Chapter I

Introduction

Tetanus is unique among the vaccine preventable disease in that it is not communicable. Instead,tetanus is acquired through environmental exposure. Many animals,in addition to human , can harbor and excrete the organisms. Also , many animal species,moreover,human are susceptable to the disease. Spores introduced under the proper (anaerobic) conditions germinate to vegetative bacilli, which elaborate toxin. The clinical presentation results from the actions of this toxin on the central nervous system (CNS). Prevention of tetanus can be achieved by use of a chemically inactivates toxin (toxoid) which induces production of neutralizing antibody(antitoxin). Tetanus can also be prevented by the introduction of exogenous antibody. The toxoid is highly immunogenic and safe and is almost 100% protective following a primary series.

However,somedeveloping countries, are unable to implementfully dose programs ,resulting in significant annual mortality from neonatal tetanus. It is possible that the use of lecithin and carboxymethyl chitin walled microcapsules, an effective long acting tetanus vaccine, nontoxic, biocompatible, biodegradableand non-antigenic substances,by interfacial polymerization technique may help alliviate some of the problems associated with widespread vaccination in developing countries.

Microcapsules, small vesicles, diameters of 1 to 500 microns,

made of nature or synthetic polymer membrane. Microcapsules those contain liquid , such as vaccine,are spherical in shape. Important functions of materials, release materials into the external environment in a sustained manner.(Ostro,and Cullis,1989) A large number of patented process are available for preparing microcapsules for vaccine (Garcon,et al.,1988)aqueous materials,interfacial polymerization technique is one appropiate. The polymerization of polymer membrane occur at the interface between droplet of emulsion,that made spherical microcapsules.

The sustained manner of tetanus toxoid microcapsules are significantly longer than adsorbed tetanus toxoid (Reuongchai 1986). However the immune response in different vesicle size of lecithin and carboxymethyl chitin walled tetanus toxoid microcapsules in mice as potency test and hemagglutination test had not yet been reported.

Microcapsules

Microcapsules (Gutcho,1976) are composed of a nature or synthetic polymeric skin or wall enclosing a liquid core or other body of material. The capsule wall is inert to the substance it contains, possesses enough strength to allow for normal handling without rupture, and is sufficiently thin to permit a high core volume to wall volume ratio. The contents of the capsule are contained within the wall until released by some means that serve to break,crush,melt,dissolve,rupture or remove the capsule shell, or until the internal phase is caused to diffuse out through the capsule wall.

The diameters of microcapsule are in a range from 1 to 500

microns and varying wall thickness. In most case microcapsules containing solid particles are of nearly the same shape as the solid , where as those that contain a liquid or gas are spherical in shape as shown in Figure 1.

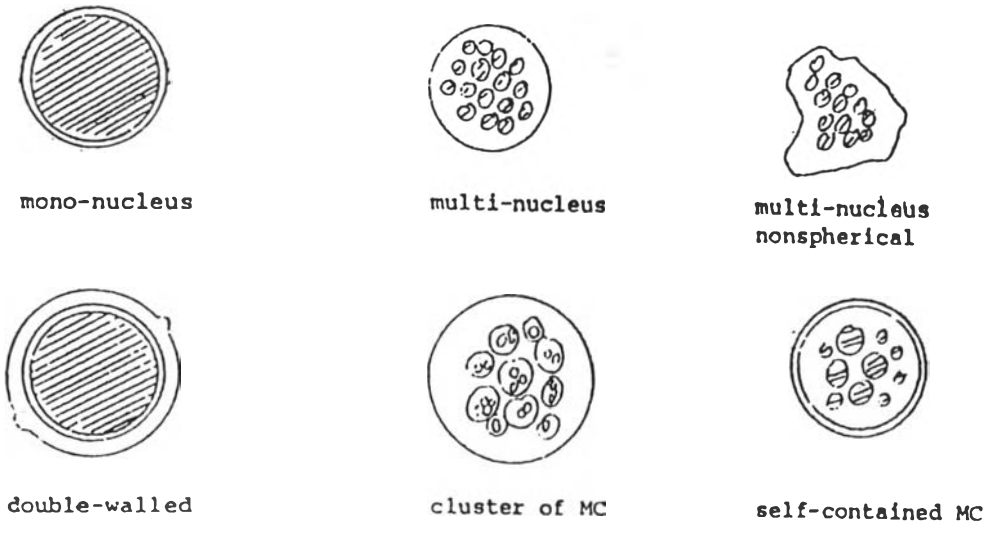


Figure 1 Various Structure of Microcapsules.

There are several important functions of microcapsules in pharmaceutical sciences,for example,to control solubilization of drugs i.e propranolol (Roger,Betageri,and Chai,1990)] ,sustained release of entrapped drugs [i.e, immunomodulator (interferon) and a peptide hormone (calcitonin) (Weiner,Martin and Riaz , 1989) ,insulin (Spangler, 1990), chloroquin phosphate and quinine hydrochloride (Chukwu , Agarwal ,and Adikwu, 1991 ; Cromelin,et al., 1991), diclofinac sodium (Lin and kaoy ,

1991), diphtheria toxoid (Singh,M.,Singh,A.,and Talwar,1991) , thyrotropin releasing hormone (Heya,et al.,1991),and triptorien(Ruiz, and Benoit,1991).

In addition, microcapsules are used in the aims of site specific drug delivery system. (Gupta ,1990) such as antineoplastic agents [i.e, doxorubicin , amphotericin B and platinum (Ostro and Cullis , 1989) , cisplatin (Crommelin ,et al., 1991)] and antiparasitic drugs [i.e, chloroquin phosphate (Crommelin ,et al.,1991)].

Other charateristics of importance are alterations to increase drug levels in blood [i.e, cisplatin (Iga,et al.,1991) , chloroquin phosphate and quinine hydrochloride (Chukwa, et al., 1991)] ,to protect the drug from biological fluid such as dichlorofenac sodium from gastric fluid (Lin and Kao,1991).

More over it has an adjuvants effect in the formation of antibodies i.e, snake venom (Alving ,Richards,et al.,1986.tetanus toxoid (Davis and Gregoriadis,1987) ,diphtheria toxoid (Singh , et al.,1991).

The name of microcapsules are always changed as their composition of the membrane;phospholipids called "liposome" ;surfactants called "neosome" etc.(Prescott,and Nimmo,1990)

1) <u>Method of Preparation</u> (Marijevic,1978)

A large number of patented processes are available for preparing microcapsules. They can be classified roughly into physicochemical,chemical and physical methods. The first group includes aqueous and organic phase separations and spray-drying methods.

Interfacial polymerization,in situ polymerization,and orific method belong to the second group. Included in the third group are the electrostatic,physical vapor deposition,and fluidized-bed spray-coating methods.Our discussion is limited to the interfacial polymerization that it is used in this research .

The interfacial polymerization method is suitable for encapsulating liquids rather than a solid phase.Our description is, therefore,devoted only to microencapsulation of liquids,this process consistes of the following steps:

1) Dispersion of an aqueous solution of a water-soluble reactant or an organic solution of an oil-soluble reactant or an organic solution of an oil-soluble reactant into an organic or an aqueous phase with the aid of an appropriate emulsifier to yield a water-in-oil emulsion or an oil-in-water emulsion.

2) Formation of polymer membrane on the surface of liquid droplets initiated by the addition of a water-insoluble reactant to the water-in-oil emulsion or a water-soluble reactant to the oil-in-water emulsion.

3) Seperation of microcapsules containing water or oil from the oil or aqueous phase.

2) Interfacial Polymerization Method

Among many existing microencapsulation processes,the interfacial polymerization is unique making direct use of polymerization reactions at interface to prepare microcapsules. Researchers at Du Pont have

developed labolatory procedures for synthesizing polyamides, polyphthalamides, polysulfonamides,polyurethanes,and polyphenyl esters by preparing microcapsules are of the condensation type.

Wittbecker and Morgan(1959) found that the Schotten Baumann reaction of diacid chlorides with compounds containing two or more active hydrogen atom (-OH,-NH,-SH) can be the basis for a simple, versatile labolatory process to prepare polymers,and they designated this process "interfacial polycondensation"

In this process the irreversible polymerization of two fast-reaction intermediates occurs near the interface of the two phases of a heterogenous liquid system. For example,if we bring an aqueous solution of hexamethylene diamine into contact with a solution of sebacoyl dichloride in carbon tetrachloride in a beaker at room temperature,we can observe at once formation of a thin polyamide film at the interface of the two solutions.When we pull the film from the interface,more polymer forms at once and we can withdraw continuously a collapsed sheet or tube of polymer. The polymer formation can be enhanced by the presence in the aqueous phase of an alkali that serves to neutralize hydrogen chloride generated during the polycondensation reaction.

In a homogeneous polycondensation the two different functional groups must be present in equivalent amounts. However, a large excess of one of the intermediates can be tolerated without limiting the molecular weight in an interfacial polycondensation because it is a heterogenous reaction and the reactive intermediates must diffuse to the interface; that is,reactant equivalence is attained in the polymerization zone though the whole system is unbalanced. Thus,two moles of diamine and one

mole of diacid chloride produce a high molecular-weight polymer,although the stoichiometric reactant ratio is unity.

We may expect that interfacial polycondensation would fail to give high-molecular-weight polymers,since hydrolysis of one of an diacid chloride just before the other reacts with an amine group or hydrolysis of an acid chloride group on the end of a growing polymer chain would lead to early termination of the polymerization even if reactant equivalence is maintained in the polymerization zone. Actually,however, high molecular weights are obtained from properly run interfacial polycondensation reactions,indicating that these side reactions are negligible. For instance,Morgan and Kwolek reported that they can prepare condensation polymers with weight-average molecular weights as high as 500,000. In the synthesis of polyamides,polyesters,and polyurethane by melt polymerization,weight average molecular weights over 50,000 are hardly obtained even when purity and equivalence requirement are rigcrously met.

Interfacial polymerization reaction should take place wherever the reactive intermediates encounter each other. The location may be exactly at the liquid interface,or in the organic or aqueous phase. Evidence shows that most water insensitive polymers form and grow on the organic solvent side of the interface. Although some reactions occur primarily at the interface,the polymer accumulates in the organic phase. No examples of polymerization in the aqueous phase are known.

The primary functions of the aqueous phase are to serve as a solvent for water-soluble reactants such as diamines and acid acceptors and to remove by-product acid from the polymerization zone. The

interface has no special orienting or aligning effect on either water or oil soluble reactants but it provides a controlled introduction of aqueous reactant into an excess of diacid halide in the organic phase adjacent to the interface through solubility difference.

Water-soluble reactants distribute between the aqueous and organic phases when the two phases come into contact with each other. Measured equilibrium partition coefficients for a water-soluble reactant in useful solvent systems vary from several thousand to less than unity. As an example,the equilibrium partition coefficients for haxamethylenediamine in several organic solvent systems are given in table 1. Here,the partition coefficient is defined as the ratio of the concentration of hexamethylenediamine in the aqueous phase to that in the organic phase. The values can be used to estimate the relative tendency of the diamine to transfer into the organic phase under polymerization conditions. Polymer formation is favorably affected by a large transfer of the diamine.

It had been reported that the transfer of piperazine is greatly affected by the pH of the aqueous phase and the partition coefficient for piperazine under conditions similar to those used for preparing microcapsules. Their results are shown graphically in Figure 2, where the logarithm of the nonequilibrium partition coefficient for piperazine between the aqueous phase and the organic phase,which consists of chloroform and cyclohexane (1:3 V/V) and containing surfactant is plotted against the pH of the aqueous phase. At any concentration of surfactant in the organic phase,the partition coefficient decreases first and then levels off as the pH of the aqueous phase increases,

Table 1 Equilibrium Partition Coefficient of

Hexamethylene Diamine in Organic Solvent Systems

solvent	Partition Coefficient K	C_{H_2O} mole/liter.
Cyclohexane	182	0.40
xylene	50	0.392
CCl_4	35	0.40
$C_6H_5NO_2$	13.8	0.391
$CHCl_3$-CCl_4 30:70(v/v)	6.4	0.39
$ClCH_2CH_2Cl$	5.6	0.432
$CHCl_3$	0.7	0.457

K = $C_{H_2O}/C_{solvent}$ at 25° C at equilibrium with 2 moles of sodium hydroxide per mole of diamine in the aquous phase.

indicating that the transfer of piperazine increases initially but approaches a saturation value as the pH of the aqueous phase increases, owing to the fact that pK values of piperazine are 5.57 and 9.81. Since an increase in the pH of the aqueous phase causes an increase in the number of unprotonated piperazine molecules,a transfer is favored. The transfer stops increasing when all the piperazine molecules in the

aqueous phase take the unprotonated form above pH 10.0 Figure 2 shows also that the presence of surfactant in the organic phase enhanced the transfer of piperazine irrespective of the pH of the aqueous phase and the amount transferred increases as the surfactant concentration increases. Although the exact role of surfactant in promoting the transfer of piperazine is not fully stood,a portion of piperazine transferred should exist in the solubilized form in surfactant micells since the surfactant concentrations they used lie well above the critical concentration for micell formation of the surfactant.

More recently,the chemical structure of the reactants has been found to affect the formation of polymer. In the formation of polyphenyl esters from bisphenols and diacid chlorides, The percentage of reacted biphenols showed a tendency to deminish with increasing surfactant concentration in the organic phase if p-phthaloyl dichloride was used, where as the reverse tendency was observed when sebacoyl dichloride was employed despite the fact that the nonequilibrium partititon coefficients of the bisphenols were the same in the both cases. Since the two diacid chlorides are equally highly reactive with the bisphenols, the discrepancy should arise from the difference in the molecule structure between the two diacid chlorides. It would be easy for sebacoyl dichloride molecules to take favorable conformations to react with bisphenol molecules in the organic phase even in the presence of high concentrations of surfactant because they are flexible owing to their linear chain.However,the presence of a rigid aromatic ring is supposed to impede the reaction of p-phthaloyl dichloride with the bisphenols if the surfactant concentrations is high because the chance

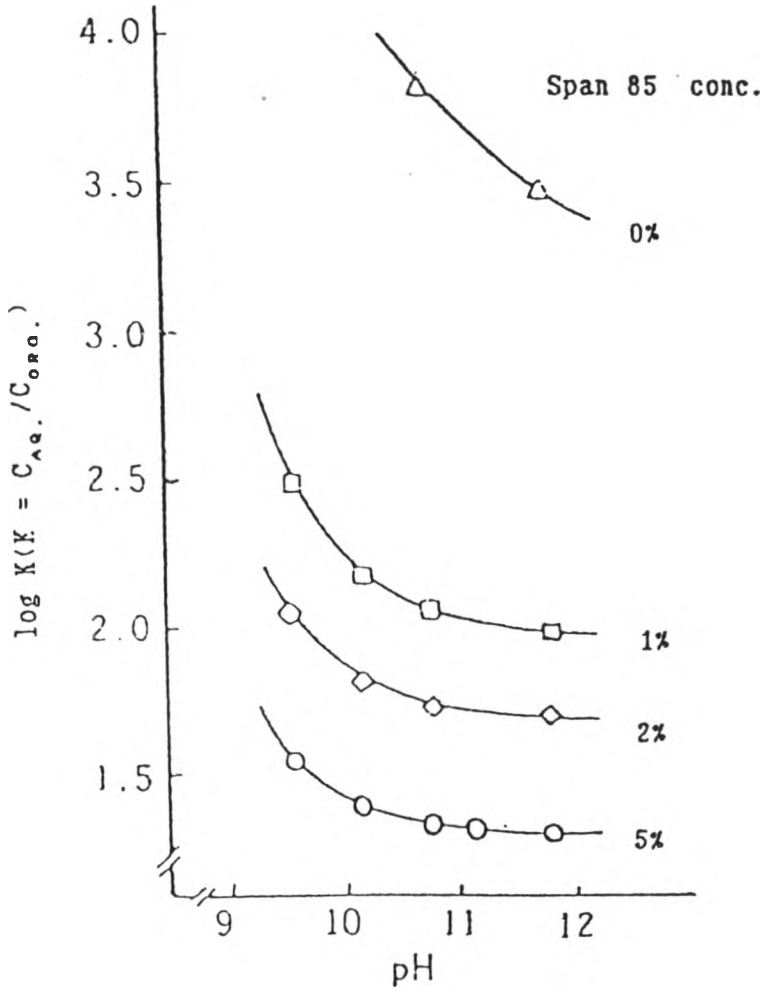


Figure 2 Dependence of nonequilibrium partition coefficient of piperazine.

for molecules of diacid chloride to assume advantageous orientations to the reactions with molecules of bisphenols is lower due to the presence of a large number of surfactant molecules.

3) Liposome

When phospholipids are dispersed in water they spontaneously form closed structures with internal aqueous compartment bounded by phospholipid bilayer membranes;these structures are called "liposomes" . Recently,a number of workers have prepared phospholipid analogs capable of undergoing polymerization reactions,which can be used to form liposomes and which provide some unusual properties (Ostro , and Cullis,1989) Nonphospholipid compounds including sterol esters and even certain amphiphilic polymers can form vesicle structures which resemble phospholipid liposomes. Thus, a wide variety of amphiphilic substances can be used in the formulation of liposomes. The choice of chemical constituents will,of course, influence the charge,stability, chemical reactivity and biological properties of the liposomal preparation.

Due to the high degree of biocompatibility (Wada, 1990), liposomes were initially conceived as delivery systems for intravenous delivery. It has since become apparent that liposomes can also be useful for delivery of drugs by other routes of administration (Ostro,and Cullis,1989). The formular can use strategies to design liposomes for specific purposes,thereby improving the therapeutic index of a drug by increasing the percent of drug molecules that reach the target tissue,or

alternatively,decreasing the percent of drug molecule that reach sites of toxicity. Clinicals trial now underway utilize liposomes to achieve a variety of therapeutic objectives including enhancing the activity and reducing toxicity of a widely used antineoplastic drug (doxorubicin) and antifungal drugs(amphotericin B) delivered intravenously. Other clinical trials are evaluating the ability of liposomes to deliver intravenously immunomodulators to macrophages and imaging agents (^{111}Indium) to tumors. Recent studies in animals have reported the delivery of water-insoluble drug into the eye,and the prolonged release of antigens (Davis, D.,Gregoriadis,G.,1989) (tetanus toxoid,influenza virus subunit proteins etc.) and immunomodulators (interferon). these trials and animals study provide evidence of the versatility of liposomes.

3.1) Nature of Lipid Bilayer Vesicle

Liposomes consist of amphiphilic lipids (Brajtburg, 1990) typically, phospholipid (eg.lecithin) having a polar headgroup and two long fattyacid chains. They are spherical vesicles,30-100 nm in diameter, formed by one or several concentric lipid bilayers ,4-5 nm thick as shown in Figure 3, enclosing an aqueous core. The double-chain amphiphiles include various phospholipids such as phosphatidyl-choline, and the corresponding serine,ethanolamine and inositol derivatives as shown in Figure 4. When derived from natural sources(soy bean,egg yolk, or brain tissue),these lipids contain unsaturated fatty acid groups. Natural lipids may be hydrogenated to obtain more stable liposomes. Other two-chain amphiphiles include ammonium salts such as di-hexadecyl

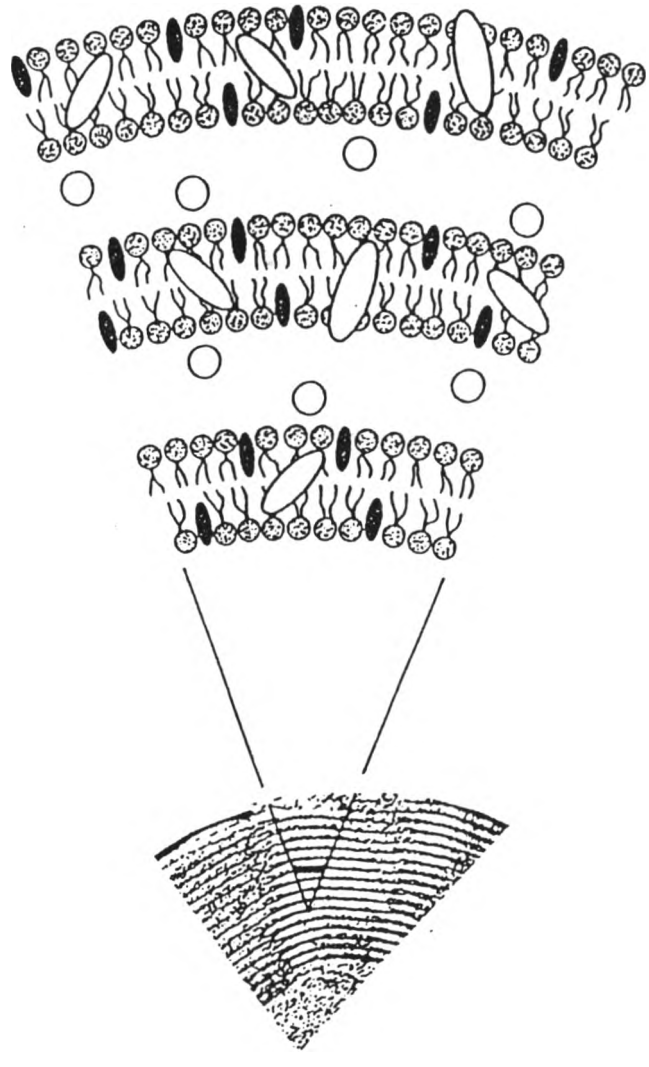


Figure 3 A section of electron micrograph of a negatively stained multilamellar liposomes.

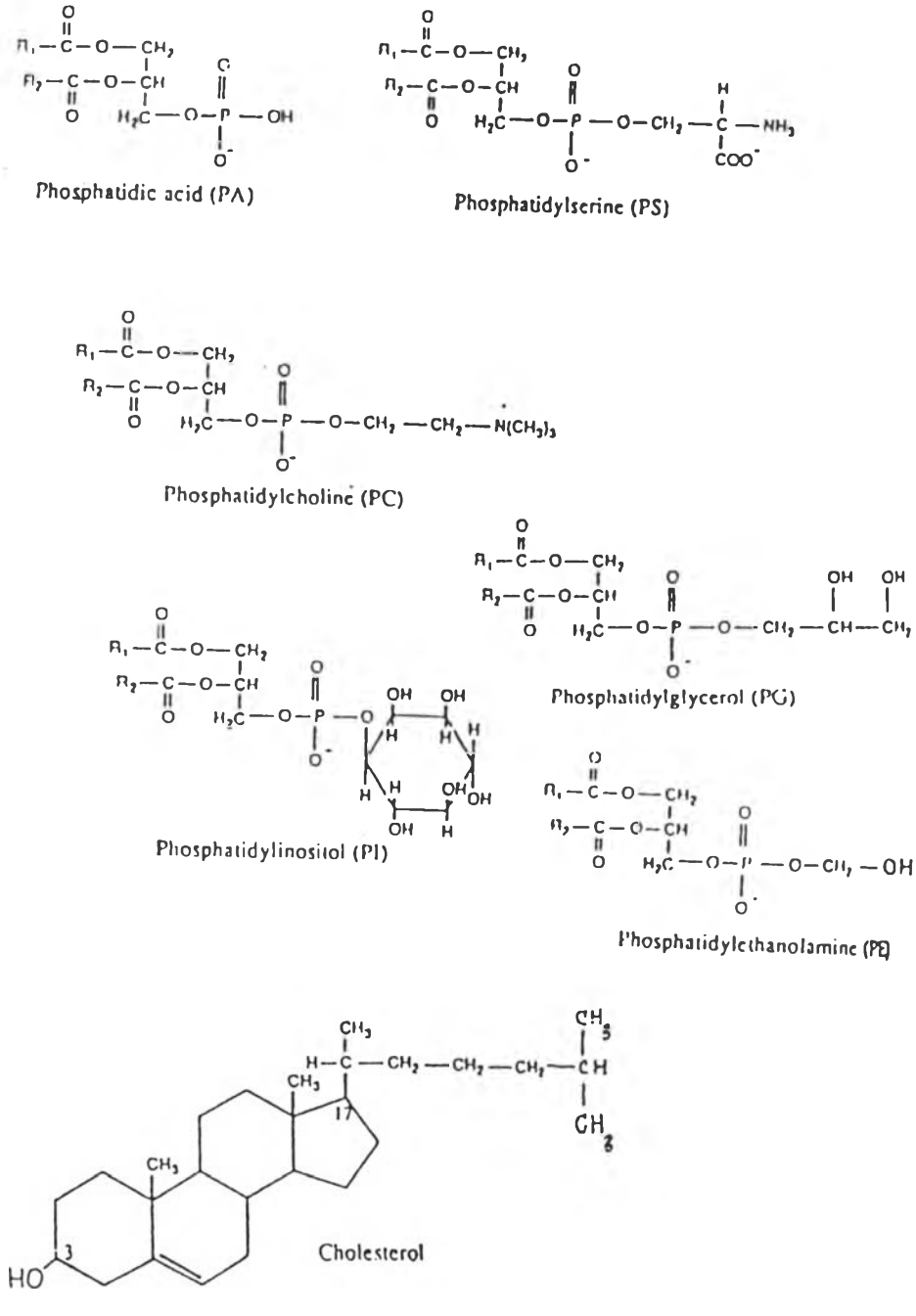
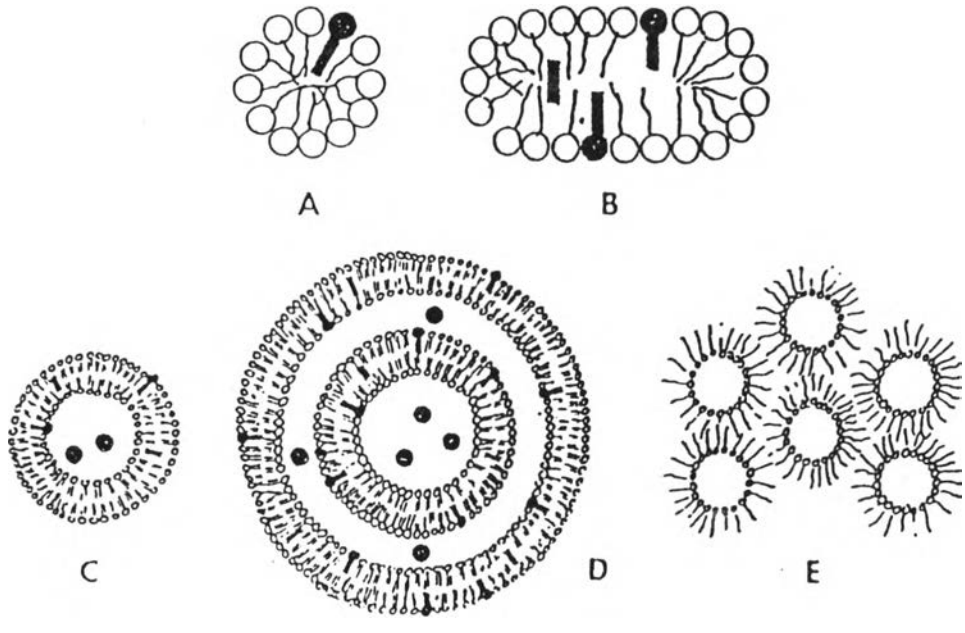


Figure 4 Chemical structure of phospholipids commonly used to prepared liposomes. (Weiner, Martin, and Riaz. 1989)

dimethyl ammonium chloride as shown in Figure 5.

Figure 5 Dihexadecyl dimethyl ammonium chloride.

Bilayer vesicle, however , represent only one type of several possible assemblies formed by amphiphilic lipids. Figure 6 show the type of lipid assemblies that can form spontaneously under the right conditions; spherical and cylindrical micells, unilamellar and multilamellar vesicles, flat bilayers, and inverted structures. The assembly type actually formed is largely determined by the shape of the amphiphilic molecule. Single-chain lipids such as sodium dodecyl sulfate and cetyl trimethyl ammonium bromide can be thought of as cones ,with a bulky head group at the base and a single chain leading to the apex. For such molecules the energetically most favorable assembly is the micell. Two-chain lipids resemble truncated cones or cylinders. These form bilayers, a flat bilayer would be the most stable forms ; due to itself , forming vesicles.



Solute Types: ● Hydrophilic ▬ Lipophilic ⊶ Amphiphilic

Figure 6 Assemblies formed by lipid amphiphiles.

3.2) Phospholipid

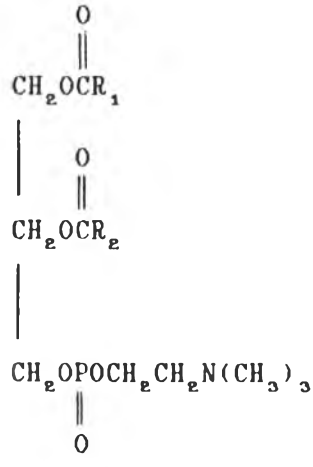
The phospholipids include all lipoidal constituents that contain phosphorus in their molecules. They appear to be essential components of every plant and animal cell and have been categorized as lecithin , cephalines and sphingomyelins.

The chemical composition in all cases is revealed through quantitative measurement of the products resulting from hydrolysis under various conditions. The only phospholipid with pharmaceutical application are the lecithin.

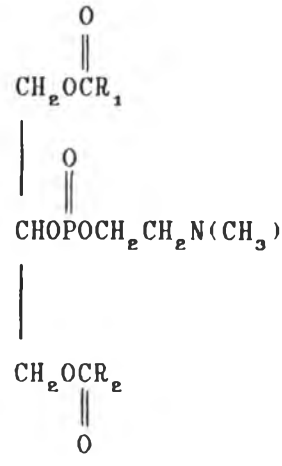
The Lecithins

When completely hydrolysed, each molecule of a lecithin yields two moleculesof fatty acid and one molecule each of glycerol, phosphoric acid and a biasic nitrogenous compound (usually choline).

The fatty acids obtained from lecithin on hydrolysis are usually oleic, palmitic and stearic. The phosphoric acid may be attached to the glycerol in either an α- or the β- position,forming α-glycerophosphoric acid or β-glycerophosphoric acid,respectively,and producing the corresponding series of lecithins which are known as α- and β-lecithins. The representations below are in the zwitterion (internal salt) form. Each series of lecithin may differ in the fatty acid attached to the glycerol. The naturaly occuring lecithins are of the α-variety.



α-Lecithin



β-Lecithin

Commercially, lecithin is obtained by extraction processes from egg yolk, brain tissue or soybeans. Ovolecithin (vitellin) from calves brains, areused as emulsifiers (most antioxidants and stabilizers in foods and pharmaceutical preparation). Lecithin oxidize readily on exposure to air and,simultaneously,darken in color.

3.3) Preparation of Carboxymethylchitin

Flaky chitin (Namyo Kasie Chemicals,Ltd.,Tokyo) was washed with a methanolic HCL solution to remove the coloured materials,and the decolorized chitin was wash three times with ethanol and dried. After 50 g of the dried chitin had been mixed throughly with 200 g of 11 N NaOH solution, the mixture was stored in a refrigerator at -20° C to make it swell. The swollen chitin was added with stirring to isopropanol

containing 100 g of sodium monochloroacetate,and the temperature was kept at 4°C for 2 hours;the reaction was then allowed to proceed at room temperature for 24 hours. At the end of this period, isopropanol was removed by filtration and the reaction product,CM Chitin,was dissolved in 1500 ml of deionized water. The solution was neutralized with HCl solution and dialysed against deionized water for 48 hours. Acetone was added to the dialysed solution to separate CM chitin and the precipitated polymer was dried and pulverized. The chemical structure of CM chitin is shown in Figure 7.

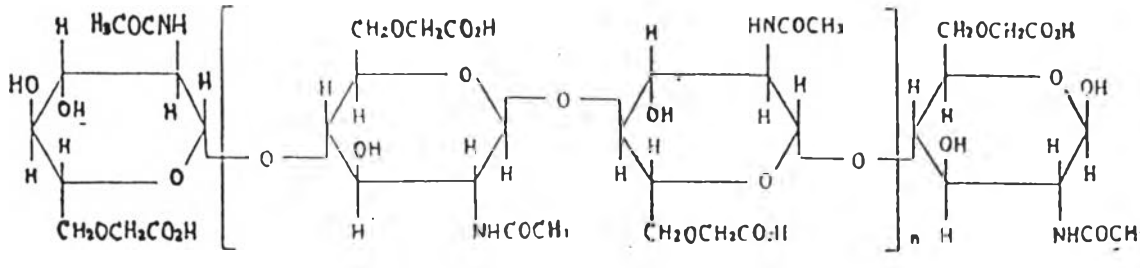


Figure 7 Carboxymethyl chitin.

3.4) Tetanus Toxoid Microcapsules Using Lecithin as Polymeric Membrane and Carboxymethylchitin as Stabilizer

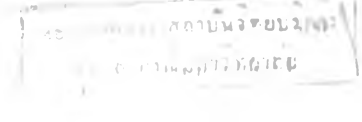
Using microcapsule for human , the biophysical and biochemical properties had been considered in comparison with those of natural mammalian cells because it is feared that the chemicals used in the preparation may be trapped in the human body or may cause immunoligical reactions before they are degraded in the body. Thus , the use of such

non toxic , biocompatible , biodegradable and non antigenic substance as lecithin,one type of phospholipid,is required to prepare microcapsules as polymeric membrane and carboxymethyl chitin as stabilizer.

As the interfacial polycondensation technique, two step of emulsification required for preparation of these microcapsules. Tetanus toxoid was dispersed as fine droplets in a lecithin solution in dichloromethane , a volatile organic solvent to yield a w/o type emulsion. Then , these w/o emulsion was dispersed in as aqueous carboxymethyl chitin solution to give a w/o/w type complex emulsion. Removal of the organic solvent by evaporation from the complex left an aqueous suspension of the tetanus toxoid microcapsules.

4) Factor Affect Size Distribution

The size of microcapsules is never uniform and varies over a fairy wide range. The size distribution of microcapsules has been investigated by microscopy and in some cases by use of a Coulter counter. The factors affecting the size distribution have been studied. In an effort to control the mean diameter of hemoglobin loaded poly (hexamethylene sebacamide) microcapsules, Chang,MacIntosh,and Mason founded that the mean diameter is strongly affected by the concentration of the emulsifier and mechanical agitation used in the emulsification step of the preparation though they did not present a size distribution curve. However,the presence of hemoglobin at high concentration in the solution to be encapsulated prevented formation of the microcapsules with diameters less than 20 micron even at high concentration of



emulsifier and made it difficult to analyze the results because the exact role of hemoglobin in forming the microcapsules is still unknown.

In order to surmount this difficulty,an attempt was made to set up size distribution curves for water-containing microcapsules prepared under various conditions. The results are described below. Increasing mechanical agitation in the absence of an emulsifier makes the size distribution narrower and sharper,whereas the presence of an emulsifier at concentrations over a certain value reduces the effect of mechanical agitation. At a constant mechnical agitation,the size distribution curve becomes steeper and narrower first and then remains almost unchanged as the emulsifier concentration increases. Figure 8 and 9 give some of the size distribution curves for poly(hexamethylene phthalamide) microcapsules in aqueous dispersions. These findings seem to indicate that the size distribution of microcapsules prepared by interfacial polymerization technique is determined by the emulsification conditions since the effects of emulsifier concentration and mechanical agitation on the microcapsule size are quite similar to those on the droplet size in mechanically prepared emulsions.

Interestingly enough , however,it was demonstrated that varying the chemical structure of intermediate reactants used in the polymerization step also greatly exerts an influence on the size distribution. Figure 10 shows the size distribution curves for water-containing poly(phthaloyl piperazine),poly(hexamethylene sebacomide), and poly(diethyl ether piperazine) microcapsules prepared under exactly the same conditions. Therefore,the difference in size distribution must have arisen from the different polymerization characteristics for each

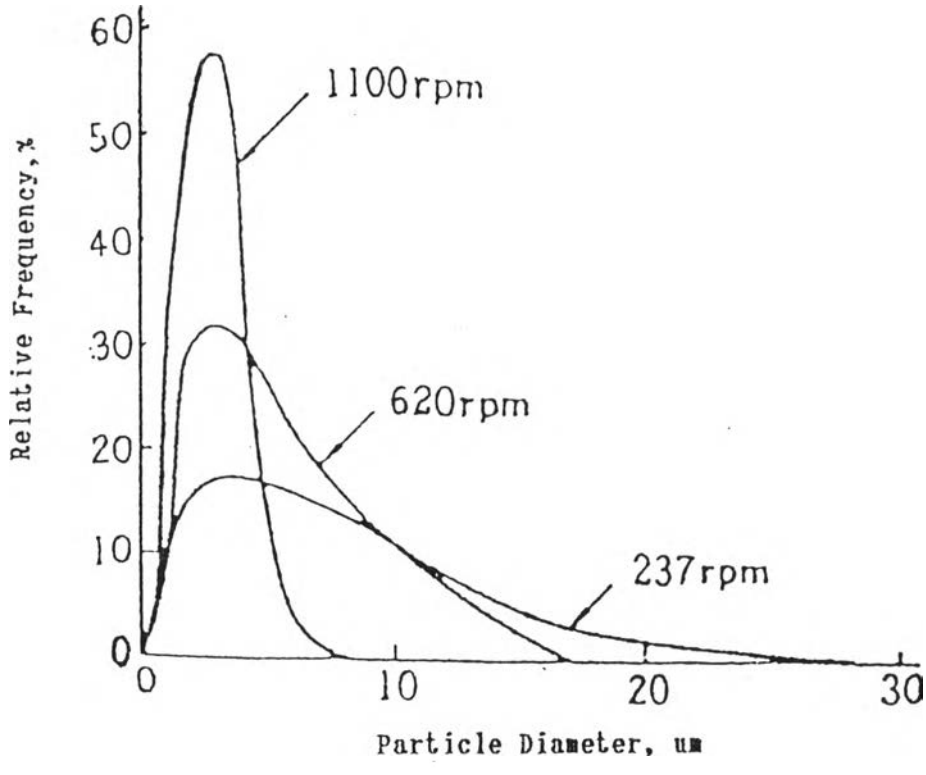


Figure 8 Effect of stirring on the size distribution of microcapsules.

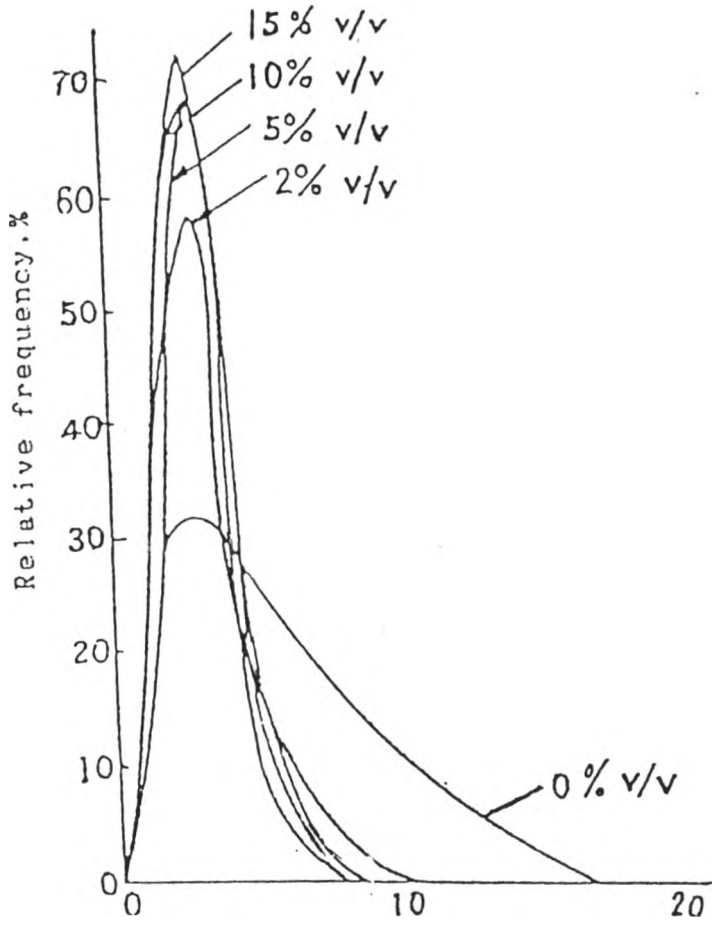


Figure 9 Effect of the concentration of sorbitan trioleate on the size distribution of microcapsules at constant stirring.

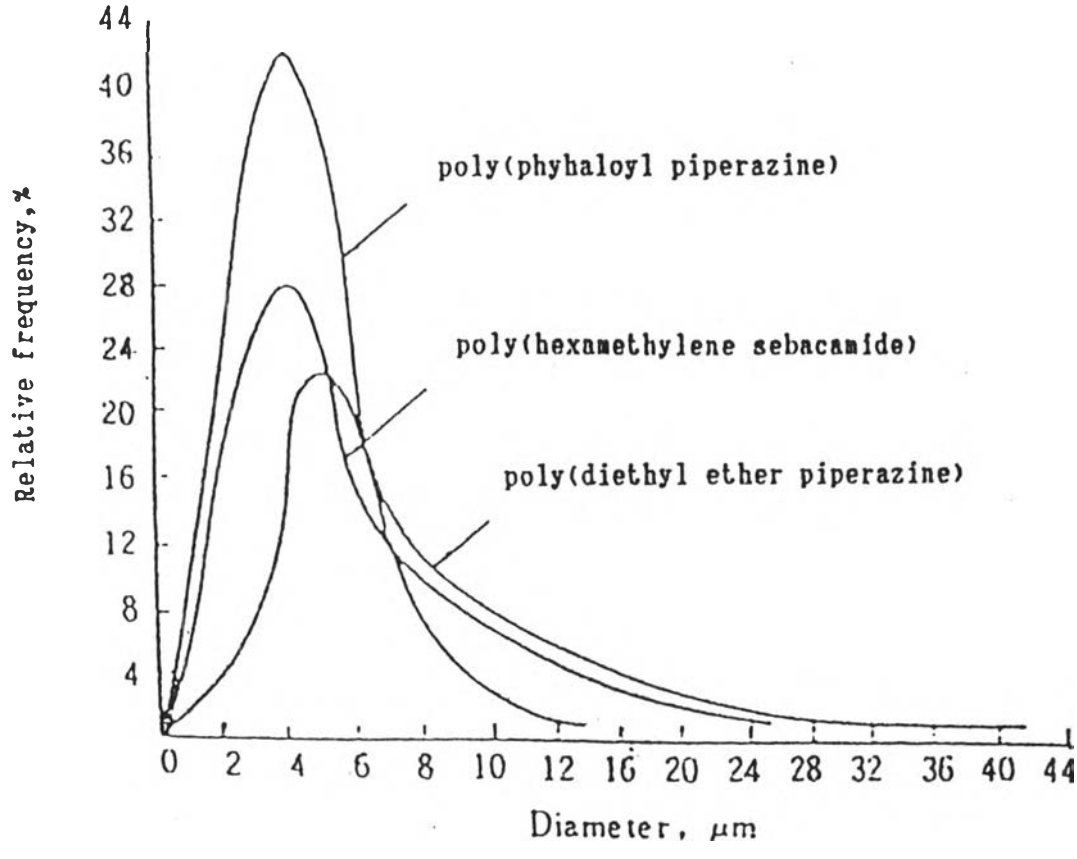


Figure 10 Size distribution curves of poly(phthaloyl piperazine),poly(hexamethylene sebacomide), and poly(diethyl ether piperazine)microcapsules.

combination of reactants. However,no comparative study of polymerization characteristics of the three combination of reactants has so far been carried out, and consequently,no conclusion can be drawn at present on the mechanism by which the size distribution is determined.

In Figure 11 a significant change in the size distribution of poly(diethylether piperazine)microcapsules can be seen caused by an increase in the polymerization temperature. Since the polymerization rate increases with temperature,an increase in the molecular weight of the membrane polymer is expected. In fact,the intrinsic viscosity of the polymer constituting poly(diethly ether piperazine) microcapsules, a measure of the molecular weight of this polymer,was found to double when the polymerization temperature rose from 3° to $30^{\circ}C$. It was argued, therefore,that the microcapsule size is closely related to the intrinsic viscosity of membrane polymer. Similary,a reduced intrinsic viscosity gave rise to a rise in the microcapsule size. This was accomplished by decreasing the reactant concentration or adding a monofunctional reactant (a terminating agent of polymerization)to the solution of the difunctional reactant.

As a consequence,a hypothesis was put forward that the broadening of the size distribution is produced by formation of large microcapsules at an early stage of the polymerization reaction through coalesence of primary emulsion particles with membrane of insufficient strength and coverage to prevent them from uniting with each other to form large secondary particles,provided the polymerization rate is not high. Since the polymerization reaction could be still going on at this and later stages,the membrane on the secondary particle surface might

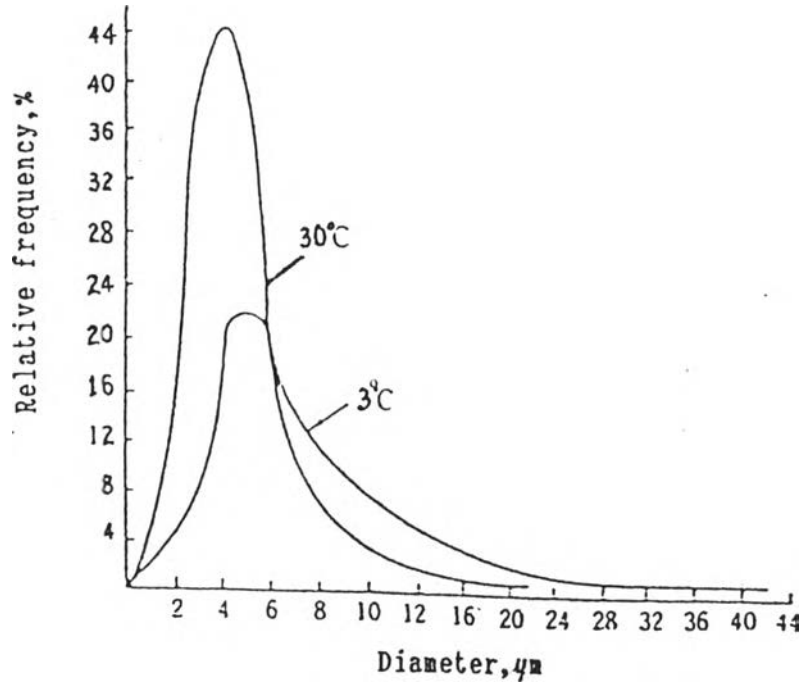


Figure 11 Size distribution curves of poly(diethly ether piperazine) microcapsules prepared at 30°C and 3°C.

have rapidly thickened enough to resist further coalescence from occuring.

The hypothesis seems to account for the process of formation of frequently observed double microcapsules,that is those containing a number of a small microcapsules inside them. Shiba *et al.* gave the following explanation:as the ratio of surface area to volume is larger for small liquid droplets than for the large ones,the transfer of diamine from the aqueous to the organic phaseis greater for the former than for the latter, thereby leading to accumulation of diamine on and around the droplet surface. The accumulation of diamine at the interface should facilitate formation of polymer. Accordingly,newly formed small microcapsules have a relatively thick membrane and can penetrate on collision into large ones with a very thin membrane. The membrane of large microcapsules is capable of growing enough to patch up the poles formed by the penetration of small microcapsules as the polymerization reaction still continues. Although the hypothesis is attractive,there are many problems to be solved,such as the role of the emulsifier,before it becomes acceptable.

5) Membrane Thickness

A relatively large number of papers deal with the membrane thickness of microcapsules prepared by phase separation and interfacial polymerization methods.The membrane thickness of microcapsules depends on the method by which they are prepared.Microcapsules obtained by phase seperation have a membrane thickness of the order of micrometers,whereas

those prepared by interfacial polymerization possess a thickness in the nanometer range since the membrane thickening in the latter case is restricted by the limited solubility of reactants in the phase to be encapsulated.

Miyano and Kondo, who measured the wall thickness with an electron microscope,reported that gelatin-gum arabic microcapsules containing dibutyl phthalate of the average of 26.6 microns have a wall thickness of 0.4 microns. Those microcapsules were prepared by aqueous phase separation using a system consisting of 6.0 gm. each of gelatin and gum arabic,10.0 gm. of dibutyl phthalate,and 1000 gm. of aqueous acetic acid solution at pH 4.3. The capsules were imbedded in an epoxy resin mass after hardening with formaldehyde and spray drying. The epoxy resin mass was then sliced with a microtome to make specimens for electron microscopic observations. It should be noted,however,that the authors determined the wall thickness of the microcapsules in a dry state. In water the wall thickness may be larger due to swelling of gelatin-gum arabic complexs,which depend on the degree of hardening with formaldehyde.

Recently, Luu *et al.* have estimated the wall thickness of gelatin gum arabic microcapsules containing eprazinone, a liquid organic base. First they calculated the wall thickness from the mean microcapsule radius,the ratio of the encapsulated liquid mass to the total mass of the microcapsules,and the densities of the encapsulated liquid and of the coating. Second,direct measurements of wall thickness were made on the microcapsules imbedded in a gelatin mass under an optical microscope fitted with a micrometer and an immersion objective.

The gelatin mass was sliced prior to the measurements with a congelation microtome after hardening. The results obtained by these indirect and direct methods with sieve separated samples are given in Table 2 and indicate that the wall thickness is fairly constant whatever the microcapsuls size.

Table 2 Wall Thickness of Oil-Containing Gelatin-Gum arabic Microcapsules

	Mean thickness,micron	
Mean radius,micron	Indirect method	Direct method
140	6.4	6.7
200	5.0	6.8
300	6.8	7.0
375	5.9	6.6

From S.N. Luu *et al.*, by permission of the American Pharmaceutical Association,Washington,D.C.

For several kinds of water-loaded polyamide microcapsules prepared in a way similar to that used by the procedure; the average menbrane thickness , h , could be calculated (Takamura ,et al.,1973) by following equation ;

$$h = W_m P / P_d A$$

There W_m is the weight of microcapsules membranes in unit volume of aqueous dispersion , assuming the complete reaction of diamine with diacid chloride. A , The total surface area of microcapsules in unit volume of aqoeous dispersion. P , percentage of diamine reacted. P_d, the density of the microcapsules membrane was obtained by multiplying the specific gravity of the membrane by the density of water.

The specific gravity was determined pycnometrically in the conventional way using dried powder samples prepared by collecting , washing , and drying (under reduced pressure) of the membrane after breaking down microcapsules by centrifugation.

Tetanus

Tetanus is a global disease that accounts for approximately 50% of neonatal deaths and 25% of infant deaths .(Stanley,J.,and Cryz,S. ,1990) Current estimates of worldwide mortality are 800,000 deaths per year from neonatal tetanus and 120,000 to 300,000 deaths per years from nonneonatal tetanus. This high incidence is additionally tragic because the disease is largely preventable by appropriate immunization. The WHO Expanded Program on Immunization(EPI) has been working toward greater vaccine coverage particularly in developing countries,in the hope that tetanus will someday become a rare disease.

Tetanus is probrably recognized as district ones early in

human history because of the constancy of symptom presentation. The first medicals description of tetanus appears in the writing of Hippocrates , but the ethiology of tetanus was unknown until 1884. Carle and Rottone demonstrated that the contents of a pustule from a fatal human case led to typical symptoms in rabbits when injected into their sciatic nerves; the disease could subsequently be passed to other rabbits from infected nervous tissue. Inoculation of soil samples into animals also resulted in tetanus. Gram-positive bacilli were often noted in the exudate at the inoculation site but generally not in nervous tissue, leading Nicolaier to hypothesize that a poison produced at the site of inoculation led to the nervous system symptoms. In 1886, spored-forming bacilli were observed in the exudate obtained from a human case. In 1889,the spores of the causative organism, *Clostridium tetani* (Fairweather et al.,1990),in contrast to the vegetative organisms,were shown to survive heating and germinate under anaerobic conditions: injection of pure cultures caused reproducible disease in animals. After indentification and purification of the toxin in 1890,it was shown that repeated inoculation of animals with minute quantities of toxin led to the production of antibodies in survivors that neutralized the effects of toxin. Preparations of these antibodies derived from animal sera, particularly of horses,became the first means of preventing and treating the cause of tetanus. Further research culminated in the preparation of "antitoxin"-chemically inactivated toxin,now termed"toxoid" in 1924. These preparetions induced active immunity against the disease prior to exposure.

Tetanus is usually associated with the introduction of

environmentally ubiquitous spores of *C.tetani* into wounds or infection of the umbilicus of infants(neonatal tetanus). The spores germinate in an anaerobic environment resulting in the production of tetanus toxin. After an incubation period of typically several days, the toxin produces a disinhibition of the central nervous system resulting in violent spasms of the skeletal muscles of the body. Tetanus may involve local muscle groups near the site of infection or be generalized. Trismus or lockjaw is a common finding. Generalized tetanus is particularly serious and may require therapy with strong muscle relaxants and assisted ventilation. Antibiotics to prevent proliferation of the bacteria and tetanus immune globulin are also components of therapy.

Tetanus toxin is synthesized by the bacterium as a single polypeptide chain of approximately 150,000 Da. (Helting ,and Fugelhad, 1979) molecule can be cleaved by clostridial proteases to form two chains linked by a disulfides bond. The heavy chain binds to gangliosides present on neural cells and is thought to be involved in receptor recognition and internalization of the toxin. As has been found with other bacterial toxins,the light chain appears to be the active chain. The molecular mechanism of action of the toxin is not yet understood.

1) <u>Manufacturing</u>

Production of a typical tetanus toxoid begins with the growth of a high-yielding strain of *Clostridium tetani* in a liquid, nonantigenic medium. After a suitable period, the culture supernatant fluid

(extracellular toxin) and/or cells -(intercellular toxin) are harvested and are usually purified further,often by alcohol or salt fractionation. Most manufacturers detoxify (toxoid) the crude toxin before purification, in part for the safety of production personnel.

The toxoiding process involves treatment of the toxin with a dilute solution of formaldehyde for a period of days or weeks,during which time the product may be tested for toxicity in animals. The product is labeled as tetanus toxoid only when it no longer exhibits any signs of tetanus toxicity. When the bulk toxoid has been prepared,it may be mixed with other antigens (as in the case of DTP(diphtheria,tetanus, pertussis),for example)and/or combined with an insoluble,inorganic salt (e.g.,aluminium phosphate) to enhance its immunogenicity. Numerous variations in the process outlined here are used commercially and result in acceptable vaccines. The WHO has prepared a detailed manual for the production and control of tetanus toxoid (WHO,1977). Common requirements include assessment of sterility,potency in animal tests,purity,freedom from toxicity,freedom from reversion to toxicity,and product stability.

2) Immune Testing of Tetanus Toxoid

Immune testing , the most important to evaluate the toxoid to be valuable enough for immunization usage. The method in this way is occupied by potency testing and determinating of antibody.

2.1) Potency Testing (WHO,1990)

Each final bulk of tetanus toxoid shall be tested for immunizing potency by comparison with a national reference material

calibrated against the appropriate international standard. The test shall involve the inoculation of groups of guinea-pigs (weight 250-350 g) or mice (weight 14-20 g provided that, in a single test, the individual weights of the mice shall not vary by more than 3 g). Three dilution of both the final bulk and reference material shall be used. After immunization,the animals shall be challenged with a lethal or paralytic challenge dose of toxin given by the subcutaneous route. Standard statistical method shall be used to calculate the potency of the final bulk. The methos adopted and its interpretation shall be approved by the national control authority.

2.2) Antibody Determination

There are several serological tests had been developed to determine the antibody level. Among these are passive hemagglutination (PHA) , enzyme immunoassays (EIA) , radioimmunoassays (RIA), immunofluoroassay (IFA) , latex agglutinatin and a variety of methodds using agar gel precipitation (Hardegru,et al ,1970; Wang,et al.,1982 ; and Sedgwick,et al.,1983) All tests can be specific, but sensitivity varies. IFA ,agar gel diffusion ,and latex agglutination are the least sensitive. In genaral any one of the techniques is useful provided correlation with toxin neutralization has been performed. The evaluation of these most often involved passive hamagglutination techniques, that has a good correlation with toxin neutralization, particularly at high titers. In regard to specificity , a PHA measures both IgG and IgM. Studies indicate that IgG is a better neutralizer of tetanospasmin than IgM. Hence ,titers detected early in an immunization by PHA,

particularly after the first dose, may not represent neutralizing antitoxin. EIA , RIA and IFA can measure specific immunoglubulin but at low levels of antitoxin, they may indicate higher levels than those by neutralization assays in mice. This finding has been in part attributed to more ready detection by EIA, RIA, and IFA of low affinity IgG, which may not be neutralizing .

a) Passive Hemagglutination Test

The hemagglutination method is a convenient procedure to detect and ascertain the concentration of IgG and IgM agglutinating antibodies to proteins, blood group substances, and haptans. It is particularly sensitive in detecting IgM since small concentrations of these antibodies may yield significant titers, disproportionately high in comparison with hemagglutionation by IgM. The hemagglutination reaction may detect antigen- antibody complexs.

In passive hemagglutination, red cells are agglutinated by antibodies directed against antigens that have been coupled chemically to the red cell surface. Thus the red cell now serves simply as a convinient ,visible indicator of an antigen-antibody interaction.

The first step in the assay is to "sensitize" the red cells to couple the desired antigen to them. Because it is generally recognized that protein antigens cannot be adsorbed directly on to normal erythrocytes. This step greatly increase the sensitivity of the agglutination reaction.

General methods available for coating antigens on red blood cells are using bifunctional reagents, such as tannic acid or

bisdiazotized benzidine (BDB) to achieve covalent bonding between the antigens and the red cells.

This assay is performed in tubes or microtiter plates by adding a fixing number of the sensitized red cells to doubling dilutions of the antiserum. The recipocal of the highest dilution to give a visible agglutination reaction defines the titer of the antiserum as shown in Figure 12(Kimball,1986). The reaction is strongly positive in the first 5 wells;the bottom of each of these wells is covered with a sheet of agglutinated red cells. Wells 6-9 show increasingly weak reactions. The solid "botton" of settles red cells in well 10 is a negative reaction.

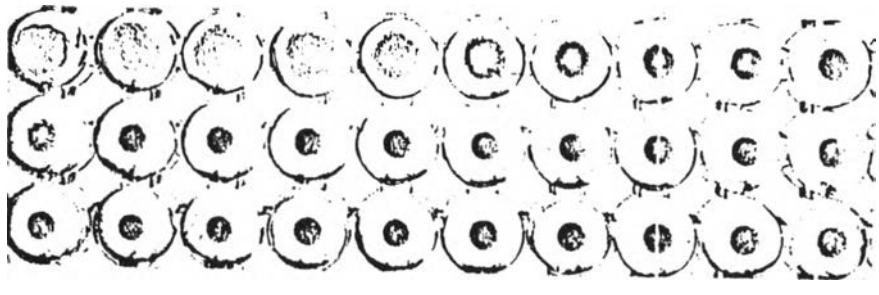


Figure 12 Passive hemagglutination test

It is frequently observed in hemagglutination titration that the first few tube with the most concentrated antiserum,fail to agglutinate the test cells while higher dilutions agglutinate successfully. This phenomenon is call the prozone effect. A mechanism may account that at high concentrations of antibodies, each of the antigenic sites on the red cells may be occupied by a unshared antibody molecules, preventing

cross-linking of the cells (Wier,1967) However the use of standard serial dilutions eliminates this difficulty (Stites,et al.,1982), and the units/ml of the test sampls are calculated.

b) Coupling of Diazonium Compounds to Red Blood Cell Reaction

The coupling reaction been used widely for attaching the antigen to red blood cells in the BDB passive hemagglutination reaction. It is employed for detection of antibodies in immune sera produced in experimental animals or in sera of allergic individuals .

In reaction, the diazonium functional group reacts readily with the phenolic (tyrosyl),histidyl , amino , and carboxyl group of proteins. The simple organic chemical benzidine,on treatment with nitrous acid ($NaNO_2$ and HCl), is converted to the bifunctional bis-diazotizedbenzidine (BDB), which can react with the appropriate group in the different protein molecules.

From this reaction the aromatic amines react with nitrous acid to form diazonium salt. At slightly alkaline pH a diazonium salt will couple to side chains of a protein molecule. If the number of moles of diazonium salt used is comparable to the number of moles of tyrosine , histidine, and lysine residues in the protein , the reaction yields mainly monosubstituted tyrosine and histidine and disubstituted lysine groups;coupling occurs through linkages in each case. Some reaction with arginine and tryptophan may also take place.

The reaction with tyrosyl side chains proceeds as shown in Figure 13.

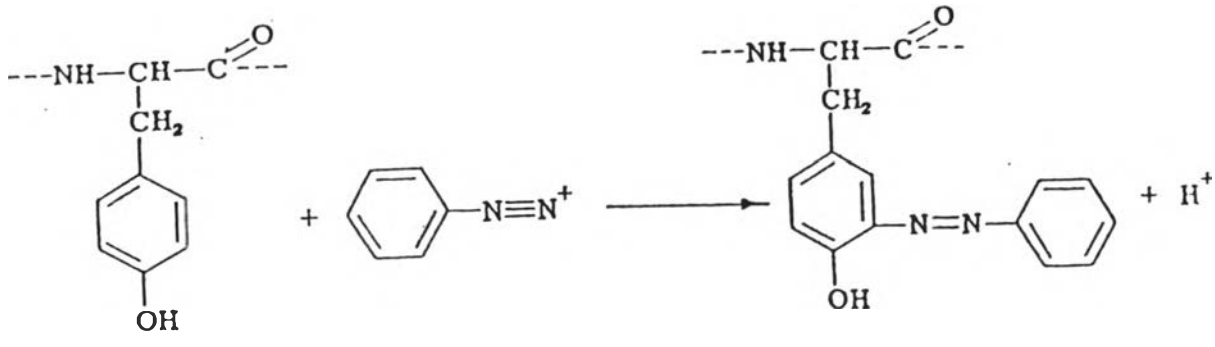


Figure 13 Couple reaction with tyrosyl side chain.

When relatively large amounts of the diazonium salts are employed, disubstituted of tyrosine and histidine and more extensive reaction with arginine and tryptophan residues may occur.

c) Precautions in Interpretation of Results Obtained with the Hemagglutination Procedure.

General precaution to be taken in all serological experiments have been adequately describes elsewhere. It is necessary to pay particularly attention in all hemagglutination assays to false positive and occasional false negative reaction. Inclusion of appropriate controls and a standard in each test will guard against this being caused by faulty reagents. Some putative inhibitors, however, do not act by entering into a complementary reaction with the antibody combinding site, but rather render the erythrocytes inagglutinable by changing

there surface properties. Frequently,specificity of lack of it can be ascertained by sufficient dilution of the substance tested. However some acidic polymers, lipids, or aldehydes will unspecifically prevent agglutionation even at high dilutions. Such an effect given by substances as different as the Vi antigen of gram negative bacteria , formaldehyde , and some phospholipids can usually be recognized by the similar activity of these substances in a large number of different hemagglutination system.Other substances may unspecifically combine with certain classes of antibodies and thus prevent their attachment to specific structure on the erythrocyte surface. False negative reaction occur less frequently but may caused by heavy metals and other cations if they are bi- or multivalent. These false negative reactions may be detected by incubation of red cells with the putative inhibitor agglutination will then occur already in the absence of agglutinins.

3) Clinical Use

The WHO EPI stresses the immunization of all women of child bearing age in tetanus endemic areas, including pregnant women regardless of month of pregnancy. In many developed countries where the risk of neonatal tetanus is low, immunizations are often deferred during pregnancy. It has been reported that immunization during the fifth and eighth month of pregnancy results in the formation of antibodies in the infants and also enhances the response of these infants to subsequent immunization;this phenomenon has been termed transplacental immunization.

Tetanus toxoids are routinely injected intramuscularly or subcutaneously. The intramuscular route is preferred for adsorbed

products. Injections are usually in the deltoid or upper thigh. The thigh is often preferred in infants because of the larger muscle mass there,but for 18-month-old children,the deltoid has also been recommended because of fewer and milder adverse reactions(Gold,R.,et al.,1989) . The buttock should not be routinely used because of the risk of injury to the sciatic nerve. Recently,the subject of ideal needle length has been restudied. A 1-inches(25mm.)needle has been recommended based on study of the fat layer over the anterolateral thigh of infants and on the finding of reduced swelling and erythema with 25-mm compared with 16-mm needles. A separate needle and syringe should be used for each injection. Jet gun injections generally result in more local reactions and have been implicated in disease transmission. The usual dose of tetanus toxoid products is 0.5 ml. For immunization of infants and young children, tetanus toxoid is administered at 2, 4, 6 and 15 to 18 months and again at 4 to 6 years of age.

Tetanus toxoid is a safe and effective vaccine. There are, however,some adverse reactions associated with its use. Local reaction such as redness or pain are fairly common but usually resolve in a few days. The serious reactions associated with DTP immunization are often attributed to the pertussis component,but peripheral neuropathies have been reported following tetanus toxoid only. Fortunately,these and other serious reactions are rare,and the proper use of tetanus toxoid can virtually eliminate the threat of tetanus.

Purpose of the Study

1) To be the way to develop a new ,most effective, long acing tetanus toxoid microcapsules to reduce the frequency of immunization by using only a single dose instead of triple dose at 2, 4 and 6 month. Lecithin and carboxymethylchitin walled tetanus toxoid microcapsules was developed by interfacial polymerization method.

2) To produce various vesicle sizs preparations of tetanus toxoid microcapsules.

3) Testing the quality of tetanus toxoid microcapsules.

a) Particle size analysis of the microcapsules.

b) Scanning electron microscopy.

c) Animal testing.

1) To determine and compare the potency of various vesicle size of tetanus toxoid microcapsules preparations in mice during 180 days after immunization.

2) To determine and compare the antibody level of tetanus toxoid microcapsules preparations in mice during 180 days after immunization by hemagglutination method.

Application for the study

1) To reduce the problem of incomplete immunizing program of tetanus toxoid.

2) To reduce the incidence and death that cause by tetanus.

3) To implement the healthy of the world.

4) To be a conception of developing other pharmaceutical products.