in long terms, one should be awared about the genotoxicity because it can be inherited to next generation.

An alteration in any part of the DNA structure that results in permanent inheritable change is called mutation, and the agent that cause such mutation is known as a genotoxic agents or genotoxin (Johnson *et al.*, 1993).

There are three major types of genotoxic effect, gene mutation, chromosomal aberration and DNA effect. Gene mutation and chromosomal aberration test the DNA molecule, while DNA effects detect events that may lead to cell damage.

1.2.1 General purpose of genotoxicity testing

Genotoxicity tests can be defined as in vitro and induce genetic damage directly or indirectly by various mechanism. These tests should enable a hazard identification with respect to damage to DNA and its fixation. Fixation of damage to DNA in the form of gene mutation which is generally considered to be essential for heritable effect. Compounds which are positive in tests that detect such kind of damage have the potential to be human carcinogens and /or mutagen. They may induce cancer or heritable defects.Genotoxicity test have been used mainly for the prediction of carcinogenicity. In addition , the test may be valuable for the interpretation of carcinogenicity studies.

1.2.2 Standard procedures for *in vitro* genotoxicity test (ISO 10993-3)

The *Samonella typhimurium* reverse mutation test is rapid, inexpensive and relatively easy to perform. Many of the tester strains have several features that make them more sensitive for the detection of mutations, including responsive DNA sequences at the reversion sites and elimination of DNA repair systems or enhancement of error-prone DNA repair processes. The specificity of the tester strains can provide some useful information on the types of mutations that are induced by genotoxic agents. A very large data base of results for a wide variety of chemical structures is available for bacterial reverse mutation tests and well-established procedures have been developed for testing chemicals with different physicochemical properties, including volatile substance.

Registration of pharmaceuticals products requires assessment of their genotoxic potential. It is appropriate to assess genotoxicity in bacterial reverse mutation test. This test has been shown to detect relevant genetic changes and the majority of genotoxic. When the genetic toxicity of a medical device be experimentally assessed , a series of *in vitro* shall be used. Test shall be performed either using extracts or the dissolved material using appropriate solvent.

1.3 Reverse mutation assay of *Samonella thyphimurium*

The *Salmonella typhimurium* reverse mutation test was first validated in a study of 300 chemicals, most of which are known carcinogens (McCann *et al,* 1975). It was subsequently validated in studies by the Imperial Chemical industries (Purchase and Ashby, 1976) and National Cancer Center Research Institute in Tokyo (Sugimura *et al* , 1976). Nearly 90 % of the carcinogens tested were mutagenic (Rinkus and leagtor , 1979).

Bacterial mutagenicity assays , especially the Ames test (*Samonella typhimurium his* reversion assay), have been used worldwide in research laboratories. Their application is motivated by several aims such as identification of chemical exposures and biochemical mechanism of mutagenesis . The assay is rapid , inexpensive and reliable (Maron and Ames, 1983). In addition , Samonella assay information for numerous complex environmental mixture has been published . The test is used as a

screen for mutagenic activity of pure compound complex mixture and body fluids(Claxton *et al*,1984)

1.3.1 Characteristic of some Samonella strains

At present the most commonly used *Salmonella* strains are TA 1535, TA 1537 , TA 1538 , TA 98 and TA 100 (Serres and Shelby , 1979). The number and type of strains used depend upon the availability and type of sample. The tester strain having a mutation in one of the genes of the histidine operon. One mutation, (*rfa*) leads to the defective lipopolysaccharide coat . Another is a deletion of genes involved in the synthesis of the vitamin biotin (bio) and in the excision repair of DNA damage (*uvr B*). The rfa mutation increases the permeability of the strains to large molecules, there by increasing the mutagenicity or toxic effects of these chemicals. The uvrB mutation leads to a reduced level of error-free repair of some type of DNA damage and there by enhances the strainssensitivity to certain chemical and physical mutagens. Strain TA 100 has been derived from TA 1535 by the introduction of the plasmid pKM101 which increases the sensitivity of mutagen detection by enhancing error-prone DNA repair. Strain TA 1538 carries a different frameshift mutation his D3052 . Strain TA 98 is derived from TA 1538 by the introduction of plasmid pkM101

Within laboratory each strain is maintained as a frozen permanent culture. They are opened only to subculture the strains for additional frozen permanent cultures or to prepare master plates . Master plates are stored refrigerated at 4 C°, and they serve as a convenient source for the more frequently used strains in the mutagenicity assay . Minimal-glucose aga ,enriched with histidine and biotin , is used to prepare master plates. Some list of genotypes is shown in Table 1.3

Table 1.3 Genotypes of the TA strain used for mutation testing (Ames *at al.*, 1975)

Histidine mutation		LPS	Repair	R-factor
hisD3052	hisG46			
TA1538	TA1535	*rfa*	*uvrB*	- R
TA98	TA100	*rfa*	*uvrB*	+R
TA1978	TA1975	*rfa*	+	- R
TA94	TA92	+	+	+R
TA1534	TA1950	+	*uvrB*	+R
-	TA2410	+	*uvrB*	+R

1.3.2 Metabolic activation by S9

Bacteria should be exposed to the test substance both in the presence and absence of an appropriate metabolic activation system. Many chemicals that are not direct-acting mutagens will yield with metabolic activation , mutagenic metabolic by-products . In order to test for these indirect-acting mutagen in bacteria , a representative mammalian enzyme system is added to prepare a metabolically active liver fraction. The most commonly used system is a cofactor-supplemented post-mitochondrial fraction (S9) prepared from the livers of rodents (usually rats) treated with enzyme-inducing agents such as Aroclor 1254 (Ames *et al*., 1975) polybiphenyl(PCB) (Elliott *et al.,* 1992). The post-mitochondrial supernatant fraction is usually used at concentrations in the range from 10 to 30 percent v/v in the S9 mix. The choice and concentration of a metabolic activation system may depend upon the class of chemical being tested. For azo dyes and diazo compounds, using a reductive metabolic activation system may be more appropriate (Matsushima, 1980). Liver S9 should be prepared using aseptic techniques so that subsequent filter-sterilization is not required. Filtration of the S9 or S9 mix may lead to loss of enzyme activity (Maron *et al*., 1983). Each batch of S9, whether produced by the testing laboratory or obtained commercially, should be tested for activatiing mechanism.

1.3.3 Plate incorporation method

Plate incorporation test (Maron and Ames, 1983) consists of combining the test compound, the *Samonella thyphimurium* and presence/absence S9 mix in soft agar poured onto a minimal glucose agar plate. Positive and negative controls are also included in each assay. After incubation at 37 C° for 48 hours, revertant colonies were counted. The test was positive when the number of revertant colonies was at least twice of the negative control. The concept of testing method is shown in Figure 1.7

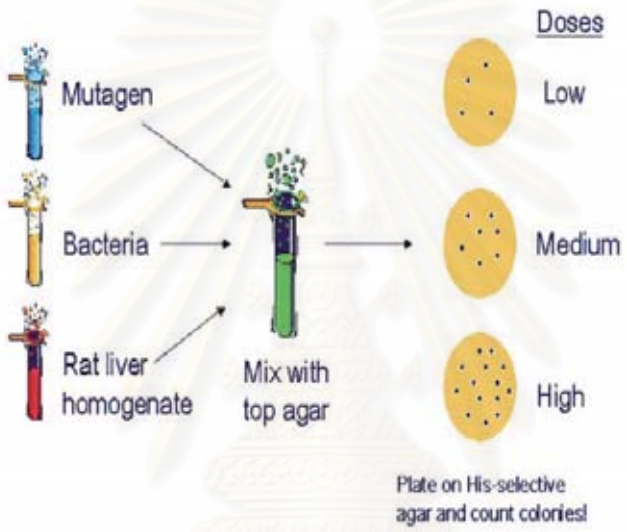


Figure 1.7 Schematic diagram of testing method

1.3.4 Spontaneous reversion

Spontaneous reversion of the tester strains to histidine is usaually happened in mutagenicity experiments and is expressed as the number of spontaneous revertants per plate.The revertant colonie are clearly visible in a uniform background lawn of auxotrophic bacteria . Each tester strain reverts spontaneously at a frequency that is characteristic of the strain . The number of revertants that arise spontaneously during the 48 h incubation is dependent on the final number of auxotrophs on the plate and that number is a function of the histidine concentration. (Green and Muriel , 1976).

The revertant colonies of *Samonella typhimurium* is shown in Figure 1.8

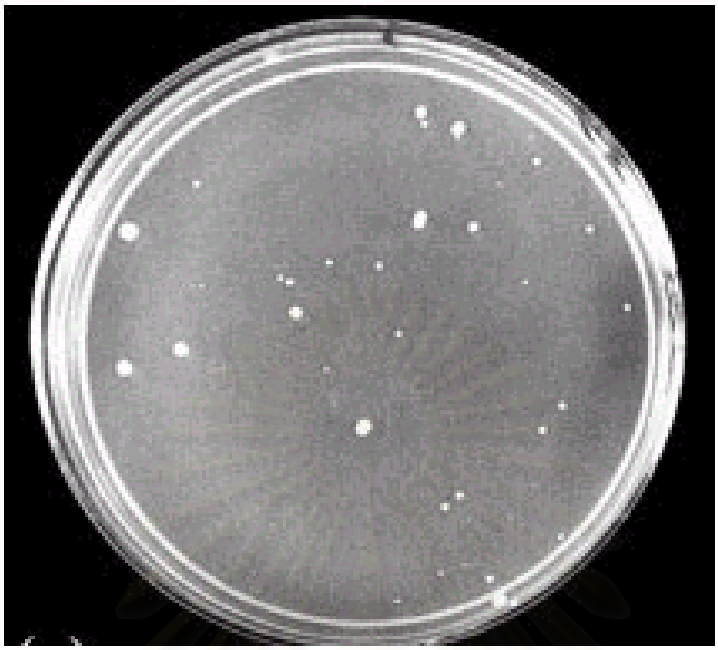


Figure 1.8 Revertant colonies of *Samonella typhimurium*

1.3.5 Positive control (standard mutagen)

In each experiment, it is essential to have positive control.The effective standard mutagen is used to confirm the reversion properties and specificity of each strain and the efficacy of S9 mix (Zeiger, 1985). There are various standard mutagens that are used in reverse mutation of *Samonella typhimurium* e.g. Benz[o]pyrene, 2-Aminofluorene (AF-2), Sodium azide, Mitomycin C, Dexon etc.

1.4 The rationale and aim of this study

The medical devices such as surgical glove, urinary catheters, dental device, face mask, injection port etc. were made from natural and synthetic rubber. All of these products must pass the process called "vulcanization" which is the process of crosslinking rubber molecules. It is a chemical reaction, which imparts elastic properties to rubber. Many chemicals may be added in vulcanization process such as vulcanizing agent, accellerator, activator, antioxidant, filler to improve the physical property.

Conventional vulcanization system consists of a variety of chemicals so it is a risk to leave residual chemicals in the products.

Some chemicals such as dithiocarbamate zinc oxide, sulfur etc. have been reported to be carcinogenic and nitrosamine producing and hence unsuitable for the manufacture of articles which may come into contact with human tissue (Jacob and Vijayakumar, 1997). The medical devices must pass both the physical and biological property. The most important biological property is genotoxicity which can be inherited to next generation and may lead to cancer and genetic disorder.

The peroxide and radiation vulcanized natural rubber products have definite technical advantage over conventional sulfur-cured latex in some specialized areas, namely less nitrosamine, low cytotoxicity. Hence it can be used in areas of body contact.

The aims of this research are to develop the method for testing the genotoxic effect of vulcanized rubber and chemicals used in vulcanizing systems. Testing method was developed from reverse mutation assay of *Samonella typhimurium* (Maron and Ames, 1983). This method is used widely to identify genetic effect. This research is in response to the demand of rubber industry which need to test the genotoxicity of pharmaceutical rubber products. Good quality NRL products can be exported and can compete in rubber world's market.

The objectives of this research are

1. To test the genotoxicity of chemicals used in curing processes.

2. To find suitable solvent for extraction of the chemicals from vulcanized rubber

3. To develop the method for testing genotoxicity of NRL products from three vulcanizing systems; sulfur vulcanization, peroxide vulcanization and gamma radiation vulcanization.

CHAPTER II

MATERAILS AND METHODS

2.1 Materials

2.1.1 Bacterial strains

Salmonella typhimurium strain TA 98 and TA 100 were obtained from National Cancer Institute(NCI). *Salmonella typhimurium* strain TA 98 detects mutagen that cause frame shift mutation and strain TA 100 detects for base-pair substitution mutation.

2.1.2 Fresh field latex

Fresh field latex was kindly provided by Pan Asia Biotechnology Co., Ltd., it was preserved with 0.3 % ammonia, TMTD and ZnO

2.1.3 Concentrated latex 60%

Concentrated latex 60% was prepare according to Oranoot Haowuttikul, 2003 (see page 28) at Pan Asia Biotechnology Co., Ltd.,Rayong

2.1.4 Solid rubber

STR 5L, STR XL and polyisoprene rubber was kindly provided by S.K. Polymer Co.,Ltd.,

2.1.5 Chemicals

All vulcanizing chemicals were obtained from SK. Polymer Co., Ltd., except n-butyl acrylate was kindly provided by Assoc. Prof. Chayagrit Siri-Upathum , Department of Nuclear Technology, Faculty of Engineering, Chulalongkorn University

2.1.5.1 chemical for sulfur vulcanization

Butylhydroxytoluene (BHT)

Dipentamethylene thiuram tetrasulfide (DPTT)

Hisil233

Irganox

Mercapto benzothiazole (MBTS)

Stearic acid

Zinc cabonate

Zinc dibenzyl dithiocarbamate (ZBEC)

Zinc mercaptobenzothiazole (ZBMT)

Zinc dibutyldithiocarbamate (ZDBC)

Zinc oxide

Zinc stearate

2.1.5.2 Chemicals for peroxide vulcanization

Butylhydroxytoluene (BHT)

Hisil233

Irganox

Dicumylperoxide 98% (DCP)

Stearic acid

ZnO

2.2.5.3 Chemical for gamma radiation vulcanization

Normal butyl acrylate (n-BA)

2.2.5.4 Chemical for testing genotoxicity

AF-2 was perchased from Sigma

Benzo[a]pyrene was perchased from Sigma

D-Biotin was perchased from Sigma

D-Glucose was perchased from BDH

D-glucose-6-phosphate was perchased from Sigma

Citric acid monohydrate was perchased from MERCK

Potassium phosphate , dibasic (anhydrous) was perchased from BDH

Sodium ammonium phosphate was perchase from BDH

L-Histidine.HCl was perchased from Sigma

Sodium chloride was perchased from MERK

Potassium chloride was perchased from BDH

Magnesium chloride was perchased from BDH

Sodium hydrogen phosphate was perchased from MERK

Disodium hydrogen phosphate was perchased from MERK

B-Nicotinamide adedine dinucleotide phosphate was perchased from Sigma

Bacto agar was perchased from Difco

Sodium hydroxide was perchased from Sigma

2.2 Apparatus

2.2.1 Apparatus were kindly provided by the National Cancer Institute (NCI)

Incubator shaker model SW23 United instrument, USA

Colony countermodel CC-2 Q.N.C., Thailand

pH meter model 360 pH , temp,mV meter

Plate maker model Wheaton unispense, USA

Evaporator model R111 Rotavapour, Zwitzerland

Incubator model Imperial II , Lab-Line Instrument, Inc, USA

Freezer model 958 Thermoforma, Becthai Co. Ltd

2.2.2 Apparatus availabled at Department of Biochemistry Faculty of science, Chulalongkorn University

Centrifuge, microcentrifuge high speed model MC-15 A, Tomy Seiko Co. Ltd, Japan

Centrifuge , refrigerated centrifuge model J-21 C, Beckman Instrument Inc, USA

Incubator shaker model G 76D, New Brunswick Scientific Co., Inc Edison, N. J. USA

UV visible spectrophotometer model UV-240, Shimadzu, Japan

2.2.3 Apparatus from Pan Asia Biotechnology Co., Ltd. Rayong

Centrifuge, (Alfa Laval, 7000xg) and 200-liter latex storage tank

2.2.3 Apparatus from Banpan Research Bankok Rubber Co., Ltd.

Tensile tester, Instron Calibration Laboratory, Thailand

2.2.4 Apparatus from Department of Nuclear Technology, Faculty of Engineering Chulalongkorn University

Latex irradiator BSV-60, Institute of Isotope, Hungary

2.2.5 Apparatus at Prosthodontics Department, Faculty of Dentistry, Chulalonkorn University

Durometer (shore A) model 471, Zwick, Germany

2.3 Method

2.3.1 Preparation of chemical samples, positive(std. Mutagen) and negative control

All chemical samples were dissolved in dimethyl sulfoxide (DMSO). For each sample concentration was prepared at 1, 10, 100 μg/30 ml (1plate). Kept at 4 C° in a sterile vial.

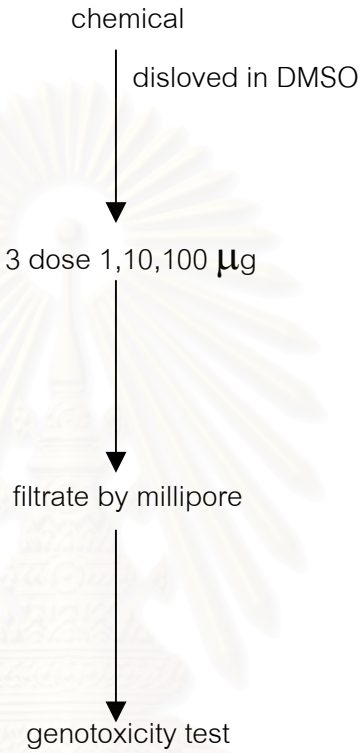


Figure 2.1 preparation of chemical samples for genotoxicity test

The Positive standard mutagens used in this research are 2-Aminofluorene (AF-2) and Benz[o]pyrene (BP). AF-2 is a direct mutagen and BP is an indirect mutagent must be metabolized by S9. Both standard mutagens were dissolved in dimethyl sulfoxide (DMSO).

The solvent used as negative control was Dimethysulfoxide (DMSO)

The doses of standard mutagens used were shown below

Samonella typhimuriumTA98 , + S9 mix : Benzo[a]pyrene 5 μg /plate

Samonella typhimuriumTA98, - S9mix : AF-2 0.1 μg/plate

Samonella typhimuriumTA100, + S9mix : Benzo[a]pyrene 0.625 μg/plate

Samonella typhimuriumTA100 , -S9mix : AF-2 0.01 μg/plate

2.3.2 Preparation of sulfur vulcanized natural and isoprene rubber (SV-NR, SV-IR)

2.3.2.1 Sulfur vulcanized natural rubber (SV-NR)

First , add solid rubber into the two roll mill machine. From this step , the rubber particle will be decresed. Then add following chemicals to the rubber and mix them together. In this research was produced SV-NR by using 2 formulations shown in Table 2.1 and 2.1

Table 2.1 Chemical formulation 1 of NR in sulfur curing system

Component	Function
NR- STR 5L	Rubber
ZBEC	Accelerator
ZMBT	Accelerator
Zinc Stearate	Activator
$ZnCO_3$	Activator
BHT	Antioxidant
Hisil 233	Filler
PEG	Filler
Sulfur	Vulcanizing agent

Table 2.2 Chemical formulation 2 of NR in sulfur curing system

Component	Function
NR-STR XL	Rubber
DPTT	Accelerator
ZDBC	Accellerator
Stearic acid	Activator
$ZnCO_3$	Activator
BHT	Antioxidant
Sulfur	Vulcanizing agent

After mixing, sulfur was added to rubber compound and vulcanization at 150 C for 8.3 minute.

2.3.2.2 Sulfur vulcanized polyisoprene rubber (SV-IR)

First , add polyisoprene rubber into the two roll mill machine. From this step, the rubber particle will be decrease. Then add following chemicals to the rubber and mix them together. This research was aim to produced SV-IR by using 2 formulations.

Table 2.3 Chemical formulation 1 of IR in sulfur curing system

Component	Function
IR-2005	Rubber
MBTS	Accellerator
ZDBC	Accellerator
Stearic acid	Activator
ZnO	Activator
Hisil	Filler
PEG	Filler
BHT	Antioxidant
Sulfur	Vulcanizing agent

Table 2.4 Chemical formulation 2 of IR in sulfur curing system

Component	Function
IR-2205	Rubber
MBTS	Accelerator
ZDBC	Accelerator
Stearic acid	Activator
ZnO	Activator
Hisil	Filler
PEG	Filler
IRGANOX	Antioxidant
Sulfur	Vulcanizing agent

After mixing , sulfur was added to rubber compound and vulcanized at 150C° for 9.2 minute.

2.3.3 Preparation of peroxide vulcanized natural rubber (PV-NR)

First , add solid rubber into the two roll mill machine. From this step , the rubber particle will be decresed. Then add following chemicals to the rubber and mix then together. This research was aim to produced PV-NR by using 2 formulations.

Table 2.5 Chemical formulation 1 of NR in peroxide curing system

Component	Funtion
NR–STR 5L	Rubber
ZnO	Activator
BHT	Antioxidant
Hisil 233	Filler
DCP	Vulcanizing agent

Table 2.6 Chemical formulation 2 of NR in peroxide curing system

Component	Function
NR-STR XL	Rubber
Stearic acid	Activator
ZnO	Activator
Hisil	Filler
Irganox	Antioxidant
DCP 98%	Vulcanizing agent

After mixing, dicumyl peroxide was added to rubber compound and vulcanized at 150 C°for 7.8 minute.

2.3.4 Preparation of Concentrated latex 60 % as raw material for RVNRL

Fresh field latex was diluted with water and preserved with 0.3 %(v/v) NH_3. The ammoniated latex was determined for dry rubber content (DRC), Total solid content(TSC), Volatile fatty acid (VFA), and Mg content. Ammoniated latex was adjusted by

15 % NH_3 to final concentration 0.35 % w/w .Then add diammonium hydrogen phosphate(DAP) to reduced Mg content.The DAP-ammoniated latex was added 32% w/v of luaric acid in 0.3 % NH_3 for making final concentration of 0.05 %. The latex was kept over night.The overnigth latex was added 2 % alginate for making final concentration of 0.01 phr. The alginated latex was added 40 % KOH for making final concentration of 0.1 % w/w. Then it was centrifuged for making 60 %concentrated latex Concentrated latex was determined for dry rubber content (DRC). Total solid content(TSC), Volatile fatty acid (VFA), KOH and Mg content .

2.3.5 Testing of concentrated latex specification (ISO-2004-1979 (E))

2.3.5.1 Determination of total solid content (TSC)

Weighed the empty petridish and record the empty petridish weight. An aliquot of 5 g of latex was pipetted into the petridish and dried in an oven at 60°C for 10 - 12 hours. Dried rubber in pretridish was reweighed and calculated for TSC content by the equation below.

$$\% \text{ TSC} = W_1 / W_0 \times 100$$

Where W_1 = weight of the dry rubber in pretidish (g)

W_0 = weight of the latex after subtraction the weight of petidish (g)

2.3.5.2 Determination of dry rubber content (DRC)

An aliquot of 5 ml of latex was pipetted into a petridish and coagulated with 5 % acetic acid in ethyl alcohol. After complete coagulation the coagulum was then removed, washed with water, creped and dried in an oven at 60°C for 10 - 12 hours. Dried coagulum was weight and calculate DRC content by the equation below.

$$\% \text{ DRC} = W_1 / W_0 \times 100$$

Where W_1 = weight of the dry rubber (g)

W_0 = weight of the latex taken (g)

2.3.5.3 Determination of non rubber content (NR)

Non rubber content was calculated by the equation below.

TSC - DRC = (%) NR

2.3.5.4 Determination of volatile fatty acid (VFA)

Weighed 50 g of latex in a stainless beaker and added 50 ml of 30 % $(NH_4)_2SO_4$ Heat the solution in the water bath at the temperature of 100°C and squeeze for the serum. Filtrated the serum through Whatman number 1 filter paper.Pipetted 25 ml of serum into a flask containing 5 ml of 50 % H_2SO_4. Passed steam through the steam jacketed distillation apparatus for at least 15 minutes with steam through the steam

jacketed distillation apparatus for at least 15 minutes with steam outlet open, introduce into the innertube 10 ml of acidified serum by pipette. Place a 100 ml graduated cylinder under the tip of condenser to obtain 100 ml of distilled serum. Degassed for 3 minutes and titrated with standard 0.01 N Ba $(OH)_2$ using phenolphthalene as indicator. The % VFA was calculated by the equation below.

$$\text{Volatile fatty acid, VIF (\%)} = \frac{[67.32xNxV]}{mxTSC}x50 + \frac{m(100-DRC)}{100xP}$$

Where N : Normal of $Ba(OH)_2$ m : weight of latex (g)

V : Volume (ml) of $Ba(OH)_2$ used in titration TSC : % TSC of latex

P : Serum density = 1.02 megagram/m^3 DRC : % DRC of latex

2.3.5.5 Determination of alkalinity (NH_3)

Weighed 10 g of latex into the beaker diluted with 200 ml distilled water and added non-ionic stabilizer such as Teric 16A. Titrated with standard 0.1 N $(NH_4)_2SO_4$ with methyl red indicator. Calculated in g NH_3 per 100 g latex.

2.3.5.6 Determination of Mg content (%Mg)

Weighed 10 g of latex into the beaker, added 10 ml of distilled water and 5 ml of 25 % (v/v) acetic acid. Squeezed for the serum and poured on to the 80 mesh filter. Pipetted filtrated 10 ml serum into 50 ml beaker adjusted pH to 10.5 by NH_4Cl or NH4OH then pipetted 4 ml of 4 % KCN into the serum. Added 0.1 g of Erichrome Black T into the serum and the serum would turn violet. Titrated with 0.05 M EDTA. End point was the violet color turned blue.

Calculation : the Mg content was expressed in percent (w/w)

$$\text{Percentage Mg} = \frac{24.32xBxDx10,000}{1,000 \quad 10 \quad C}$$

$$\text{Where B : EDTA factor} = \frac{\text{burette reading}}{(v)} x \frac{(M)EDTA}{(M)}$$

C : Value of solid in 10 g of latex (g) $C = \frac{AxTSC}{100}$; A : Weightoflatex(g)

D : Total volume of serum in sample (ml) ; D = (A - C) + 15

(15, 10 ml of water added + 5ml of 25 % acetic acid)

24.32 = Mw of Mg

2.3.5.7 Determination of potassium hydroxide (KOH)

Added formaldehyde solution into 50 % TSC latex and diluted to 30 % TSC with water and titrated with standard KOH indicated end -point by pH meter. Plotted graph of 1) pH or 2) dpH/dV or 3) d^2pH/dv^2. V is volume of KOH at end-point.

2.3.5.8 Determination of nitrogen content (RRIM, 1992)

This specification is the rubber specification added to this research because of the requirement from glove manufacturers. The concentrated latex sample was dried as described on Methods 2.4.2 Rubber specimen was weighed accurately about 0.1 - 0.2 g into a micro Kjeldahl tube and 0.65 g of catalyst mixture (K_2SO_4 : Cu_2SO_4, 5HO : SeO ; 30 : 4 : 1) and 2.5 ml of concentrated sulfuric acid were added. The mixture was boiled gently in the digestion utill the solution becomes clear green or colorless with no yellow tint. Cool the digest and transfer to distillation until followed by three washing with to the distillation vessel, and pass steam through the distillation apparatus until the volume of distillate in the receiving flask reach 150 ml which take about 5 minutes. Immediately titrate the distillate with standardized 0.01N H_2SO_4. Blank can be prepared by adding all the reagents but omitting the sample.

Calculation : Total nitrogen content was calculated as follows :

$$\% \text{ Total nitrogen} = \frac{(V1 - V2) \times M \times 1.4}{W}$$

Where V1 = Volume of blank (ml)

V2 = Volume of titrant (ml)

M = concentration of H_2SO_4(N)

W = weight of sample (g)

2.3.7 Gamma radiation vulcanization

Concentrated latex 60% was diluted up to 50% dry rubber content (DRC) using 1% ammonia solution, stabilized by 0.5 phr KOH as 10 % solution. The 5 phr of n-butyl acrylate (n-BA)was added as the sensitizer to the latex while stirring. Gamma-rays irradiation from a Co-60 source was carried out at various dose rate 10, 12, 14, 16, 18 and 20 KGy. Rubber film were prepared by casting on the glass plates, dried in air until it became transparent. Post drying of films as done by in an oven at 80 C^{o} for 3 hour.

2.3.8 Testing physical property of vulcanized rubbers curring by sulfur, peroxide and gamma radiation

2.3.8.1 Hardness test (ASTM D1415,1988)

The international hardness test is based on measurement of the penetration of a rigid ball into the rubber specimen under specified condition. Rubber vulcanized was prepared as flat and smooth sheet having thickness sufficient to fit the gap of type A durometer . The plunger of durometer was pressed with the minor force on to the specimen; the scale was pointed and read as the hardness in shore A at room temperature. The median valve of 5 different point distributed over the specimen was record.

2.3.8.2 Tear strength test (ASTM D624,2989)

Five test piece for tear resistant were cut out from vulcanized rubber by punching with dye using a single stroke of press the thickness of the test piece was measured by micrometer dial gauge. The highest force required to tear the test piece was recorded and calculated as follows.

$$\text{Tear strength} = \frac{\text{Highest force (Kg)}}{\text{Thickness of test piece (cm)}}$$

2.3.8.3 Determination of tensile strength , 300% modulus and Elongation at break (ASTM D412 , 1987)

The 5 dumbell test pieces (Figure 2.2) were cut out from the rubber vulcanized punching with a die using a single stroke of press. Sample thickness at gauge length was measured by micrometer .

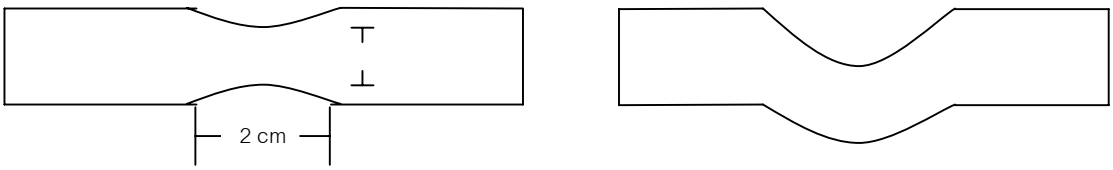


Figure 2.2

a) For tensile strength , % elongation at break and 300% modulus test

b) For tear strength test

The two ends of the test piece were clamped into the two grips of the testing machine , The test piece was stretched at a constant rate of moving grip of 500+/-50 mm the force required to stretch the sample to 300% of reference mark length and to breakage were recorded and modulus and tensile strength were calculate as follows.

$$\text{300 \% modulus} = \frac{\text{Force at 8 cm (Kg)}}{\text{Cross-Sectional area (cm}^2\text{)}}$$

$$\text{Tensile strength} = \frac{\text{Force at break (Kg)}}{\text{Cross –Sectional area (cm}^2\text{)}}$$

$$\text{\% Elongation at break} = \text{length of reference mark at break}$$

2.3.9 Extraction of residual chemicals in vulcanized rubbers (SV-NR, SV-IR, PV-NR, RVNRL)

A pieces of vulcanized rubber was cut to 1x1 cm^2. Vulcanized rubbers pieces were was extractd with following solvent

Toluene	2 days
Chloroform:Methanol (1:!)	2 days

(Ratio of rubber : solvent = 1 g / 10 ml)

After extraction , the solvent was removed by evaporation then add 10 ml of DMSO. The sample was filtered with millipore . Keep sample solution in vial.

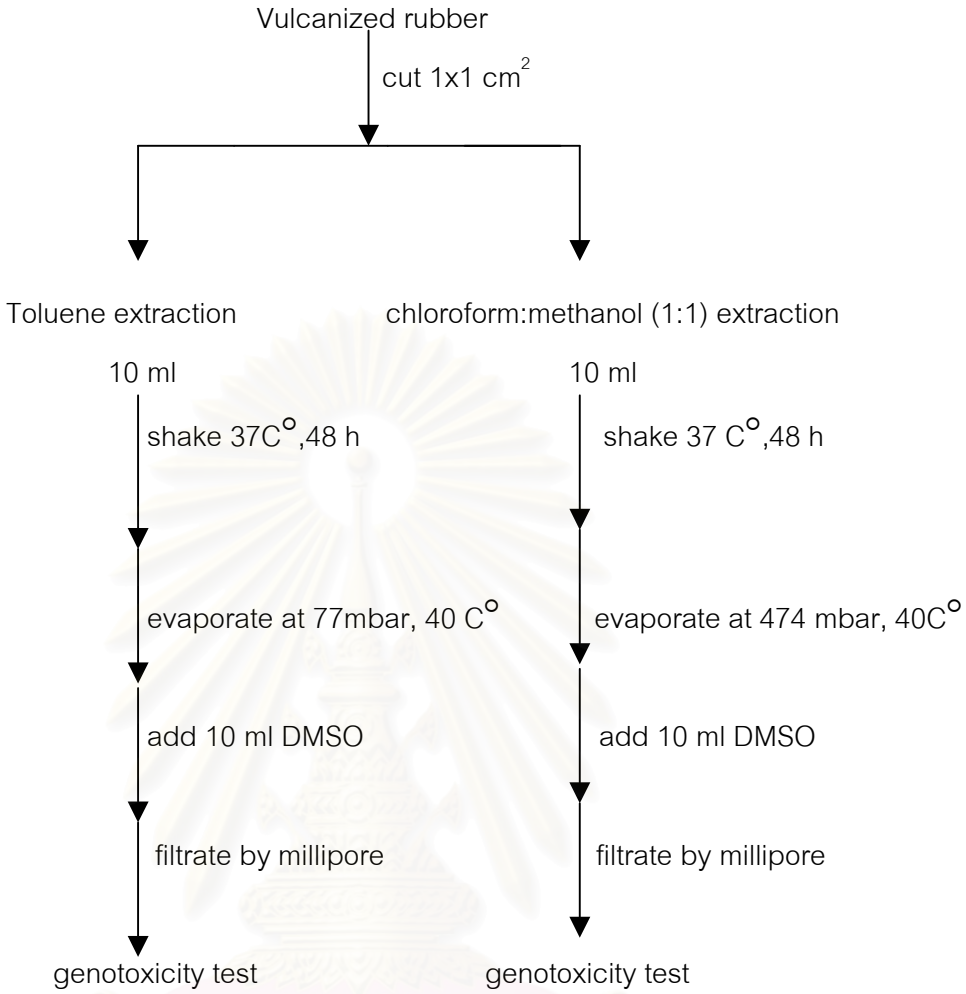


Figure 2.3 Preparation of test solution for genotoxicity test

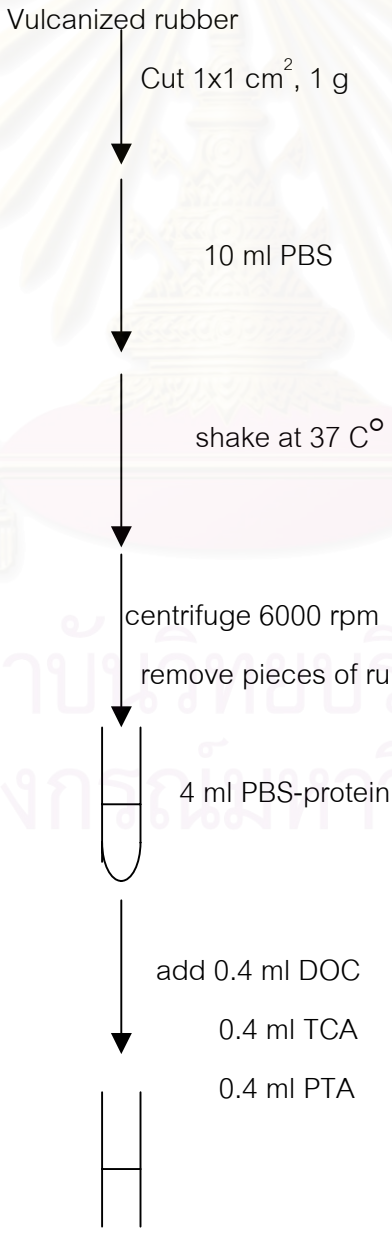
2.3.10 Protein determination

2.3.10.1 Extraction of water extractable proteins in vulcanized rubber(ISO/DIS 12243)

A piece of latex film was cut from each sample with 1x 1 cm square size, weighed and transferred to a 200 ml flask and added 10 ml of phosphate buffered saline (PBS) per gram of specimen. The flask was sealed with sealing film; extraction was at 37 °C .After that decant off the extract and remove any particulate matter, by cenrifuging at 6,000 rpm for 15 minutes.The extract was precipitated and concentrated.

2.3.10.2 Precipitation and concentration of protein (ISO DIS 12243)

Accurately transfer 4 ml each, of extract to the 10 ml polypropylene tubes. Add 0.4 ml of Deoxycholate (DOC), mix and allow to stand for 10 minutes than add 0.4 ml of Trichoroacetic acid (TCA) and mix. Add 0.4 ml Phosphotungstric acid (PTA), mix and allow to stand for a further 30 min. After that centrifuge at 4500 rpm for 45 minutes. Decant the supernatant liquid and drain by inverting each centrifuge tube on an absorbent paper towel. Add 0.8 ml of 0.2 M sodium hydroxide solution to each tube, including the blank, to redissolve the precipitated protein.



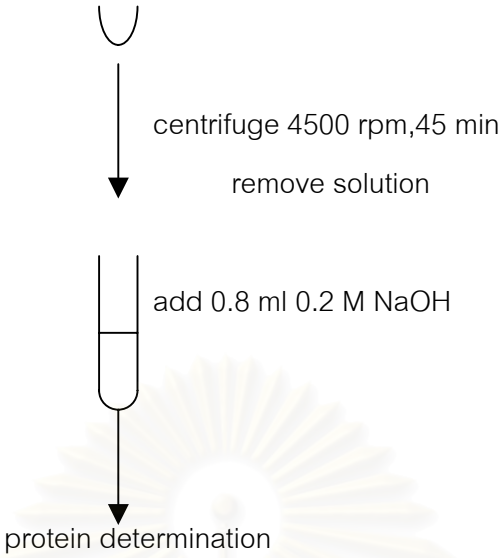


Figure 2.4 (continue) Preparation of sample for protein determination

2.3.10.3 Determination of water extractable protein by modified Lowry method (ISO DIS 12243)

0.8 ml of the redissolved protein solutions including the blank, add 0.3 ml alkaline copper sulfate, Reagent A, mix well. Add 0.1 ml of dilute Folin solution, Reagent B,mix and allow to stand at least 15 minutes and no longer than 1 hour before measuring the absorbance. Transfer the solutions to cuvettes and measure the absorbance at 750 nm.

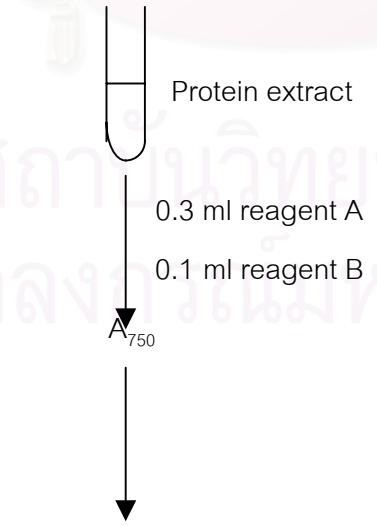


Figure 2.5 Protein determination by modified Lowry method

2.4 Genotoxicity Test of *Samonella typhimurium* strain TA 98 and TA 100

2.4.1 Preparation of *Salmonella typhimurium* tester strains

50 µl of tester strains , TA 98 and TA 100 were grown in 12 ml of Oxoid nutrient broth No 2. and incubated overnight (about 16 hrs.) in 37 C° in shaking water bath . These cultures were re-isolated by steaking on minimal glucose agar plates which the surfaced were spreaded with 0.1 ml of 8 mg/ml ampicillin , 0.3 ml of 0.1 M histidine and 0.1 ml of 1 mm biotin.

2.4.2 Preparation of a Minimal Glucose Agar Plate

Bacto agar was solubilized in distilled water and autoclaved . Then it was mixed with sterile glucose and Vogel – Bonner medium E. See the proportion for making it in Appendix 4. About 30 ml of agar was poured on to the sterile plate . When it solidified , keep in the incubator for 4 days.

2.4.3 Preparation of Top Agar

Top agar containing 0.6 % Bacto agar and 0.5 % Sodium chloride was autoclaved . Mix thoroughty and transfer to glass bottle . Before use it was heated by microwave and added 10 % (v/v) of 0.5 mM histidine- biotin .

2.4.4 Preparation of S9 fraction

10 wista male rats can be prepare 300 ml S9 fraction. Each rat was induced by injection of 500 mg/kg polychlorinated biphenyl (PCB) for 5 days before Kill (Alvares et al., 1973 ; Kler et al., 1974). Then remove liver from the rats. All steps of procedure are carried out at 0 – 4 Cº. The rat's liver was washed by cold sterile 0.15 M KCl then minced with sterile scissors. The liver was centrifuged for 10 min at 9000rpm. The supernatant is S9 fraction. Keep 2 ml S9 in cryotube at -80°C.

2.4.5 Preparation of S9 mix

S9 mix is rat liver microsomal enzymes plus cofactors.

S9 mix fraction compose of 0.15M KCl, 0.1 M Glucose -6- phosphate, 0.1 M NADP, 0.1 $MgCl_{2,}$ 0.2 M phosphate-KCl buffer pH 7.4, 0.1M NADP. All ingrediend

should be chilled and prepare fresh for each experiment. The proportion of each solution for making S9 mix shown in Appendix 2

2.4.6 Plate-incorporation assay

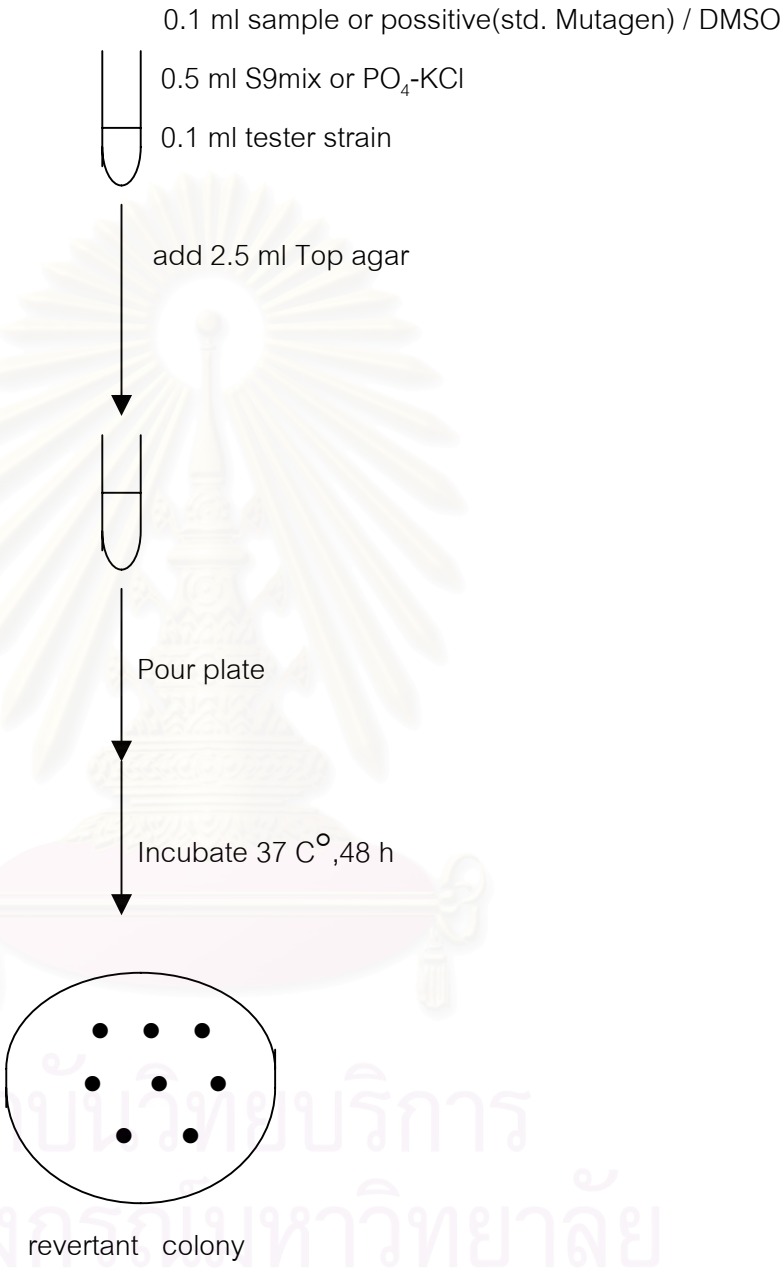


Figure 2.6 Step in genotoxicity evaluation using *Salmonella typhimurium* TA 98 and TA100

For each experiment for testing genotoxicity. Each sample , prepare

4 groups

Group 1. Samonella typhimurium strain TA98 , - S9

Negative control is DMSO and positive control is AF-2 0.1 μg

Group 2. Samonella typhimurium strain TA98 , + S9

Negative control is DMSO and positive control is Benzo[a]pyrene 5 μg

Group 3. Samonella typhimurium strain TA100 , - S9

Negative control is DMSO and positive control is AF-2 0.01 μg

Group 4. Samonella typhimurium strain TA100 , + S9

Negative control is DMSO and positive control is Benzo[a]pyrene 0.625 μg

Start each experimental group by following steps

Group 1.

Add 100 μl sample to sterile tube

↓

Add 0.5 ml PO_4 – KCl buffer

↓

Add 100 μl bacterial strain TA98

↓

Add 2.5 ml top agar

↓

Mix and pour to minimal glucose agar plate

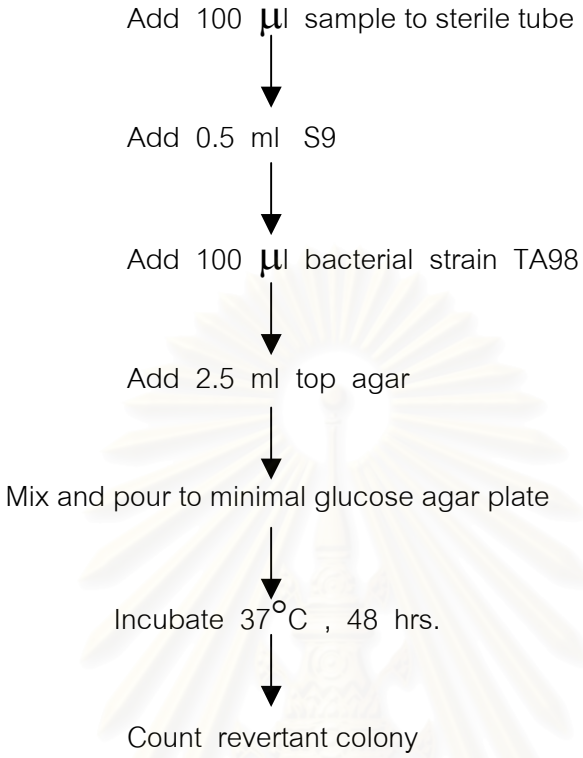
↓

Incubate 37°C , 48 hrs.

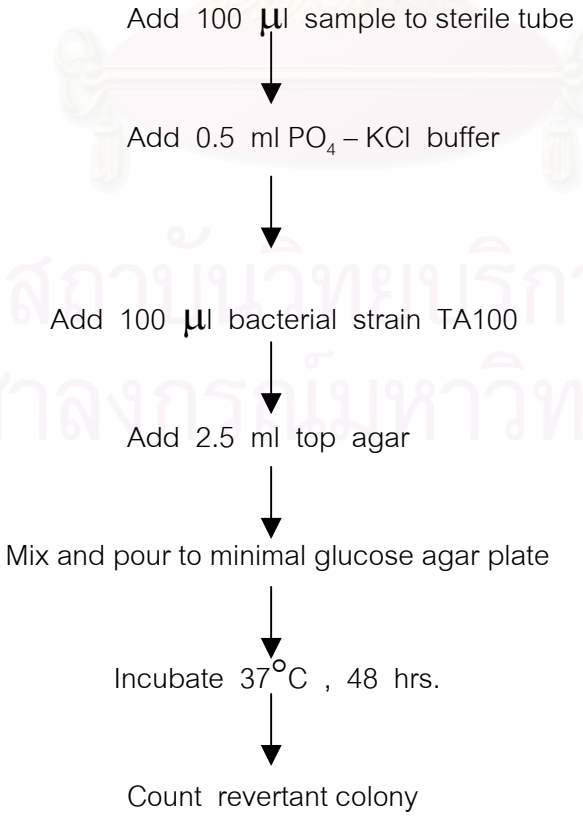
↓

Count revertant colony

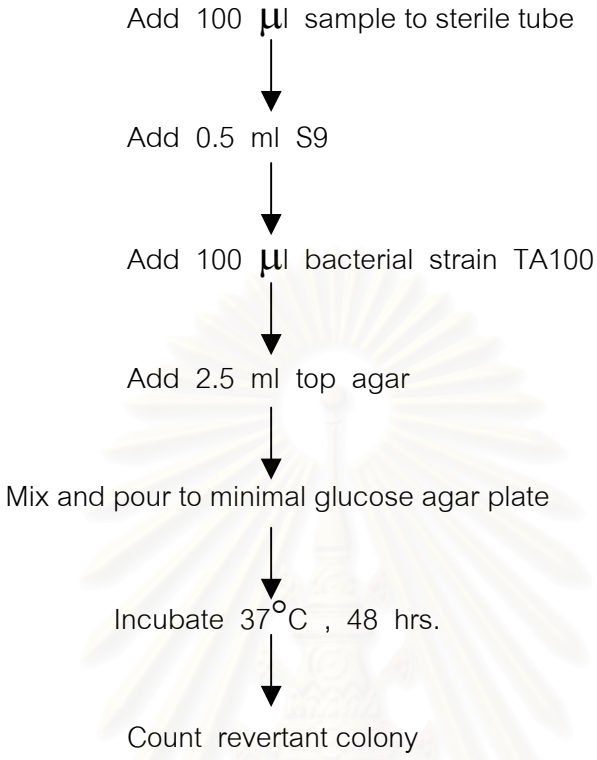
Group 2.



Group 3.



Group 4.



2.4.7 Spontaneous reversion

Spontaneous reversion of the tester strains to histidine independence is measured routinely in mutagenicity experiments and is expressed as the number of spontaneous revertants per plate . The revertant colocies are clearly visible in a uniform background lawn of auxotrophic bacteria . Each tester strain reverts spontaneously at a frequency that is characteristic of the strain .

0.1 ml of DMSO was added to capped culture tube . 0.5 ml of $NaPO_4$ – KCl buffer pH 7.4 and 0.1 ml of fresh overnight culture of TA98 or TA100 was added . The mixture was incubated in shaking water bath at 37°C in 20 min . After that 2.0 ml of molten top agar was added to the mixture , mixed and then poured on the minimal glucose agar plate . Plates were rotated and left it to become harden and incubated at 37°C for 48 hours . The his revertants colonies that grown on the mimimal glucose agar plate were counted.

CHAPTER III

RESULTS

3.1 Dose response studies of chemicals used in sulfur and peroxide vulcanizing system

3.1.1 Dose response studies of chemicals used in sulfur vulcanizing system

Both NR and synthetic rubber (isoprene, IR) are vulcanized by sulfur vulcanization. There are several types of chemicals used in this system

The results of the genotoxicity test of chemicals used as accelerator and activator are presented in Table 3.1 and 3.2. All the experiments were run in duplicate using 2 plates per dose. Each chemical was examined at 3 dose 1, 10 and 100 μg/plate. The positive test of each chemical exhibited when the number of revertant colonies was double of the negative control dimethyl sulfoxide(DMSO), and showed trend of increasing revertant colonies with increasing dose.

3.1.1.1 Accellerators and Activators

Table 3.1 Dose response study of chemical used as accellerators and activators in sulfur curing system

Function	Chemical	Dose (μg/plate)	Number of revertants observed using *Salmonella typhimurium*			
			strain TA98		strain TA100	
			-S9	+S9	-S9	+S9
Negative control	DMSO		25	27	103	127
Positive control	AF-2	0.1	402	-	-	-
	AF-2	0.001	-	-	418	-
	BP	5	-	403	-	-
	BP	0.625	-	-	-	446
Accelerator	DPTT	1	32	34	132	155
	DPTT	10	37	39	142	177
	DPTT	100	43	47	154	194

Table 3.2 Dose response study of chemical used as accellerators and activators in sulfur curing system

Function	Chemical	Dose (μg/plate)	Number of revertants observed using *Salmonella typhimurium*			
			strain TA98		strain TA100	
			-S9	+S9	-S9	+S9
Negative control	DMSO		29	31	156	167
Positive control	AF-2	0.1	453	-	-	-
	AF-2	0.001	-	-	516	-
	BP	5	-	466	-	
	BP	0.625	-	-	-	519
Accellerator	MBTS	1	33	38	167	176
	MBTS	10	50	47	175	181
	MBTS	100	61	55	184	181
	ZBEC	1	32	40	147	142
	ZBEC	10	45	46	170	154
	ZBEC	100	61	45	196	176
	ZDBC	1	33	48	162	164
	ZDBC	10	41	52	172	173
	ZDBC	100	43	63	169	182

Table 3.2 continue

Function	Chemical	Dose (μg/plate)	Number of revertants observed using *Salmonella typhimurium*			
			strain TA98		strain TA100	
			-S9	+S9	-S9	+S9
Accelertor	ZMBT	1	55	37	160	169
	ZMBT	10	39	38	183	170
	ZMBT	100	41	37	184	174
Activator	Stearic acid	1	32	48	126	139
	Stearic acid	10	34	50	133	140
	Stearic acid	100	35	52	147	142
	Zinc carbonate	1	45	44	149	174
	Zinc carbonate	10	45	44	170	146
	Zinc carbonate	100	51	37	194	141
	Zinc oxide	1	53	34	176	162
	Zinc oxide	10	47	46	162	167
	Zinc oxide	100	47	44	192	160
	Zinc stearate	1	30	42	118	143
	Zinc stearate	10	33	50	122	147
	Zinc stearate	100	35	54	139	152

Table 3.1 and 3.2 showed that the dosage 100 microgram of zinc dibenzyl dithiocarbamate (ZBEC), zinc dibuthyldithicarbamate (ZDBC), and mercapto benzothiazole (MBTS) that used in sulfur vulcanization showed direct genotoxic effect as evident by double revertant colonies with increasing dose (Figure 3.3, 3.5 and 3.8). Other accelerators and activators did not show direct genotoxic effect. Zinc dibuthyl dithicarbamate (ZDBC) exhibited genotoxic effect with metabolic activation (+S9) in *Salmonella typhimurium* strain TA98 . The number of revertant colonies was 63 which was more than double when compared with negative control dimethyl sulfoxide, the number of revertant colonies was 31. Zinc dibenzyl dithiocarbamate (ZBEC), Zinc dibenzyl dithiocarbamate (ZBEC), Mercapto benzothiazole (MBTS) exhibited the genotoxic effect without metabolic activation (-S9) in *Salmonella typhimurium* strain TA98 . The number of revertant colonies was 61 which was more than double when compared with negative control dimethyl sulfoxide, the number of revertant colonies was 29.

Figure 3.1-3.18 show the dose response curve of the accelerators and Activators.

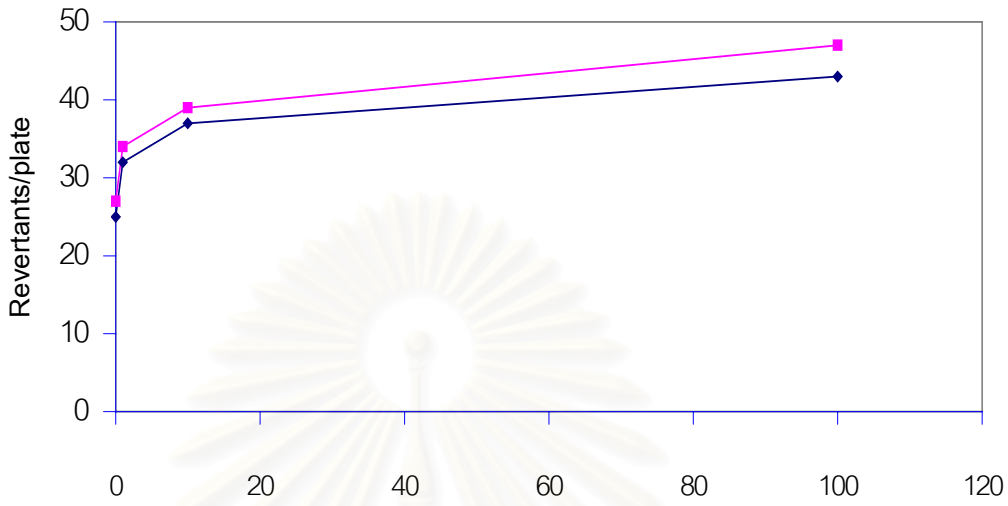


Figure 3.1 Dose response curve of DPTT with *Salmonella typhimurium* strain TA98

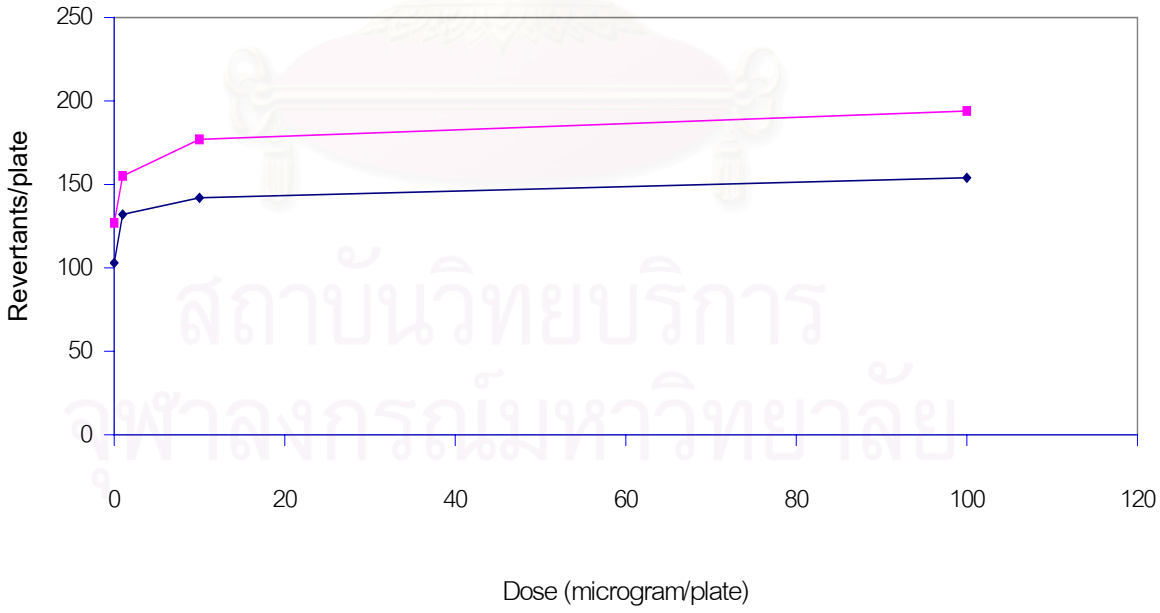


Figure 3.2 Dose response curve of DPTT with *Salmonella typhimurium* TA100

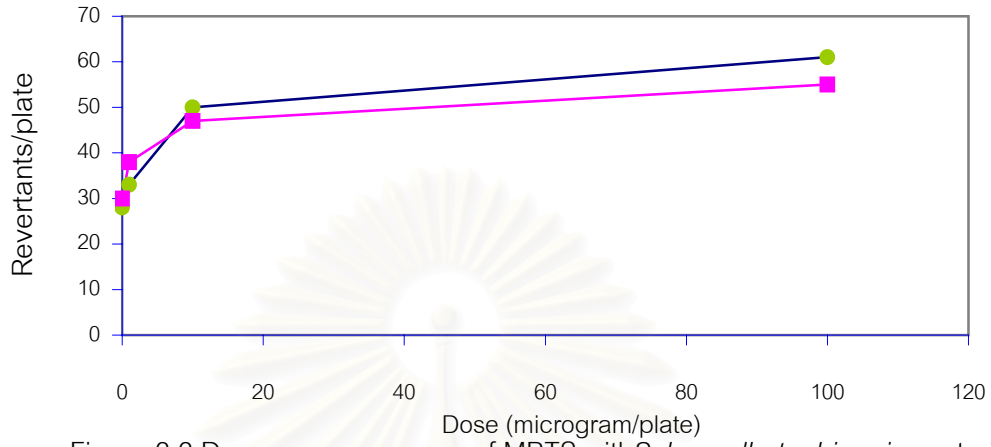


Figure 3.3 Dose response curve of MBTS with*Salmonella typhimurium* strain

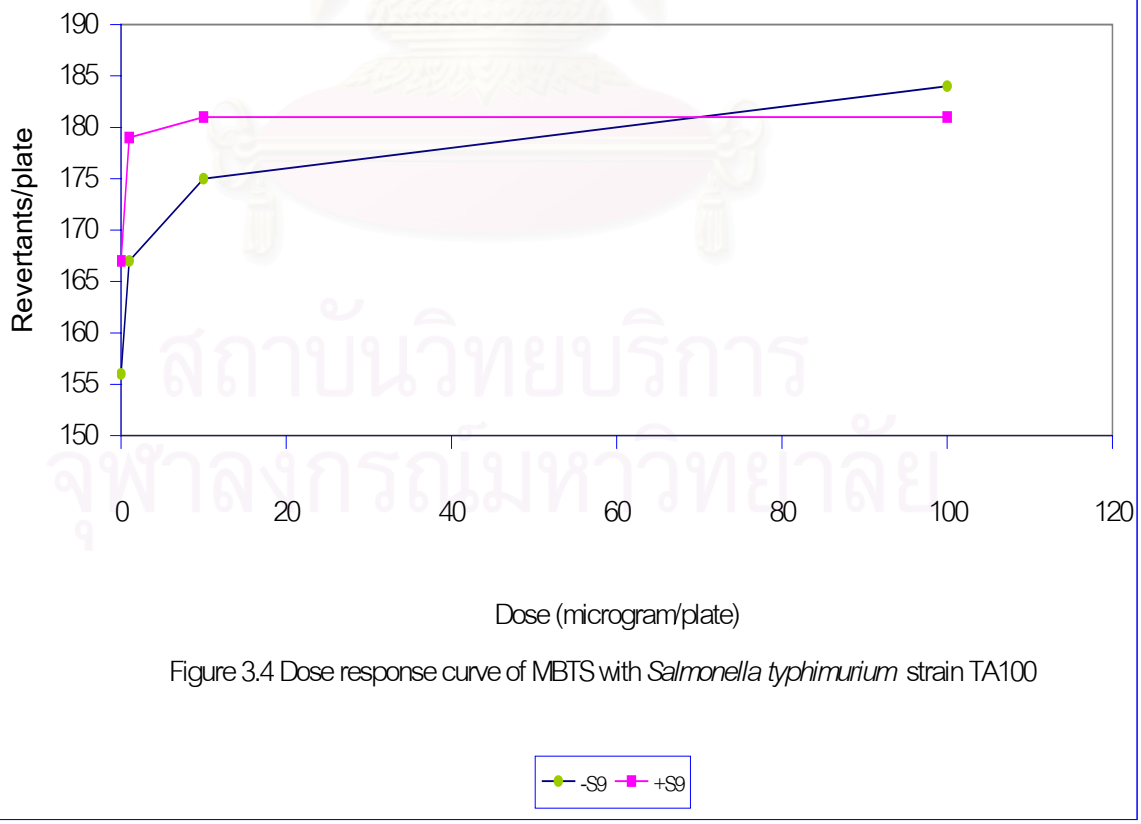


Figure 3.4 Dose response curve of MBTS with *Salmonella typhimurium* strain TA100

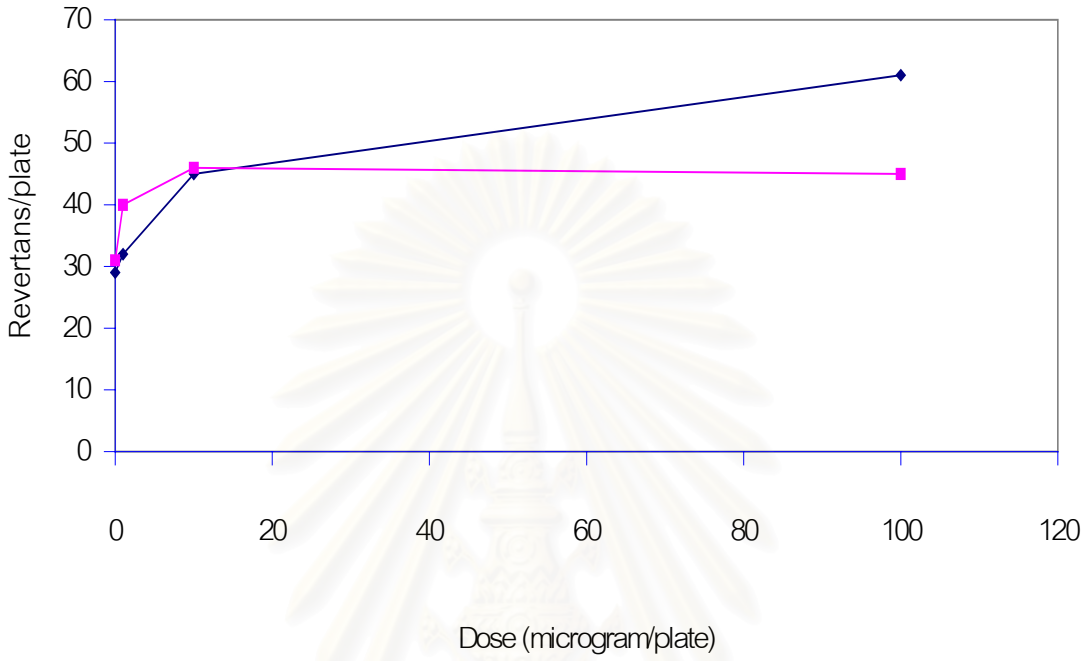


Figure 3.5 Dose response curve of ZBEC with *Salmonella typhimurium* strain TA98

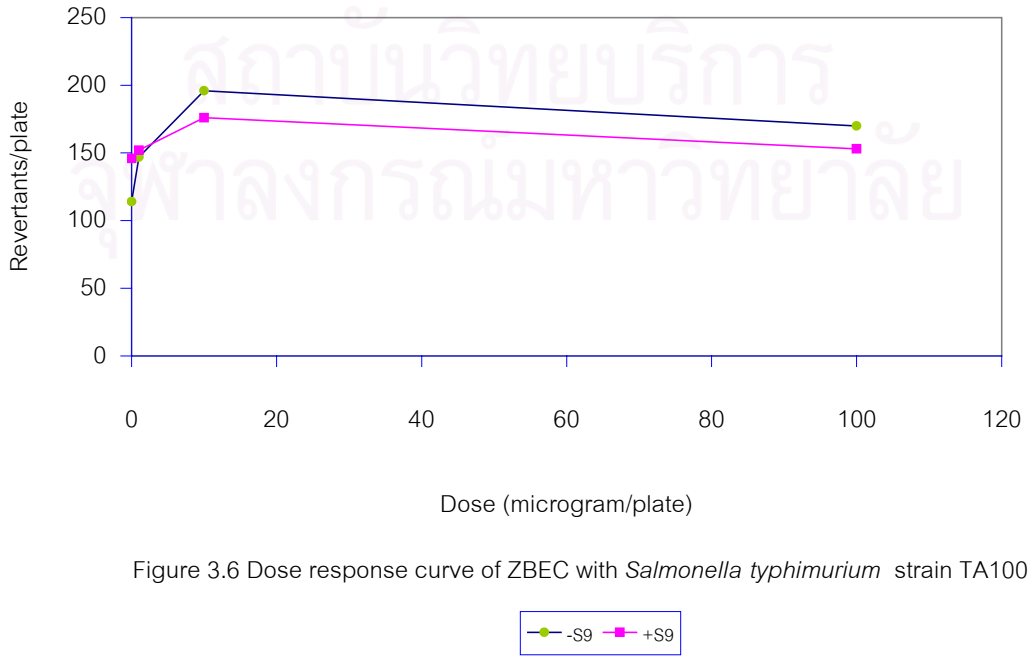


Figure 3.6 Dose response curve of ZBEC with *Salmonella typhimurium* strain TA100

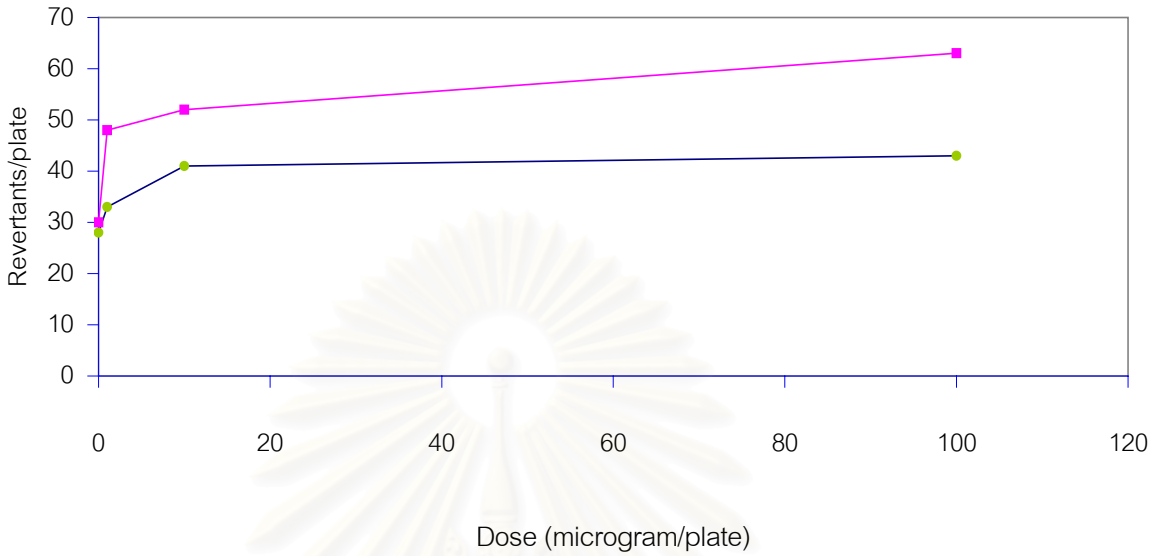


Figure 3.7 Dose response curve of ZDBC with *Salmonella typhimurium* strain TA98

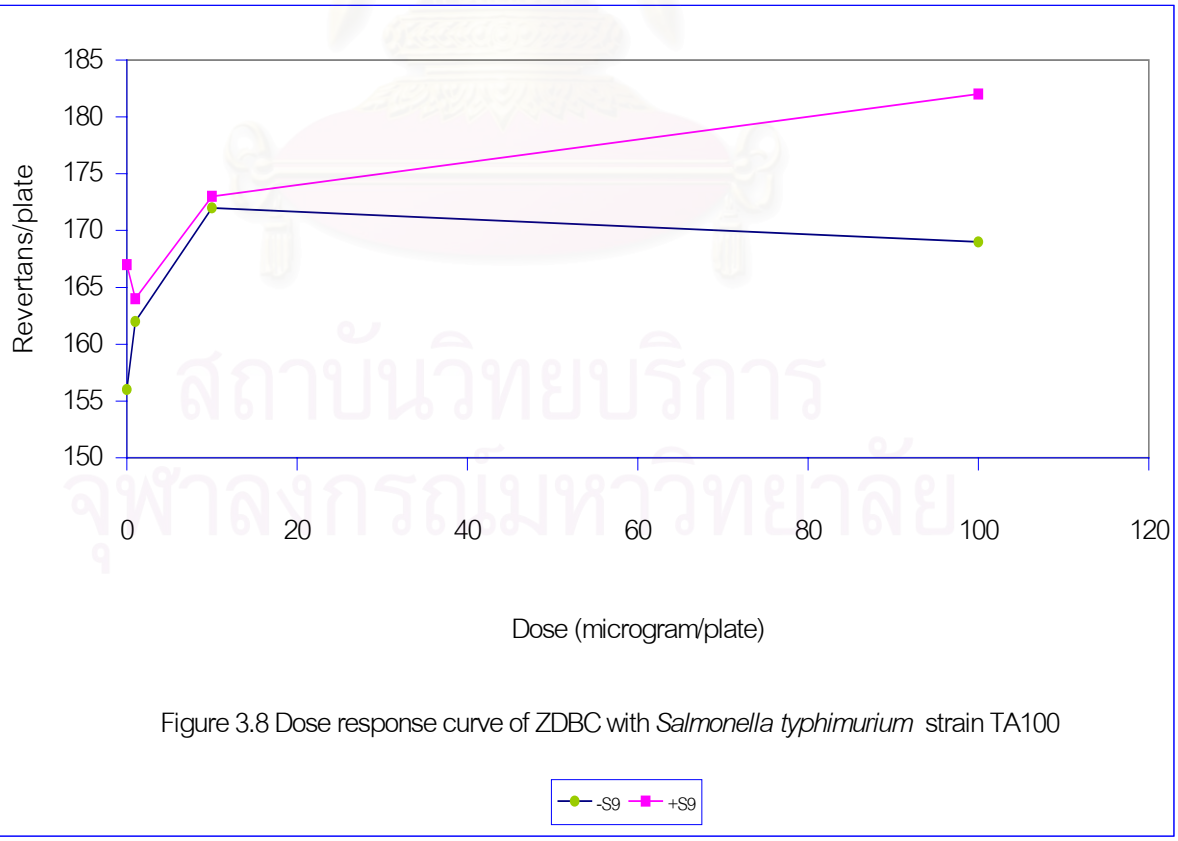


Figure 3.8 Dose response curve of ZDBC with *Salmonella typhimurium* strain TA100

The number of revertant colony of Mercaptobenzothiasole(MBTS), Zinc dibutyl dithiocarbarmate (ZDBC) and Zinc dibenzyl dithio carbamate (ZBEC) increase when their concentration increased . The dose response curve of these chemicals are linear (figure 3.3, 3.5 and 3.7) so that we can conclude that both chemicals have genotoxic potential.

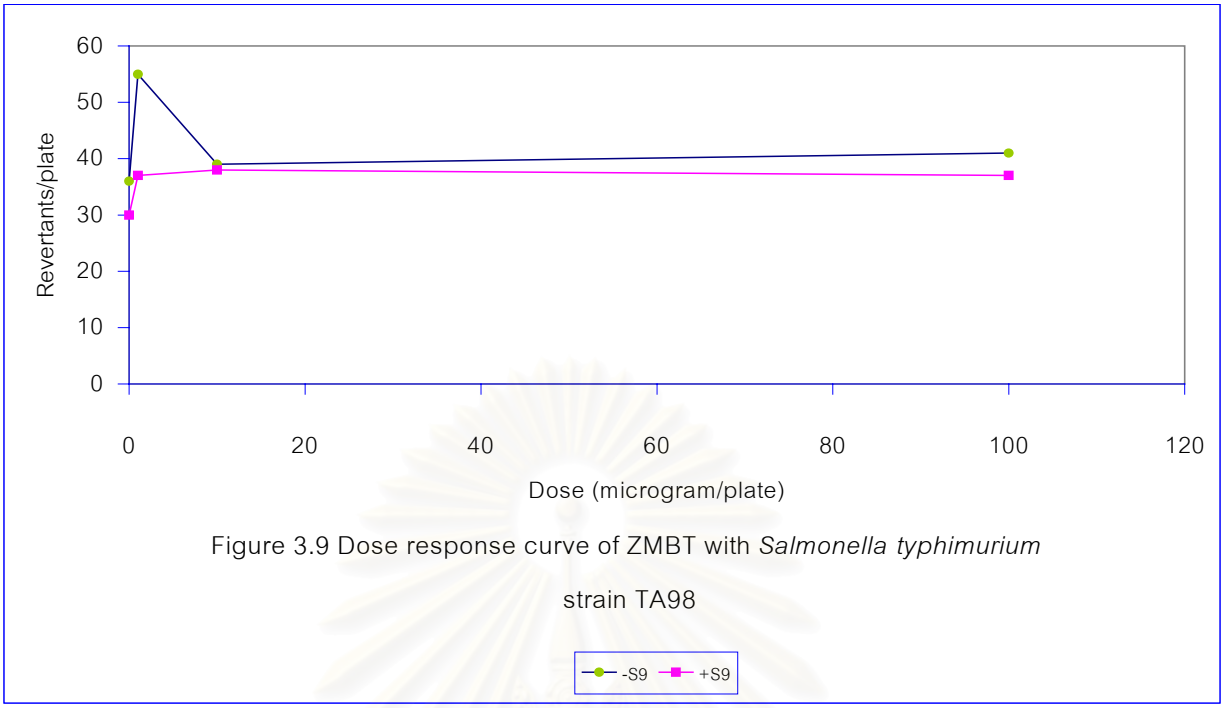


Figure 3.9 Dose response curve of ZMBT with *Salmonella typhimurium* strain TA98

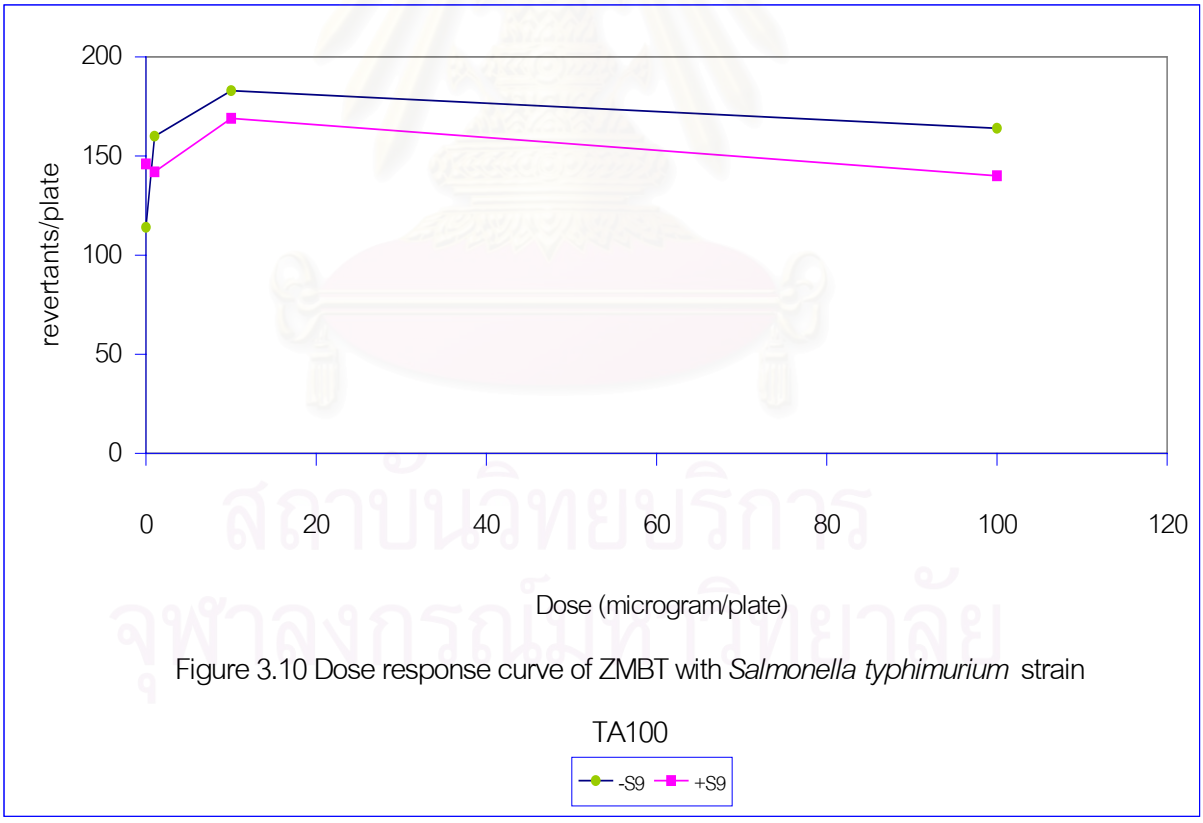


Figure 3.10 Dose response curve of ZMBT with *Salmonella typhimurium* strain TA100

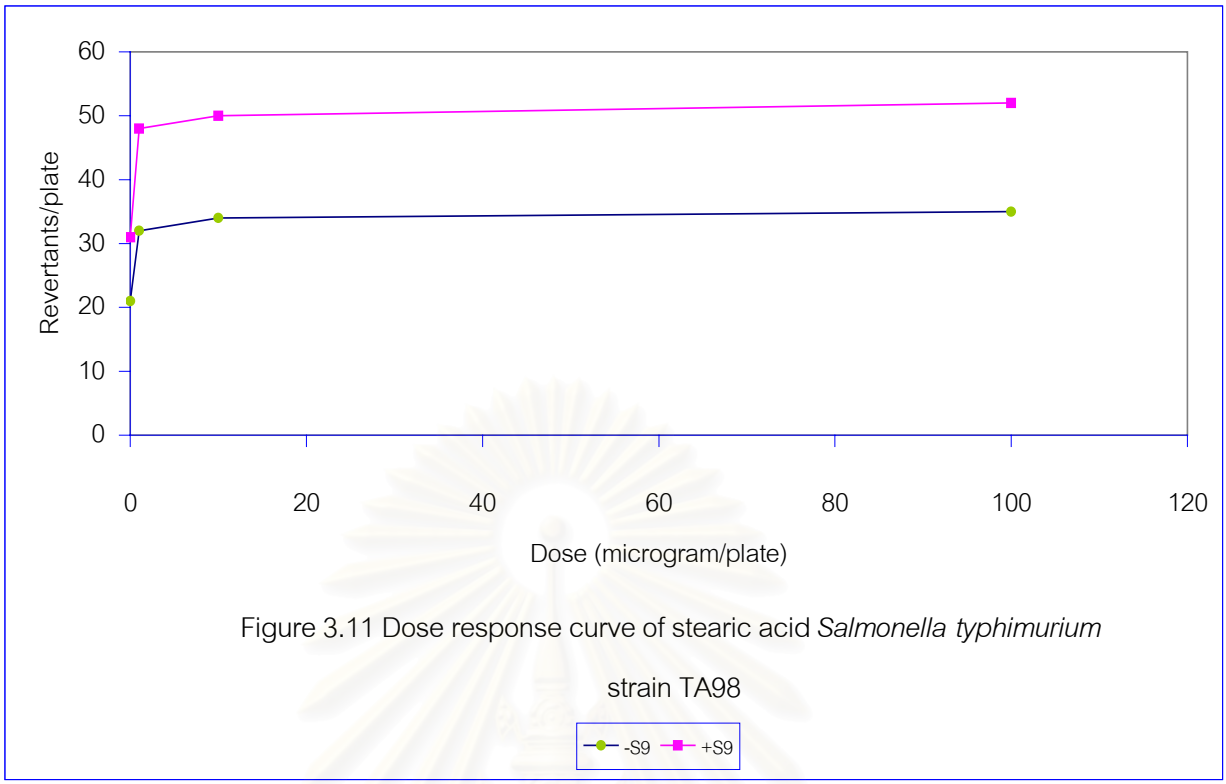


Figure 3.11 Dose response curve of stearic acid *Salmonella typhimurium* strain TA98

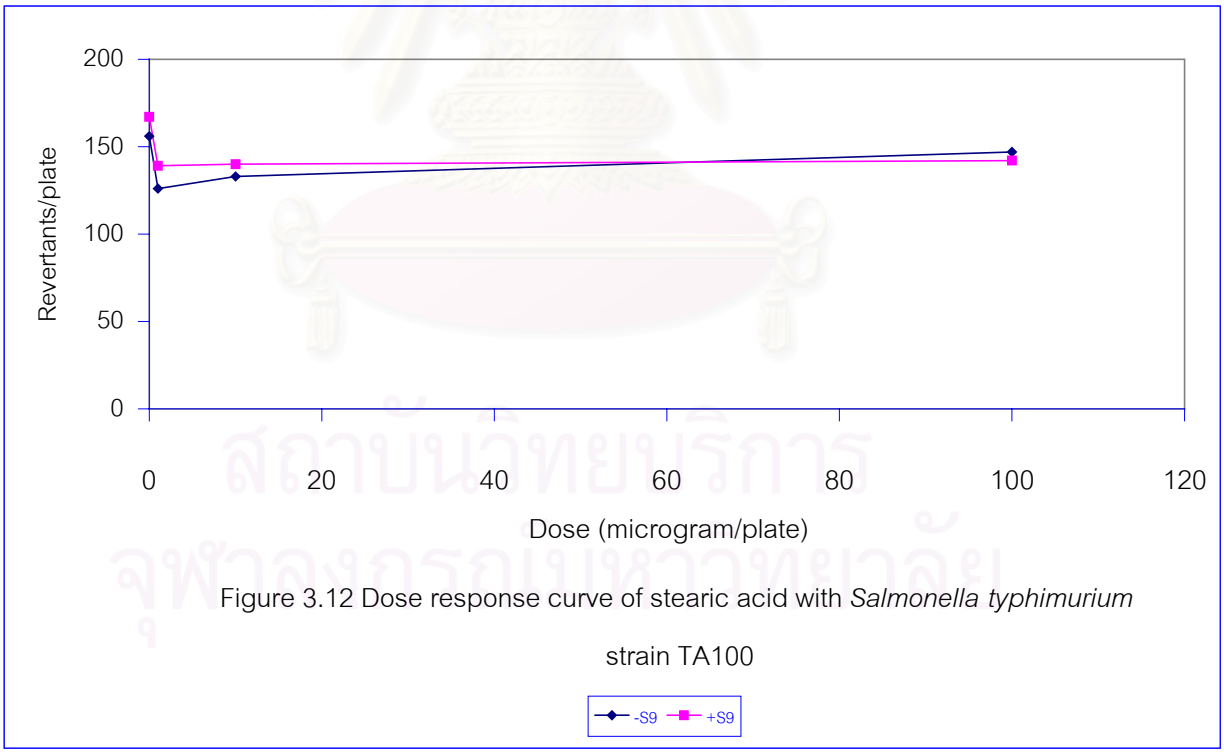


Figure 3.12 Dose response curve of stearic acid with *Salmonella typhimurium* strain TA100

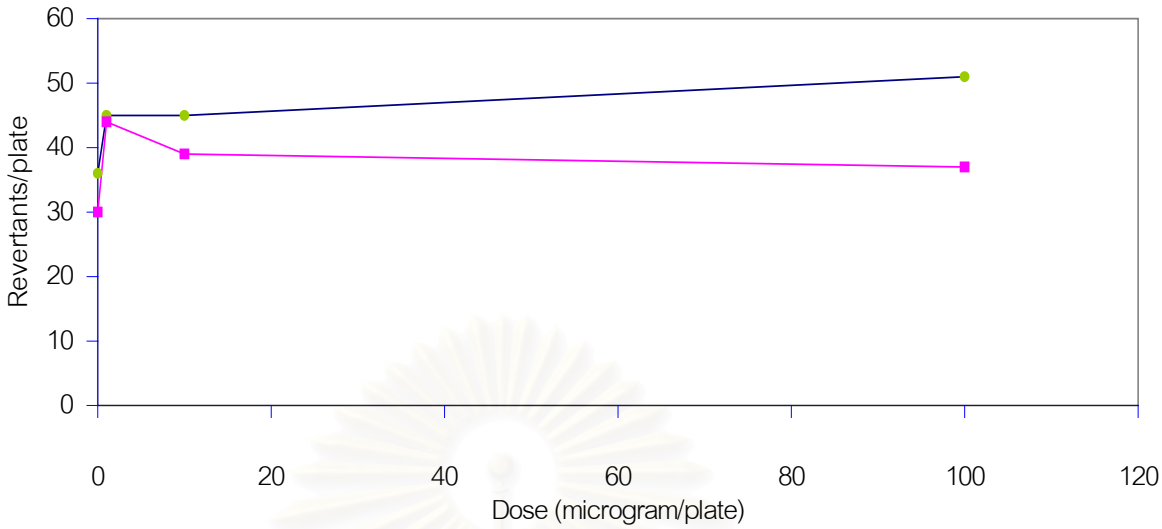


Figure 3.13 Dose response curve of Zinc carbonate with *Salmonella typhimurium* strain TA98

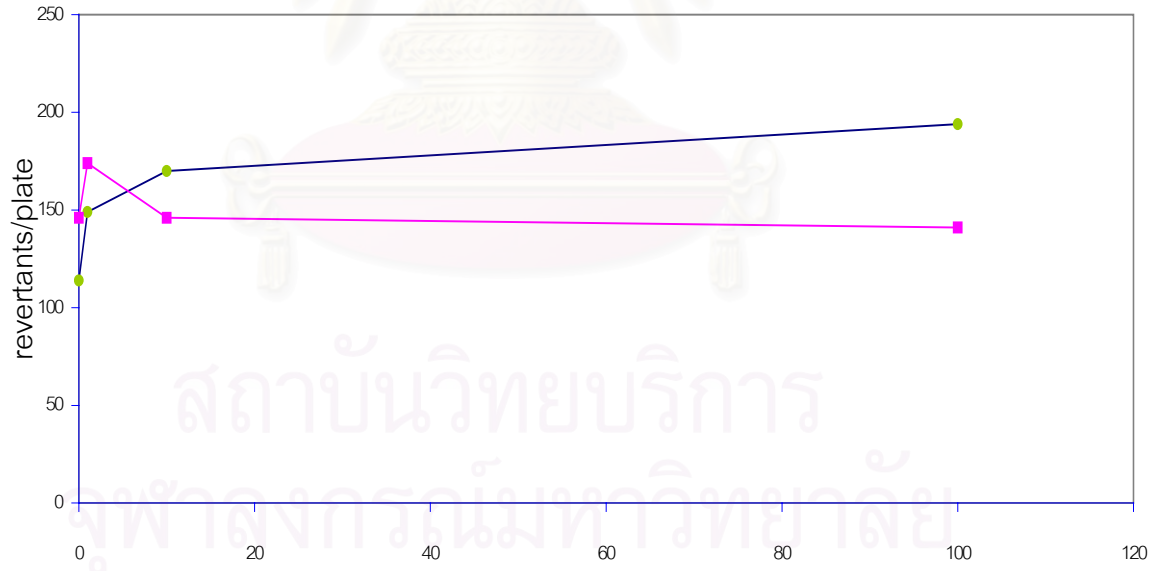


Figure 3.14 Dose response curve of zinc carbonate with *Salmonella typhimurium* strain TA100

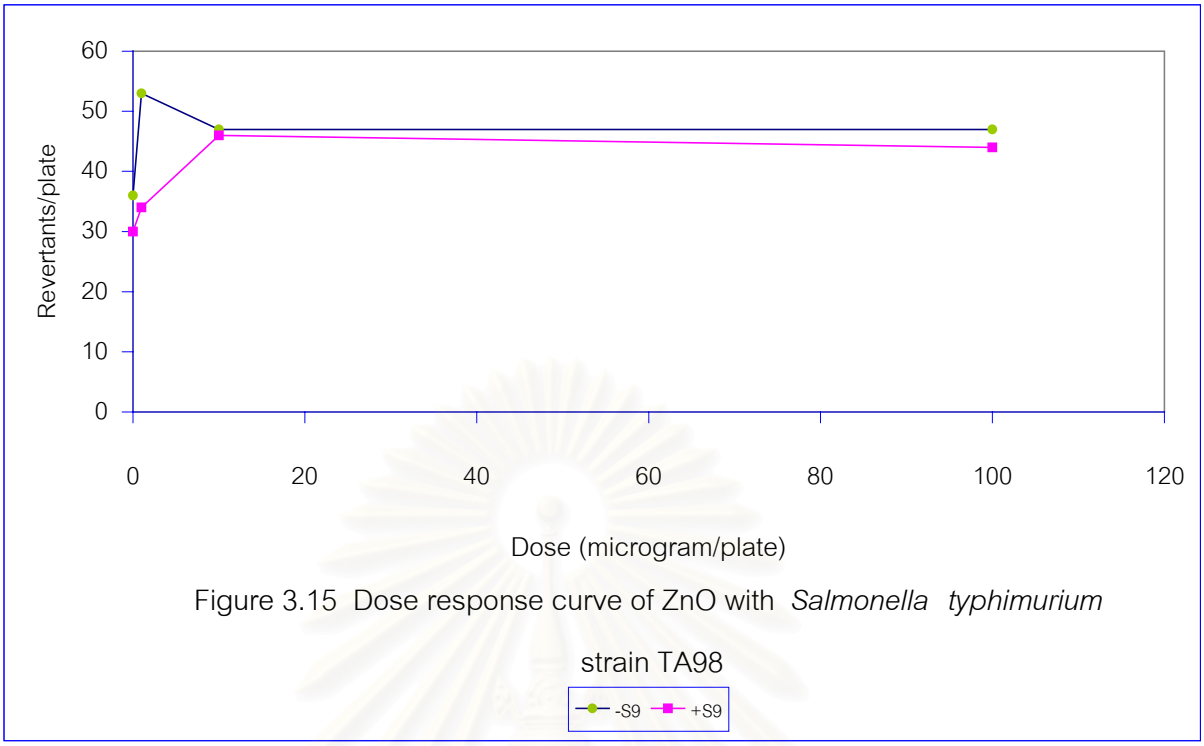


Figure 3.15 Dose response curve of ZnO with *Salmonella typhimurium* strain TA98

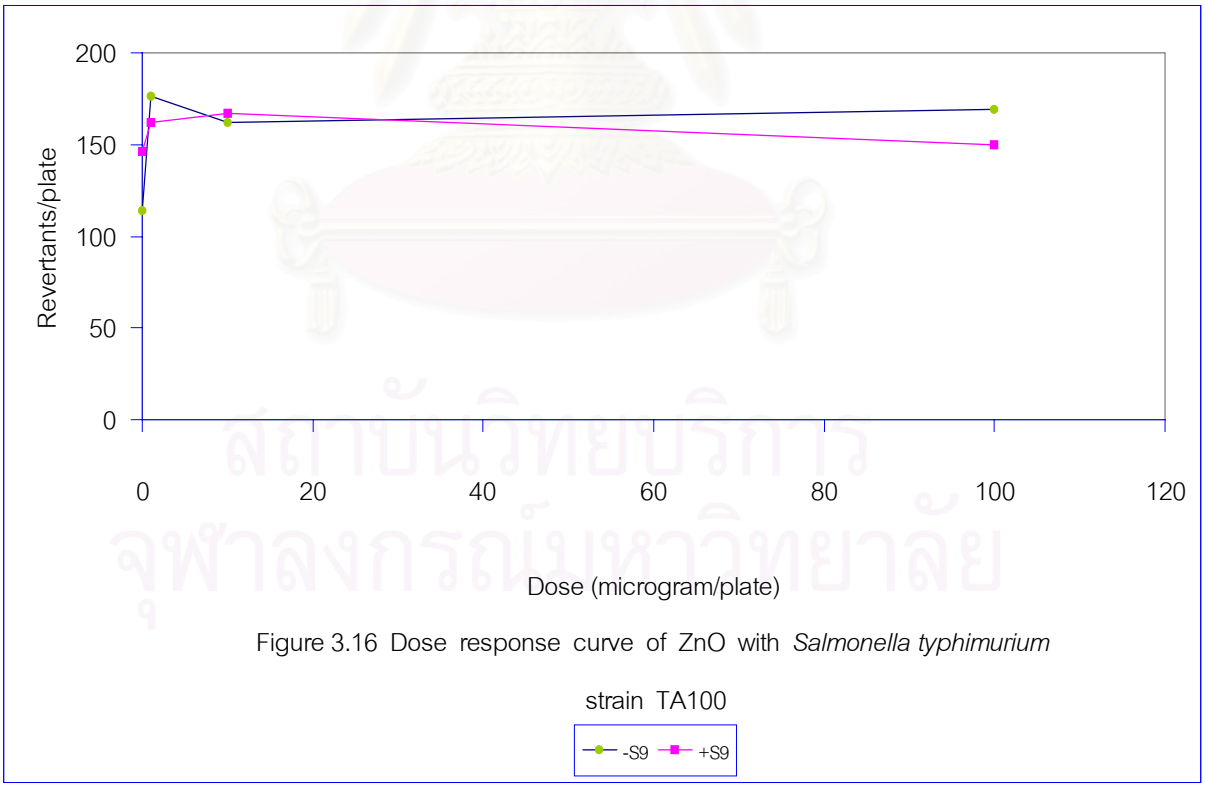


Figure 3.16 Dose response curve of ZnO with *Salmonella typhimurium* strain TA100

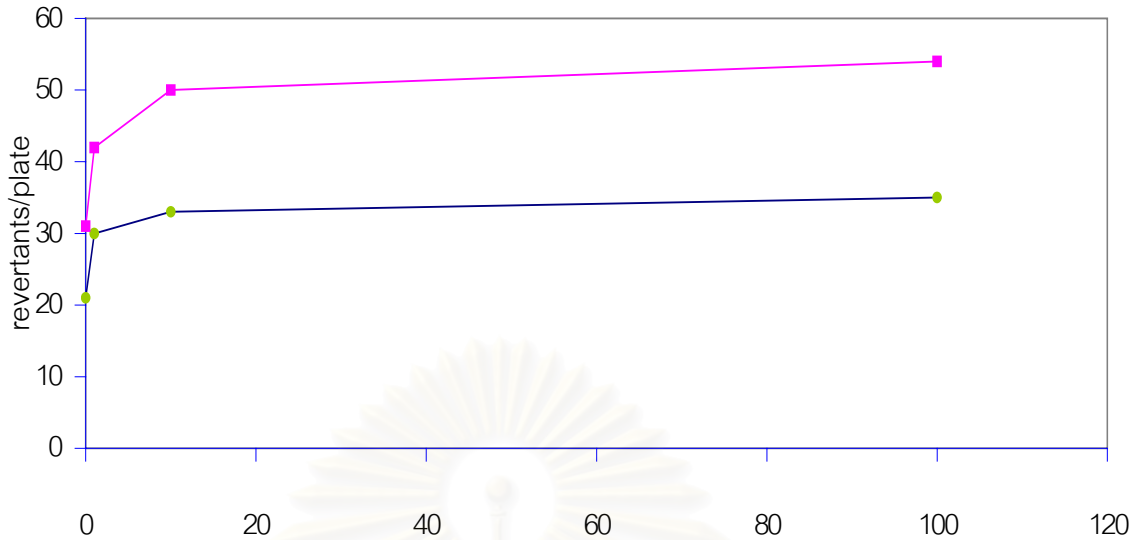


Figure 3.17 Dose response curve of Zinc stearate with *Salmonella typhimurium* strain TA98

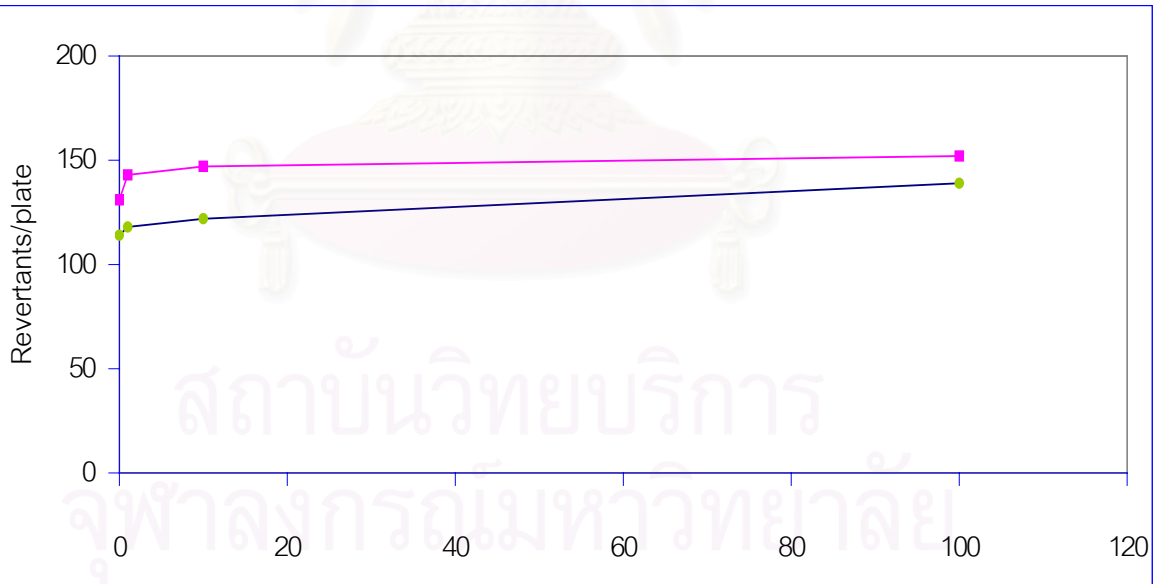


Figure 3.18 Dose response curve of zinc stearate with *Salmonella typhimurium* strain TA100

Table 3.3 Dose response study of chemical used as antioxidant in sulfur curing system

Function	Chemical	Dose (μg/plate)	Number of revertants observed using *Salmonella typhimurium*			
			strain TA98		strain TA100	
			-S9	+S9	-S9	+S9
Negative control	DMSO		38	52	156	163
Positive control	AF-2	0.1	516	-	-	-
	AF-2	0.001	-	-	553	-
	BP	5	-	529	-	--
	BP	0.625	-	-	-	605
	BHT	1	47	85	165	186
	BHT	10	63	67	174	201
	BHT	100	no revertants	no revertants	no revertants	no revertants

Table 3.4 Dose response study of chemical used as antioxidant in sulfur curing system

Function	Chemical	Dose (μg/plate)	Number of revertants observed using *Salmonella typhimurium*			
			strain TA98		strain TA100	
			-S9	+S9	-S9	+S9
Negative control	DMSO		25	27	103	127
Positive control	AF-2	0.1	402	-	-	-
	AF-2	0.001	-	-	418	-
	BP	5	-	403	-	--
	BP	0.625	-	-	-	446
	Irganox	1	32	33	130	140
	Irganox	10	37	37	138	158
	Irganox	100	41	42	153	167

Table 3.3 and 3.4 showed that both Butylhydroxytoluene (BHT) and Irganox do not exhibit genotoxic effect in *Samonella typhimurium* strain TA98And TA 100. The number of revertant colonies is less than double when compared with negative control dimethy sulfoxide (DMSO).

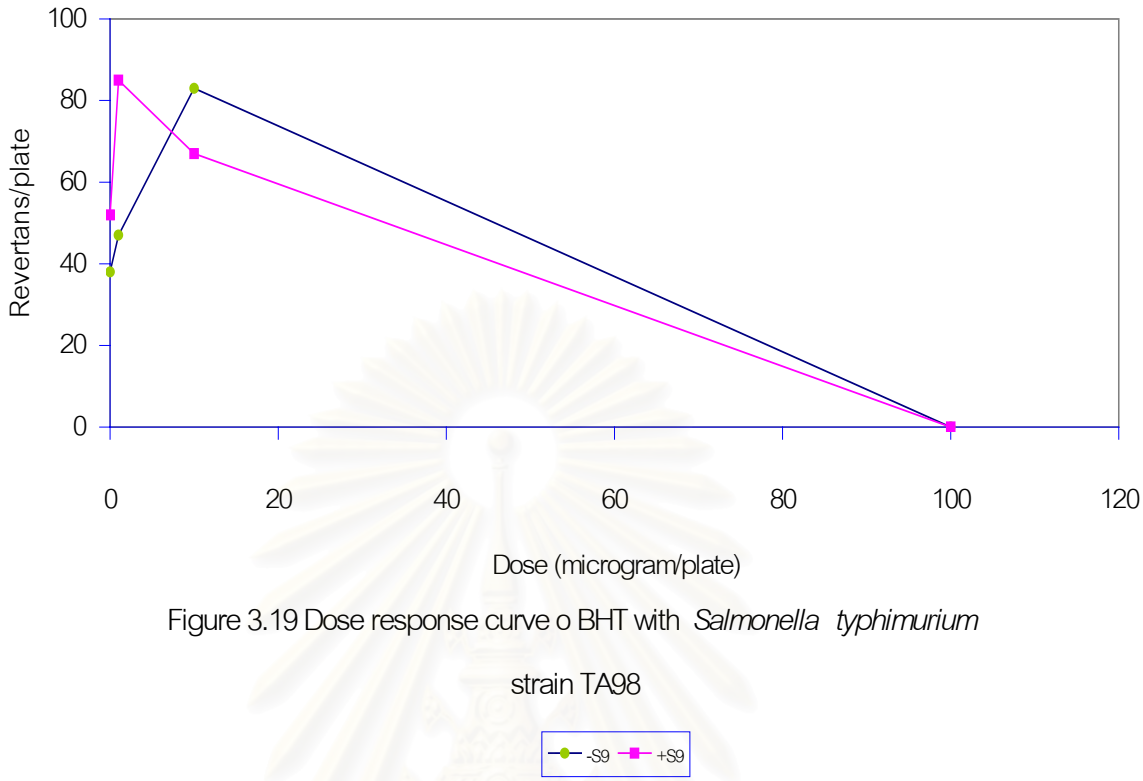


Figure 3.19 Dose response curve o BHT with *Salmonella typhimurium* strain TA98

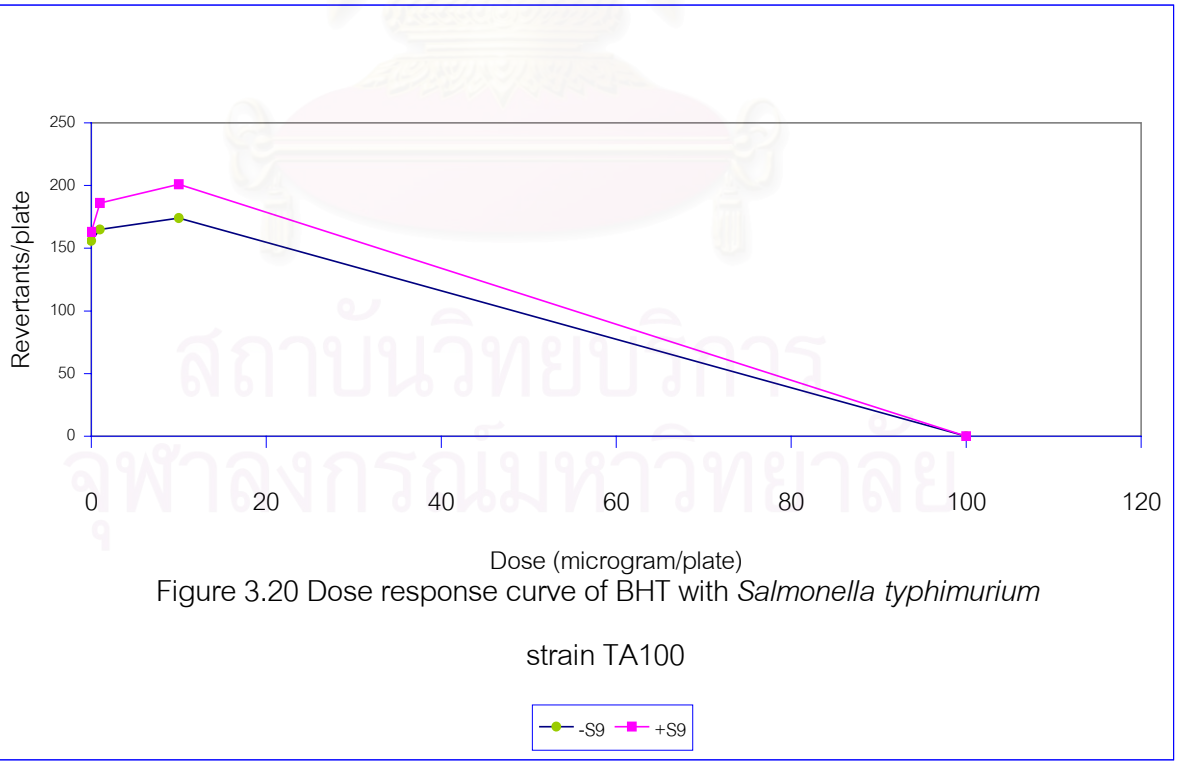


Figure 3.20 Dose response curve of BHT with *Salmonella typhimurium* strain TA100

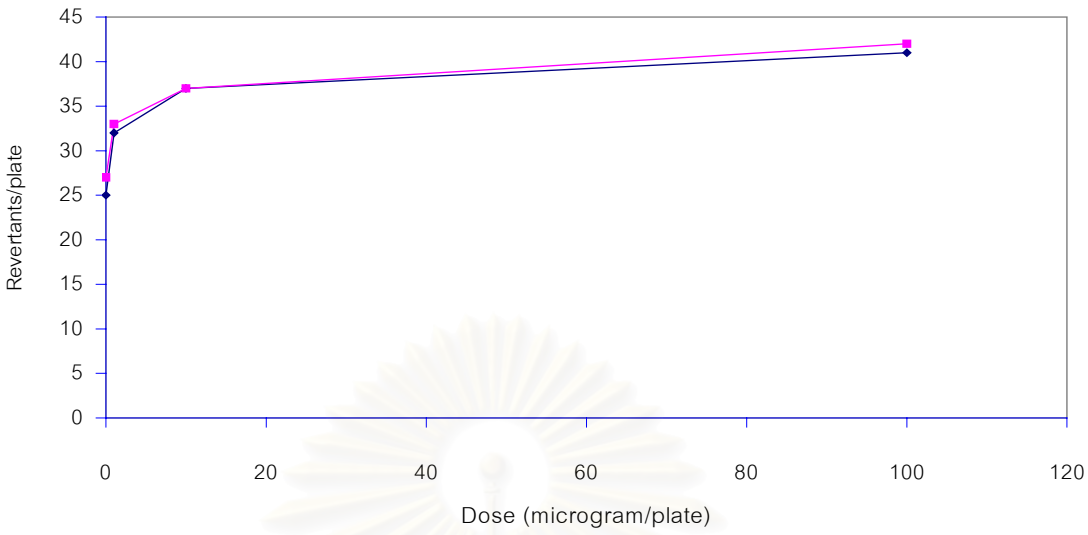


Figure 3.21 Dose response study of Irganox with *Salmonella typhimurium* strain TA98

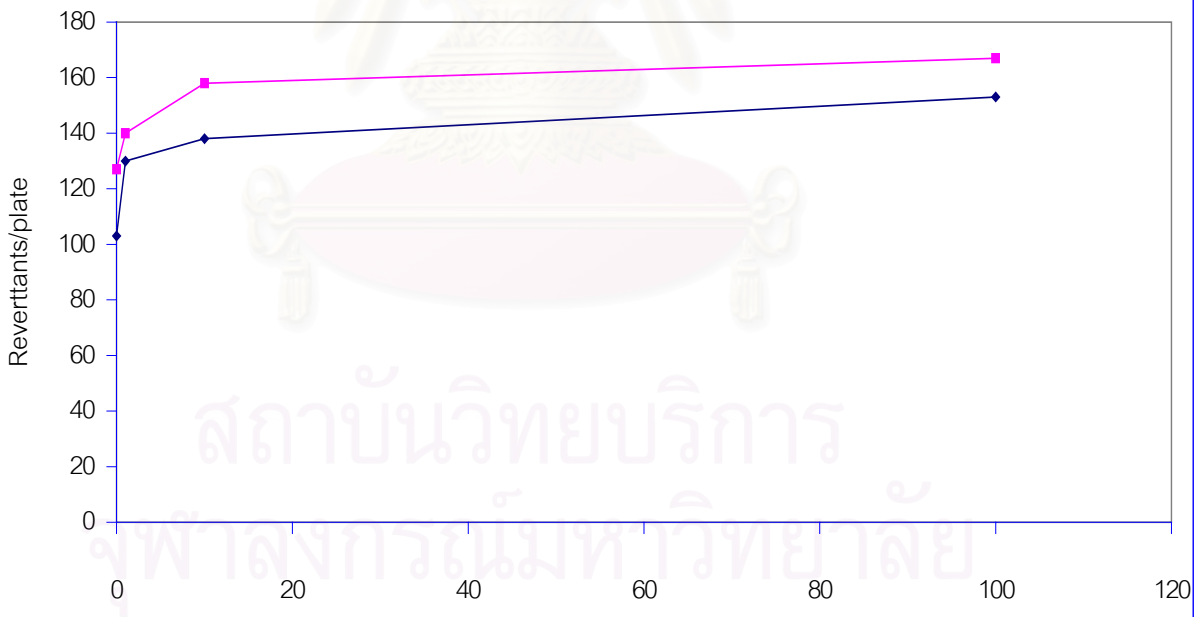


Figure 3.22 Dose response study of Irganox with *Salmonella typhimurium* strain TA100

3.1.1.3 Filler

Table 3.5 Dose response study of chemical used as filler in sulfur curing system

Function	Chemical	Dose (μg/plate)	Number of revertants observed using *Salmonella typhimurium*			
			strain TA98		strain TA100	
			-S9	+S9	-S9	+S9
Negative control	DMSO		28	30	114	118
Positive control	AF-2	0.1	426	-	-	-
	AF-2	0.001	-	-	406	-
	BP	5	513	-	-	
	BP	0.625	-	-	-	448
	Hisil233	1	42	41	168	161
	Hisil233	10	49	43	180	160
	Hisil233	100	53	49	184	186
	PEG	1	32	46	119	163
	PEG	10	36	49	147	169
	PEG	100	36	52	154	175

Table 3.5 showed that both Hisil233 and Polyethyleneglycol (PEG) which used as filler in vulcanizing system do not exhibit genotoxic effect in Samonella typhimurium strain TA98 and TA100. The number of revertant colonies is less than double when compared with negative control dimethyl sulfoxide (DMSO).

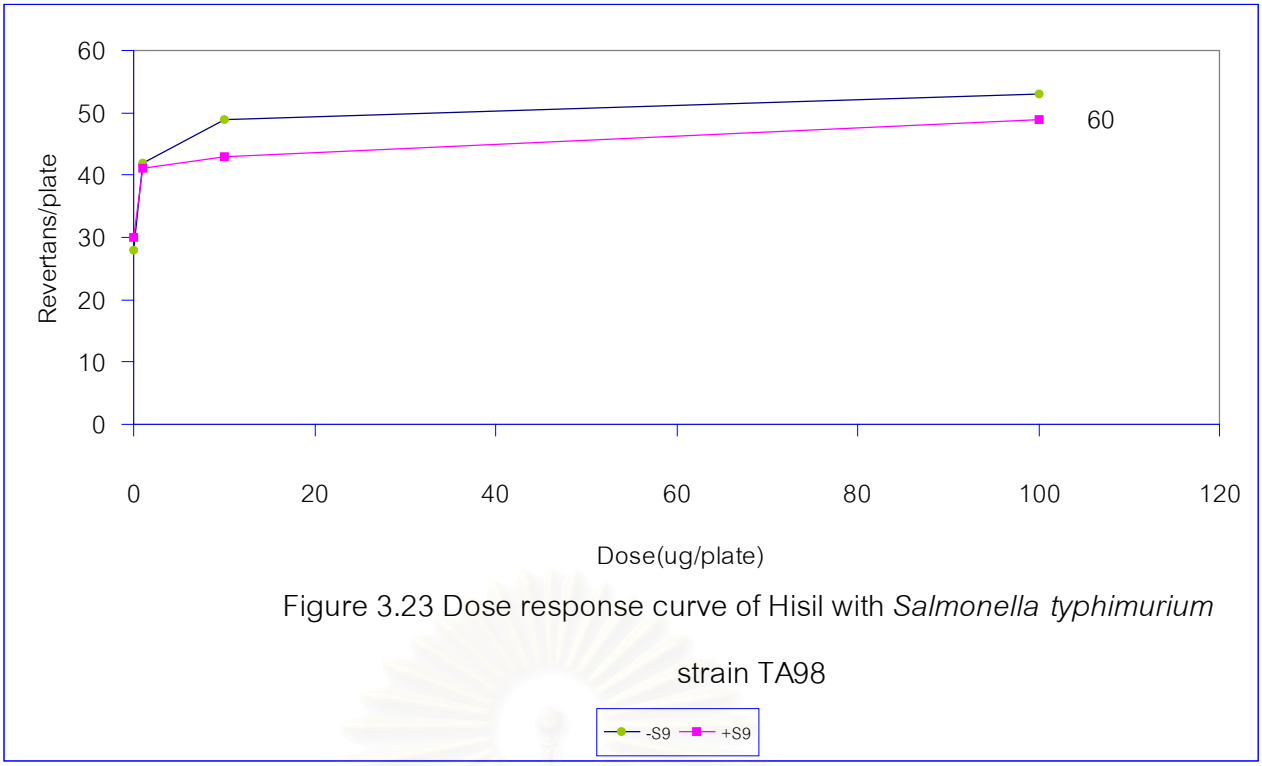


Figure 3.23 Dose response curve of Hisil with *Salmonella typhimurium* strain TA98

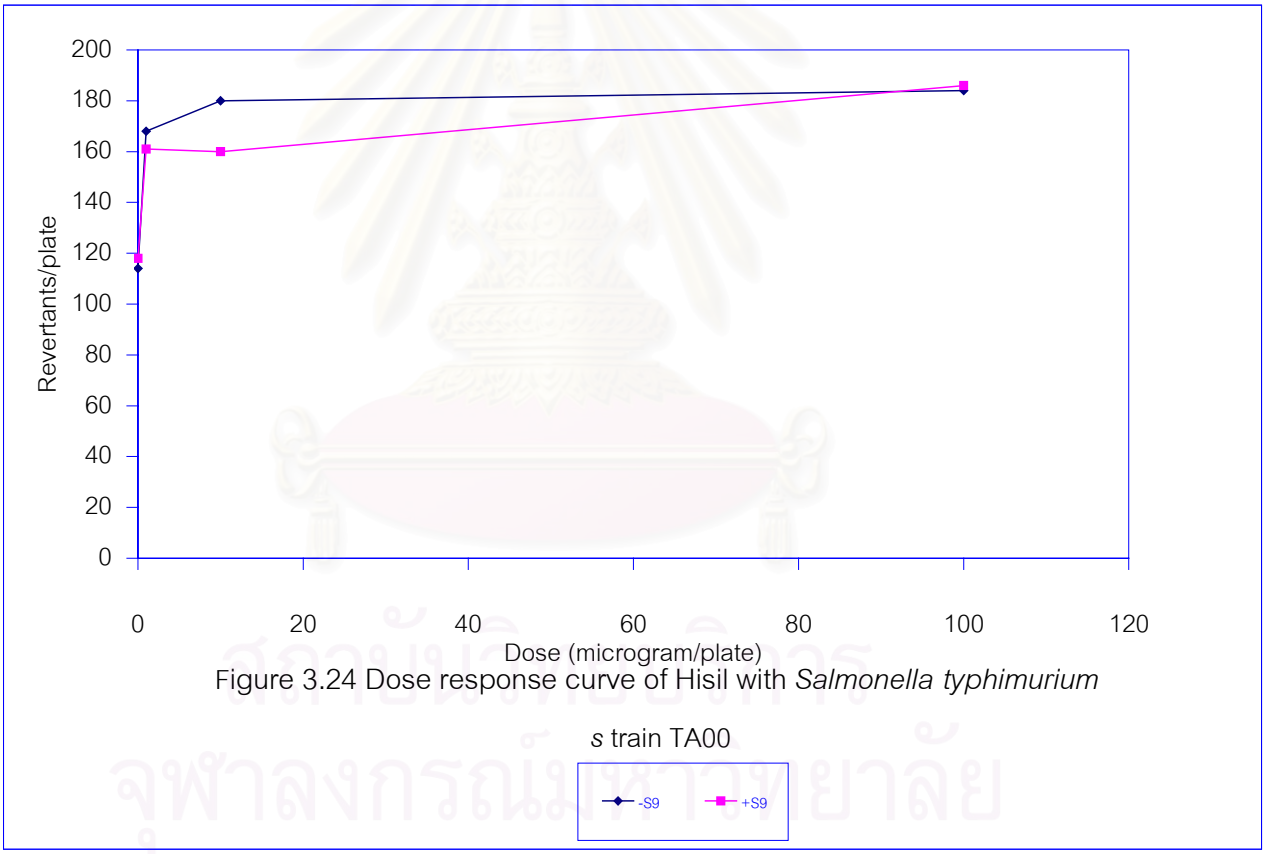


Figure 3.24 Dose response curve of Hisil with *Salmonella typhimurium* s train TA00

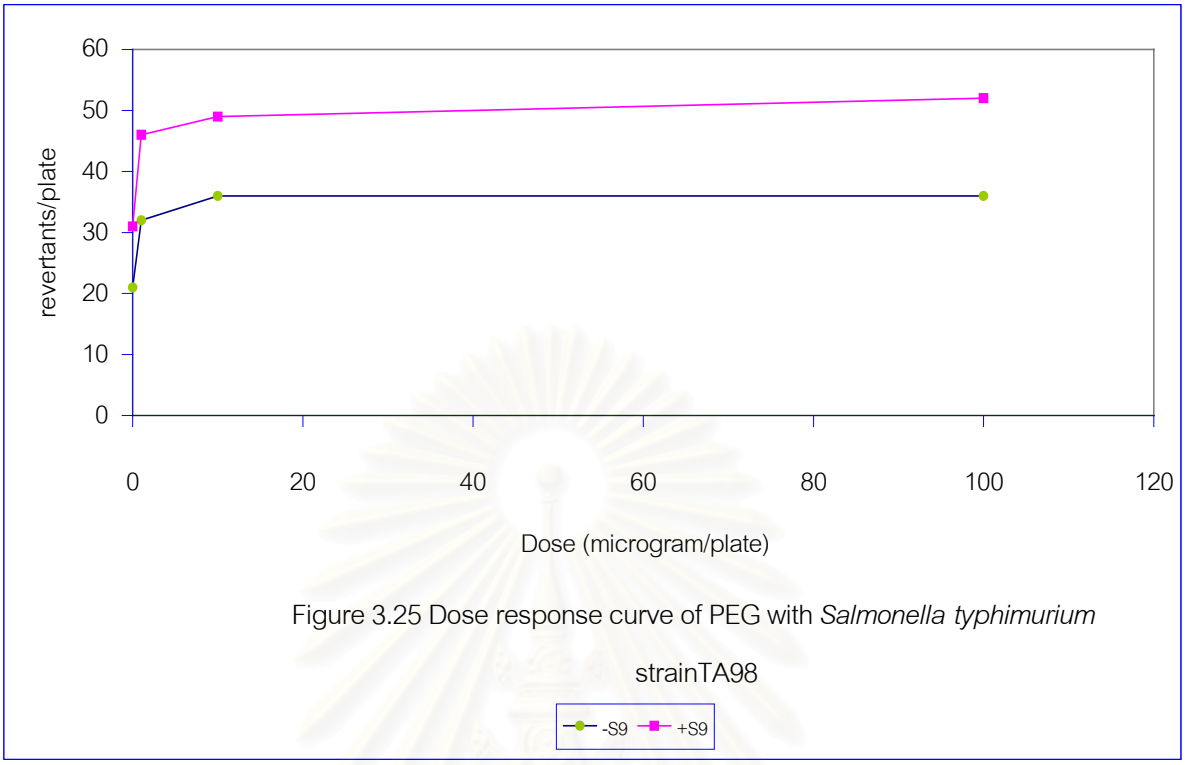


Figure 3.25 Dose response curve of PEG with *Salmonella typhimurium* strainTA98

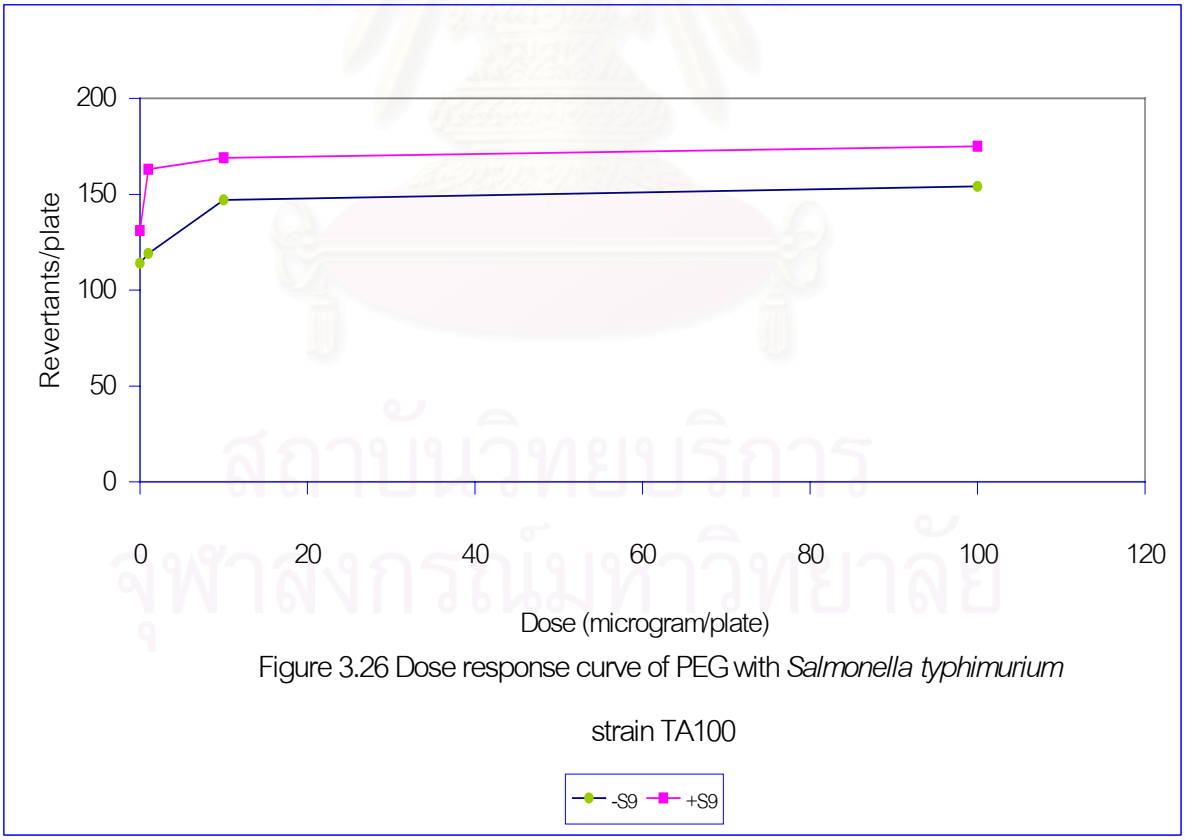


Figure 3.26 Dose response curve of PEG with *Salmonella typhimurium* strain TA100

3.1.4 Vulcanizing agent

Table 3.6 Dose response study of chemical used as vulcanizing agent in sulfur curing system

Function	Chemical	Dose (μg/plate)	Number of revertants observed using *Salmonella typhimurium*			
			strain TA98		strain TA100	
			-S9	+S9	-S9	+S9
Negative control	DMSO		21	31	114	131
Positive control	AF-2	0.1	426	-	-	-
	AF-2	0.001	-		406	-
	BP	5	-	513	-	
	BP	0.625	-	-	-	448
	sulfur	1	23	53	123	159
	sulfur	10	26	52	137	165
	sulfur	100	19	55	no revertants	172

Table 3.6 showed that sulfur which used as vulcanizing agent in sulfur vulcanizing system do not exhibit genotoxic effect in Samonella typhimurium strain TA98 and TA100. The number of revertant colonies is less than double when compared with negative control dimethyl sulfoxide (DMSO).

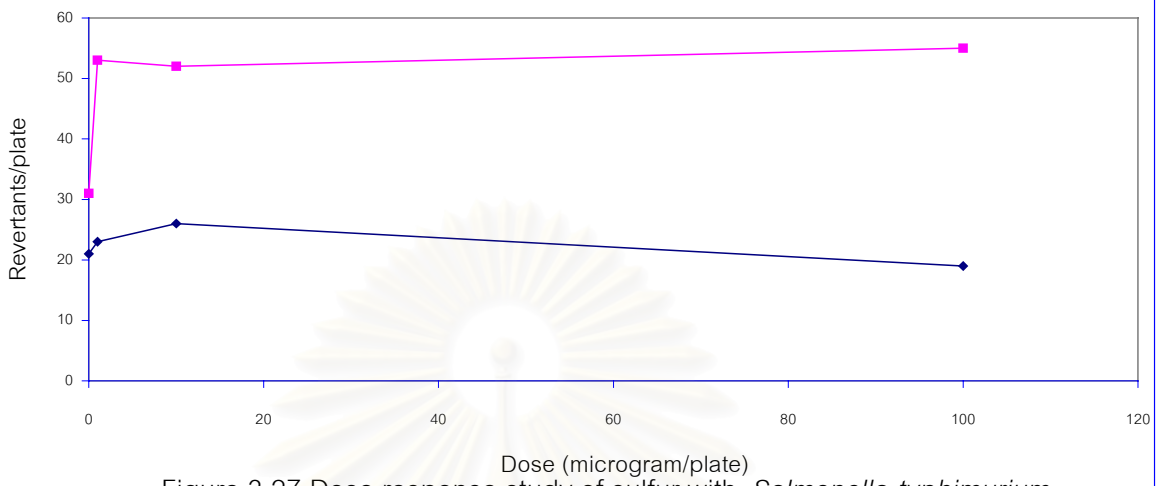


Figure 3.27 Dose response study of sulfur with *Salmonella typhimurium* strain TA98

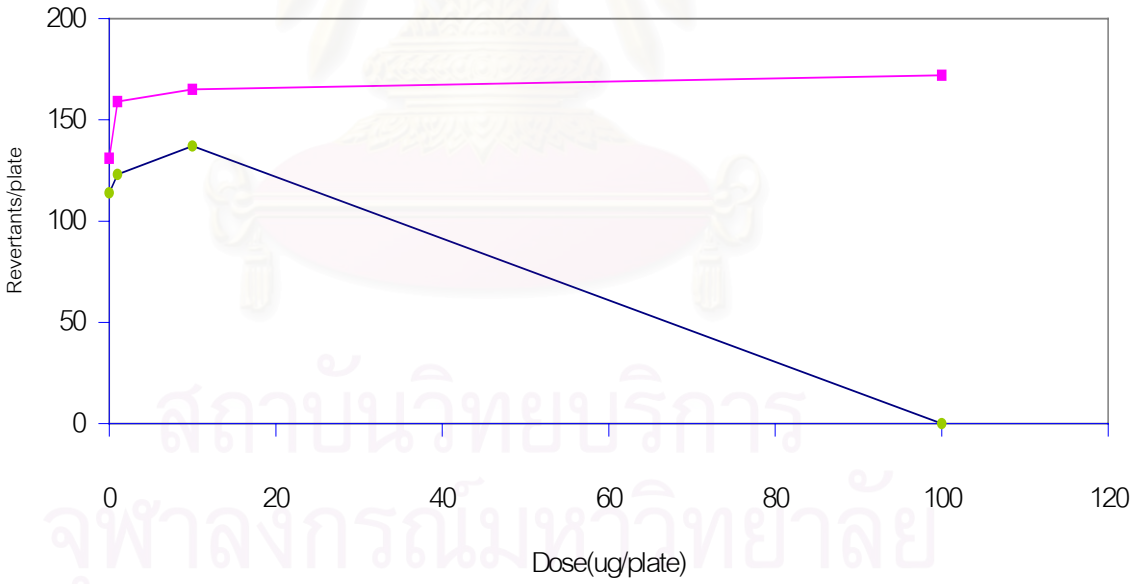


Figure 3.28 Dose response curve of sulfur with *Salmonella typhimurium* strain TA100

3.1.2 Dose response studies of chemicals used in peroxide vulcanizing system

Peroxide vulcanization used BHT as an antioxidant , Hisil as filler and ZnO as accelerator in the same way as sulfur vulcanization but Dicumyl peroxide 98% was used as vulcanizing agent

Table 3.7 Dose response study of chemical used as vulcanizing agent in peroxide curing system

Function	Chemical	Dose (ug/plate)	Number of revertants observed using *Salmonella typhimurium*			
			strain TA98		strain TA100	
			-S9	+S9	-S9	+S9
negative control	DMSO		28	30	156	167
Positive control	AF-2	0.1	453	-	-	-
	AF-2	0.001	-	-	516	-
	BP	5	-	466	-	-
	BP	0.625	-	-	-	519
	DCP	1	37	37	161	162
	DCP	10	49	45	167	173
	DCP	100	53	49	173	185

Table 3.7 showed that Dicumyl peroxide 98 %(DCP)which is used as vulcanizing agent in peroxide vulcanizing system does not exhibit genotoxic effect in *Samonella typhimurium* strain TA98And TA 100. The number of revertant colonies is less than double when compared with negative control dimethy sulfoxide (DMSO)

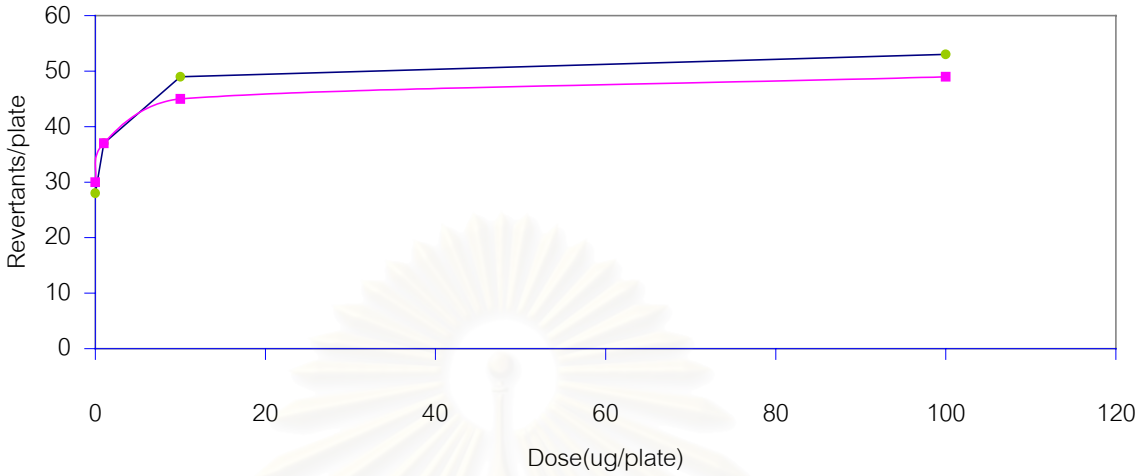


Figure 3.29 Dose response curve of DCP with *Salmonella typhimurium* strain TA98

3.2 rubber vulcanizates produced by sulfur and peroxide curing system

3.2.1 Compounding formulation of NR and IR in sulfur curing system

Table 3.8 Chemical formulation of NR in sulfur curing system formulation 1

Material name	Function
NR- STR 5L	Rubber
ZBEC	Accellerator
ZMBT	Accellerator
Zinc Stearate	Activator
$ZnCO_3$	Activator
BHT	Accellerator
Hisil 233	Antioxidant
PEG	Filler
Sulfur	Vulcanizing agent

Table 3.9 Chemical formulation of NR in sulfur curing system formulation 2

Material name	Function
NR-STR 5L	Rubber
DPTT	Accelerator
ZDBC	Acellerator
Stearic acid	Acticator
$ZnCO_3$	Activator
BHT	Antioxidant
Sulfur	Vulcanizing agent

Table 3.10 Chemical formulation of IR in sulfur curing system formulation 1

Material name	Function
IR-2005	Rubber
MBTS	Accellerator
ZDBC	Accellerator
Stearic acid	Activator
ZnO	Activator
Hisil	Filler
PEG	Filler
BHT	Antioxidant
Sulfur	Vulcanizing agent

Table 3.11 Chemical formulation of IR in sulfur curing system formulation 2

Material name	Function
IR-2205	Rubber
MBTS	Accelerator
ZDBC	Accelerator
Stearic acid	Activator
ZnO	Activator
Hisil	Filler
PEG	Filler
IRGANOX	Antioxidant
Sulfur	Vulcanizing agent

3.2.2 Compounding formulation of NR in peroxide curing system

Table 3.12 Chemical formulation of NR in peroxide curing system formulation 1

Material name	Funtion
NR–STR 5L	Rubber
ZnO	Activator
BHT	Antioxidant
Hisil 233	Filler
DCP	Vulcanizing agent

Table 3.13 Chemical formulation of NR in peroxide curing system formulation 2

Material name	Function
NR-STR XL	Rubber
Stearic acid	Activator
ZnO	Activator
Hisil	Filler
Irganox	Antioxidant
DCP 98%	Vulcanizing agent

3.2.3 Physical properties of vulcanizate

Table 3.14 Physical properties of natural rubber curing by sulfur

Formulation 1	Formulation 2	Psysical property
40	38	Hardness (Shore A)
125.27	81.20	Tensile Strength (Kgf/cm^2)
800	906	Elongation (%)
15.63	6.98	Modulus 300 % (Kgf/cm^2)
28.85	28.66	Tear strength (Kgf/cm^2)

Table 3.15 Physical properties of isoprene rubber curing by sulfur

Formulation 1	Formulation 2	Psysical property
31	33	Hardness (Shore A)
70.57	88	Tensile Strength (Kgf/cm^2)
800	800	Elongation (%)
9.19	8.97	Modulus 300 % (Kgf/cm^2)
23.78	22	Tear strength (Kgf/cm^2)

Table 3.16 Physical properties of natural rubber curing by DCP

Formulation 1	Formulation 2	Psysical property
32	50.4	Hardness (Shore A)
96.78	53.73	Tensile Strength (Kgf/cm^2)
500	569	Elongation (%)
17.20	16.84	Modulus 300 % (Kgf/cm^2)
25	17.64	Tear strength (Kgf/cm^2)

3.2.4 Protein allergenic potency (μg/g rubber)

Table 3.17 Protein concentration in vulcanizate[a]

Sample	Formulation table	Protein (μg/g rubber)
CL[b]		472
SV-NR	3.8	238
SV-NR	3.9	234
SV-IR	3.10	6.3
SV-IR	3.11	4
PV-NR	3.12	249
PV-NR	3.13	248

[a] No leaching with 70 C° distilled water

[b] Lot no. 22/12/47

* All data are given as mean of triplicate

Table 3.18 Protein concentration in vulcanizate[a]

Sample	Formulation table	Protein (μg/g rubber)
CL[b]		462
SV-NR	3.8	101
SV-NR	3.9	95
SV-IR	3.10	0
SV-IR	3.11	0
PV-NR	3.12	108
PV-NR	3.13	97

[a] Leached with 70 C° distilled water

[b] Lot no. 22/12/47

* All data are given as mean of triplicate

3.2.5 Genotoxic potential of NR and IR vulcanizate produced by sulfur and peroxide Curing system

Table 3.19 The number of revertant caused by residual chemicals in vulcanizate

Function/ Formulation	Chemical	Solvent extraction	Dose (μg/plate)	Number of revertants observed using *Salmonella typhimurium*			
				strain TA98		strain TA100	
				-S9	+S9	-S9	+S9
Negative control	DMSO			27	29	193	196
Positive control	AF-2		0.1	475	-	-	-
Positive control	AF-2		0.001	-	-	440	-
Positive control	BP		5	449	-	-	-
Positive control	BP		0.625	-	-	-	468
3.8	SV-NR	Toluene		46	29	212	229
3.12	PV-NR	"		26	34	213	224
3.10	SV-IR	"		38	30	215	232
3.8	SV-NR	Chloroform:Methanol(1:1)		36	39	238	230
3.12	PV-NR	"		39	37	233	232
3.10	SV-IR	"		41	37	324	262

* All data are given as mean of four plate from 2 separate experiment

Table 3.20 The number of revertant caused by residual chemicals in vulcanizate

Function/ Formulation	Chemical	Solvent extraction	Dose (ug/plate)	Number of revertants observed using *Salmonella typhimurium*			
				strain TA98		strain TA100	
				-S9	+S9	-S9	+S9
Negative control	DMSO			22	25	101	115
Positive control	AF-2		0.1	454	-	-	-
Positive control	AF-2		0.001	-	-	484	-
Positive control	BP		5	498	-	-	-
Positive control	BP		0.625	-	-	-	502
3.9	SV-NR	Toluene		31	41	124	134
3.13	PV-NR	"		32	40	122	127
3.11	SV-IR	"		34	35	132	142
3.9	SV-NR	Chloroform:Methanol(1:1)		33	46	129	143
3.13	PV-NR	"		32	41	122	133
3.11	SV-IR	"		35	40	136	143

* All data are given as mean of triplicate

3.3 Evaluation for physical and biochemical properties of natural rubber vulcanizates produced by various dose of gamma radiation (10 - 20 kGy)

3.3.1 Prevulcanization of 50 % concentrated latex by gamma radiation (dose 10, 12, 14, 16, 18, 20 kGy)

Concentrated latex was diluted up to 50 % dry rubber content (DRC) using 1 % ammonia solution, stabilized by 0.5 phr KOH as 10 % solution, 5 phr of n-butyl acrylate (n-BA) was added as the sensitizer to the latex while stirring. Gamma-rays irradiation from a co-60 source was carried out at vary dose 10, 12, 14, 16, 18, 20 kGy. Rubber film were prepared by casting on clean the glass plates, dried in air until it become transparent. Post drying, films were heated in an oven at 80^{o}C for 3 hours.

3.3.2 Physical properties of vulcanizate.

Table 3.21 physical properties of RVNRL

Physical property	RVNRL (kGy)					
	10	12	14	16	18	20
Hardness (shore A)	33.9	34.0	33.8	34.4	34.8	34.4
Tensile strength (kg/cm^2)	3.66	3.68	4.28	4.08	7.11	7.15
% Elongation	892	852	790	786	750	744
Modulus 300 % (Kgf/cm^2)	2.30	2.42	3.18	4.24	5.78	5.68
Tear strength (Kgf/cm^2)	6.21	6.10	6.19	7.69	7.84	7.92

*All data are given as mean of five test pieces

3.3.3 Protein allergenic potency (ug/g rubber)

Table 3.22 Protein concentration in volcanizate

Vulcanizate	Radiation Dose (kGy)	protein (μg/g rubber)
CL[a]		448
RVNRL[b]	10	340
	12	311
	14	268
	16	231
	18	177
	20	125

[a] Lot no. 22/12/47

[b] No leaching in 70°C distilled water, 30 min

*All data are given as mean of triplicate

Table 3.23 Protein concentration in vulcanizate

Sample	Radiation Dose (kGy)	protein (μg/g rubber)
CL[a]		459
RVNRL[b]	10	250
	12	278
	14	120
	16	90
	18	76
	20	68

*All data are given as mean of triplicate

[a] Lot no. 22/12/47

[b] Leaching in 70°C distilled water, 30 min

3.3.4 Genotoxic potential of RVNRL

Table 3.24 The number of revertants caused by residual chemical in vulcanizate

Function	Chemical/ Vulcanizate	Dose (μg/ plate)	Solvent extraction	Number of revertant observed using *Salmonella typhimurium*[a]			
				Strain TA98		Stran TA100	
				-S9	+S9	-S9	+S9
negative control	DMSO			29	34	121	137
positive control	AF-2	0.1		498	-	-	-
positive control	AF-2	0.01		-	-	500	-
positive control	BP	5		-	497	-	-
positive control	BP	0.625		-	-	-	501
positive control	RVNRL 10 kGy		Toluene	43	49	149	172
	RVNRL 12 kGy			40	48	150	169
	RVNRL 14 kGy		Toluene	37	44	149	162
	RVNRL 16 kGy		Toluene	37	43	149	150

	RVNRL 18 kGy		Toluene	33	43	147	150
	RVNRL 20 kGy		Toluene	36	42	144	147
	RVNRL 10 kGy		Chloroform : methanol (1 : 1)	46	54	156	170
	RVNRL 12 kGy		Chloroform : methanol (1 : 1)	47	54	154	168
	RVNRL 14 kGy		Chloroform : methanol (1 : 1)	47	51	152	165
	RVNRL 16 kGy		Chloroform : methanol (1 : 1)	45	51	149	165
	RVNRL 18 kGy		Chloroform : methanol (1 : 1)	44	49	145	159
	RVNRL 20 kGy		Chloroform : methanol (1 : 1)	40	48	143	152

[a] All data are given as mean of triplicate

CHAPTER IV

DISCUSSION

4.1 Dose response study of chemicals used in sulfur and peroxide vulcanizing system

In this study, 13 chemicals which were used as accelerator, activator, antioxidant, filler and vulcanizing agent in rubber vulcanization were tested for their genotoxic potential as an individual chemical at 3 concentration 1, 10, 100 µg/plate. The method for testing genotoxic effect has been developed from the reverse mutation assay of *Salmonella typhimurium* 2 strains, TA 98 and TA 100. The chemical may exert genotoxic potential by itself (direct genotoxin) or may pass the metabolic activation by enzyme and then change to become genotoxic chemical (indirect genotoxin) so that the metabolic activation by rat-liver enzyme plus cofactors (S9mix) were added in this testing condition. Maron and Ames, 1983 recommended that for initial testing of chemical, at least 3 log dose range should be observed. The dose response curve of genotoxic chemical should be linear Occasionally, non-linear dose-response curve were obtained such as

9-amionoacridine, diethylsulfate and ethylmethanesulfonate (McCann *et al.,* 1975).

The results of genotoxic potential of some accelerators and activators (Table 3.1 and 3.2) indicated that there are only 3 accelerator namarlly used in sulfur-vulcanizing system genotoxic potential at 100 µg/plate which were Zinc dibutyl dithiocarbarmate (ZDBC), Zinc dibenzyl dithio carbamate (ZBEC) and Mercaptobenzothiazole(MBTS). Zinc dibutyl dithiocarbarmate (ZDBC) exhibited indirect genotoxic potential in *Salmonella typhimurium* strain TA 98 because it need metabolic activation (+S9).

The number of revertant colonies increased with 3 log dose to 63 which was double when compared with negative control dimethyl sulfoxide (DMSO), where the number of mutant colonies was 31 (Table 3.2 and Figure 3.7). The dose response curve of Zinc dibutyl dithiocarbamate (ZDBC) with *Salmonella typhimurium* strain TA98 and TA 100 (Figure 3.7 and 3.8) increase when the concentration of testing chemical is increasing. Potential human health hazards associated with dithiocarbamates namely, zinc dimethyldithiocarbamate (ZDMC), zinc dibutyl dithiocarbamate (ZDBC) include genotoxicity and possible carcinogenicity were previously reported in Medical Devices Agency, MDA 1998. Beside Dithiocarbamates such as zinc dimethyldithiocarbamate (ZDMC), zinc diethyldithiocarbamate (ZDBC) and amines such as dimethylamine (DMA), diethylamine (DEA) and piperidine (PIP) were also causative agents for chemical allergy (Type IV) in cases from surgical rubber gloves (Kaniwa *et. al*., 1994). Zinc dibutyl dithio carbarmate (ZDBC) may cause slight eye/skin irritation (Turjanmaa. *et. al., 1987*) while Wim H *et al*,. 2002 failed to detect allergenic potency of ZDBC in a modified local lymph node assay (LLNA) with ex vivo ^{3}H-TdR labelling of the proliferating lymph node cells.

Zinc dibenzyl dithio carbamate (ZBEC) at the concentration of 100 μg/plate was direct genotoxic in *Salmonella typhimurium* strain TA 98 without metabolic activation (-S9) at all 3 dose range, showing hight revertant colonies with increasing dose (Figure 3.5 and 3.7). The number of revertant colony is 61 which was double when compared with negative control dimethyl sulfoxide (DMSO) where the number of mutant colony is 29. Zinc dibenzyl dithio carbamate (ZBEC) was frameshift genotoxin in *Salmonella typhimurium* strain TA 98. Zinc dibenzyl dithio carbamate (ZBEC) was negative reported for genotoxic test was negative at concentration 100 μg in the chinese hamster ovary cells (CHO) (Soloneski *et al*., 2002), which may be less sensitive than TA98.

Mercaptobenzothiazole (MBTS) at concentration 100 μg/plate was direct genotoxic in *Salmonella typhimurium* strain TA 98 without metabolic activation (+S9). The number of revertant colony is 61 which was double when compared with negative control dimethyl sulfoxide (DMSO), the number of mutant colony is 29. The number of revertant colony of Mercaptobenzothiazole(MBTS) increased when its concentration increased, indicating that mercaptobenzothiazole (MBTS) was a frameshift genotoxin in *Salmonella typhimurium* strain TA 98

Zinc dibenzyldithiocarbamate (ZBEC) contains two benzyl group and mercaptobenzothiazole(MBTS) contains benzene ring in its structure while zinc dibutyldithiocarbamate (ZDBC) contains two butyl group. Based on their planair structure zinc dibenzyldithiocarbamate (ZBEC) and mercaptobenzothiazole(MBTS) may easily intercalate at the base-pairs and finally caused frameshift mutation of *Salmonella typhimurium strain* TA98.

In the case of butlyl hydroxy toluene (BHT) when the concentration/plate of butlyl hydroxy toluene (BHT) was increasing, the number of revertant colonies decreased (Figure 3.19 and 3.20) which was contrast to dose response curve of Irganox (Figure 3.21 and 3.22) . No revertants coloniy was observed at concentration 100 μg/plate implying that BHT may be cytotoxic and caused bacterial cell death (Mckee and Tometsko, 1979).

Due to cytotoxic effect of BHT, the rubber factory should use Irganox as antioxidants in chemical formulation for medical device products.

Hisil233 and Polyethylene glycol (PEG) are chemicals that nolmally used as filler in vulcanization. Both chemicals do not exhibit genotoxic potential in *Salmonella typhimurium* strain TA 98 and TA 100 with (+S9)/ without metabolic activation (-S9). No data of genotoxic potential of both chemicals were reported. When the concentration of both chemicals increased, the number of revertant colonies increased only slightly (Figure 3.23 – 3.26).

Sulfur is a chemical used as vulcanizing agent in sulfur vulcanization. Table 3.6 shows that at concentration100 μg/plate the number of revertant colonies of *Salmonella typhimurium strain* TA98 and TA100 with metabolic activatiob (+S9) is decreased when the dosage of chemical was increased. This result may suggest that sulfur exhibit cytoxic effect and can cause bacterial death. we clound not detect the genotoxic effect of sulfur by using reverse mutation assay of *Salmonella typhimurium*.

Dicumyl peroxide (DCP) is a chemical used as vulcanizing agent in Peroxide vulcanization. This chemical does not exhibit genotoxic potential in *Salmonella typhimurium* strain TA 98 and TA 100 with (+S9)/ without metabolic activation (-S9). When the concentration of DCP increased, the number of revertant colonies also increased too, but not high enougth to be considered genotoxic at100 μg/plate (Figure 3.29 – 3.30). Several aldehyde and peroxide were tested for mutagenicity using *Salmonella typhimurium* tester strains TA100 and TA102 in the presence and absence of S9 mix. Dillon *et. al.,* 1998 also reported that acetaldehyde and dicumyl peroxide gave no mutagenic effect in *Salmonella typhimurium* strain TA100 and TA 102 with S9.

When most of chemical concentration increased, the number of revertant colony increased, except zinc dibenzyl dithio carbamate(ZBEC) (+S9) and Zinc dibutyl dithio carbarmate (ZDBC) (-S9) contrast to Zinc mercaptobenzothiazole(ZMBT) , no significant different of the number of revertant colony when the chemical concentration was in testing condition .

Using of reverse mutation assay of *Salmonella typhimurium* have many advantages, because this assay is rapid , inexpensive and reliable (Maron and Ames, 1983), although the butyl hydroxy toluene and sulfur could not be observed because they were cytotoxic to bacterail cells .

Using cell culture such as chinese hamster ovary cells (CHO) for detection of genotoxic effect is another choice in case of chemicals that are cytotoxic to bacterail cell. This method is reliable (Soloneski *et al*., 2002) because using of mamalian cells are used, which are nearly the same condition as human cells but this method is more expensive than using of bacterial cells.

However using of reverse mutation assay of *Salmonella typhimurium* should be have more advantage for rubber factory to screenning the genotoxic effect of the chemicals and vulcanized rubber.

4.2 Genotoxicity of vulcanizate produced by sulfur, peroxide and gamma radiation

In this research, genotoxic potential of vulcanizate produced by sulfur, peroxide and gamma radiation were assayed because after compounding some chemicals may react among each other and develop genotoxic effect . The solvents used to extract residual chemicals were toluene and chloroform: methanol(1:1). The results showed that all of them were not genotoxic to *Salmonella typhimurium* strain TA98 and TA 100 with (+S9) or without metabolic activation (-S9) (Table 3.19-3.20 and 3.24). Yamazaki *et. al*., 1986 and Knudsen *et. al*., 2000 have used choroform : acetone (1:1) for extraction of chemicals in baby botttle. The results showed that this solvent system can only extract dimethyl dithiocarbamate . In this research we found that extraction of residual chemicals in vulcanizate by chloroform:methanol (1:1) can extract residual chemicals better than using toluene (Table 3.19-3.20 and 3.24). Mix solvents of Chloroform:methanol (1:1) have both polar and non-polar functional groups so that they should be more effective to extract residual chemicals in the rubber vulcanizate better than only one solvent.

There are many possibilities that genotoxic effect of rubber vulcanizate could not be detected. First reason is some genotoxic chemical may react with other chemicals and become inactive. The second reason is that in the sulfur vulcanizing system,elemental sulfur is predominant vulcanizing agent for general-purpose rubbers. It is used in combination with one or more accelerators and an activator system such as zinc oxide and a fatty acid (normally stearic acid). The crosslinks among rubber molecules block the residual chemicals leaching out from vulcanizate .

The third possibility is the solvent system or its ratio was not suitable for extracting residual chemicals in vulcanizate. Besides, most chemical itself does not have genotoxic effect and may inhibit the reactive ZBEC/ZDBC so that their genotoxic effect was weaken in all vulcanizate.

Conventional vulcanization system is achieved througth sulfur linkage (Jacob and Vijayakumar, 1997) and the sulfur-sulfur linkage is stronger than carbon-carbon linkage. In this it is observed that using of choroform : methanol (1:1) effective to extract residual chemicals in rubber vulcanizate produced by sulfur and peroxide. The number of revertants colonies of *Salmonella typhimurium* were not no significant different (Table 3.19-3.20). Chloroform:methanol (1:1) is very effective to extract residual chemical in RVNRL the number of revertant colonies is higher when compared with SV-NR and PV-NR (Table 3.24 and Table 3.19-3.20).

The number of revertant colonies caused by residual chemicals in RVNRL were less than SV-NR and PV-NR at 18 and 20 KGy were used (Table 3.24). This results suggest that using gamma radiation at high dose may make crosslink density increasing so that it is not easy to extract residual chemicals in RVNRL.

4.3 Effect of leaching on water extractable protein (WEP) of vulcanized rubber

Table 3.17-3.18 and Table 3.22-3.23 shows that leaching of vulcanized rubber produced by sulfur, peroxide and gamma radiation with 70 C° distilled water 30 min can reduced water extractable protein(WEP) in vulcanizate as reported by Ghazaly, 1994 and Ngamlert, 2002. This result shows that degraded water extractable protein (WEP) are washed out by 70 C°distilled water 30 min about 50 % and easy to be washed out with increasing leaching time (Haowuttikul, 2003).

CHAPTER V

CONCLUSION

1. There are only 3 individual chemicals that used as accelerator in sulfur curing system namely zinc dibutyl dithiocarbarmate (ZDBC), zinc dibenzyl dithio carbamate (ZBEC) and mercaptobenzothiazole(MBTS) that exhibiting genotoxic effect and show dose response curve.
2. Zinc dibutyl dithiocarbarmate (ZDBC) at concentration 100 μg/plate was indirect genotoxic effect in *Salmonella typhimurium* strain TA 98 with metabolic activation (+S9). Mercaptobenzothiazole(MBTS) and Zinc dibenzyl dithiocarbamate(ZBEC) at concentration 100 μg/plate were direct genotoxic in *Salmonella typhimurium* strain TA 98 without metabolic activation (-S9).
3. Zinc dibutyl dithiocarbarmate (ZDBC), zinc dibenzyl dithio carbamate (ZBEC) and mercaptobenzothiazole (MBTS) were frameshift genotoxin in *Salmonella typhimurium* strain TA 98
4. The dose response of ZDBC, ZBEC and MBTS do not have significant different because the number of revertant colonies in the same range of 61-63 colonies at 100 μg/plate.
5. Butyl hydroxy toluene and sulfur were cytotoxic *to Salmonella typhimurium* strain TA98 and TA 100.
6. Using chloroform : methanol(1:1) is more suitable than toluene for extracting residual chemicals in rubber vulcanizate because the number of revertant colonies when extract by chloroform : methanol(1:1) is higher The study of genotoxic potential of vulcanizate produced by sulfur peroxide and gamma radiation show that all vulcanizate do not show genotoxic potential.
7. The vulcanizate produced by sulfur peroxide and radiation vulcanization altogether 6 formulations.
8. Radiation vulcanized natural rubber (RVNR) and peroxide vulcanization may be safer for medical devices, because there were no chemical residues that may cause both genotoxic and cytotoxic effects

9. For RVNR water extractable protein allergens were degedraded and easily washed out resuting in vulcanizate with low protein allergen, chemical and not genotoxic.
10. Reverse mutation assay should be used to test individual chemical in the vulcanizing system before and after curring.

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APPENDIC 1

1. Preparation of Stock Solution and Media

1.1 Vogel – Bonner medium E stock salt solution (VB salt)

Ingredient	500 ml
Distilled H_2O	335 ml
Magnesium sulfate ($MgSO_4.7H_2O$)	5 g
Citric acid monohydrate	50 g
Potassium phosphate , dibasic (anhydrous) (K_2HPO_4)	250 g .
Sodium ammonium phosphate ($NaNH_4PO_4.4H_2O$)	87.50 g

Salts were added to water in the order that indicated and allowed each salt to dissolve completely before adding the next . The solutions were filtered and then autoclaved at 121°C for 20 min .

1.2 Minimal glucose agar plate

Ingredient	3 L
Bacto agar	45 g
Distilled H_2O	2800 ml
VB salts	60 ml
30 %glucose	200 ml

Agar was added to distilled water in a glass bottle and then autoclaved at 121°C for 20 min . When the solution has cooled slightly , sterile VB salts and sterile glucose were added , mixed and poured 30 ml into each sterile petri plate . Minimal glucose agar plates were kept in at 37°C before using .

1.3 Oxoid nutrient broth No . 2

2.5 g of nutrient broth No.2 was dissolved in 100 ml distilled H2O and 12 ml of nutrient broth was transferred into each flask (covered with sterile gauze) . They were autoclaved at 121°C for 20 min

1.4 Top agar

Ingredient	**100**	**ml**
Bacto agar	0.6	g
Sodium chloroide (NaCl)	0.5	g
Distilled H_2O	100	ml

All ingredients were dissolved in water and stored in a glass bottle . The solution was autoclaved for 20 min at 121°C. 10 ml of 0.5 mM histidine HCl – 0.5 mM biotin was added for 100 ml of top agar .

1.5 0.1 M L – histidine HCl stock

Ingredient	100	ml
L – histidine HCl	2.096	g
Distilled H_2O	100	ml

2.096 g of L – histidine HCl (MW 209.63) was dissolved in 100 ml distilled water and then it was prepared to 1 mM l – histidine HCl . The solution residue was autoclaved at 121°C for 20 min and stored in a glass bottle for stock .

1.6 1 mM L – histidine HCl stock

Ingridient	100	ml
0.1 M L – histidine HCl	1	ml
Distilled H_2O	99	ml

1 ml of 0.1 M l – histidine HCl was diluted in 99 ml of distilled water and then it was propared to 0.5 mM L – histidine HCl – 0.5 mM biotin .

1.7 1 mM biotin stock

Ingridient	100	ml
Biotin	24.43	mg
Distilled H_2O	100	ml

Biotin (MW 244.3) was dissloved in distilled water , warmed and stirred until dissolve completely and then it was prepared to 0.5 mM L – histidine HCl – 0.5 mM biotin .

1.8 0.5 mM L – histidine HCl – 0.5 mM biotin

Ingridient	200	ml
1 mM L – histidine HCl	100	ml
1 mM biotin	100	ml

The ingridients were mixed and then it was autoclaved at 121°C for 20 min .

1.9 $NaPO_4$ – KCl buffer

Ingredient	330	ml
0.5 M $NaPO_4$ pH 7.4	100	ml
1 M KCl	16.50	ml
Distilled H_2O	213.50	ml

The ingredients were mixed and autoclaved at 121°C for 20 min .

1.10 1 M KCl

Ingredient	1,000	ml
Potassium chloride	74.56	g
Distilled H_2O	1,000	ml

Potassium chloride was dissolved into water and autoclaved at 121°C for 20 min .

APPENDIC 2

Ingradient of S9 mix

S9 mix (ml)	PO-KCL (ml)	0.16M MgCl (ml)	0.1MG-6-P (ml)	0.1M NADP (ml)	S9 (ml)	0.15MKCL (ml)
10	6.60	0.50	0.50	0.40	1.00	1.00
15	9.90	0.75	0.75	0.60	1.50	1.50
20	13.20	1.00	1.00	0.80	2.00	2.00
30	19.80	1.50	1.50	1.20	3.00	3.00
35	23.10	1.75	1.75	1.40	3.50	3.50
40	26.40	2.00	2.00	1.60	4.00	4.00
45	29.70	2.25	2.25	2.20	4.50	4.50
50	33.00	2.50	2.50	2.00	5.00	5.00

APPENDIC 3

Protein determination by modified Lowry method

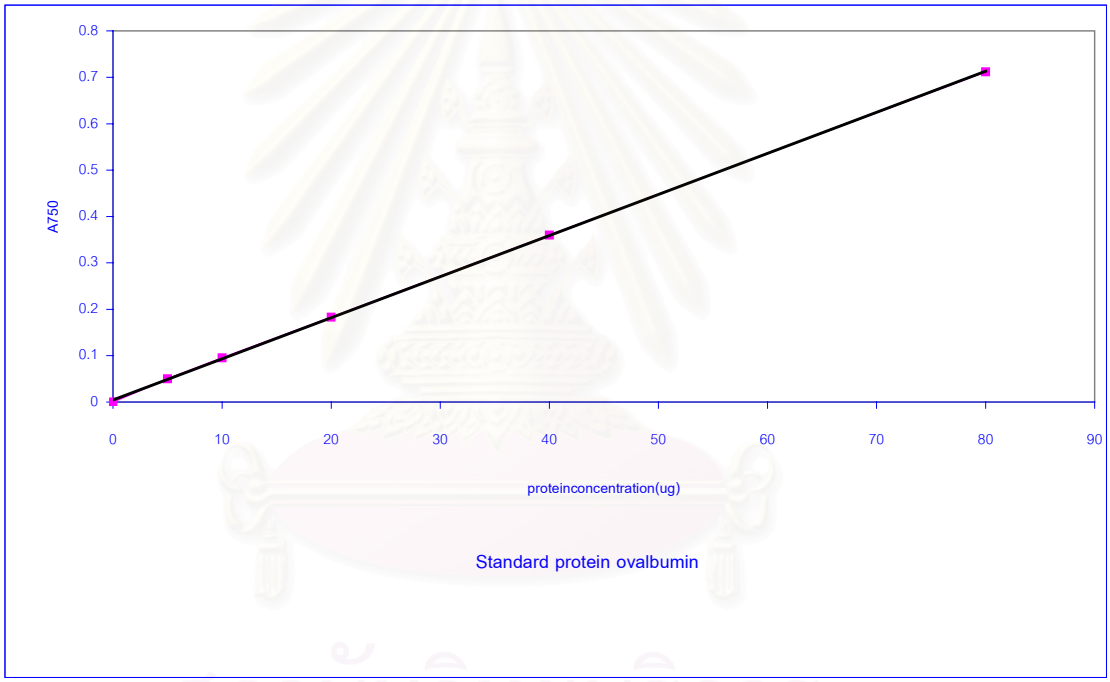
Solution for modified Lowry method

solution C : 6 % w/v of sodium carbonate

solution D : 1.5 % w/v of copper sulfate in 3 % w/v of sodium citrate

reagent A : Alkali copper sulfate (10 parts of C : 0.2 part of D)

reagent B : Diluted Folin Reagent



APPENDIC 4

Dose response of revertants caused by chemicals used in sulfur and peroxide vulcanizing system

Dose response of revertants caused by chemicals used as accellerators and activator in sulfur curing system

Function	Chemical/ Vulcanizate	Dose (ug/plate)	Number of revertant observed using *Salmonella typhimurium*			
			Strain TA98		Strain TA100	
			-S9	+S9	-S9	+S9
negative control	DMSO		30	32	121	151
negative control	DMSO		28	30	120	161
Positive control	AF - 2	0.1	445	-	-	-
Positive control	AF - 2	0.1	460	-	-	-
Positive control	AF - 2	0.01	-	-	510	-
Positive control	AF - 2	0.01	-	-	521	-
Positive control	BP	5	-	470	-	-
Positive control	BP	5	-	461	-	-
Positive control	BP	0.625	-	-	-	515
Positive control	BP	0.625	-	-	-	523
Accelerator	DPTT	1	32	33	130	158
Accelerator	DPTT	1	31	35	134	151
Accelerator	DPTT	10	35	39	140	175
Accelerator	DPTT	10	38	39	144	183
Accelerator	DPTT	100	44	48	151	192
Accelerator	DPTT	100	41	46	156	196
Accelerator	MBTS	1	32	36	162	171

Accelerator	MBTS	1	33	39	171	171
Accelerator	MBTS	10	48	45	170	179
Accelerator	MBTS	10	51	48	174	182
Accelerator	MBTS	100	60	50	180	182
Accelerator	MBTS	100	62	50	189	179
Accelerator	ZBEC	1	34	39	135	131
Accelerator	ZBEC	1	30	40	158	152
Accelerator	ZBEC	10	58	47	184	153
Accelerator	ZBEC	10	31	44	156	155
Accelerator	ZBEC	100	62	48	194	180
Accelerator	ZBEC	100	59	41	197	172
Accelerator	ZDBC	1	30	49	160	165
Accelerator	ZDBC	1	35	47	164	163
Accelerator	ZDBC	10	41	50	175	177
Accelerator	ZDBC	10	40	54	169	169
Accelerator	ZDBC	100	41	67	168	128
Accelerator	ZDBC	100	44	60	170	186
Accelerator	ZMBT	1	68	40	164	168
Accelerator	ZMBT	1	42	33	165	172
Accelerator	ZMBT	10	37	35	191	167
Accelerator	ZMBT	10	40	41	174	173
Accelerator	ZMBT	100	41	38	176	125
Accelerator	ZMBT	100	41	35	190	154
Activator	stearic acid	1	31	50	122	136

Activator	stearic acid	1	33	46	130	138
Activator	stearic acid	10	35	50	131	139
Activator	stearic acid	10	33	50	135	140
Activator	stearic acid	100	35	49	149	140
Activator	stearic acid	100	35	55	144	144
Activator	Zinc carbonate	1	44	40	140	180
Activator	Zinc carbonate	1	46	38	158	167
Activator	Zinc carbonate	10	43	50	192	147
Activator	Zinc carbonate	10	46	37	147	145
Activator	Zinc carbonate	100	54	34	186	137
Activator	Zinc carbonate	100	48	39	201	145
Activator	Zinc Oxide	1	49	36	181	148
Activator	Zinc Oxide	1	57	31	171	176
Activator	Zinc Oxide	10	46	47	174	156
Activator	Zinc Oxide	10	47	49	210	177

Activator	Zinc Oxide	100	43	44	193	155
Activator	Zinc Oxide	100	50	44	191	165
Activator	Zinc stearate	1	30	40	121	145
Activator	Zinc stearate	1	29	43	115	140
Activator	Zinc stearate	10	39	49	117	147
Activator	Zinc stearate	10	32	50	126	147
Activator	Zinc stearate	100	35	55	140	149
Activator	Zinc stearate	100	34	52	139	154

Dose response of revertants dosed by chemicals cused as antioxidant in Sulfur curring system

Function	Chemical/ Vulcanizate	Dose (μg/plate)	Number of revertant observed using *Salmonella typhimurium*			
			Strain TA98		Strain TA100	
			-S9	+S9	-S9	+S9
negative control	DMSO		35	50	134	161
negative control	DMSO		40	53	158	164
Positive control	AF - 2	0.1	512	-	-	-
Positive control	AF - 2	0.1	520	-	-	-
Positive control	AF - 2	0.01	-	-	546	-
Positive control	AF - 2	0.01	-	-	560	-
Positive control	BP	5	-	525	-	-
Positive control	BP	5	-	532	-	-
Positive control	BP	0.625	-	-	-	611
Positive control	BP	0.625	-	-	-	599
Antioxidant	BHT	1	48	55	161	171
Antioxidant	BHT	1	45	59	169	176
Antioxidant	BHT	10	59	65	172	195
Antioxidant	BHT	10	66	69	176	206
Antioxidant	BHT	100	no revertant	no revertant	no revertant	no revertant
Antioxidant	BHT	100	no revertant	no revertant	no revertant	no revertant
negative control	DMSO		24	26	101	125
negative control	DMSO		25	28	104	126

Positive control	AF - 2	0.1	398	-	-	-
Positive control	AF - 2	0.1	405	-	-	-
Positive control	AF - 2	0.01	-	-	422	-
Positive control	AF - 2	0.01	-	-	413	-
Positive control	BP	5	-	407	-	-
Positive control	BP	5	-	399	-	-
Positive control	BP	0.625	-	-	-	452
Positive control	BP	0.625	-	-	-	439
Antioxidant	Irganox	1	33	33	128	138
Antioxidant	Irganox	1	31	32	131	141
Antioxidant	Irganox	10	38	35	135	156
Antioxidant	Irganox	10	36	38	140	160
Antioxidant	Irganox	100	42	40	151	165
Antioxidant	Irganox	100	40	43	154	168

Function	Chemical/ Vulcanizate	Dose (μg/plate)	Number of revertant observed using *Salmonella typhimurium*			
			Strain TA98		Strain TA100	
			-S9	+S9	-S9	+S9
negative control	DMSO		30	28	112	129
negative control	DMSO		28	32	115	133
Positive control	AF - 2	0.1	420	-	-	-
Positive control	AF - 2	0.1	431	-	-	-
Positive control	AF - 2	0.01	-	-	401	-
Positive control	AF - 2	0.01	-	-	410	-
Positive control	BP	5	-	507	-	-
Positive control	BP	5	-	518	-	-
Positive control	BP	0.625	-	-	-	445
Positive control	BP	0.625	-	-	-	450
filler	Hisil233	1	41	40	170	158
filler	Hisil233	1	43	41	165	163
filler	Hisil233	10	47	44	182	154
filler	Hisil233	10	50	41	178	165
filler	Hisil233	100	51	50	151	172
filler	Hisil233	100	54	47	179	191
Filler	PEG	1	33	45	121	165
filler	PEG	1	30	47	116	161
filler	PEG	10	37	52	143	161
filler	PEG	10	35	46	150	170
filler	PEG	100	37	54	151	172
filler	PEG	100	35	50	156	178

Dose response of revertants dosed by chemicals cused as vulcanizing agent in Sulfur curring system

Function	Chemical/ Vulcanizate	Dose (μg/plate)	Number of revertant observed using *Salmonella typhimurium*			
			Strain TA98		Strain TA100	
			-S9	+S9	-S9	+S9
negative control	DMSO		20	29	114	129
negative control	DMSO		22	32	113	133
Positive control	AF - 2	0.1	420	-	-	-
Positive control	AF - 2	0.1	431	-	-	-
Positive control	AF - 2	0.01	-	-	401	-
Positive control	AF - 2	0.01	-	-	410	-
Positive control	BP	5	-	507	-	-
Positive control	BP	5	-	518	-	-
Positive control	BP	0.625	-	-	-	445
Positive control	BP	0.625	-	-	-	450
Vulcanizing agent	Sulfur	1	22	51	425	160
Vulcanizing agent	Sulfur	1	24	54	120	158
Vulcanizing agent	Sulfur	10	24	54	138	168
Vulcanizing agent	Sulfur	10	28	50	136	162
Vulcanizing agent	Sulfur	100	20	56	no revertant	173
Vulcanizing agent	Sulfur	100	18	54	no revertant	170

Dose response of revertants dosed by chemicals cused as vulcanizing agent in peroxide curring system

Function	Chemical/ Vulcanizate	Dose (μg/ plate)	Number of revertant observed using *Salmonella typhimurium*			
			Strain TA98		Strain TA100	
			-S9	+S9	-S9	+S9
negative control	DMSO		30	28	151	157
negative control	DMSO		28	32	160	177
Positive control	AF - 2	0.1	445	-	-	-
Positive control	AF - 2	0.1	460	-	-	-
Positive control	AF - 2	0.01	-	-	510	-
Positive control	AF - 2	0.01	-	-	521	-
Positive control	BP	5	-	470	-	-
Positive control	BP	5	-	461	-	-
Positive control	BP	0.625	-	-	-	515
Positive control	BP	0.625	-	-	-	523
Vulcanizing agent	DCP98%	1	35	36	164	158
Vulcanizing agent	DCP98%	1	39	38	158	165
Vulcanizing agent	DCP98%	10	48	45	163	170
Vulcanizing agent	DCP98%	10	50	44	170	176
Vulcanizing agent	DCP98%	100	51	50	174	180
Vulcanizing agent	DCP98%	100	55	48	171	189

APPENDIC 5

Genotoxic potential of vulcanizate (SV-NR, SV-IR ,PV-NR and RVNRL)

The number of revertants caused by residual chemical in vulcanizate[a]

Function/ Formulation table	Chemical/ Vulcanizate	Dose (ug/plate)	Solvent extraction	Number of revertant observed using Salmonella typhimurium			
				Strain TA98		Strain TA100	
				-S9	+S9	-S9	+S9
negative control	DMSO			26	31	190	198
negative control	DMSO			28	30	198	200
Positive control	AF-2	0.1		494	-	-	-
	AF-2	0.1		451	-	-	-
Positive control	AF-2	0.01		-	-	-	-
	AF-2	0.01		-	-	-	-
Positive control	BP	5		-	487	-	-
	BP	5		-	399	-	-
Positive control	BP	0.625		-	-	-	540
	BP	0.625		-	-	-	512
3.8	SV - NR		Toluene	40	33	266	257
3.8	SV - NR		Toluene	44	31	274	327
3.10	SV - IR		Toluene	39	36	304	303
3.10	SV - IR		Toluene	37	28	280	323
3.12	PV - NR		Toluene	35	28	265	304
3.12	PV - NR		Toluene	29	31	296	255

3.8	SV - NR		Chloroform methanol (1:1)	36	34	189	257
3.8	SV - NR		Chloroform methanol (1:1)	42	40	211	276
3.10	SV - IR		Chloroform methanol (1:1)	41	38	191	362
3.10	SV - IR		Chloroform methanol (1:1)	39	43	180	287
3.12	PV - NR		Chloroform methanol (1:1)	42	41	202	277
3.12	PV - NR		Chloroform methanol (1:1)	38	40	193	245

[a] experiment no. 1

The number of revertants caused by residual chemical in vulcanizate[a]

Function/ Formulation table	Chemical/ Vulcanizate	Dose (ug/plate)	Solvent extraction	Number of revertant observed using *Salmonella typhimurium*			
				Strain TA98		Strain TA100	
				-S9	+S9	-S9	+S9
Negative control	DMSO			25	28	195	245
Negative control	DMSO			29	23	203	248
Positive control	AF-2	0.1		485	-	-	-
	AF-2	0.1		451	-	-	-
Positive control	AF-2	0.01		-	-	501	-
	AF-2	0.01		-	-	495	-
Positive control	BP	5		-	498	-	-
	BP	5		-	487	-	-
Positive control	BP	0.625		-	-	-	501
	BP	0.625		-	-	-	49.9
3.8	SV - NR		Toluene	47	39	266	257
3.8	SV - NR		Toluene	51	32	274	327
3.10	SV - IR		Toluene	38	26	304	303
3.10	SV - IR		Toluene	36	31	280	323
3.12	PV - NR		Toluene	29	28	265	304
3.12	PV - NR		Toluene	50	32	296	255
3.8	SV - NR		Chloroform methanol (1:1)	42	43	266	257

3.8	SV - NR		Chloroform methanol (1:1)	37	47	284	276
3.10	SV – IR		Chloroform methanol (1:1)	44	30	264	362
3.10	SV – IR		Chloroform methanol (1:1)	39	55	261	287
3.12	PV - NR		Chloroform methanol (1:1)	39	26	265	277
3.12	PV - NR		Chloroform methanol (1:1)	36	38	269	245

[a] experiment no. 2

Genotoxic potential of RVNRL

Function	Chemical/ Vulcanizate	Dose (μg/plate)	Number of revertant observed using *Salmonella typhimurium*			
			Strain TA98		Strain TA100	
			-S9	+S9	-S9	+S9
Negative control	DMSO		29	34	121	137
Negative control	DMSO		30	32	130	131
Negative control	DMSO		29	36	125	134
Positive control	AF - 2	0.1	498	-	-	-
Positive control	AF - 2	0.1	503	-	-	-
Positive control	AF - 2	0.1	491	-	-	-
Positive control	AF - 2	0.01	-	-	501	-
Positive control	AF - 2	0.01	-	-	490	-
Positive control	AF - 2	0.01	-	-	509	-
Positive control	BP	5	-	483	-	-
Positive control	BP	5	-	510	-	-
Positive control	BP	5	-	498	-	-
Positive control	BP	0.625	-	-	-	507
Positive control	BP	0.625	-	-	-	493
Positive control	BP	0.625	-	-	-	501
	RVNRL 10 kGy		43	49	149	170

	RVNRL 10 kGy		45	50	150	175
	RVNRL 10 kGy		41	47	148	171
	RVNRL 12 kGy		42	50	151	172
	RVNRL 12 kGy		40	48	153	163
	RVNRL 12 kGy		38	45	146	170
	RVNRL 14 kGy		35	45	148	166
	RVNRL 14 kGy		36	42	147	160
	RVNRL 14 kGy		40	44	150	159
	RVNRL 16 kGy		39	43	147	152
	RVNRL 16 kGy		34	42	149	148
	RVNRL 16 kGy		37	42	150	150
	RVNRL 18 kGy		38	43	147	148
	RVNRL 18 kGy		35	45	144	149

	RVNRL 18 kGy		32	41	148	152
	RVNRL 20 kGy		34	43	143	147
	RVNRL 20 kGy		36	40	146	144
	RVNRL 20 kGy		37	43	142	149

[a] extract by toluene

The number of revertants caused by residual chemical in RVNRL[a]

Function	Chemical/ Vulcanizate	Dose (ug/plate)	Number of revertant observed using *Salmonella typhimurium*			
			Strain TA98		Strain TA100	
			-S9	+S9	-S9	+S9
negative control	DMSO		28	31	125	135
negative control	DMSO		29	33	131	131
negative control	DMSO		29	35	133	135
Positive control	AF - 2	0.1	409	-	-	-
Positive control	AF - 2	0.1	415	-	-	-
Positive control	AF - 2	0.1	420	-	-	-
Positive control	AF - 2	0.01	-	--	507	-
Positive control	AF - 2	0.01	-	--	499	-
Positive control	AF - 2	0.01	-	--	490	-
Positive control	BP	5	-	495	-	-
Positive control	BP	5	-	501	-	-
Positive control	BP	5	-	489	-	-
Positive control	BP	0.625	-	-	-	491
Positive control	BP	0.625	-	-	-	502
Positive control	BP	0.625	-	-	-	510
	RVNRL 10 kGy		47	52	159	173
	RVNRL 10 kGy		44	55	155	166

	RVNRL 10 kGy		45	53	152	170
	RVNRL 12 kGy		49	54	158	173
	RVNRL 12 kGy		45	55	153	168
	RVNRL 12 kGy		46	51	151	161
	RVNRL 14 kGy		47	51	157	169
	RVNRL 14 kGy		48	48	153	161
	RVNRL 14 kGy		44	54	152	165
	RVNRL 16 kGy		44	52	150	170
	RVNRL 16 kGy		45	50	145	163
	RVNRL 16 kGy		44	50	148	161
	RVNRL 18 kGy		46	50	145	161
	RVNRL 18 kGy		44	48	140	160
	RVNRL 18 kGy		41	47	148	155

	RVNRL 20 kGy		40	48	142	155
	RVNRL 20 kGy		41	50	140	149
	RVNRL 20 kGy		39	46	147	152

[a] extract by chloroform : methanol (1 : 1)

Genotoxic potential of vulcanizate (SV-NR, SV-IR and PV-IR)

The number of revertants caused by residual chemical in vulcanizate

Function/ Formulation table	Chemical/ Vulcanizate	Dose (ug/plate)	Solvent extraction	Number of revertant observed using *Salmonella typhimurium*			
				Strain TA98		Strain TA100	
				-S9	+S9	-S9	+S9
Negative control	DMSO			21	25	101	110
negative control	DMSO			20	22	97	114
negative control	DMSO			23	28	104	119
Positive control	AF-2	0.1		451	-	-	-
Positive control	AF-2	0.1		439	-	-	-
Positive control	AF-2	0.1		470	-	-	-
Positive control	AF-2	0.01		-	-	481	-
Positive control	AF-2	0.01		-	-	472	-
Positive control	AF-2	0.01		-	-	498	-
Positive control	BP	5		-	498	-	-
Positive control	BP	5		-	505	-	-
Positive control	BP	5		-	491	-	-
Positive control	BP	0.625		-	-	-	507
Positive control	BP	0.625		-	-	-	497
Positive control	BP	0.625		-	-	-	501
3.9	SV - NR		Toluene	28	41	125	128
3.9	SV - NR		Toluene	32	40	129	135
3.9	SV - NR		Toluene	31	40	118	139
3.11	SV - IR		Toluene	37	36	131	140

3.11	SV - IR		Toluene	33	33	129	145
3.11	SV - IR		Toluene	31	34	133	139
3.13	PV-NR		Toluene	35	37	117	127
3.13	PV-NR		Toluene	30	41	120	130
3.13	PV-NR		Toluene	29	40	128	122
3.9	SV - NR		Chloroform methanol (1:1)	35	49	131	139
3.9	SV - NR		Chloroform methanol (1:1)	33	43	129	147
3.9	SV - NR		Chloroform methanol (1:1)	30	44	125	143
3.11	SV - IR		Chloroform methanol (1:1)	38	37	131	148
3.11	SV - IR		Chloroform methanol (1:1)	32	42	135	141
3.11	SV - IR		Chloroform methanol (1:1)	35	39	140	139
3.13	PV-NR		Chloroform methanol (1:1)	31	41	119	133
3.13	PV-NR		Chloroform methanol (1:1)	29	43	120	129
3.13	PV-NR		Chloroform methanol (1:1)	34	38	127	137

BIOGRAPHY

Miss Pattamawadee Sankheangaew was born on June 7, 1979. She graduated with the degree of Bachelor of Science in Biochemistry and Biochemical technology from Chiangmai University in 2001. She continue her study in the Master Program of Biochemistry, Faculty of Science at Chulalongkorn University.