ผลของตัวแปรสูตรตำรับต่อการดูดซับของ ดีทีพี-เจ อี แอนติเจน บนแอดจูแวนท์

นางสาว วรนุช ศรีพงษ์สาร

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาเภสัชศาสตรมหาบัณฑิต
สาขาวิชาเภสัชอุตสาหกรรม ภาควิชาเภสัชอุตสาหกรรม
คณะเภสัชศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2548

ISBN 974-17-4437-4

EFFECT OF FORMULATION VARIABLES ON ADSORPTION OF DTP-JE ANTIGENS ON ADJUVANTS

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A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science in Pharmacy Program in Industrial Pharmacy
Department of Manufacturing Pharmacy
Faculty of Pharmaceutical Sciences
Chulalongkorn University
Academic Year 2005
ISBN 974-17-4437-4

Thesis Title	EFFECT OF FORMULATION VARIABLES ON ADSORPTION OF DTP-JE ANTIGENS ON ADJUVANTS
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วรนุช ศรีพงษ์สาร : ผลของตัวแปรสูตรตำรับต่อการดูดซับของ ดีทีพี-เจอี แอนติเจนบนแอดจูแวนท์ (EFFECT OF FORMULATION VARIABLES ON ADSORPTION OF DTP-JE ANTIGENS ON ADJUVANTS) อ. ที่ปรึกษา : ศ. ดร. กาญจน์พิมล ฤทธิเดช, อ. ที่ปรึกษาร่วม : รศ. ดร. วิมลมาศ ลิปิพันธ์, 181 หน้า. ISBN 974-17-4437-4.

อลูมิเนียมไฮดรอกไซด์เจลและอลูมิเนียมฟอสเฟตเจลต่างถูกดูดซับด้วยดิบทีเรียทอกซอยด์ เตตานัสทอกซอยด์และเจอี แอนติเจนที่สภาวะต่างกัน คือ อุณหภูมิ 37 และ 9 องศาเซลเซียส, ใน 0.01 โมลาฟอสเฟตบัฟเฟอร์ที่พีเอช 6.0 ถึง 7.4 และปั่นเหวี่ยงที่ความเร็วรอบ 3000 รอบต่อนาที พบว่าปริมาณการดูดซับของแอนติเจนแต่ละชนิดบนอลูมิเนียมไฮดรอกไซด์ของดิบทีเรียและเจอีมีการดูดซับที่อุณหภูมิ 9 องศาเซลเซียส มากกว่าที่ 37 องศาเซลเซียส ในขณะที่เตตานัสจะดูดซับที่ 37 องศาเซลเซียส ได้มากกว่า ยิ่งไปกว่านั้นค่าการดูดซับของแอนติเจนทุกชนิดจะค่อยๆลดลงเมื่อพีเอชเพิ่มขึ้น การดูดซับของแอนติเจนบนอลูมิเนียมฟอสเฟสจะมีค่าน้อยกว่าบนอลูมิเนียมไฮดรอกไซด์ ที่อุณหภูมิ 9 องศาเซลเซียสเจอีจะดูดซับได้มากในขณะที่ดิบทีเรียและเตตานัสจะดูดซับที่ 37 องศาเซลเซียสได้ดีกว่า ปริมาณที่คำนวณได้ของอลูมิเนียมที่จะต้องใช้ในสูตรรวมของอลูมิเนียมไฮดรอกไซด์มีค่าน้อยกว่าของอลูมิเนียมฟอสเฟต ดังนั้นสภาวะที่เลือกคือ การดูดซับบนอลูมิเนียมไฮดรอกไซด์ที่อุณหภูมิ 9 องศาเซลเซียส พีเอช 6.5 อลูมิเนียมไฮดรอกไซด์และเจอีมีประจุที่ผิวเป็นบวก ส่วนอลูมิเนียมฟอสเฟต ดิบทีเรียและเตตานัสมีประจุเป็นลบ เปอร์ทุสซิสมีประจุลบเล็กน้อยหรือค่อนข้างเป็นกลางในน้ำ แอนติเจนต่างๆอาจจะถูกดูดซับบนอลูมิเนียมไฮดรอกไซด์และอลูมิเนียมฟอสเฟตด้วยแรงประจุทางไฟฟ้าและกลไกการแลกเปลี่ยนลิแกนอลูมิเนียมไฮดรอกไซด์และอลูมิเนียมฟอสเฟตที่ถูกดูดซับแล้วจะมีขนาดอนุภาคที่ใหญ่ขึ้น ลักษณะรูปร่างของอลูมิเนียมไฮดรอกไซด์เป็นอนุภาคทรงกลมที่ซับซ้อนส่วนอลูมิเนียมฟอสเฟตจะเกาะกันเป็นกลุ่มก้อนรูปร่างไม่แน่นอน ลักษณะรูปร่างของอลูมิเนียมไฮดรอกไซด์ที่ถูกดูดซับสามารถดูได้ด้วยวิธีเอสอีเอ็ม ในขณะที่ลักษณะรูปร่างของอลูมิเนียมฟอสเฟตสามารถดูได้ด้วยวิธีเอเอฟเอ็ม พีคหลักของการวิเคราะห์ด้วยวิธีไอออาร์และเอกซเรย์ของอลูมิเนียมไฮ ดรอกไซด์และอลูมิเนียมฟอสเฟตที่ถูกดูดซับแล้วยังคงปรากฏอยู่แสดงว่าการดูดซับไม่ทำให้ลักษณะโครงสร้างหลักของอลูมิเนียมทั้งสองเปลี่ยนแปลงไป ปริมาณแอนติเจนของสูตรตำรับรวมหลังจากเก็บที่สภาวะอุณหภูมิ 2-8 องศาเซลเซียส เป็นเวลา 3 เดือนที่ถูกวิเคราะห์ด้วยวิธีอีไลซ่าแสดงให้เห็นว่า ปริมาณดิบทีเรีย เตตานัสและเจอีแอนติเจนจากสูตรที่เตรียมโดยวิธีแยกดูดซับสารแต่ละชนิดมีมากกว่าสูตรที่เตรียมโดยวิธีการแย่งการดูดซับ เวลาที่ใช้ในการผสมจะมีผลต่อปริมาณดิบทีเรีย เตตานัสและเจอีแอนติเจนในขณะที่ทั้งวิธีการเตรียมและเวลาในการผสมจะไม่มีผลต่อปริมาณเปอร์ทุสซิส

ภาควิชา เภสัชอุตสาหกรรม ลายมือชื่อนิสิต วรนุช ศรีพงษ์สาร

สาขาวิชา เภสัชอุตสาหกรรม ลายมือชื่ออาจารย์ที่ปรึกษา

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##4576630533 MAJOR : MANUFACTURING PHARMACY
KEYWORD : DIPHTHERIA / TETANUS / PERTUSSIS / JE / ADJUVANTS / ADSORPTION

WORANUCH SRIPONGSARN: EFFECT OF FORMULATION VARIABLES ON ADSORPTION OF DTP-JE ANTIGENS ON ADJUVANTS. THESIS ADVISOR: PROF. GARNPIMOL C. RITTHIDEJ, Ph.D., THESIS CO-ADVISOR: ASSOC. PROF. VIMOLMAS LIPIPUN, Ph. D., 181 pp. ISBN 974-17-4437-4.

Aluminium hydroxide gel (AH) and aluminium phosphate gel (AP) were individually adsorbed with diphtheria toxoid (DT), tetanus toxoid (TT) or JE antigen at different conditions: temperature 37 °C and 9 °C; pH 6.0 to 7.4 in 0.01 M phosphate buffer and centrifuged to precipitate the adsorbed adjuvant at 3,000 rpm. The extent of adsorption of adsorbed single antigen AH suggested that DT and JE favored to adsorb at 9 °C than 37 °C, whereas TT preferred at 37 °C. In addition, a gradual decrease in an adsorption of all antigens was observed as the pH was raised. For AP, poor adsorption of all antigens was noted. At 9 °C, JE displayed a higher degree of adsorption, while DT and TT favored at physiological temperature. The calculated quantity of AH used in combined preparations were less than that of AP. Therefore, AH was chosen to adsorb at 9 °C, pH 6.5. The surface charge in deionized water of AH and JE were positive while AP, DT and TT were negative and PT was slightly negative or neutral charge. The antigens possibly adsorbed on AH and AP by electrostatic force and ligand exchange mechanism. The particle size of adsorbed AH and AP were larger than blank adjuvants. Morphology of AH and AP by SEM were spherical complex particle and aggregate irregular shape, respectively. Adsorbed AH could be observed by SEM, while adsorbed AP showed three-dimensional surface morphology from AFM. The principle peak of IR spectra and X-ray diffraction bands of adsorbed AH and AP indicated that the adsorption had no effect on their chemical structures. The antigen contents combined preparations after storage at 2-8 °C for 3 months and analyzed by ELISA showed more DT, TT and JE content from formulation of separate adsorption than that of competitive method. Mixing time affected the antigen content of DT, TT and JE whereas adsorption process and mixing time did not affect PT content.

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Field of study Industrial Pharmacy Advisor's signature

Academic year 2005 Co-advisor's signature Vimolmas Lipipun

ACKNOWLEDGEMENTS

First, and foremost, I would like to express my sincere gratitude to my thesis advisor, Professor Garnpimol C. Ritthidej, Ph.D. for not only her invaluable advice, guidance, encouragement, hearten and understanding throughout my investigation but also her kindness and cheerfulness that make me feel deeply appreciated.

I am also indebted to Associate Professor Vimolmas Lipipun, Ph. D., my co-advisor, for her kind assistance, helpful consultation and everlasting support.

I wish to express appreciation to Associate Professor Poj Kulvanich, Ph.D., Narueporn Sutanthavibul, Ph. D. and Sit Thirapakpoomanunt, M. Sc. to as members of thesis committee for their valuable suggestion and comments.

Special thanks are deeply appreciated to Bacteria division, Biological product department, GPO for the supplied diphtheria toxoid, tetanus toxoid, *Bordetella pertussis* and aluminium phosphate adjuvant; JE division, Biological product department, GPO for the supplied JE antigen and ELISA reagent; Bureau of Veterinary Biologic, Department of Livestock Development for the supplied aluminium hydroxide adjuvant; Warisada Sila-on, Ph. D., Faculty of Pharmaceutical Sciences, Ubon Rajathanee University for the permission to use the equipments and her assistance also Associated Professor Teerakiet kertjarern, Ph.D., Center for Protein Structure and Function, Faculty of Science, Mahidol University for the permission to use the equipments and his help, kindness and encouragement.

The other special thanks were expressed to all staffs of Faculty of Pharmaceutical Sciences, Chulalongkorn University; especially the staffs of the Department of Manufacturing Pharmacy and other people whose names have not mentioned for their assistance and encouragement firmly deserve acknowledgements.

Ultimately, I would like to express my infinite thanks and deepest gratitude to my family especially my parents for their endless love, care, understanding, encouragement, and support throughout these past years.

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ABBREVIATIONS

%	percentage
° C	degree Celsius (centrigrade)
µl	microlitre(s)
Ab	antibody
Ag	antigen
AH	aluminium hydroxide adjuvant
AP	aluminium phosphate adjuvant
BCA	bicinchoninic acid
BP	British Pharmacopoeia
BSA	bovine serum albumin
DT	Diphtheria toxoid
DT-AH	diphtheria toxoid adsorbed on aluminium hydroxide adjuvant
DTaP	diphtheria, tetanus and acellular pertussis
DT-AP	diphtheria toxoid adsorbed on aluminium phosphate adjuvant
DTwP	diphtheria, tetanus and whole cell pertussis
e.g.	for example, exempli gratia
ELISA	enzyme linked immunosorbent assay
et.al.	et alli, and others
FT-IR	fourier transform infrared
g	gram(s)
HRP	horseradish peroxidase conjugate
ICP	inductively coupled plasma spectrometry
IgG	Immunoglobulin G
JE	Japanese Encephalitis antigen
JE-AH	JE antigen adsorbed on aluminium hydroxide adjuvant
JE-AP	JE antigen adsorbed on aluminium phosphate adjuvant
L	litre(s)
Lf	limit of flocculation
mg	milligram(s)

ml	millilitre(s)
MW	molecular weight
N	normality
O.U.	opacity units
o/w	oil in water
OPD	O-Phenylenediamine
pH	the negative logarithm of the dissolution constant
pI	isoelectric point
PI	polydisperse index
PT	*Bordetella pertussis* antigen
PXRD	Powder X-ray diffractrometry
rpm	round per minute
SEM	scanning electron microscope
TEM	transmission electron microscope
TT	Tetanus toxoid
TT-AH	tetanus toxoid adsorbed on aluminium hydroxide adjuvant
TT-AP	tetanus toxoid adsorbed on aluminium phosphate adjuvant
USP	The United State Pharmacopoeia
UV	ultraviolet
v/v	volume by volume
w/o	water in oil
w/v	weight by volume

CHAPTER I
INTRODUCTION

Vaccination is recognised as being the most cost effective medical intervention when used in properly implemented rational population-wide programmes. The ability of vaccines to control the considerable disease burden inflicted by infectious diseases is well documented. The most acclaimed victory of medical science, the eradication of smallpox, was achieved through the use of the first vaccine developed over 200 years ago. However, it is also clear that, currently, the potential of disease control, elimination and eradication offered by vaccines is not being fully exploited in the world. The reasons for this failure are obviously very complex. They include political and socio-economic traditions that tend to favour, in healthcare as in other areas, short-term therapeutic approaches over the more fundamental but less glamorous long-term approach of prevention. Other barriers to optimal implementation of vaccination programmes are the complicated and expensive logistics, including the maintenance of a cold chain, required to administer to all children many thermolabile vaccines according to multi-injection schedules. The number of injections required to fully immunize a child against all the diseases for which vaccines exist has already reached a level which is becoming unacceptable to parents and healthcare personal. Unjustified fear of side effects is also affecting acceptance.

The current boom in vaccine research, fuelled by the spectacular breakthroughs in immunology, molecular biology and genomics, will lead to many new vaccines that it will be hard to add to the already overcrowded immunization calendar for young children. The obvious solution to this growing problem is combination, in a multivalent vaccine, of antigens that induce immunity against several diseases. This will reduce the number of inoculations and medical visits required to achieve full immunization.

Combined vaccines are not new and combinations like DTP (triple vaccine against diphtheria, tetanus and pertussis), trivalent oral (OPV) and injectable (IPV)

polio, measles/mumps/rubella (MMR), trivalent influenza, polyvalent pneumococcal and meningococcal vaccines have been extensively used for many decades.

The advantages of combined vaccines include increased convenience for all users, higher compliance by recipients, wider coverage of the population, better disease control and, because of the simplified logistics of vaccine delivery, reduced administrative costs. Fewer inoculations will be needed to protect against more diseases, thus enhancing the acceptance of immunization programmes by both the general public and medical profession. These cumulative favourable factors will boost the effectiveness and success of immunization programmes by increasing vaccine coverage, while at the same time creating cost savings in healthcare budgets.

Over the last ten years, SmithKline Beecham Biologicals (SB BIO), the vaccine manufacturer has been engaged in developing new paediatric vaccines using DTP as the cornerstone on which to build more polyvalent-vaccines. The difficulties encountered, progress made, results obtained and lesson learned will be surveyed, in chronological fashion (André, 1999). Combined vaccines based on mixtures of killed antigens are conceptually more promising. Substantial attention is being given to the killed combined diphtheria, tetanus and pertussis (DTP) vaccine which has been administered to infants for over five decades as a building block for many future combined vaccines. Either the classical DTP with a whole-cell pertussis component (DTPw), or a new less reactogenic and more immunogenic DTP with an acellular pertussis component (DTPa), could be used as the foundation for new combined vaccines. Antigens that have been added to this DTP core include those for hepatitis B (DTPw-HBV), inactivated polio vaccine (DTPw-IPV), already used for some time in some countries), Haemophilus influenzae type B (HIB), (DTPw-HIB), or eventually a combination of all of these (DTP-HBV-IPV-HIB). The combined vaccines will become even more polyvalent as and when new vaccines are developed in the future, possibly against diseases such as hepatitis C, respiratory syncytial virus (RSV), cytomegalovirus, Lyme, herpes or Japanese encephalitis (JE) virus.

The number of different vaccine combinations that can be created with just a few additional antigens is considerable. By adding 1 to 4 other antigen components

(e.g. HIB, HBV, IPV, HAV) to either DTPw or DTPa, there are 44 possible different vaccine combinations that can be generated. This number would increase to thousands if individual components from different manufacturers were considered. As every individual new combined vaccine (taking into account differences in components according to source) must be developed separately to demonstrate safety, stability, compatibility and efficacy, the development of all these vaccines becomes prohibitive in term of costs (André, 1994). No information was found in the literature of combination DTP with JE antigen which JE vaccine is widely used in Asia for childhood immunization, and also used for travelers to Asia from other parts of the world and because of DTP and JE vaccine are the vaccines which were produced in country. In this study, the adsorption variables were investigated for DTP-JE preparation.

The objectives of this study are the following

1. To conceptually develop combined DTP-JE vaccine.
2. To determine the adsorption of diphtheria toxoids, tetanus toxoids and JE antigens on aluminium hydroxide and aluminium phosphate adjuvants by investigate the effect of pH and temperature on the adsorption of these antigens on the different aluminium containing adjuvants.
3. To compare the antigens content from different formulation processes of combined preparations and observe for the antigens content, characterization and morphology after formulate.
4. To evaluate the physical stability of combined DTP-JE preparations.

CHAPTER II
LITERATURE REVIEWS

I. Diphtheria

Diphtheria is an acute communicable upper respiratory illness caused by *Corynebacterium diphtheriae*, a gram positive bacillus. The illness is characterized by a membranous inflammation of the upper respiratory tract, usually of the pharynx but sometimes of the posterior nasal passages, larynx, and trachea, and by widespread damage to other organs, primarily the myocardium and peripheral nerves. Extensive membranes and organ damage are caused by local and systemic action of a potent exotoxin produced by some strains of *C. diphtheriae*. A cutaneous form of diphtheria also occurs (Gross and Rappuoli, 1990).

1. Clinical description

Symptoms of diphtheria are initially nonspecific and mild; throughout the course of the disease, fever does not usually exceed 38.5 °C (101.3 °F). Other early symptoms in children include diminished activity and some irritability. At the very onset of symptoms, the pharynx is injected on examination but no membrane is present. About a day after onset, small patches of exudate appear in the pharynx. Within 2 or 3 days, the patches of exudate spread and become confluent and may form a membrane that covers the entire pharynx, including the tonsillar areas, soft palate, and uvula. This membrane becomes grayish, thick, and firmly adherent. Efforts to dislodge the membrane result in bleeding. Anterior cervical lymph nodes become markedly enlarged and tender. In a proportion of patients, the lymph node swelling is associated with considerable inflammation and edema of the surrounding soft tissues, giving rise to the so called bull neck appearance, which is associated with a higher morbidity and mortality. In untreated patients, the membrane begins to soften about a week after onset and gradually sloughs off, usually in pieces but sometimes as a single unit. As the membrane detaches, acute systemic symptoms, such as fever, begin to disappear (Wharton and Vitek, 2004).

2. Bacteriology and pathogenesis

Corynebacterium diphtheriae is a slender gram positive bacillus, usually with one end being wider, thus giving the often described club-shaped appearance. The organisms are resistant to environmental changes, such as freezing and drying. There are four biotypes of *C. diphtheriae (gravis, mitis, belfanti, and intermedius),* which historically were identified by colonial morphology and biochemical differences; however, in practice, only the *intermedius* biotype can be distinguished reliably by colonial morphology. No consistent differences are found in severity of disease caused by different biotypes.

Identified features of *C. diphtheriae* that are important in the pathogenesis of the disease in humans comprise certain cell wall antigens and in particular the organism's exotoxin. The cell wall contains a heat stable O antigen, which is found in all corynebacteria. The cell wall also contains K antigens, which are heat labile proteins that differ among strains of *C. diphtheriae* and therefore permit categorization of the organisminto a number of types. The K antigens play two roles in relation to humans: first, they appear to be important in the establishment of infection; and second, they produce local type specific immunity.

3. Diphtheria Toxin

The exotoxin produced by *C. diphtheriae* is by far the most important pathogenetic factor. Diphtheria toxin is a polypeptide with a molecular weight of about 58,000. The toxin is secreted as a proenzyme, requiring enzymatic cleavage into two fragments (fragments A and B) to become active. Fragment B is responsible for attachment to and penetration of the host cell. Although nontoxic by itself, fragment B appears to be the antigen responsible for clinical immunity.

On mucous membranes, the toxin causes local cellular destruction, and the accumulated debris and fibrin result in the characteristic membrane. More important, absorbed toxin is responsible for remote manifestations affecting various organs,

including the myocardium, nervous system, kidneys, and others (Gross et al., 1990; Wharton et al., 2004).

4. Epidemiology

Active immunization of children with diphtheria toxoid has markedly altered the epidemiology of diphtheria, reducing diphtheria to extremely low levels in both developed countries and many developing countries. However, diphtheria continues to produce substantial childhood morbidity and mortality in developing countries with incompletely implemented childhood immunization programs.

Preschool and school-age children are most often affected by respiratory diphtheria. Diphtheria was rare in infants younger than 6 months, presumably because of the presence of maternal antibody, and rare among adults, especially those living in urban areas, as a result of acquired immunity. Transplacental antitoxic immunity to diphtheria is present at birth in most infants but declines to nonprotective levels during the second 6 months of life.

Although diphtheria has become a rare disease in most developed countries, a major epidemic of diphtheria began in the Russian Federation in 1990 and subsequently spread throughout the countries of the former Soviet Union, with more than 157,000 cases and 5,000 deaths reported between 1990 and 1998 (Wharton et al., 2004).

Diphtheria vaccine is a preparation of diphtheria toxoid. Usually it is available as a preparation adsorbed with aluminium hydroxide or phosphate and often combined with other toxoids or vaccine (Clements and Griffiths, 2002).

II. Tetanus

Tetanus is unique among diseases for which immunization is routinely recommended because it is not communicable. *Clostridium tetani*, the causative agent of tetanus, is widespread in the environment; many animals in addition to

humans can harbor and excrete the organism and its spores. When spores of *C. tetani* are introduced into the anaerobic/hypoaerobic conditions found in devitalized tissue or punctures, they germinate to vegetative bacilli that elaborate toxin. The clinical presentation results from the actions of this toxin on the central nervous system (CNS). Many animal species besides humans are susceptible to the disease (Habig and Tankersley, 1990; Wassilak, Roper Murphy and Orenstein, 2004).

1. Clinical description

Although the incubation period for tetanus has been reported to vary from 1 day to several months following a wound, the majority of cases occur within 3 days to 3 weeks after inoculation of spore. In the United States during 1998 to 2000, the median interval between the injury and onset of tetanus was 7 days (range 0 to 112 days) for 89 nonneonatal cases with reported information. The time between injury and the onset of symptoms was 30 days or less for 94% of the cases, and 2 days or less for 12% of the cases.

There is a direct relationship between the site of inoculation and the incubation period, with the longest intervals occurring after injuries farthest from the CNS; injuries of the head and trunk generally are associated with the shortest incubation periods. Incubation periods of 10 days or more tend to result in mild disease, whereas incubation periods within 7 days of injury tend to result in more severe disease.

Three clinical syndromes are associated with tetanus infection: (1) localized, (2) generalized, and (3) cephalic. Localized tetanus, which is unusual in humans. More than 80% of cases of tetanus are generalized. The most common initial sign is spasm of the muscles of mastication - trismus, or lockjaw - occurring in more than 50% of the cases. Trismus may be followed by spasm of other muscles in the neck, thorax and back, abdomen, and extremities. Spasm of the glottis can result in immediate death. Temperature elevations of 2 °C to 4 °C are often associated with severe spasms. Cephalic tetanus is a rare manifestation of the disease generally associated with lesions of the head or face, especially in the distribution of the facial nerve and the orbits (Wassilak et al., 2004).

2. Bacteriology

Clostridium tetani is a gram positive, spore-forming, motile, anaerobic bacillus. Typically measuring 0.3 to 0.5 μm in width and 2 to 2.5 μm in length, the vegetative form often develops long filament-like cells in culture. Flagellae are attached bilaterally on non-spore forming bacteria. With sporulation, *C. tetani* takes on the more characteristic drumstick-like appearance. Spores usually form in the terminal position. *C. tetani* is considered a strict anaerobe that grows optimally at 33 °C to 37 °C; however, depending on the strain, growth can occur at 14 °C to 43 °C.

Sporulation is dependent on a variety of factors that include pH, temperature, and media composition. The germination of spores requires anaerobic conditions and is enhanced by the presence of lactic acid and chemicals toxic to cells.

The most common source of environmental exposure to *C. tetani* bacilli and spores is the soil, where the organism is widely but variably distributed. Soil is not the only reservoir of the organism. Animals, both herbivores and omnivores, can carry *C. tetani* bacilli and spores in their intestines and readily disseminate the organism in their feces. *C. tetani* spores also have been detected in street dust and the dust and air of surgical operating theaters (Habig et al., 1990; Wassilak et al., 2004).

3. Pathogenesis

Clostridium tetani produces two exotoxins, tetanolysin and tetanospasmin. The toxin has an approximate molecular weight of 150,000 and is synthesized as a single polypeptide prototoxin chain. Tetanus toxin is one of the most potent known poisons on a weight basis. The estimated minimum human lethal dose is less than 2.5 ng/kg. Various species have different levels of responsiveness to the toxin.

Infection usually begins with the inoculation of spores through the epithelium. Wounds accompanied by tissue injury and necrosis (with or without the presence of aerobic organisms) leading to anaerobic or hypoaerobic conditions are generally necessary for the spores to germinate and bacilli to replicate.

Transport of toxin from the injured site into the CNS is complex. Toxin injected under the skin appears to enter underlying muscle; infiltration of muscle with antitoxin before subcutaneous toxin injection can block the development of tetanus. Once in the muscle, some toxin makes its way to the CNS directly by intra-axonal transport; the major portion is transported by the lymphatics to the bloodstream and then disseminated to a variety of tissues (Habig et al., 1990; Wassilak et al., 2004).

4. Epidemiology

In 1984, estimates based on mortality surveys suggested that there were approximately 1 million annual deaths caused by neonatal tetanus alone. Tetanus generally follows a distinct seasonal trend with a midsummer or “wet” season peak, which may reflect soil and spore conditions as well as more frequent injuries during the warmer months. The global distribution of tetanus generally focuses in areas with a moist, warm climate and fertile soil. The highest rates of tetanus remain in the developing world, particularly in countries near the equator.

Aside from neonatal tetanus, the largest proportion of cases in developing countries is among male older children and young adults. Wherever immunization programs are in place, the rates of tetanus decline, and the sex and age distributions shift to mirror the underimmunized population. In the 1950s, more than one third of the deaths from tetanus in the United States were among neonates and infants less than 1 year old. In contrast, from 1998 to 2000, no deaths from tetanus occurred among neonates or children, and three fourths of the deaths occurred among persons 60 years of age or older (Wassilak et al., 2004).

Tetanus toxoid is available in a plain (unadsorbed) liquid form, or adsorbed with aluminium phosphate or hydroxide, alone or in combination with other toxoids or vaccines (Clements et al., 2002).

III. Pertussis

Pertussis (whooping cough) is a bacterial respiratory infection caused by *Bordetella pertussis*, a gram negative bacillus, Its major manifestation is a protracted cough illness that lasts many weeks. The disease is most severe in infants and young children, many of whom suffer the intense paroxysmal coughing that terminates in an inspiratory “whoop”. The epidemic primarily affected infants and young children and resulted in high mortality. Worldwide, pertussis remains an important killer of children. The World Health Organization (WHO) estimates that 45 million cases occur worldwide annually, with 400,000 deaths (Granstrom, Blennow and Winberry, 1990; Locht, 1999; Edwards and Decker, 2004).

1. Clinical description

The incubation period of pertussis averages 9 or 10 days (rang, 6 to 20 days). The onset is insidious, and symptoms are indistinguishable from those of minor upper respiratory infection. Fever is usually minimal throughout the course of infection. Cough, initially intermittent, progresses within 1 or 2 weeks to become paroxysmal. The paroxysms increase in both frequency and severity and then gradually subside, rarely lasting longer than 2 to 6 weeks.

It is during the paroxysmal stage, when the cough is most severe, that the characteristic whoop occurs. The whoop is caused by forced inspiration through a narrowed glottis immediately after a paroxysm of a dozen or more rapid, short coughs without intervening inspiration. The paroxysms apparently result from difficulty in expelling thick mucus from the tracheobronchial tree. During a paroxysm, cyanosis may occur and vomiting may ensue. Paroxysms may be induced by eating, laughing, crying, and a variety of other stimuli and are usually worse at night. Recovery is gradual. The paroxysms become less frequent and milder, and the whoop disappears. Nonparoxysmal cough may persist for many weeks. During the convalescent phase, intercurrent respiratory infections may trigger a recurrence of the paroxysmal cough.

Children less than 6 months of age were noted to have complications more frequently than older children (23.8% and 5.1%, respectively). Minor complications of pertussis include subconjunctival hemorrhages and epistaxis secondary to the paroxysms. Suppurative otitis media frequently occurs. Major complications, which are sometimes fatal, are of three types: pulmonary, encephalitic, and nutritional. Of these, pulmonary complications are the most frequent (Granstrom et al., 1990; Edwards et al., 2004).

2. Bacteriology

The causative agent of pertussis is *B. pertussis*, a small, gram negative, pleomorphic bacillus. Two closely related organisms in the genus *Bordetella* are *B. parapertussis* and *B. bronchiseptica*. The former is responsible for a pertussis-like syndrome in humans. The latter produces respiratory illnesses in domestic animals. Of all the *Bordetella* species, only *B. pertussis* synthesizes PT (Locht, 1999).

Bordetella pertussis has a marked tropism for and attaches strongly to ciliated respiratory tract epithelial cells. The bacteria may be internalized by epithelial cells but do not penetrate submucosal cells or invade the blood stream. However, toxins produced by the organism can enter the blood stream and produce systemic effects (Granstrom et al., 1990; Edwards et al., 2004).

3. Pertussis toxin

PT, previously termed lymphocytosis promoting factor, is a major contributor to the pathogenesis of pertussis and is generally believed to play an important role in the induction of clinical immunity. PT is an oligomeric structure composed of five different subunits, Sl through S5 (Fig. 1) molecular weights; 26,024 Dalton S1, 21,924 Dalton S2, 21,873 Dalton S3, 12,058 Dalton S4 and 11,013 Dalton S5 (Locht and Keith, 1986). Structurally it belongs to the A-B class of bacterial toxins. The S1 component (A protomer) catalyzes the ADP ribosylation of GTP binding regulatory proteins involved in signal transduction in the eukaryotic cell. The A protomer is largely responsible for the recognized biologic activities of PT, including promotion

of lymphocytosis, stimulation of islet cells, sensitization to histamines, clustering of Chinese hamster ovary cells, and adjuvant properties. The B oligomer is a ring shaped structure that consists of one copy each of subunits S2, S3 and S5 and two copies of S4. S5 serves to link the two dimers, S2-S4 and S3-S4. The primary function of the B oligomer is to facilitate the attachment of PT to the ciliated cells of the respiratory tract (Edwards et al., 2004).

PT appears to play two major roles in the pathogenesis of pertussis. First, it facilitates the attachment of *B. pertussis* to ciliated respiratory cells. Second, it appears to be of major importance in cell toxicity. PT is a strong immunogen.

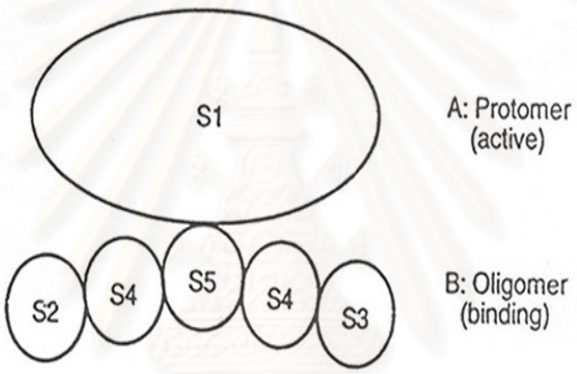


Figure 1 Diagrammatic representation of the pertussis toxin (Edwards et al., 2004)

4. Pathogenesis

Transmission occurs when airborne bacteria from symptomatic patients reach the ciliated respiratory epithelium of a susceptible host. The organisms attach strongly to the ciliated cells through several adhesions. The bacteria do not invade beyond the epithelial layers of the respiratory tract, but PT enters the bloodstream and exerts its biologic effects on systemic sites (Granstrom et al., 1990; Edwards et al., 2004).

5. Epidemiology

Pertussis is an endemic disease with epidemic peaks occurring every 2 to 5 (typically, 3 to 4) years. Widespread pertussis vaccination of children and the consequent reduction in the incidence of disease do not appear to have altered these

intervals, suggesting that ongoing endemic circulation of the organism in the community continues. There is no consistent seasonal pattern.

Pertussis may occur at any age. Infants are susceptible to pertussis within the first few weeks or months of life, when mortality from whooping cough is highest. For many years, it was assumed that one attack of pertussis provided lifelong immunity. Before widespread vaccination, this belief was reflected by the age distribution of pertussis: approximately 20% of all whooping cough cases occurred in infants younger than 1 year, and nearly 60% occurred among children ages 1 to 4 years (Edwards et al., 2004).

Duchén et al. (1997) investigated about the response of immunoglobulin E and G to pertussis toxin in children immunized with adsorbed and non-adsorbed whole cell pertussis vaccines. The results showed that the adsorbed vaccine influenced the IgG response but not the IgE response to pertussis toxin.

Two classes of pertussis vaccine are currently available: whole-cell vaccines and acellular vaccines. The whole-cell vaccines are suspensions of killed *Bordetella pertussis* organisms at a concentration of more than 4 IU. The vaccine is adsorbed onto aluminium phosphate or aluminium phosphate sulphate (Clements et al., 2002).

IV. Japanese Encephalitis

Japanese encephalitis (JE), a mosquito borne flaviviral infection, is the leading recognized cause of childhood encephalitis in Asia. Approximately 20,000 cases and 6,000 deaths are reported annually, but in many locations the disease is not under systematic surveillance, and official reports undoubtedly underestimate the true number of cases. Although the disease is transmitted only in Asia, because the region contains more than 3 billion people and 60% of the world's population, regional JE associated morbidity may exceed worldwide morbidity from herpes encephalitis, the latter estimated at 5 cases per 1 million population per year, or approximately 30,000 cases worldwide. JE now is the continent's leading cause of childhood viral neurologic infection. By any standard, JE is a major public health problem that

potentially can be controlled by proven effective vaccines (Halstead and Tsai, 2004; Rao, 2004).

During the first half of this century, JE was recognized principally in temperate areas of Asia in the form of perennial outbreaks in Japan, Korea, and China. In Japan, Korea, and Taiwan, the introduction of national immunization programs after 1965 led to the near elimination of the disease; however, the absence of reported cases is disarming because enzootic transmission of the virus in its enzootic cycle continues in these locations, and periodic outbreaks, as in Korea in 1982, have occurred. Although sporadic viral encephalitis cases had been noted in northern Thailand, JE was not recognized as a major public health problem in Southeast Asia until 1969, when an epidemic of 685 cases was reported from the Chiang Mai Valley. Yearly outbreaks producing thousands of cases and hundreds of deaths followed in the northern region, and JE became recognized as a leading cause of childhood mortality and disability. The continued public health impact of JE in the region has led to efforts in Thailand and, more recently, in Vietnam to implement programs of childhood immunization and vaccine production (Halstead et al., 2004).

1. Clinical description

The great majority of infections are not apparent, and only 1 in approximately 250 infections results in symptomatic illness in susceptible Asians. The principal clinical manifestation of illness is encephalitis. Milder clinical presentations, such as aseptic meningitis and simple febrile illness with headache, may sometimes occur but usually escape recognition. The incubation period is 5 to 15 days. Illness usually begins with abrupt onset of high fever, change in mental status, gastrointestinal symptoms, and headache, followed gradually by disturbances in speech or gait or other motor dysfunction. Irritability, vomiting, and diarrhea or an acute convulsion may be the earliest signs of illness in an infant or child. Seizures occur in more than 75% of pediatric patients and less frequently in adults.

A substantial proportion of patients become totally unresponsive and require ventilatory assistance. Generalized weakness and changes in muscle tone, especially

hypertonia and hyperreflexia, are common, but focal motor deficits cranial nerve palsies (especially central facial palsy); and abnormal reflexes. Signs of extrapyramidal involvement, including tremor, mask like facies, rigidity, and choreoathetoid movements, are characteristic of JE, but these signs may be obscured initially by generalized weakness.

Five to 30% of cases are fatal, with some deaths occurring after a brief prodrome and fulminant course lasting a few days and others occurring after a more protracted course with persistent coma. Young children (< 10 years) are more likely than adults to die, and, if they survive, they are more likely to have residual neurologic deficits. Overall, approximately one third of surviving patients exhibit serious residual neurologic disability. In children, motor abnormalities frequently improve or eventually resolve, but behavioral changes and psychological deficits have been detected 2 to 5 years after recovery in up to 75% of pediatric cases (Halstead et al., 2004; Rao, 2004).

2. Virology

JE virus is one of 70 viruses in the *Flavivirus* genus of the Flaviviridae family. Morphologically, flaviviruses are spherical, approximately 40 to 50 nm in diameter, with a lipid membrane enclosing an isometric 30 nm diameter nucleocapsid core comprising a capsid (C) protein and a single stranded messenger (positive) sense viral RNA. Membrane surface projections are composed of a glycosylated envelope (E) and membrane (M) protein, a mature form of the premembrane (prM) protein (Heinz and Mandl, 1993; Halstead et al., 2004; Rao, 2004).

3. Pathogenesis

After an infectious mosquito bite, viral replication occurs locally and in regional lymph nodes. Virions disseminate to secondary sites, where further replication contributes to a viremia. Invasion of the CNS probably occurs from the blood by antipodal transport of virions through vascular endothelial cells. Infection in

the CNS spreads by viral dissemination through the extracellular space or by direct intercellular spread (Halstead et al., 2004).

4. Epidemiology

JE is transmitted in epidemics or in an endemic pattern, or both, in virtually every country of Asia. Transmission is seasonal, occurring approximately from May to September in temperate areas of China, Korea, Japan, and far eastern Russia. Farther south, the transmission season is somewhat longer, extending from March through October (Fig. 2). In tropical areas of Southeast Asia and India, seasonal transmission is particular to local patterns of monsoon rains and bird migration, with the possibility of two transmission intervals in a calendar year. The virus is transmitted throughout the year in some sites. JE is principally a disease of rural areas in which vector mosquitoes proliferate in close association with birds and pigs, which serve as vertebrate amplifying hosts. Humans and horses may become ill after infection, but such infections contribute minimally to the transmission cycle. Experimental observations and field studies indicate that the virus overwinters in infected adult mosquitoes. Long term persistence in tissues and blood of JEV infected vertebrate hosts, such as bats and reptiles, has been demonstrated (Halstead et al., 2004).

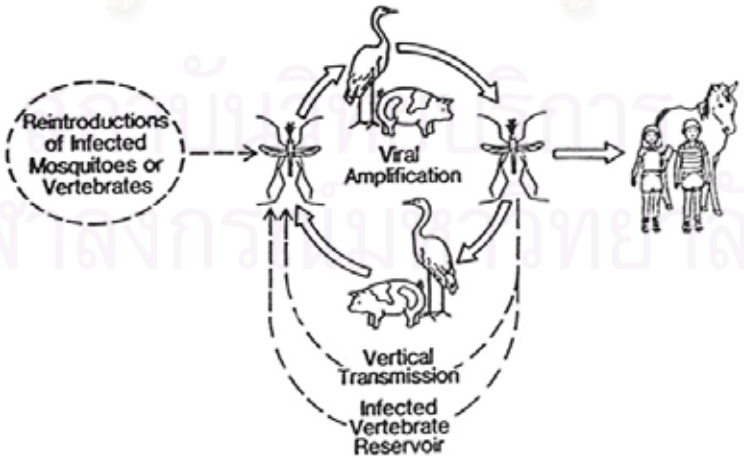


Figure 2 Transmission cycle of Japanese encephalitis (JE) virus. (Halstead et al., 2004)

Culex tritaeniorhynchus is the principle JE vector in most areas of Asia, but various other ground pool - and rice paddy - breeding species, including *C. vishnui, C. pseudovisnuris, C. gelidus, C. fuscocephala, C. bitaeniorhynchus, C. infula, C. whitmorei,* and *C.* annulus, are also important locally. Although vector abundance and risk for human infection are associated with rainfall, with the introduction of wet rice cultivation, paddy flooding schedules have come to influence vector bionomics.

In temperate regions, vector mosquitoes emerge in May, and, after several initial rounds of viral amplification, high rates of pig seroconversion are detected. This is followed almost immediately by the onset of human cases, typically in July and August. By virtue of high levels and lengthy periods of viremia after infection and their prevalence as domestic animals, pigs are the key hosts for viral amplification during pregnancy frequently results in abortions and stillbirths, with significant economic losses. In some locations, enzootic transmission of the virus is initiated among aquatic birds, and, in well characterized outbreaks in which pigs were absent, such birds have served as epidemic amplifying hosts. Other domesticated animals, such as cattle, dogs, sheep, cows, and chickens, and peridomestic rodents may become infected, but these fail to develop a sufficient viremia to support further viral amplification. JE mosquito vectors are zoophilic; consequently, cows and certain other animals can reduce risk to humans by diverting vector mosquitoes (zooprophylaxis). Immunization of pigs prevents abortion and stillbirths and also may reduce viral transmission by nullifying the role of pigs as viral amplifiers (Halstead et al., 2004; Rao, 2004).

In areas where transmission is hyperendemic, half of all cases occur in children younger than 4 years of age, and nearly all cases are found in children younger than 10 years. Usually cases in males exceed those in females, possibly reflecting greater outdoor exposure in boys.

Worldwide, three JE vaccines are in widespread production and use however, only inactivated JE vaccine produced in mouse brain is distributed commercially and is available internationally.

Table 1 Japanese Encephalitis vaccines (Halstead et al., 2004)

Vaccine Type	Substrate	Viral Strains	Manufacturers
Inactivated	Mouse brain	Nakayama, Beijing-1	*India* : Central Research Institute (currently inactive) *Japan* : Biken (Research Foundation for Microbial Disease of Osaka University), Chiba, Denka Seiken Co.,Ltd., Chemo-Sero Therapeutic Research Institute, Kitasato Institute, Saikin Kagaku Institute, Takeda *Korea* : Green Cross *Taiwan* : National Institute of Preventive Medicine *Thailand* : Government Pharmaceutical Organization *Vietnam* : National Institute of Hygiene
Inactivated	Primary hamster kidney cells	P3	*People's Republic of China*: Beijing, Shanghai, and Changchun Institutes of Biological Products
Live, attenuated	Primary hamster kidney cells	SA14-14-2	*People's Republic of China*: Chengdu, Wuhan Institutes of Biological Products

In most areas of Asia, vaccine produced from the Nakayama strain is given subcutaneously in two 0.5 ml doses 1 to 4 weeks apart (1.0 ml for people > 3 years of age) usually beginning at the age of 12 to 36 months, with a booster dose at 1 year and additional booster doses thereafter at 1 to 3 year intervals. In practice, immunization schedules are quite variable. Beijing 1 strain derived vaccine is

formulated with a higher antigen concentration, and the recommended dose is 0.5 ml (0.25 ml for children under 3 years of age).

The primary series has been administered to infants (with diphtheria and tetanus toxoids and pertussis [DTP] vaccine) as early as 2 months of age in clinical trials, but, because JE rarely occurs in infants younger than 1 year, there is no need to begin immunization at that age other than to save administration costs. In a study of infants 15 months of age, simultaneous administration of inactivated JE vaccine with measles, mumps, and rubella vaccine did not result in reduced immunogenicity of increased side effects. Under Thailand's Expanded Programme of Immunization, JE vaccine is given concurrently with the fourth dose of DTP and oral poliovirus vaccine at 18 months. A comparison of administration routes in adults showed that a 0.1 ml intradermal dose may be as immunogenic as the standard administration of 1.0 ml subcutaneously, at least when given as a booster (Halstead et al., 2004).

Rojanasuphot, Charoensook, Ungchusak, Srijaggrawalwong and Panthumachinda (1991) studied on the effectiveness of inactivated mouse brain JE vaccine produced in Thailand and in Japan in 5 to 9 year old children in Ratchaburi Province; JE vaccines produced in Thailand are as effective as vaccines made in Japan.

In general, DTP vaccine has been given to children at the age of 2, 4 and 6 months and two booster doses at 18 months and 4 years, respectively, whereas JE vaccine has been given at 12-15 months and 2 years. Rojanasuphot, Na-Chiang Mai, Srijaggrawalwong, Panthumachinda and Nimmannitya (1992) investigated the possibility, safety and immunogenicity of implementing JE vaccination simultaneously with DTP and OPV vaccine in infants at Children's hospital, Bangkok.

V. Immunization program

Table 2 Immunization program (กุลกัญญา , 2545)

Age	Vaccine
New born	♦ BCG vaccine
	♦ Hepatitis B vaccine (1st dose)
1 month	♦ Hepatitis B vaccine (2nd dose)
2 months	♦ DTP , OPV vaccine (1st dose)
4 months	♦ DTP, OPV vaccine (2nd dose)
6 months	♦ DTP, OPV vaccine (3rd dose)
	♦ Hepatitis B vaccine (3rd dose)
9 months	♦ MMR vaccine (1st dose)
12-15 months	♦ JE vaccine (1st and 2nd dose in 1-2 weeks interval)
18 months	♦ DTP, OPV (1st booster)
2 years	♦ JE vaccine (3rd booster)
4 years	♦ DTP, OPV vaccine (2nd booster)
5-6 years	♦ MMR vaccine (2nd dose)

It could be concluded that JE vaccination simultaneously with routine vaccines was safe, effective and practical. Two-and three- month intervals of a primary two dose JE vaccination were not different from the seven-day interval in inducing immunogenicity. Even the antibody one year after 2 doses of JE vaccination remained sufficiently high to confer protection against JE infection: the third dose vaccination also had a marked effect on antibody response. Srivastava et al. (2001) formulated a second generation, purified, inactivated vaccine (PIV) against JE virus with aluminium hydroxide and administered to mice by subcutaneous inoculation and to compare with the existing licensed mouse brain-derived vaccine, JE-Vax. The JE-PIV was more immunogenic than and as effective as preventing encephalitis in mice.

VI. Combination vaccines

The continuing increase in the number of effective vaccines suitable for use in infancy and early childhood has posed substantial economic and logistic difficulties. Providing these vaccines as separate injections not only is expensive but also requires multiple needle sticks, distressing parents, providers, and patients alike. Scheduling additional vaccination visits to reduce the number of injections per visit increases costs, burdens staff, and jeopardizes the entire immunization program by increasing the likelihood of missed vaccinations. The shipping, handling, and storage of a plethora of vaccines are burdensome and expensive and increase the possibility of error. These problems have stimulated continuing efforts to develop new combination vaccines.

The combining of multiple related or unrelated antigens into a single vaccine is not a new concept. Most such pediatric combination vaccines begin with a DTwP or DTaP vaccine and add such antigens as IPV, conjugate Haemophilus influenzae type b (Hib), and hepatitis B (HB). As development efforts for the DT(a)P based combinations have matured, some manufacturers have turned their efforts toward developing so called second shot combinations that incorporate conjugate pneumococcal (PnC) and conjugate meningococcal (MnC) antigens. A third developmental stream has been directed toward combination vaccines targeted principally travelers, typically based on HB or hepatitis A (HA) components (Decker, Edwards and Bogaerts, 2004). The development of combinations that are based on the currently used whole-cell pertussis antigen is limited because of the poor predictability with which combination vaccines can be made using the whole-cell pertussis antigens (Rabinovich, Mclnnes, Klein and Hall, 1994).

Mallet et al. (2004) reviewed of the immunogenicity and safety of hexavalent vaccine (Hexavac®) which was comparable with following concomitant administration of Pentavac® and monovalent hepatitis B vaccine. Hexavac® shown to be highly immunogenic and could be used by vaccination schedule.

The premium pricing of the combination or reduced provider reimbursement (as a result of administration of fewer injections) may inhibit use of the combination. Less obvious, but equally important, are the economic benefits that flow from use of a combination: savings resulting from simplified vaccine purchase, storage, and handling ; reduced costs for labor and supplies ; elimination of the need for scheduling several vaccination visits to avoid multiple injections ; and, of course increased patient satisfaction and greater compliance with vaccination recommendations (Ada, 1994).

1. Source of interference between different vaccine preparations

1.1 Antigen competition

Ada (1994) reported that this may arise if the peptides from different protein antigens compete with binding to a particular major histocompatibility (MHC) molecule together with the differential recognition of those complexes by the T cell receptor (TCR). One peptide, A, may preferentially bind to a given MHC molecule compared to another peptide, B. Both the MHC/A and MHC/B complexes may be seen equally well by TCRs, but as there are now very few MHC/B complexes to be recognised, there will be a poor response to the protein supplying peptide B. For example, immunizing mice with myelin basic protein in the presence of ovalbumin results in a much decreased response to the myelin basic protein compared to the normal level obtained in the absence of ovalbumin.

It is usually not possible to predict such interference in advance. Theoretically, it becomes more likely as the number of proteins in a mixture increases. It may in part be overcome by administration of different components at different sites-not an ideal practical solution.

1.2 The effect of different adjuvants

They may be cases where for example a mucosal immune response (Th2) to one vaccine component of a mixture would be beneficial whereas a systemic

response favouring a Th1 response is most advantageous for another component and a cytotoxic T-lymphocyte (CTL) response to a third component of the mixture (Ada, 1994). Careful thought would need to be paid to the choice of adjuvant, but it is unlikely optimum responses to each would be obtained.

1.3 Interference with the replication of different infectious agents

It is well known that in the case of the three subtypes of attenuated polioviruses, adjustments to the amount of each subtype in the final vaccine need to be made to suit regional circumstances. A similar effect has recently been noticed with mixtures of cold adapted, live influenza virus vaccines. The factors that are potentially important in these or other cases are the varying susceptibilities of different cells to infection, the rates of replication of the different infectious agents and a variety of host factors, such as malnutrition, the extent of existing infections by other agents, etc (Ada, 1994).

VII. Adjuvants

The term of “adjuvant” is derived from the Latin word *adjuvare* which means to help. Any material that helps the antigens or increases the humoral and/or cellular immune response to an antigen is referred to as an adjuvant. Adjuvants have been in use to augment the immune response to antigens for about 70 years since Ramon showed increased antitoxin response to tetanus and diphtheria toxoids injected together with other compounds such as agar, tapioca, lecithin, starch, oil, saponin or even breadcrumbs. During the last 70 years, many adjuvant formulations have been developed and a few of these have been evaluated in clinical trials. However, most of these were never accepted for routine vaccines, mainly due to their toxicity and side effects.

The adjuvanticity of most of the formulations developed so far is associated with adverse side effects of varying degree. Some of the adverse effects are ascribed to mechanisms involved in the adjuvanticity of these formulations. For example, local reactions may be due to depot formation at the site of injection which is a major

mechanism of adjuvanticity of several adjuvant formulations such as mineral compounds, oil emulsions, liposomes and biodegradable polymer microspheres. Another mechanism of adjuvant action is to stimulate the cells of immune system to secrete various cytokines which may lead to systemic side effects depending upon the type and amount of cytokines elicited. Other side effects may be due to general adverse pharmacological reactions of various formulations. Usually a compromise or balance between toxicity and adjuvanticity is accepted base upon risk benefit analysis. For example, for routine childhood vaccines safety is the biggest concern and adjuvanticity may be restricted to the antigens because these vaccines are injected to normal, healthy babies. An adjuvant for routine immunization of healthy infants, children and adults must have a very low rate of adverse side effects. Severe side effects occurring as rarely as once in several thousand doses would not be acceptable for routine vaccines. On the other hand, for high risk groups like cancer and AIDS patients or for therapeutic vaccines, a certain level of toxicity may be acceptable based upon benefits (Gupta et al., 1993; Gupta and Siber; 1995; Matheis, Zott and Schwanig, 2002; Singh and O'Hagan, 2002; Kenny and Edelman; 2004).

1. Mode of action

Adjuvants may act in one or more of five ways (Gupta et al., 1995; Cox and Coulter, 1997; Kenny et al., 2004).

1.1 Immunomodulation

This refers to the ability of many adjuvants to modify the cytokine network. In general, only immunomodulatory adjuvants will exert an adjuvant effect when presented at a separate time or site to the immunogen. Immunomodulation may result in a general up regulation of the entire immune system, but most commonly results in up regulation of certain cytokines and a concomitant down regulation of others. Two majors subset of $CD4^+$ T cells, viz Th1 and Th2 have been well described for mouse and man and their existence is postulated for other animal species. Th1 responses typically induce complement fixing antibody and strong delayed-type hypersensitivity (DTH) reactions and are associated with γ-IFN, IL-2 and IL-12 whilst

Th2 responses result in high circulating and secretory antibody levels, frequently IgE and the cytokines IL-4, IL-5, IL-6 and possibly IL-10. Th1 and Th2 responses are mutually inhibitory.

1.2 Presentation

This refers to the ability of an adjuvant to preserve the conformational integrity of an antigen and to present this to appropriate immune effector cells. This will occur when an adjuvant is able to interact with an antigen in such a way that conformational epitopes are more effectively maintained. The main benefits are an improved *in vivo* activity and an increased shelf life.

Three major sets of interactions are required to achieve an effective antibody response. The first interaction is with professional antigen presenting cells (APC), typically dendritic cells (DC) and Langerhans cells (LC), and possibly macrophages, although their role is still in dispute. The second interaction involves antigens and B cell, and recognition is primarily between surface immunoglobulin and antigen. The third interaction is partly speculative though there is increasing supportive evidence that follicular dendritic cells (FDC) can provide a long term reservoir of native antigen which is essential both for effective affinity maturation of the immune response and for persistence of biologically relevant antibody production.

1.3 Induction of $CD8^+$ cytotoxic T-lymphocyte (CTL) responses

The induction of CTL responses generally requires that antigen be processed within the cell cytosol (the endogenous pathway) where peptides, generally 9-mers, become incorporated within the close-end groove of the MHC class 1 molecule and are then expressed on the cell surface. For an adjuvant to be useful for CTL induction, it must facilitate incorporation or persistence of appropriate peptide into MHC-1. The most effective way to achieve this is for the adjuvant to interact in some way with cell membranes so that antigen associated with the adjuvant is deposited within the cytosal in a form suitable for normal processing in the proteasome.

1.4 Targeting

This defines the ability of an adjuvant to deliver an immunogen to immune effector cells, generally via APCs. This form of adjuvant activity may not modify the type of immune response but rather will affect the amount of immunogen required to achieve a given effect that is the efficiency of generation of the immune response. There are several ways in which an adjuvant can achieve this effect. The most common is to interact with antigen in such a way as to form multimolecular aggregates.

1.5 Depot generation

This can be achieved as a short term or long term depot, the latter giving either a continuous or pulsed release. Short term depots are typified by aluminium salts and w/o emulsions, where antigen is trapped at the injection site and therefore cannot be lost by liver clearance. Excision of the injection site 8-10 days after dosing has little if any effect on magnitude or duration of response suggesting antigen has either been removed or walled-off by that stage.

Long term depots are best achieved using synthetic polymers such as polylactide coglycolide (PLG) to produce microspheres which degrade to yield a pulsed delivery. These microspheres are preferably of a size >10 μm so that they must remain at the injection site until biodegradation permits removal of their contents (immunogen and preferably adjuvant) by APC. Release times from 1 to 6 months can be achieved with reasonable precision.

2. Classification of adjuvants

There are a number of different criteria which can be used to group adjuvants to permit their rational comparison. Gupta et al. (1995) and Cox et al. (1997) have been allocated adjuvants into two broad groups: particulate and non-particulate.

Table 3 Mode of adjuvant action (Cox et al., 1997)

Action	Adjuvant type	Benefit
Immunomodulation	Generally small molecules or proteins which modify the cytokine network	Upregulation of immune response. Selection of Th1 or Th2
Presentation	Generally amphipathic molecules or complexes which interact with immunogen in its native conformation	Increased neutralizing antibody response. Greater duration response
CTL induction	- Particles which can bind or enclose immunogen and which can fuse with or disrupt cell membranes	Cytosolic processing of protein yielding correct class 1 restricted peptide
	- w/o emulsions for direct attachment of peptide to cell surface MHC-1	Simple process if promiscuous peptide know
Targeting	- Particulate adjuvants which bind immunogen. Adjuvants which saturate Kupffer cells	Efficient use of adjuvant and immunogen
	- Carbohydrate adjuvants which target lectin receptorson macrophages and DCs	As above. May also determine type of response if targeting selective
Depot generation	- w/o emulsion for short term	Efficiency
	- Microspheres or nanospheres for long term	Potential for single-dose vaccine

2.1 Particulate adjuvants

Adjuvants which exist as microscopic particles and owe at least some of their adjuvant activity to this property. Generally the benefits of particulate adjuvants are only fully realized when immunogen is able to be incorporated into or at least associated with the particle.

2.2 Non-particulate adjuvants

These are adjuvants where activity does not depend upon any particulate or multimeric nature. They are generally immunomodulators though some improve targeting. Most benefit from association with a particulate adjuvant.

Table 4 Characteristics of particulate adjuvants (Cox et al., 1997)

Adjuvant	Immunomodulation	Targeting	Presentation	CTL	Depot
Aluminium salts	Strong Th2, IgE	+	-	-	+ST[a]
w/o emulsions	Weak Th1 and Th2	-	-	- or +++[b]	+++ST
o/w emulsions	Weak Th1 and Th2	+	+++	-	-
ISCOM™	Strong Th1 and Th2	+++	++++	++++	-
Liposomes	-	++	+++	++	-
Microparticles					
< 10 µm	-	++++	-	-	-
> 10 µm	-	-	-	-	+++LT[c]
Calcium salts	-	+	-	-	+ST
Proteosomes / virosomes	-	++	+++	-	-
Stearyl tyrosine	Mod Th1 and Th2	-	-	-	+ST
γ-Inulin	Mod Th1	-	-	-	-
Algammulin	Mod Th1 and Th2	+	-	-	+ST

[a] ST, short term(≤ 2 weeks); [b] Good CTL response for externally applied peptide only; [c] LT, long term (weeks to months)

Table 5 Characteristics of principal non-particulate adjuvants (Cox et al., 1997)

Adjuvant	Immunomodulation	Targeting	Presenting	CTL	comments
MDP-hydrophilic	Strong Th2	-	-	-	use in w/o emulsions
MDP-lipophilic	Strong Th1	-	-	-	use in o/w emulsions
Non-ionic block copolymers	?	- or +++[a]	+++	-	use in w/o or o/w emulsions
saponins	Strong Th1, Th2	-	-	+	from ISCOMs, use with liposomes, MPL
Lipid A (MPL)	Strong Th1	-	-	-	use with o/w emulsions, liposomes, saponins
Cytokines	Various	-	-	-	use preferably with some particulate adjuvant
Carbohydrate polymers	Mod Th1, IL-1 induction	+++	-	-	preferably conjugate?
Derivatized polysaccharides	?	+++	-	-	

[a]For self-aggregating copolymers, e.g. CRL 1005

Table 6 Further non-particulate adjuvants (Cox et al., 1997)

Adjuvant	Action	Comments
Avridine DNA	Th1 induction, Presentation (in liposome or o/w emulsions)	Unacceptable toxicity
CWS (cell wall skeleton)	Th1 induction ?	Use with MPL in o/w emulsions
DHEA (dehydroepi-androsterone)	Th1 induction ?	Administration difficult
Vitamin D3	Th2, secretory IgA induction ?	Administration difficult
TDM (Trehalose dimycolate)	Th1 induction	Administration difficult Toxicity unacceptable
P_3CSS	Targeting, potent CTL induction	Potentially toxic
Poly I:CPoly ICLC	Both Th1 (γIFN) and Th2 (IL-4) induction	Poly I:C toxic
Poly A:U	Th2 induction (IL-6)	

3. Characteristics of an “ideal” adjuvant

Since safety of the adjuvant formulations is the biggest concern, particularly for routine childhood vaccines, a number of criteria to ensure the safety of adjuvant vaccines were listed (Gupta et al., 1995). In addition to safety with regard to local reactions, systemic reactions (general toxicity, pyrogenicity), autoimmune diseases, hypersensitivity reactions, carcinogenicity, teratogenicity, etc., an ideal adjuvant would be chemically defined so that is can be manufactured consistently. The preparation would elicit a protective immune response with weak antigens including polysaccharide-protein conjugates with lower doses of antigens and with fewer injections. The adjuvant would be effective in infants and young children, ideally at birth, and elicit a more persistent response of high quality (high affinity antibodies or desired type of IgG isotype). The adjuvant would be stable with regard to

adjuvanticity and toxicity without any interaction with the antigen. It would be biodegradable and non-immunogenic by itself. None of the adjuvants available at present meet these criteria.

4. Safety

The absolute safety of adjuvanted vaccines, or any vaccine, cannot be guaranteed, so the risks must be minimized. Undesirable reactions can be grouped as either local or systemic. The most frequent local adverse effects are tenderness and swelling, with the most severe ones involving the formation of painful abscesses and nodules at the inoculum site. The mechanisms for such severe local reactions include the following: 1) contamination of the vaccine at the time of formulation with reactogenic chemicals and microbial products; 2) instability of the vaccine on storage with breakdown into reactogenic side products; 3) formation of inflammatory immune complexes at the inoculation site by combination of the adjuvanted vaccine with preexisting antibodies resulting in an Arthus-type reaction; and 4) poor biodegradability of the adjuvanted vaccine resulting in prolonged persistence in the tissues and reactive granuloma formation. Such local reaction are of special concern for depot-type adjuvants, such ad aluminum salts, oil emulsions, liposomes, biodegradable polymer microspheres, and living vectors such as BCG. To date, vaccine adjuvants have caused few severe acute systemic adverse effects (Kenny et al.; 2004).

5. Adjuvant combinations

The purpose of adjuvant combinations is to combine various adjuvant components to achieve the desired mix of immunological responses. The best -known adjuvant combination is Freund's complete adjuvant (FCA) which combines the immunomodulatory properties of *Mycobacterium tuberculosis* (essentially TDM and MDP) along with the short-term depot effect of w/o emulsions. This adjuvant generates very strong Th1 and Th2 responses and is especially suited to hydrophilic immunogens. The Ciba-Geigy adjuvant formulation is a modification of FCA which uses a metabolizable oil (squalene) and nor-MDP. It has been used successfully in

clinical trial. Despite the success of w/o formulations as a basis for adjuvant combinations (especially FCA and TiterMax™) they do not normally induce CTL responses and require multiple doses for effective immunization i.e. long-term depots are not established.

Selection of the “best” adjuvant combination requires some knowledge of the chemical nature of the protective immunogen(s) and some idea of the nature of the immune response which is likely to be protective. However, even where knowledge of both these issues is minimal, rational selection of a small number of basic formulations and additives should permit selection of an effective adjuvant system (Cox et al., 1997).

6. Aluminium adjuvant

Aluminium adjuvants have a long history of use with routine childhood vaccines which was found on the discovery that a suspension of alum precipitated diphtheria toxoid had much higher immunogenicity than the soluble toxoid. Aluminium compounds, including aluminium phosphate ($AlPO_4$), aluminium hydroxide ($Al(OH)_3$) and alum precipitated vaccines, historically referred to as protein aluminate, are currently the most commonly used adjuvants with human and veterinary vaccines. These adjuvants are often referred to as “alum” in the literature, which is misleading, because (1) alum, chemically potassium aluminium sulfate ($KAl(SO_4)_2.12H_2O$); (2) aluminium hydroxide and aluminium phosphate have different physical characteristics and differ in their adjuvant properties. Alum was originally used to partially purify protein antigens, mainly tetanus and diphtheria toxoids by precipitating them in the presence of anions including phosphate, sulphate, and bicarbonate ions resulting in a mixture of compounds, mainly aluminium phosphate and aluminium hydroxide (Gupta et al., 1995; Gupta, 1998; Baylor et al., 2002).

6.1 Physico-chemical characteristics

Aluminium in the form of aluminium hydroxide, aluminium phosphate or alum continues to be commonly used as an adjuvant in vaccines. Aluminium hydroxide has been identified as crystalline aluminium oxyhydroxide with a structure of the mineral boehmite. It has high surface area with an isoelectric point (pI) of 11 that is positively charged at physiological pH. In contrast, aluminium phosphate has been classified as amorphous aluminium hydroxyphosphate which is negatively charged at physiological pH (pI = 4-7). In alum-precipitated vaccines, alum is an aluminium hydroxide that contains some sulfate anions as well as anions that are used in the buffer, often phosphate. The pI depends on the precipitation process and is usually in the range of 0.3-0.6. The amorphous nature of aluminium phosphate contributes to high surface area and high protein adsorption capacity, mainly for positively charged proteins. That is the reason for poor adsorption of negatively charged diphtheria toxoid onto aluminium phosphate at neutral pH (Gupta, 1998; Baylor et al., 2002; Matheis et al., 2002; Lindblad, 2004).

6.2 Adjuvant properties

The adjuvanticity of aluminium adjuvants for human vaccines, particularly tetanus and diphtheria toxoids, was clearly established during the 1930's. The major advantage of using aluminium adjuvants was the more rapid development of high titered and long-lasting antibody responses after primary immunization. There are numerous reports in humans and animals showing the superiority of aluminium adsorbed tetanus and diphtheria toxoids over soluble toxoids, particularly after the first dose. Aluminium adjuvants are universally used with diphtheria, tetanus and pertussis (DTP) vaccines, although, the adjuvant effect on whole cell pertussis component is not clear. Although serum agglutinins to *Bordetella pertussis* produced after immunization with aluminium adjuvanted pertussis vaccine were higher than those obtained after inoculation with unadsorbed pertussis vaccine, there was no difference between unadsorbed and adjuvanted pertussis vaccine with regard to protective against disease. In a few studies, the potency of adjuvanted vaccine was higher than the non-adjuvanted pertussis vaccine but in other studies, the adjuvant did

not have any effect. Aluminium compounds are routinely used with the new acellular pertussis vaccines and which have been used with inactivated poliovaccine, human diploid cell strain rabies vaccine, hepatitis B vaccine, hepatitis A vaccine, cholera vaccine and Hib conjugate vaccine.

Aluminium adjuvants have also been widely used with a number of veterinary vaccines, including vaccines against avian infectious bronchitis, canine hepatitis, foot and mouth disease, Newcastle disease, *Bacteroides nodosus*, *Bordetella bronchiseptica, Pasteurella multocida, Leptospira interrogans, Cooperia punctata, Nematospiroides dubius, Trichinella spiralis*. Thus, aluminium adjuvants have wide applications with both human and veterinary vaccines (Gupta, 1998).

HogenEsch (2002) reported that aluminium compounds can further enhance the immune response by direct or indirect stimulation of dendritic cells, activation of complement and by inducing the release of chemokines.

6.3 Adsorption on adjuvanticity

The immunogenicity of antigens adsorbed onto aluminium adjuvants appears to depend on the degree of antigen adsorption and the dose of adjuvant. The formulation which did not show any adsorption of diphtheria toxoid onto aluminium phosphate, due to presence of a ten-fold excess of phosphate, did not elicit an antibody response after the first injection and only a poor response after the second dose. The adsorption is considered to be a very important parameter for the function of these adjuvants (Gupta, 1998). Gupta et al. (1995) reported that the optimal pH for adsorption of tetanus and diphtheria toxoids onto aluminum phosphate was 6.0-6.3. For aluminium hydroxide in neutral aqueous solution such conditions are met with bovine serum albumin (BSA, pI 4.8) that, therefore, is often used as a model protein for determination of the adsorption properties of aluminium hydroxide. For the same reason lysozyme (pI 11) is used as a model protein for aluminium phosphate with a low pI (Matheis et al., 2002).

6.4 Dose of aluminium adjuvants

Aluminium compounds are the only adjuvants used in the manufacture of currently licensed vaccines in the United States Chapter 21 of the US Code of Federal Regulations [610.15(a)] governs the amount of aluminium permitted in the recommended single human dose of a product. The amount of aluminium is limited to no > 0.85 mg/dose if the level is assayed. The regulations were amended in 1981 to increase the permissible level of aluminium to 1.25 mg in biological products to make the regulations consistent with the World Health Organization standards per single human dose of a product. If aluminium compounds other than alum are used, the total amount of alum should not be more than the equivalent permitted as potassium alum (Gupta, 1998; Baylor et al., 2002).

6.5 Elimination

Aluminium is not biodegradable which have been found at site of subcutaneous injection in mice and guinea pigs for up to one year (Gupta, 1998). Keith et al. (2002) found 66-70% of injected aluminium was excreted in 24 hours. In a human study, Keith et al., 2002 reported that the volunteer was injected with 0.7 µg of radioactive ^{26}Al as citrate and followed blood levels and body elimination. They found that over 50% of the aluminium distributed from blood to other body tissues in 15 minutes. Long-term observation using excreta and whole body monitoring founds excretions of >50% in 24 hours, 85% at 13 days, and 96% by 1178 days. Aluminium containing adjuvants which are administered intramuscularly are dissolved in intestinal fluid, absorbed into the blood, distributed to tissues and eliminated in urine (Hem, 2002).

Verdier, Burnett, Habchi, Moretto, Groyne and Sauzeat (2005) investigated the clearance of aluminium at the vaccine injection site and the features of induced histopathological lesions. They concluded that aluminium adjuvanted vaccines administered by the intramuscular route trigger histopathological changes restricted to the area around the injection site which persist for several months but are not associated with abnormal clinical signs.

6.6 Limitations

Aluminium adjuvants cannot be frozen or easily lyophilized as both of these processes cause the collapse of gel resulting in gross aggregation and precipitation. Although tetanus toxoid with collapsed gel precipitates was found to be immunogenic, such a vaccine is not clinically acceptable. Successful lyophilization of aluminium adjuvants was reported but lyophilized vaccines containing adjuvants are not available commercially. The immunogenicity of antigens adsorbed onto aluminium adjuvants depends on several factors; however, the most important is the degree of adsorption of antigen on the adjuvant and the dose of adjuvant. As far as dose, a small amount of adjuvant may be required for complete adsorption. Even though small doses may completely adsorb the antigens, they may not show an optimal adjuvant effect. Excessive amounts of aluminium compounds may suppress immunity by covering the antigen completely with mineral compounds or the aluminium compounds may be cytotoxic to macrophages. Aluminium hydroxide has been demonstrated to have a more potent adjuvant effect than aluminium phosphate which may be due to its higher adsorption capacity and better adsorption of certain antigens at neutral pH. (Gupta, 1998; Lindblad, 2004)

6.7 Safety

Aluminium-containing vaccines have been associated with severe local reaction such as erythema, subcutaneous nodules, contact hypersensitivity and granulomatous inflammation. Some studies with aluminium-adsorbed DTP vaccine have reported fewer reactions than unadsorbed vaccine. Aluminium hydroxide has been reported to attract eosinophils to the injection site, and may increase the levels of antigen-specific and total IgE antibodies that may promote IgE-mediated allergic reactions. On the other hand, aluminium adjuvants have been used for years for hyposensitization of allergic patients without adverse results. There have also been reports, especially in patients with impaired renal function, of systemic accumulation of aluminium, which has been associated with nervous disorders and bone disease. Nonetheless, aluminium intake from vaccines is far less than that received from the diet or medications such as antacids (Baylor et al., 2002).

6.8 Comparative adjuvanticity of aluminium compounds

Aluminium hydroxide has been found to be a more potent adjuvant than aluminium phosphate. This may be due to its overall higher adsorption capacity and better adsorption properties of certain antigens at neutral pH. Aluminium hydroxide adjuvanted antigens induced antibody responses that are comparable to Freund's Complete Adjuvant (FCA). Aluminium hydroxide is a good adjuvant for weak immunogens. Aluminium compounds are also very potent adjuvants for tetanus and diphtheria toxoids in guinea pigs and mice (Gupta, 1998; Baylor et al., 2002)

Shi, HogenEsch, Regnier and Hem (2001) investigated adsorption isotherms of endotoxin and aluminium containing adjuvants at pH 7.4 and 25 °C revealed that aluminum hydroxide adjuvant has a greater adsorption capacity (283 µg/mg Al) than aluminium phosphate adjuvant (3.0 µg/mg Al) and the difference in endotoxin adsorption was related to two adsorption mechanisms: electrostatic attraction and covalent bonding.

6.9 Aluminium content in currently licensed vaccines

As previously stated, the US FDA (21 CFR 610.15(a)) allows no > 0.85 mg/dose of aluminium in vaccines. The currently licensed vaccines use alum, aluminium hydroxide, aluminium phosphate, or a combination of aluminium hydroxide and aluminium phosphate. The total amount of aluminium received from vaccines will vary depending on which brand of vaccine is given. A 1-year-old who receives a complete series of recommended vaccines may receive a minimum of 1.6 mg of aluminium or a maximum of 4.1 mg of aluminium from these vaccines. A 5 — year-old would be exposed to a similar amount of aluminium, 1.9 - 4.9 mg, if the recommended vaccines for this age were received. The amount of aluminium an adult would receive from vaccines varies greatly depending on the number of vaccines given (Baylor et al., 2002).

Table 7 Aluminium content of licensed vaccines (Baylor et al., 2002)

Vaccine	Trade name	Source	Al per dose (μg)	Chemical form of Al	No. of dose in series(mg)	Total Al for series
Childhood vaccines						
DTaP	Infanrix	SKB	≤ 625	Hydroxide	5	3.1
	Certiva	NAVA	500	Hydroxide	5	2.5
	Acelimune	Lederle	230	Hydroxide/ Phosphate	5	1.2
	Tripedia	Avent. Past Inc.	≤ 170	Alum	5	0.85
DTP	-	Bioport	≤ 600	Phosphate	5	3.0
	-	Avent. Past Inc.	≤ 170	Alum	5	0.85
Hib conjugate	Liq. Pedvax Hib	Merck	225	Hydroxide	3	0.68
Pneumo conjugate	Prevenar	Lederle	125	Phosphate	3	0.38
DTP-Hib	Tetramune	Lederle	≤ 850	Hydroxide	4	3.4
Hep B-Hib	Comvax	Merck	225	Hydroxide	3	0.68
Hep B	Recombivax B	Merck	225	Hydroxide	3	0.68
	Engerix B	SKB	250	Hydroxide	3	0.75
DT, adsorbed	-	Avent. Past Inc.	≤ 170	Alum	5	0.85
	-	MPHBL	450	Phosphate	5	2.3
	-	Bioport	≤ 600	Phosphate	5	3.0
	-	Lederle	≤ 800	Phosphate	5	4.0
	-	Wyeth	≤ 850	Phosphate	5	4.3
Adult vaccines (some may also be indicated for younger age groups)						
T, adsorbed	-	Lederle	≤ 850	Phosphate	6	5.1
	-	MPHBL	450	Phosphate	6	2.7
	-	Wyeth	≤ 850	Phosphate	6	5.1
	-	SSVI	≤ 850	Phosphate	6	5.1
	-	Avent. Past Inc.	≤ 250	Alum	6	1.5
Td, adsorbed	-	Lederle	< 800	Phosphate	6	4.8
Td, adsorbed	-	Wyeth	≤ 850	Phosphate	6	5.1
	-	PHBL	450	Phosphate	6	2.7
	-	Avent. Past Inc.	≤ 280	Alum	6	1.7
Hep A	Havix	SKB	250	Hydroxide	2	0.5
	VAQTA	Merck	450	Hydroxide	2	0.9
	VAQTA	Merck	225	Hydroxide	2	0.45
Lyme	Lymerix	SKB	≤ 500	Hydroxide	3	1.5
Anthrax	Lymerix	BioPort	≤ 830	Hydroxide	6	5.0
Rabies	RabAvent	BioPort	442	Phosphate	5	2.2

Analysis of aluminium content of aluminium hydroxide gel has been reported employing various methods such as chelatometric titration (Nail et al., 1976; Masood, White and Hem, 1994; Burrell et al., 2000; Chang et al., 2001; BP 1998; USP 25) inductively coupled plasma (ICP) spectroscopy (May et al., 1984; Burrell et al., 2001) and atomic adsorption spectrometry (AAS) (May et al., 1984; Rinella et al., 1998; Lindblad, 2004).

VIII. Adsorption process for combined vaccines

The term adsorption describes attractive interactions at surfaces without formation of covalent chemical bonds. If covalent bonds are formed, the process is called chemisorption. In the case of vaccine production, adsorption means the attraction between antigens dissolved in the medium and colloidal dispersed adsorbent particles (aluminium hydroxide or aluminium phosphate). Two primary mechanisms of antigen adsorption to aluminium containing adjuvants are the electrostatic attractive force and ligand exchange, and the adsorption mechanism of hepatitis B surface antigen (HBsAg) is predominantly due to ligand exchange (Morefield, Jiang, Romero-Mendez, Geahlen, HogenEsch and Hem, 2005). Electrostatic as well as hydrophobic interactions play an important role in this process (Matheis, Zott and Schwanig, 2002).

Iyer, HogenEsch and Hem (2003) investigated the effect of the degree of phosphate substitution in aluminium hydroxide adjuvant on the adsorption of phosphorylated proteins. The phosphorylated proteins (alpha casein, dephosphorylated alpha casein and ovalbumin) were adsorbed by ligand exchange of phosphate for hydroxyl even when an electrostatic repulsive force was present.

Iyer, Robinett, HogenEsch and Hem (2004) studied the mechanism of adsorption of hepatitis B surface antigen (HBsAg) by aluminium hydroxide adjuvant. The adsorption of HBsAg by aluminium hydroxide adjuvant exhibited a high affinity adsorption isotherm. The relatively high value of the adsorptive coefficient indicated that adsorption was due to a strong attractive force. Ligand exchange between a phosphate of the antigen and a surface hydroxyl of the adjuvant provided the strongest

adsorption mechanism. The adsorption capacity of HBsAg was not affected by increased ionic strength indicating that electrostatic attraction was not the predominant adsorption force.

Al-Shakhshir, Regnier, White and Hem (1994) examined the effect of adsorbing two model proteins, BSA and lysozyme, on the point of zero charge of aluminium-containing vaccine adjuvatns. At physiological pH, the adsorption of the negatively charged albumin (pI = 5.0) by aluminium hydroxide adjuvant resulted in a decrease in the point of zero charge. In contrast, the adsorption of positively charged lysozyme (pI = 9.6) by the negatively charged aluminium phosphate adjuvant resulted in an increase in the point of zero charge. The surface charge characteristics of the aluminium containing adjuvants dominated at low protein coverage. In contrast, the surface charge characteristics of the adsorbed protein dominated at high protein coverage.

Al-Shakhshir et al. (1995) investigated the effect of ionic strength on the adsorption of BSA or lysozyme by a commercial aluminium hydroxide or aluminium phosphate which was studies at pH 7.4 and 25 °C. The adsorption of BSA by aluminium hydroxide adjuvant and lysozyme by aluminium phosphate adjuvant was found to be inversely related to ionic strength. This indicates that electrostatic attractive forces contribute to adsorption.

Seeber, White and Hem (1991) studied the adsorption of two model proteins, albumin and lysozyme, by boehmite or amorphous aluminium hydroxyphosphate adjuvants. Electrostatic attraction has a major role in adsorption. At physiological pH, boehmite, which has a point of zero charge above 7.35, extensively adsorbed albumin but was not effective in adsorbing lysozyme. Conversely, amorphous aluminium hydroxyphosphate was effective in adsorbing lysozyme but adsorbed relatively little albumin. The results suggested that the selection of either boehmite or amorphous aluminium hydroxyphosphate as an adjuvant should be based in part on the isoelectric point of the antigen.

The adsorptive characteristics of recombinant protective antigen (rPA) and two aluminium containing adjuvants were examined in a physiological buffer with and without EDTA. It was predicted and demonstrated that rPA bound in a more efficient manner to aluminium hydroxide adjuvant than to aluminium phosphate adjuvant in the physiological buffer and the binding of the rPA to the aluminium hydroxide was decreased by increased amounts of phosphate in the buffer. These data suggested that the interaction between rPA and aluminium hydroxide was predominantly electrostatic in character (Jendrek, Little, Hem, Mitra, and Giardina, 2003).

Interactions between surfaces are strongly dependent on the surface charges of the interacting partners. Overall surface charges are quantified by measurement of the pI. This parameter is defined by the pH-value at which the overall surface charges are zero. For proteins the pI is determined by the motion of the molecule through a pH-gradient in an isoelectric focusing experiment (Matheis et al., 2002; Vogel and Hem, 2004).

Rinella, White and Hem (1996) and Wittayanukulluk, Jiang, Regnier and Hem (2004) reported that pretreatment of aluminium hydroxide adjuvant with carefully selected concentrations of phosphate anion reduces the positive surface charge which exists at pH 7.4 and treatment with higher concentrations of phosphate anion produces a negative surface charge.

Interactions between proteins and adsorbents are optimal, when their pIs differ and the pH of the medium is in between. In this case, protein and adsorbent have opposite surface charges. The diphtheria toxoids and tetanus toxoids show pI below pH 7. Therefore, the adsorbent aluminium hydroxide should be the best choice for these antigens (proteins). In some licensed vaccine preparations they are adsorbed on aluminium phosphate or on both adsorbents. Adsorption experiments with purified diphtheria toxoids or tetanus toxoids are used in vaccines, showed complete adsorption of these toxoids on aluminium hydroxide. In the case of aluminium phosphate only partial adsorption is observed, even with toxoids concentration as low as in administered vaccine (Gupta, 1998; Matheis et al., 2002; Vogel, 2004).

Gupta et al. (1998) reported that the pH and ionic strength affect adsorption by altering charge on the gel and the antigen, whereas the temperature may affect the rate of interaction between the gel and the antigen. Size of gel particles affects the surface area of gel available for adsorption: small particles have more surface area than large particles. In addition, adsorption of diphtheria toxoid resulted in higher adsorption than commercial aluminium phosphate preparation, probably due to trapping of some antigen in the gel.

Sepelyak et al. (1984) investigated a very acidic protein, pepsin (pI = 1) and its interaction with aluminum hydroxide gel [PZC = 9.4]. Over the pH range 1-10 maximal adsorption was observed at approximately pH 4.0. Infrared spectra taken of pepsin adsorbed to aluminum hydroxide suggested that a carboxylate ligand exchange reaction with the positively charged aluminum surface sites was the dominant adsorption mechanism in this case.

The study of Jiang et al. (2004) showed that calcium phosphate adjuvant which is a commercially available vaccine adjuvant identified commercial calcium phosphate adjuvant as non-stoichiometric hydroxyapatite. The surface charge was pH-dependent. It exhibited a negative surface charge at physiological pH and electrostatically adsorbed positively charged antigens. The presence of hydroxyls allows calcium phosphate adjuvant to adsorbed phosphorylated antigens by ligand exchange with surface hydroxyls.

Skea et al. (1993) examined the adhesion-mediated enhancement of the adjuvant activity of alum. Alum fails to adsorb influenza virus haemagglutinin (BHA) and is a poor adjuvant for this antigen. A specific monoclonal anti-BHA antibody adsorbed to alum promoted adhesion of the antigen to the adjuvant. The “alum-anti BHA- BHA” complex was found to be 1500-fold more immunogenic in mice and 5-fold more immunogenic in rabbits than a mixture of alum and BHA lacking the anti-BHA antibody. It indicated that the adjuvant activity of alum could be markedly enhanced by promoting a physical association between the antigen and the adjuvant.

DePaz et al. (2005) studied the adsorption mechanisms of heavy chain fragments of botulinum neurotoxin serotypes A and B. The serotype A antigen is basic, and pretreatment with phosphate anions was required for favorable adsorption conditions to aluminium hydroxide adjuvant. In contrast, the serotype B antigen displayed the high affinity to aluminium hydroxide and adsorbed to aluminium hydroxide by a ligand exchange mechanism.

The adsorption/elution behavior could be explained by the effect of pH on: (1) the ionization state of the protein, (2) the solubility of the adjuvant, and (3) the electrostatic interaction between the protein and adjuvant (Rinella, White and Hem, 1998).

1. Factor affecting adsorption (Matheis et al., 2002)

1.1 pH value

The pH value of the medium determines the real surface charge of antigen and adsorbent and, therefore, is important for the adsorption process. Practical experience showed that best results for adsorption could be obtained, when the pH is set near the pI of antigen. In this case, the interactions of antigen molecules are minimized and interaction with the adsorbent is effective. For long lasting adsorption the final pH should be between the pIs of antigen and adsorbent, respectively. If an antigen is already adsorbed and the adsorbed amount is not close to the maximum possible, small pH changes will not cause desorption. Therefore, in vaccine production the adsorption process for acidic antigens like diphtheria toxoids and tetanus toxoids be carried out at a pH below 7. For the final formulation the pH can be adjusted to a physiologically advantageous value around pH 7.

Gupta et al. (1998) reported that formulation of DTP vaccine with aluminium phosphate is usually done at pH close to 6.0 to allow maximum adsorption of diphtheria toxoid.

1.2 Ions

There are several ions in vaccine formulation that influence the adsorption capacity if they are present before, and may lead to desorption if added after adsorption. For example, highly charged ions like phosphate, sulphate, carbonate or citrate compete with antigens for adsorption sites. Thereupon the surface charges of the adsorbent shifts to values that are unfavorable for adsorption of negative charged antigens and proteins.

The presence of phosphate and citrate leads to desorption of antigens. Therefore, these ions are used at high pH values for identity test of adsorbed vaccines. Ions like chloride and nitrate are adsorbed only weekly. With respect to adsorbed vaccines phosphate and chloride are important, because they are widely used in vaccine formulations and accepted for medical treatment (phosphate buffered media and saline).

Shi, HogenEsch and Hem (2002) studied the ability of intestinal fluid to change the degree of adsorption of ovalbumin to aluminium hydroxide adjuvant or lysozyme to aluminium phosphate. Double distilled water or 0.9% NaCl did not alter the degree of adsorption while citrate at pH 7.4 and phosphate at pH 7.4 reduced the degree of adsorption after a 4 hours exposure period 75 and 45%, respectively. Dilution with sheep lymph fluid reduced the degree of adsorption 25 or 5% after exposure periods of 15 minutes or 4 hours, respectively.

1.3 Aging

Aging or maturation is a well know phenomenon in protein adsorption. The term aging is used here for the observation that protein adsorbates are stabilized by time. During the aging process the adsorbed proteins becomes more inert towards desorption. For model proteins this effect is attributed, e.g. to conformational changes during or after adsorption, to the reorientation of primarily end-on to side-on oriented molecules. For all these cases, the number of contacts between proteins adsorbent is increased during aging, and enthalpic or entropic favourable changes are the driving

forces for these process. In the cases of adsorbed vaccine especially the adsorbed components of diphtheria and tetanus vaccines maturation goes along with an increase in potency. It takes up to several months until equilibrium is reached and a product with constant high potency is formed. This process became important since the introduction of the acellular pertussis vaccines. The lack of the adjuvant effect from the whole cell pertussis components in DTP vaccines leads to a reduced potency with respect to diphtheria and tetanus in DTaP vaccines.

Nail, White and Hem (1976) investigated the aging of aluminium hydroxide gels precipitated at pH 7.0. This process results in larger particles, which were more highly ordered and resistant to attack by acid.

Burrell, Lindblad, White and Hem (1999) examined the stability of aluminium containing adjuvant to autoclaving, physical properties which were related to surface area such as protein adsorption capacity, rate of acid neutralization at pH 2.5 and point of zero charge also decrease during autoclaving for 30 or 60 minutes at 121 °C. The results suggest that autoclaving procedures which minimize exposure time to elevated temperature should be used and procedures requiring repeated autoclaving of the same sample should be avoided.

Burrell, White and Hem (2000) tested the stability of aluminium containing adjuvants during aging at room temperature, aluminium phosphate adjuvant and aluminium hydroxide adjuvant had the statistically significant decrease in adsorption capacity which was accompanied by a decrease in surface area.

Shi et al. (2002) studied the ability of intestinal fluid to change the degree of adsorption of ovalbumin to aluminium hydroxide adjuvant or lysozyme to aluminium phosphate. The results of this study indicated that the degree of adsorption upon exposure to intestinal fluid depends on the specific protein and the age of the vaccine.

IX. Immunoassay

Immunoanalytical methods, which are based on the binding of small molecules (drugs) or macromolecules by biologically derived antibodies, have revolutionized the field of biomedical analysis. Immunoassays have allowed the determination of picomolar amounts of analytes that could not be assayed in biological matrices by other techniques. They are therefore, providing information that is essential to the understanding of many biological processes. Since the original work on the analysis of insulin, immunoassay methods have been developed for the determination of a wide variety of drugs, pesticides, hormones, and biological proteins. Immunoassays are not only powerful techniques in terms of sensitivity and specificity but are also relatively simple procedurally. This has led to the development of many kit-type immunoassay systems used routinely in automated clinical laboratories.

The antibodies used as reagents in immunoassays are generally molecules of the immunoglobulin G (IgG) type. These molecules are heterogeneous, bifunctional glycoproteins in which the variable amino acid sequence in the polypeptide component provides its biologic activity. This polypeptide component is made up of two heavy or H chains (50,000 Daltons) and two light or L chains (20,000 Daltons), held together by disulfide bonds. The two binding site of the antibody molecule appear to reside on the NH_2 – terminal ends of the polypeptide chains. They are produced by white blood cells in response to foreign substances introduced into mammalian species. These glycoproteins have the unique property of combining specifically with the substances (antigens) that elicited their formation. This then triggers processes by which the foreign antigens are cleared from the organism, which is the ultimate goal of the immune process.

All immunoassay procedures take advantage of the specific reactions between antibodies and antigens. They involve measurement, directly or indirectly, of the extent of binding between antibodies (reagents) and antigens (analytes). Labels are used in conjunction with the antigens or antibodies in such a way that the concentrations of molecular species can be measured instrumentally. Labels are

chemical entities that impart some measurable signal such as radioactivity, fluorescence, or enzyme activity to the antibody or antigen to which it is attached. The extent of antibody binding is determined by measuring the amounts of labeled antigen or antibody in the complexed (bound) and in the free forms. This is generally expressed as the bound/free (b/f) concentration ratio which is related to the concentration of analyte. The measured signal can be directly or inversely proportional to the b/f ratio, depending on the chemistry of the system (Swarbrick and Boylan, 1993).

1. Enzyme Immunoassay

Quantitation is usually effected by the measurement of spectroscopic properties derived from an enzymatically transformed substrate. The importance of this discovery is reflected in the now widespread application of enzyme-substrate signals for the detection and measurement of soluble antigens, with an attained sensitivity that approaches that of a radioimmunoassay. Today this technology is used in a wide variety of enzyme-based systems both in research and routine analysis. Enzyme immunoassays (EIAs) can be divided into two major classes: homogeneous and heterogeneous immunoassay systems.

1.1 Homogeneous Methods

Homogeneous immunoassay (HOIA) does not require physical separation of the free antigen and antibody-bound antigen because the measured physical signal derived from the antibody-bound, labeled material may by significantly different from that of the unbound entity. There may be an enhancement or an inhibition of enzyme activity upon the binding of Ab to Ag. Homogeneous immunoassay methods are simple to perform and easily automated. Elimination of the separation step avoids a major source of imprecision, but may compromise selectivity since interfering substances are not eliminated in the separation step.

1.1.1 Homogeneous Immunoassays Using Enzyme-Labeled Antigen

Rubenstein et al. described an HOIA method for morphine using lysozyme as the enzyme label. The covalent enzyme-labeled antigen (Ag^E) competes with sample antigen (Ag) for a limited concentration of antibody (Ab) to form a complex. The resultant complex exhibits very little enzyme activity because of steric hindrance or allosteric inhibition caused by the bound antibody. In the presence of Ag there is competition for the Ab, leaving more Ag^E uncomplexed and free to catalyze the conversion of substrate to product. Thus, the enzyme activity which can be measured by the appearance of product (P) or disappearance of substrate (S) is directly proportional to the amount of free antigen in the sample.

1.1.2 Homogeneous Immunoassays Using an Antigen-Labeled Enzyme Modulator

This method is based on the ability of an antigen labeled with an enzyme modulator (Ag^M) to modulate the activity of an indicator enzyme. The Ag^M competes with free antigen (Ag) for a limited amount of antibody (Ab). On binding with Ab the Ag^M is unable to modulate the activity of the indicator enzyme. As the concentration of the analyte increases, it competes successfully for binding sites on the antibody, leaving more Ag^M free to complex with indicator enzyme, thereby modulating its activity. Based on this principle, practical assays for human serum thyroxine and theophyline have been developed; three distinct classes of modulators have been investigated.

1.1.3 Homogeneous Immunoassay Using an Antigen Labeled with a Fluorogenic Enzyme Substrate

The antigen is linked to a fluorogenic enzyme substrate (Ag^{FS}) to form a stable conjugate, which competes with the sample antigen (Ag) for a limited concentration of antibody (Ab). The antigen-conjugated substrate is a fluorogenic substrate for the enzyme which reacts only when it is not bound to the Ab. Thus, at

high concentrations of sample antigen, more of the Ag^{FS} would remain free to act as the substrate for the enzyme (E) and more products would be formed. Thus, fluorescence intensity increases with increasing concentration of the sample antigen. A derivative of umbellifery-β-galactoside serves as a flourogenic substrate for *E. coli* β-galactosidase in this system, and solid-phase reagent strips based on this method have been developed.

1.1.4 Homogeneous Immunoassay Using Liposome - Trapped Enzyme

In this technique, the antigen is labeled with a cytolysin (Ag^{CYT}) capable of lysing a cell membrane or a liposomal membrane. The lytic activity is, however, lost when the conjugate binds to an antibody (Ab) specific for the antigen. The Ag^{CYT} conjugate competes with sample antigen (Ag) for antibody complex formation. An increase in sample antigen concentration, therefore, leads to increased displacement of Ag^{CYT}, leaving the cytolysin conjugate free to lyse the enzyme-containing liposome (L), and thereby releasing more enzyme (E) to form product (P). The enzyme activity is directly proportional to the amount of free sample antigen.

1.2 Heterogeneous Methods

Heterogeneous immunoassays (HEIA) have at least one separation step which allows the differentiation of bound from free material. The enzyme-linked immunosorbent assay (ELISA), is a heterogeneous immunoassay which has been extensively reviewed; either antigen or antibody is immobilized on a solid phase. An essential difference from HOIA is that in HEIA the enzyme label is designed to retain its activity even after its reaction with the antibody. These ELISA typically demonstrate accuracies of 95 to 110% with relative standard deviations (RSD) of 5 to 20%; these values are more than adequate for their intended purpose. The relative lack of control of enzyme-labeling reactions is a limitation of this technique, however, when compared to radiolabeling procedures. In an addition, it is sometimes difficult to purify enzyme-labeled substances.

Heterogeneous methods can be divided into competitive and noncompetitive assays.

1.2.1 Competitive Assays

1.2.1.1 Enzyme-Labeled Antigen Conjugate

The enzyme-labeled antigen (Ag^E) competes with sample antigen for a limited amount of antibody which has been immobilized on a solid phase, for example, polystyrene (Ab^{SP}). After incubation, the unbound Ag^E is separated by washing with a detergent solution. The solid-phase Ab^{SP}, containing bound labeled and unlabeled antigen, is incubated with a substrate (S), and the product concentration is determined with a colorimeter or fluorimeter. The enzyme activity or product concentration is inversely proportional to the concentration of sample antigen. With this competitive method, picogram quantities of hormones and other substances can be measured accurately.

1.2.1.2 Enzyme-Labeled Antibody

This assay employs enzyme-labeled antibody (Ab^E), and the antigen is attached to the solid phase (Ag^{SP}). The binding of Ag^{SP} to Ab^E is competitively decreased by the addition of sample Ag. The enzyme activity is inversely proportional to the concentration of sample Ag. Human IgG at the picomole level has been quantified in less than 1.5 h with this method.

1.2.2 Noncompetitive Assays

1.2.2.1 Enzyme-Labeled Antigen

The sample is first incubated with a moderate excess of solid-phase immobilized antibody (Ab^{SP}). After washing, excess enzyme-labeled antigen (Ag^E) is allowed to bind to unreacted Ab^{SP}. The enzyme product concentration is inversely proportional to the concentration of standard or test antigen.

1.2.2.2 Enzyme-Labeled Antibody

The sample antigen (Ag) is incubated with a moderate excess of enzyme-labeled antibody (Ab^{E}). The mixture is added to an excess of immobilized antigen (Ab^{SP}) to remove unreacted Ab^{E}. The enzyme activity is inversely proportional to the concentration of sample and the procedure has been used to measure α-fetoprotein.

1.2.2.3 Sandwich or Double Antibody

This method is used with antigens having multiple antibody-binding sites (epitopes). Immobilized unlabeled antibody (Ab^{SP}), in excess, is incubated with sample or antigen. After washing, the antibody-antigen complex is incubated with an excess of enzyme-labeled antibody (Ab^{E}) which binds to one or more antigenic sites to form a sandwich-type complex. In this case, the concentration of enzyme product is directly proportional to the concentration of sample antigen.

Sandwich-type assay is well suited for quantifying antigens with multiple antigenic determinants, such as antibodies, rheumatoid factors, a polypeptide hormone, proteins, and hepatitis-B surface antigens. The results obtained are comparable to those obtained with radiolabels in terms of precision, convenience, and sensitivity. Macromolecular antigens at attomole levels have been quantified with this technique.

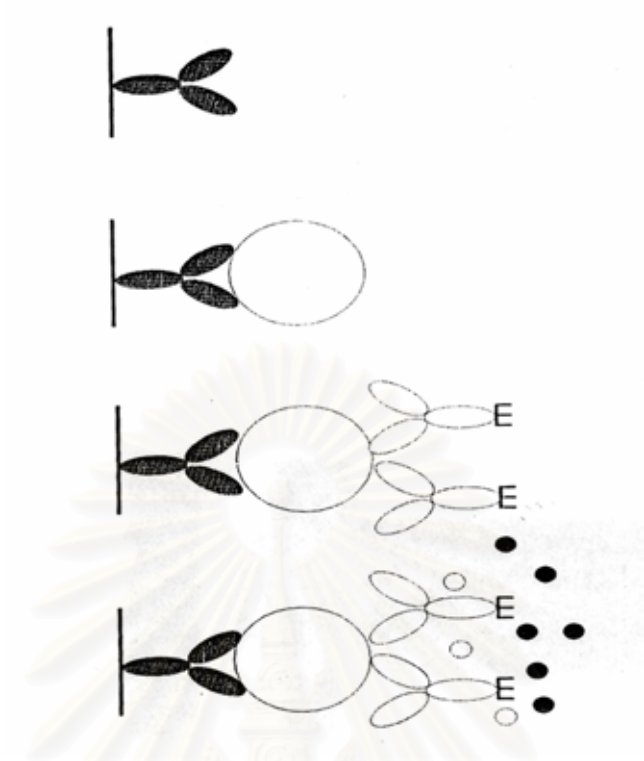


Figure 3 Sandwich ELISA (Crowther, 1996).

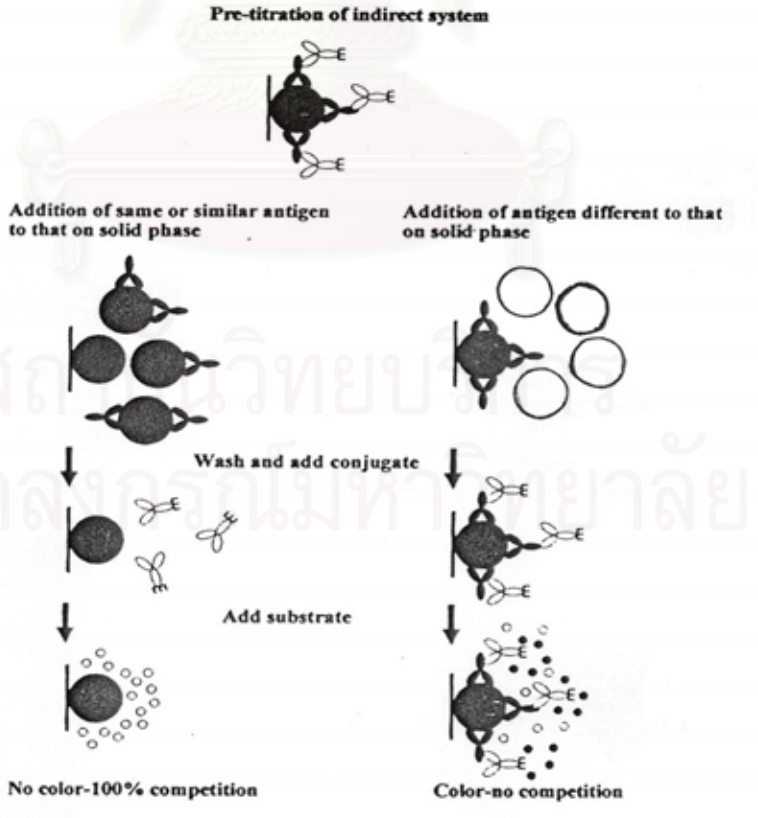


Figure 4 Competition ELISA (Crowther, 1996).

CHAPTER III
EXPERIMENTAL

Materials

All chemicals and reagents were analytical or pharmaceutical grades and were used as received.

1. Aluminium hydroxide gel (Bureau of Veterinary Biologic, Department of Livestock Development, Thailand)
2. Aluminium phosphate gel (Lot R3-46/00691, Government Pharmaceutical Organization, Thailand)
3. Ammonium acetate (Lot F3B273, Ajax Chemicals, Australia)
4. Ammonium hydroxide (Lot 1336-21-6, J.T. Baker, USA)
5. Anti-Beijing mouse IgG (JE) (Lot pool A1+A3, , Government Pharmaceutical Organization, Thailand)
6. Bicinchoninic acid disodium AR (BCA, Lot 053K532V, Sigma, USA)
7. Bicinchoninic acid kit (Sigma, USA)
8. *Bordetella pertussis* antibody (Lot 9L36401, Biodesign International, USA)
9. Citric acid AR (Lot 0086978, Fisher Scientific, UK)
10. Diphtheria Toxoid (Lot DV46002, Government Pharmaceutical Organization, Thailand)
11. Diphtheria antibody (Lot 1H21303, Biodesign International, USA)
12. di-Sodium hydrogen phosphate AR (Lot FZG096, Ajax Chemicals, Australia)
13. Dithizone AR (Lot 434553/1, Fluka, Switzerland)
14. Etylenedinitrilo (EDTA) AR (Lot 7727 KMTP, Mallinckrodt Chemical, USA)
15. Gelatin (medium gel strength) (Lot 424331/1, Fluka, Switzerland)
16. Glacial acetic acid (Merck, Germany)
17. Horseradish peroxidase-goat anti-mouse IgG (H+L) conjugate (Lot 40286774, Zymed Laboratories, USA)
18. Horseradish peroxidase-labeled anti-Beijing mouse IgG (Lot 181202, Government Pharmaceutical Organization, Thailand)

19. Hydrochloric acid solution 37%, AR grade (Labscan Asia Co., Ltd., Thailand)
20. Hydrogen peroxide 30% AR (Lot 749938D8, Panreac Quimica SA, Spain)
21. Japanese Encephalitis virus antigen (Lot JVJ48013 , Government Pharmaceutical Organization, Thailand)
22. Japanese Encephalitis virus reference antigen (Lot 197, Government Pharmaceutical Organization, Thailand)
23. O-Phenylenediamine (Lot 40788994, Zymed Laboratories, USA)
24. Potassium chloride AR (Lot F1G253, Ajax Chemicals, Australia)
25. Potassium dihydrogen phosphate AR (Lot F1F125, Ajax Chemicals, Australia)
26. Rabbit anti-horse IgG (whole molecule) peroxidase conjugate (Lot 034K4780, Sigma, USA)
27. Rehydragel HPA (Lot 4122401, Reheis Incorporated, USA)
28. Sodium carbonate AR (Lot 479307, Carlo Erba, Italy)
29. Sodium chloride AR (Lot F2C273, Ajax Chemicals, Australia)
30. Sodium ethylmercurithiosalicylate (thimerosol) AR (Lot 436657/1, Fluka, Switzerland)
31. Sodium hydrogen carbonate AR (Lot AF 310196, Ajax Chemicals, Australia)
32. Sodium hydroxide pellets AR (Lot B131198 214, Merck, Germany)
33. Sodium tartrate dihydrate dibasic AR (Lot 3176, Riedel-deHaën, Germany)
34. Sulfuric acid (Merck, Germany)
35. Tetanus antitoxin (Lot TC44002, Government Pharmaceutical Organization, Thailand)
36. Tetanus Toxoid (Lot 063, Government Pharmaceutical Organization, Thailand)
37. Tween 20 AR (Lot 448707/1, Fluka, Switzerland)
38. Whole cell *Bordetella pertussis* antigen (Lot PV45006, Government Pharmaceutical Organization, Thailand)
39. Zinc chloride (Lot F3D341, Ajax Chemicals, Australia)
40. Zinc sulfate AR (Lot F2K005, Ajax Chemicals, Australia)

Instruments

1. Analytical balance (Mettler Toledo AG204, USA)
2. Atomic force microscope (SPA 400-DFM, Seiko Instruments Inc., Japan)
3. Autoclave (Hirayama MFG. Corp., Tokyo, Japan)
4. Centrifuge (centrifuge 5810, Eppendorf, Germany)
5. Fourier transform Infrared spectrometer (model 1760X, Perkin Elmer, USA)
6. Freeze dryer (model FD-6-850MP0, Dura - Dry™, FTS System, Inc., USA)
7. Inductively coupled plasma atomic emission spectrometer (Plasma-1000, Perkin Elmer, USA)
8. Laser diffractrometer (Mastersizer2000, Malvern Instruments, UK)
9. Magnetic stirrer (Variomax multipoint, Komet, Taiwan)
10. Micropipet (Socorex, Switzerland)
11. Microplate reader (VICTOR^3, Perkin Elmer, USA)
12. Multichannel pipet (Socorex, Switzerland)
13. Optical microscope (E200, Nikon Eclipse, Japan)
14. pH meter (model 210, Thermo Orion, USA)
15. Photon correlation spectrophotometer (Zetasizer nanoseries, Nano-ZS, Malvern Instruments, UK)
16. Powder X-ray diffractrometer (JDX-8030, JEOL, Japan)
17. Scanning electron microscope (JSM-5410LV, JEOL, Japan)
18. Shaking Incubator (Lab Tech, USA)
19. Shaking water bath (PolyScience, USA)
20. Transmission electron microscope (JEM-1230, JEOL, Japan)
21. UV-VIS spectrophotometer (Jasco V-530, Schimidzu, Japan)
22. Vortex mixer (model G-560E, Scientific Industries, USA)

Methods

All antigens were used as received, while aluminium adjuvants were prepared as stock solution. Prior to the preparation of the adjuvant stock solution, the content of aluminium in each adjuvant had to be determined.

1. Aluminium content assay

1.1 Aluminium hydroxide adjuvant

Aluminium hydroxide (AH) was assayed for aluminium content by back titration method as aluminum hydroxide gel (USP 25). Briefly, the gel of about 1.5 g of AH and hydrochloric acid were gently heated until solution was completed. After cooling, the solution was diluted with water to 500 ml. The solution of 20 ml was added with 25 ml of edetate disodium titrant and 20 ml of acetic acid-ammonium acetate buffer TS. After heating to near the boiling point for 5 minutes, the solution was cooled and added with 50 ml of alcohol, 2 ml of dithizone TS, and finally, titrated with 0.05 M zinc sulfate VS until the color changed from green-violet to rose-pink.

1.2 Aluminium phosphate adjuvant

Aluminium phosphate (AP) was assayed for aluminium content by back titration method as dried aluminium phosphate (BP 1998). Briefly, 10 ml of AP of 0.008 g/ml in 2 M hydrochloric acid was added with 25 ml of 0.05 M edetate disodium VS, and then 13.5 M ammonia was added dropwise until the solution was just alkaline to litmus paper. After boiling gently for 5 minutes, cooling, the solution was added with 0.154 g/ml of ammonium acetate solution, 6 ml of glacial acetic acid and sufficient water to produce 100 ml. The pH of solution was adjusted to 4.5 with glacial acetic acid and added with 2 ml of 0.025% dithizone in ethanol (96%). Then, ethanol was added to double the volume and the solution was titrated with 0.05 M zinc chloride VS until the color changed to red.

2. Separation of unadsorbed antigens by centrifugation

This examination was conducted in order to choose the speed of centrifugation to distinguish the adsorption of single antigen on aluminium containing adjuvants. The speeds of centrifugation were performed at various rpm, each for 20 minutes to determine the aluminium content in the supernatant of solution. Aluminium stock solution as pipetted into microtube to produce aluminium content of about 0.6 mg aluminium/ml (from preliminary observation of the optimal aluminium content which could be analyzed by BCA method). Then aluminium preparations were centrifuged 20 minutes by Centrifuge®. AH was centrifuged at various rpm, 200, 600, 1,000, 1,400, 1,800, 2,200, 2,600, 3,000 and 3,400 rpm and AP was centrifuged at 200, 500, 800, 1,100, 1,400, 1,700, 2,000, 2,300, 2,600, 2,900 and 3,200 rpm. Preliminary experiments indicated that the supernatant was clear around 3,000 rpm by visual inspection. After that the supernatant was taken to assay the amount of aluminium which remained in the supernatant by inductively coupled plasma spectroscopy (ICP) (Burrel, et al., 2001).

3. Preparation of aluminium containing adjuvant stock solutions

AH and AP stock solution were prepared to have aluminium content of 0.6 mg aluminium/ml in 0.01 M phosphate buffer of various pHs. Separately, AH and AP gels were accurately weighed equivalent to about 0.6 mg aluminium/ml in beaker and diluted with 0.01 M phosphate buffer. The final pHs were adjusted to required pHs by the addition of hydrochloric acid or sodium hydroxide. The solution was transferred to volumetric flask and adjusted volume to 25 ml with 0.01 M phosphate buffer of each pH.

4. Adsorption of single antigen on adjuvants

Since there was no difference between unadsorbed and adjuvanted pertussis vaccine with regard to protection against disease. The adjuvant effect of AP or AH on the mouse intracerebral potency of whole cell pertussis vaccine is controversial (Gupta et al., 1993; Gupta et al., 1998). Therefore, in this experiment, adsorption of

Bordetella pertussis on both aluminium containing adjuvants was not performed and studied.

For other antigens, diphtheria, tetanus, JE, various adsorption variables were investigated.

(A). Effect of type of aluminium containing adjuvants

Aluminium containing adjuvants, which were chosen to adsorb with diphtheria toxoid, tetanus toxoid and JE antigen, were AH and AP.

(B). Effect of pH on adsorption

The adsorption was performed at pH 6.0, 6.5, 7.0 and 7.4 of 0.01 M phosphate buffer.

(C). Effect of temperature on adsorption

The processing temperatures were 9 $\pm$1 °C in a shaker bath and 37 $\pm$ 1 °C in a shaking incubator. The adsorption at 9 $\pm$ 1 °C was investigated because it was the true temperature for vaccine production while at 37 $\pm$ 1 °C was conducted in order to examine the effect of temperature on the adsorption capacity. So if the antigen could adsorb on adjuvant at 37 °C, the adsorption at temperature lower than 37 °C, room temperature, could be occurred.

4.1 Adsorption procedure

AH and AP 200 µl was taken for triplicate assay from each pH aluminium stock solution and transferred to microtube. The required amount of phosphate buffer with the same pH, diphtheria toxoid, tetanus toxoid or JE antigen (as listed in Table 8) were eventually added in the microtube and swirled with vortex immediately. Then the preparations were mixed at investigated temperature, 170 rpm, 30 minutes. After that the preparations were centrifuged at the optimal speed (from section 2) for the

separation of the aluminium particles so the supernatant was collected to assay the protein content by BCA or micro BCA method (Seeber et al., 1991; Masood et al., 1994; Shakhshir et al., 1994; Shakhshir et al., 1995; Rinella et al., 1996; Heimlich et al., 1999; Shi et al., 2002; Morefield et al., 2005).

Table 8 The composition of adsorbed preparation

Aluminium adjuvant (µl)	Antigen (µl)	Phosphate buffer (µl)
200	100	1,200
200	300	1,000
200	500	800
200	700	600
200	900	400
200	1,100	200
200	1,300	-

The adsorption value of adsorbed antigen was calculated from the amount of free antigens which remained in the supernatant after centrifugation.

4.2 Protein analysis

BCA and micro BCA assay

The bicinchoninic acid (BCA) and micro BCA assay is a colorimetric assay for total protein. It is based on chemical principle similar to those of the biuret and Lowry assays but the Micro BCA assay has been an extremely sensitive and optimized for use with dilute protein samples. For BCA, Standard Working Reagent (S-WR) is prepared by mixing 100 volume of Reagent A, consisting of an aqueous solution of 1% BCA-Na_2, 2% $Na_2CO_3.H_2O$, 0.16% Na_2 tartrate, 0.4% NaOH and 0.95 % $NaHCO_3$, with 2 volume of Reagent B, consisting of 4% $CuSO_4.5H_2O$ in deionized water. For micro BCA, Micro-Reagent A (MA) consists of an aqueous solution of 8% $Na_2CO_3.H_2O$, 1.6% Na_2 tartrate, 1.6% NaOH and sufficient $NaHCO_3$ to adjust the pH

to 11.25. Micro-Reagent B (MB) consists of 4% BCA-Na_2 in deionized water. Micro-Reagent C (MC) consists of 4 volumes of 4% $CuSO_4.5H_2O$ plus 100 volume of Micro-reagent B. Micro-Working Reagent (M-WR) consists of equal volume of MC and MA. The color of S-WR and M-WR is apple green (Smith, 1985).

A standard solution containing an accurate amount of bovine serum albumin (BSA) 1 mg/ml was diluted to 7 dilutions with final concentration of 200, 300, 400, 500, 600, 700 and 800 µg / ml for BCA and 0.5, 1, 2, 5, 10, 20 and 30 µg / ml for micro BCA. The standard assay procedure of BCA and micro BCA consisted of mixing 1 volume of standard sample with 20 volume of S-WR and equal volume of standard sample with M-WR, respectively. Color development for BCA proceeded within 2 hours at room temperature, while micro BCA was 1 hour at 60º C. The absorbance was measured at the wavelength of 562 nm versus a reagent blank for BCA and versus deionized water for micro BCA. For micro BCA, the reagent blank was measured same as the sample.

The concentration versus average absorbance from triplicate assays was plotted. The relationship between absorbance and concentration was calculated. The concentration of unknown could then be determined from the plot of concentration and absorbance obtained for standard protein.

The result of the effect of aluminium containing adjuvant, the effect of pH and temperature on adsorption which had the optimal adsorption value was chosen for further study.

5. Adsorption of combined antigen on adjuvants

The compositions of combined formulation were diphtheria toxoid (DT), tetanus toxoid (TT), *Bordetella pertussis* (PT) and JE antigen were equivalent to adsorbed DT 30 Lf, adsorbed TT 6 Lf, PT 20 O.U. (2×10^{10} cell), adsorbed JE 0.35 antigen unit. The formulation also consisted of a preservative, thimerosal of 0.01% w/v and aluminium adjuvant not more than 0.85 mg aluminium / 0.5 ml (from United States Food and Drug Administration guidelines) (Baylor et al., 2002).

5.1 Effect of combination process

5.1.1 Separate adsorption

Adsorption processes of antigens on aluminium containing adjuvant in optimal condition (result from section 4) were performed by two procedures.

Each antigen was mixed with the adjuvant by magnetically stirring at the same condition of adsorption of single antigen at 170 rpm for 30 minutes and then all was transferred to another mixing container. PT and thimerosal were eventually added and the volume was adjusted with 0.01 M phosphate buffer which had the same pH with the adjuvant. The combined preparation was mixed with the same condition at various mixing time between 3 and 30 minutes. The combined preparations were collected to analyze the amount of each antigen by enzyme linked immunosorbent assay (ELISA).

5.1.2 Competitive adsorption

The optimal type and pH of aluminium adjuvant and temperature of mixing process were chosen similarly to the separate adsorption**.** DT, TT and JE antigens were taken and mixed together in a mixing container. Then aluminium adjuvant was incorporated and magnetically stirred at the same condition as in the separate adsorption. Finally, PT, thimerosol and 0.01 M phosphate buffer which had the same pH with aluminium adjuvant were added and mixed for another 3 minutes. The preparation was collected to analyze the amount of each antigen by ELISA.

5.2 Enzyme linked immunosorbent assay (ELISA)

DT, TT and PT were examined for their content by competition ELISA (Hozbort et al., 1995) whereas JE antigen was examined by direct sandwich ELISA. (Morita, 1989; WI of viral vaccine division, GPO)

5.2.1 The optimal condition of ELISA procedure

The purpose of this experiment was to determine the optimal dilution of coating antigen, primary antibody (1°Ab) and the secondary antibody (2°Ab) of DT, TT and PT. Besides, the coating methods of PT were examined for the optimal condition.

5.2.1.1 The optimal dilution of coating antigens

The total protein nitrogen of DT and TT were calculated to 3 dilutions with final concentration of about 100, 10 and 1 µg/ml to produce coating concentration of 10, 1 and 0.1 µg / well, respectively. The diluting medium was carbonate-bicarbonate coating buffer pH 9.6.

PT antigen concentration was calculated to 4 dilutions with final concentration of about 10^{12}, 10^{11} and 10^{10} cell/ml to produce coating concentration of 10^{11}, 10^{10} and 10^{9} cell/well, respectively. In practice, PT was cloudy suspension. The suspension was prepared to coat plate by centrifugation until the supernatant was clear. The supernatant was taken off and the precipitate was diluted with coating buffer and mixed until homogeneously. The suspension was then tested to study the effect of coating method.

Coating method that was investigated the effect of preparation method. The suspension was sonicated for 30 minutes before prior to coating on microtiter plate compared with the unsonicated suspension.

5.2.1.2 The optimal dilution of primary antibodies

DT antibody was diluted with diluent, 1% gelatin in phosphate buffer saline with tween 20 (PBST), to 2 dilutions; 1 : 500 and 1 : 1,000. TT antibody (tetanus antitoxin) was diluted with diluent to 2 dilutions; 1 : 5,000 and 1 : 10,000. PT antibody was diluted with diluent, to 2 dilutions; 1 : 4 and 1 : 8.

5.2.1.3 The optimal dilution of secondary antibodies

Horseradish peroxidase-Goat anti-mouse IgG (H+L) conjugate for DT was diluted with diluent to 2 dilutions; 1 : 5,000 and 1 : 10,000. Rabbit anti-horse IgG (whole molecule) peroxidase conjugate for TT was diluted with diluent to 2 dilutions; 1 : 5,000 and 1 : 10,000. Horseradish peroxidase-Goat anti-mouse IgG (H+L) conjugate for PT was diluted with diluent to 2 dilutions; 1 : 1,000 and 1 : 5,000.

5.2.1.4 Indirect antigen competition

The amount of DT, TT and PT were analyzed as followed, the 96 well ELISA microtiter plate was coated with 100 µl per well of DT optimal concentration in coating buffer overnight at 4 $\pm$ 1 °C to allow the coating antigen adsorbed to the well. The plate was thoroughly washed three times with phosphate buffer saline with tween 20 (PBST) pH 7.4 and allowed to dry. The 100 µl of blocking solution, 3% gelatin in PBST, was added to each well of the coated plate and incubated for 1 hour at room temperature.

The standard antigen and test samples, which were two fold steps serial dilutions, were incubated (pretitrated) simultaneous outside the plate with DT antibody which directed against antigen on the plate. DT antibody was diluted to optimal concentration with diluent buffer. The plate was thoroughly washed three times with PBST and allowed to dry. The 100 µl of each pretitrated serial dilution of samples and standards were added into triplicate wells of the coated plate. Diluent buffer was incubated with DT antibody and was used for control, whereas diluent buffer was used for blank by adding 100 µl per well. The plate was incubated at room temperature for 1 hour. The plate was washed with PBST for three times and allowed to dry. The 100 µl of a horseradish peroxidase-goat anti-mouse IgG (H+L) conjugate, diluted to optimal concentration with diluent, was added to each well and incubated for 1 hour at room temperature. The plate was washed again with PBST for three times. The 100 µl of the substrate, O-phenylene diamine (OPD), at a concentration of 1 mg per 12 ml of a citrate buffer plus 12 µl of 30% hydrogen peroxide, was added to

each well. The plate was covered in the dark and incubated for 30 minutes at room temperature for color development. The 50 µl of 4 N sulfuric acid was added to each well to stop the reaction. The plate was gently shaken and the optical density (OD) was read at wavelength 490 nm using a microplate reader. The procedure was shown in Figure 4. The amounts of antigen content were calculated from standard curve which was plotted between percentage of antigen binding and ln protein nitrogen concentration.

The ELISA procedures of TT and PT were the same as of DT. Standard curve of DT, TT and PT were conducted as two fold serial dilution.

$$\% \text{ antigen binding} = [\, 1 - (b/b_0) \,] \times 100 \quad \ldots\ldots\ldots\ldots [1]$$

b = sample optical density

b_0 = control optical density

The testing processes of 5.2.1.1-5.2.1.3 were performed as in section 5.2.1.4. The condition which had the maximal correlation coefficient (R^2) when the standard curve was plotted between percentage of antigen binding and ln concentration or the optimal optical density was chosen to be used for the ELISA analysis of diphtheria toxoid, tetanus toxoid and *Bordetella pertussis*.

5.2.2 Direct sandwich

The amount of JE antigen was analyzed as followed, the 96 well ELISA microtiter plate was coated with 100 µl per well of anti-Beijing mouse IgG dilution 1 : 6,500 in carbonate - bicarbonate coating buffer pH 9.6 overnight at 4 $\pm$ 1°C to allow the coating antibody adsorbed to the well. The plate was thoroughly washed three times with phosphate buffer saline with tween 20 (PBST) pH 7.4 and allowed to dry. The 100 µl of blocking solution was added to each well of the coated plate and incubated for 1 hour at room temperature. The plate was thoroughly washed three times with PBST and allowed to dry. The test samples and reference vaccines were two fold steps serially diluted with diluent and 100 µl of each sample was added

into triplicate wells of the coated plate. Diluent buffer was used for blank by adding 100 µl per well. The sample plate was incubated at room temperature for 1 hour. The plate was washed with PBST for three times and allowed to dry. The 100 µl of a horseradish peroxidase-labeled anti-Beijing mouse IgG, dilute 1 : 800 with diluent, was added to each well and incubated for 1 hour at room temperature. The plate was washed again with PBST for three times.

After that, the procedures were the same process as in section 5.2.3 except the stop reaction process which used 50 µl of 4 N sulfuric acid. The procedure was shown in Figure 3. The amounts of antigen content were calculated from standard curve which was plotted between ln optical density and ln dilution.

5.2.3 Cross reaction analysis

This analysis was conducted in order to examine the cross over reaction between the one antibody and other components in preparation (antigens, aluminium adjuvants, buffer and thimerosol).

DT was coated on 96 well ELISA microtiter plate (Maxisorb, Nunc) with optimal concentration at 4 $\pm$ 1 C°. Other antigens, AH, AP, 0.01 M phosphate buffer and thimerosol were used as samples to test with DT antibody.

TT, PT and JE antigens were individually performed similarly to DT with individually primary antibody. DT, PT and JE were used as samples of TT antibody. DT, TT and JE antigens were used as samples of PT antibody. DT, TT and PT antigens were used as samples of JE. Furthermore, both aluminium adjuvants, 0.01 M phosphate buffer and thimerosol were used to test with each antibody.

The analytical procedure was similar to the section 5.2.1.4.

6. Stability study

The combined preparations of DTP-JE which were formulated as separate adsorption (section 5.1.1) and competitive adsorption (section 5.1.2) were stored at temperature 2-8 °C for 3 months in order to evaluate the content of each antigen by randomly sampling every 4 weeks interval for analysis by ELISA method (section 5.2.1.4 for DT, TT and PT; section 5.2.2 for JE antigen). Moreover, the physical appearances of the preparations were observed every month.

7. Evaluation of materials and preparations

7.1 Optical microscopy

The physical morphology of AH, AP, PT and JE antigens were observed by optical microscope as received. DT and TT were solutions that could not be viewed under simple optical microscope.

7.2 Scanning electron microscopy

The single antigen adsorbed preparations, which DT, TT or JE antigen was adsorbed on aluminium adjuvants at optimal condition were coated with gold using ion sputtering prior to the microscopic examination. The surface morphologies of the single antigen adsorbed preparations were observed by scanning electron microscope (SEM) and compared with the surface morphology of pure AH and AP.

7.3 Transmission electron microscopy

The single antigen adsorbed preparations, which DT, TT or JE was adsorbed on adjuvants at optimal condition were prepared as negative staining method by dropping the samples on the grid which was coated with formvar and stained with 2% phosphotungstic acid (PTA) pH 7.0. Then the samples were dried in a desiccator to the examination. The surface morphologies of the single antigen adsorbed

preparations were observed by transmission electron microscope (TEM) and compared with the surface morphologies of pure AH and AP.

7.4 Atomic force microscopy

Atomic force microscope is the foremost tool for the manipulation of matter at the nanoscale. The true three-dimensional surface morphologies of the single antigen adsorbed preparations which diphtheria toxoid, tetanus toxoid or JE antigen was adsorbed on AP adjuvant at optimal condition were observed by atomic force microscope (AFM) and compared with that of pure AP adjuvant. The samples were centrifuged for clear supernatant. The precipitates were collected and pasted as thin film on a clear and smooth platform. The samples were measured using a laser spot reflected from the top of the cantilever into an array of photodiodes. The results showed as the true three-dimensional surface profile as the topography of the sample.

7.5 Fourier Transform Infrared spectrometry

The single antigen adsorbed preparations, which DT, TT or JE antigen was adsorbed on aluminium containing adjuvants at optimal condition, AH and AP were centrifuged until the supernatant was cleared. The supernatant was taken off and the precipitates were sampled to assay. The infrared spectra of both adjuvants were used to identify and characterize these materials. Infrared spectra of the single antigen adsorbed preparations were measured by pasting the samples on the potassium bromide cell and performed in the range of 4000 - 400 cm^{-1} and compared with those of pure AH and AP adjuvant.

7.6 Powder X-ray diffractrometry

The X-ray diffraction bands of both aluminium-containing adjuvants were used to identify and characterize these materials. X-ray diffraction patterns of the single antigen adsorbed preparations which DT, TT or JE was adsorbed on aluminium adjuvants at optimal condition were investigated by using an X-ray diffractrometer and compared with the X-ray diffraction patterns of plain AH and AP adjuvant.

The samples were prepared as random powder mounts after gently grinding freeze-dried samples in a mortar (Shirodkar, Hutchison, Perry, White and Hem, 1990). The powders were firmly packed in the cavity of a thin rectangular quarts slide by the other slide. The glass slide was taken off and the prepared sample was exposed to the X-ray beam in the X-ray diffraction chamber. The samples were irradiated with monochromatized Cu Kβ radiation at the speed of 0.04° per minute from 5º - 60º in the term of 2θ angles. The voltage, and current used were 45 kV, and 35 mA, respectively.

7.7 Particle size distribution analysis

The particle size of the single antigen adsorbed preparations which DT, TT or JE was adsorbed on the adjuvants at optimal condition were performed by laser diffractrometry (LD) with pump speed 1500, ultrasonic displacement 15, and by photon correlation spectrophotometry (PCS) and compared with particle sizes of pure AH and AP adjuvant.

The ultrapure water was used as the medium for measured the particle size. The single antigen adsorbed preparations which just had been prepared, AH and AP were added in the medium. The samples were triplicate measured and showed as average particle size. The results of LD reported the percentile sizes for 10%, 50% and 90% which expressed as d(0.1), d(0.5) and d(0.9) respectively and the width of the distribution, which was the measure of the absolute deviations from the median expressed as uniformity, while the results of PCS reported the mean particle size with polydisperse index (PI).

7.8 Surface charge analysis

The surface charge of DT, TT, PT and JE antigens were measured by PCS in deionized water and phosphate buffer various pHs. The surface charge of the single antigen adsorbed preparations which DT, TT or JE antigen was adsorbed on aluminium adjuvants at optimal condition in various medium; deionized water, phosphate buffer various pHs, were measured by PCS and compared with the surface

charge of pure AH and AP. Furthermore, the isoelectric points of AH and AP were determined of aliquots diluted 1:80 with supernatant. The results reported the mean surface charge which express as mean zeta potential at temperature, 25 °C.

8. Statistical analysis

The means adsorption values of AH and AP between 37±1 °C with 9±1 °C, and the antigen content of stability were studied by analysis of variance (ANOVA). The means particle size distribution of single antigen adsorbed preparations, aluminium hydroxide and aluminium phosphate adjuvants were statistically evaluated by paired student's *t* - test. Results were considered statistically significant if $p<0.05$.

CHAPTER IV
RESULTS AND DISCUSSION

1. Aluminium content assay

The percentage of aluminium content in AH and AP calculated triplicately by back titration method (USP 25 and BP 1998, respectively) were 1.94 $\pm$ 0.03 and 2.25 $\pm$ 0.02 %Al, respectively.

2. Centrifugation rate for separation of unadsorbed antigens

This examination was conducted in order to choose the speed of centrifugation which was used to determine the adsorption of single antigen on AH and AP. The quantities of aluminium content by inductively coupled plasma spectroscopy analysis after centrifugation at various speeds for 20 minutes shown in Figure 5-6 that the percentages of aluminium in supernatant of both adjuvants gradually decreased as the speed increased. At the speed of 500 and 3000 rpm, the percentage of aluminium in supernatant of AP and AH were less than 1% (0.99%Al and < 0.12%Al for AH and AP, respectively). Therefore, the speed of centrifugation which was chosen for both adjuvants was 3,000 rpm for 20 minutes.

There were many studies about the adsorption of the different proteins on aluminium containing adjuvant. The dispersion was centrifuged at different speeds to precipitate the adsorbed adjuvant and to obtain the clear supernatant (Seeber et al., 1991; Al-Shakhshir et al., 1995; Rinella et al., 1996; Chang et al., 2001; Morefield et al., 2004). Al-Shakhshir et al. (1995) studied the effect of dilution on protein distribution. Samples of the BSA-AH and the lysozyme-AP were prepared and centrifuged at 4500 rpm for 20 minutes whereas Morefield et al. (2005) chose the different speeds and time of centrifugation; 10,000 rpm for 15 minutes to evaluate the adsorption of ovalbumin with AH and AP.

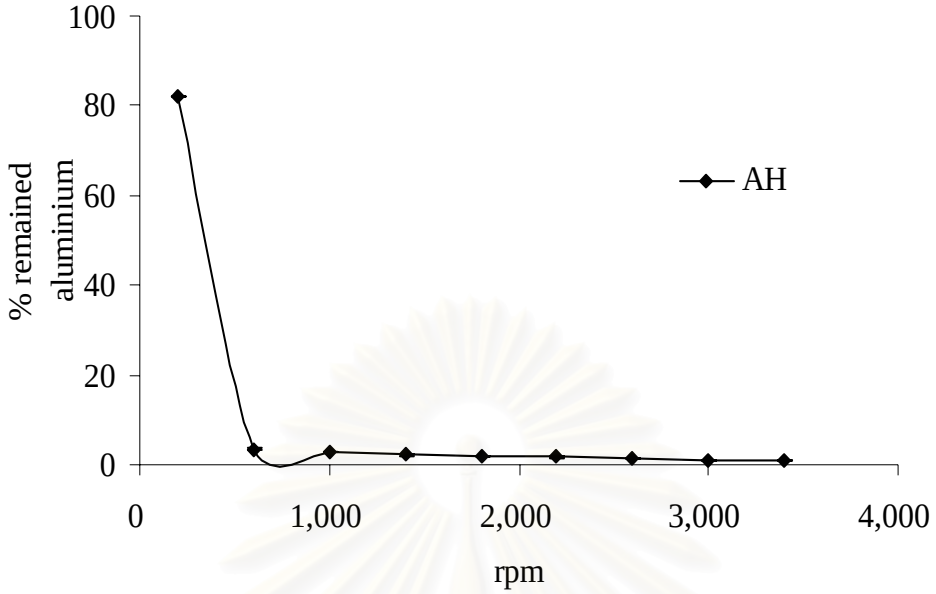


Figure 5 Percentage of aluminium content in supernatant of aluminium hydroxide after centrifugation at various speeds for 20 minutes.

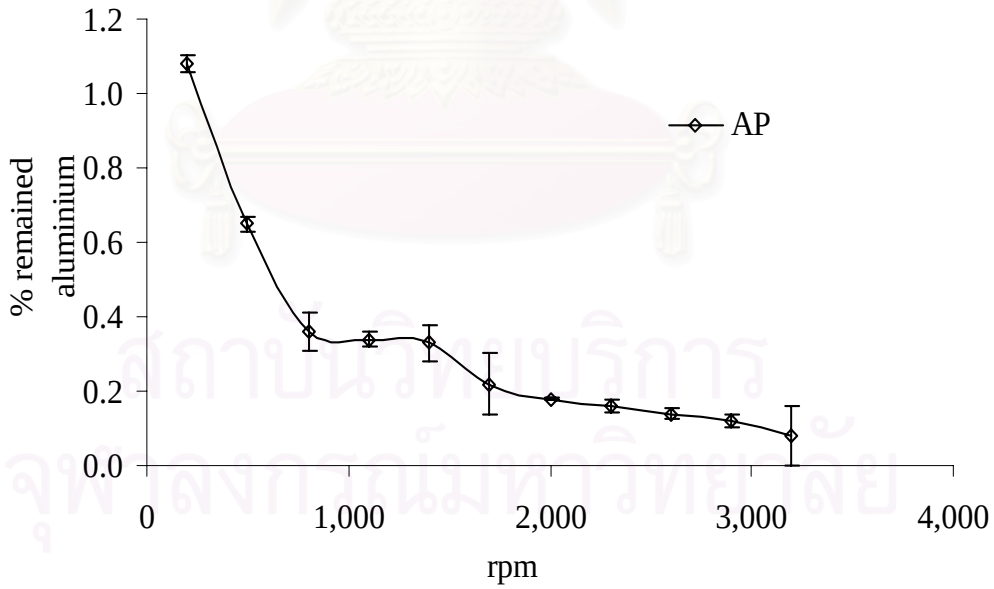


Figure 6 Percentage of aluminium content in supernatant of aluminium phosphate after centrifugation at various speeds for 20 minutes.

The clear supernatant from these experiments were assayed for protein by BCA method. Therefore, the optimal condition should be determined for each individual antigen.

3. Adsorption of single antigen on adjuvants

The concentration unit of DT, TT and JE were transformed from Lf (diphtheria, tetanus) and antigen unit (JE) to µg. The measurement as µg was calculated by BCA analysis and referred to the original concentration as Lf or antigen unit (Table 26, in Appendix B). The results were used to calculate the amount of aluminium adjuvants from the adsorption values. They are shown by the following.

Diphtheria toxoid ;	1	Lf	=	2.73 µg
Tetanus toxoid ;	1	Lf	=	4.55 µg
JE antigen ;	1	antigen unit	=	201.01 µg

3.1 Antigens adsorbed with aluminium hydroxide adjuvant

Basically vaccine preparation processes of adsorbed antigens on adjuvants are formulated in cold room. In this experiment, the samples were mixed at 170 rpm, 30 minutes. In many studies, the mixing time was 20 to 60 minutes (Al-Shakhshir et al., 1994; Al-Shakhshir et al., 1995; Rinella et al., 1995; Heimlich et al., 1999; Chang et al., 2001; Shi et al., 2002; Iyer et al., 2003; Morefield et al., 2004; Morefield et al., 2005).

Figures 7-9 show the adsorption isotherm of antigens on AH at various pHs, 37 °C. The effect of concentration of DT revealed the fluctuated adsorption pattern on AH at every pH used. It might be affected by pH that antigen could adsorb or desorb with adjuvant. There were statistically significant differences between the adsorption of DT at concentration above 600 µg/ml, pH 6.0 with pH 7.0-7.4 ($p<0.05$).

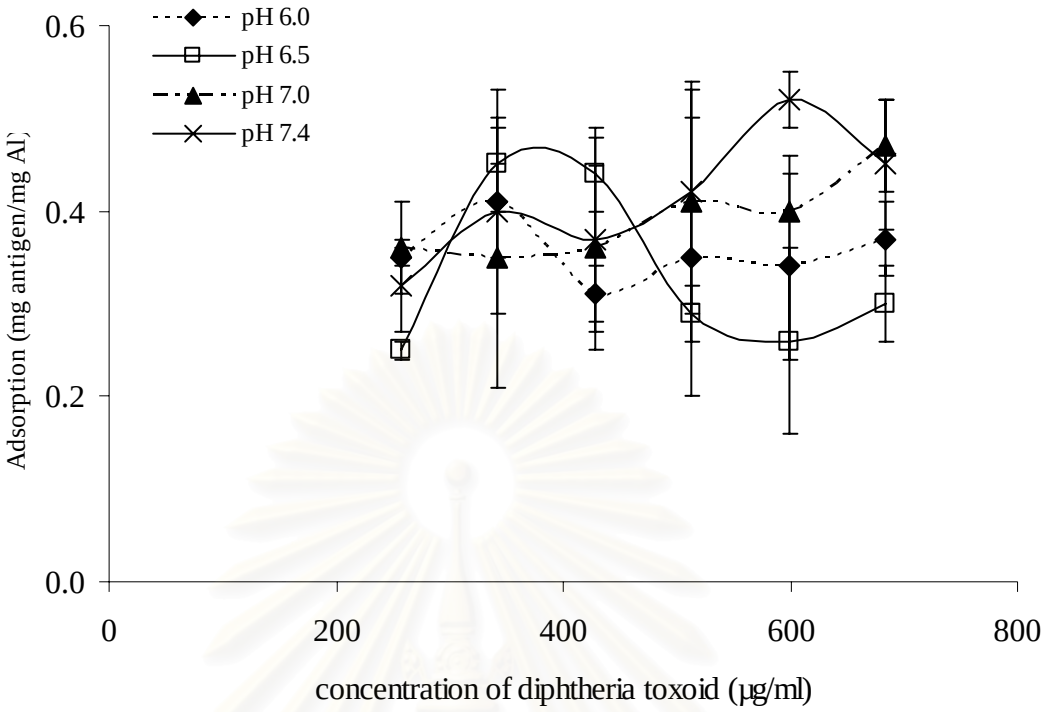


Figure 7 Effect of concentration of diphtheria toxoid on the adsorption onto aluminium hydroxide at various pHs and 37 °C

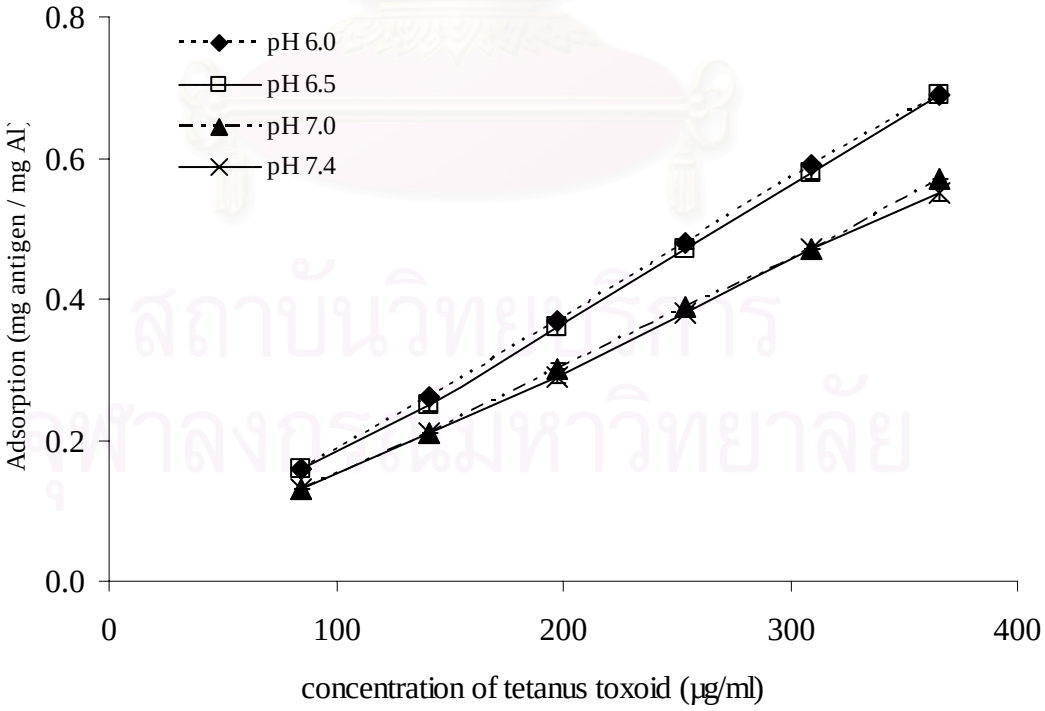


Figure 8 Effect of concentration of tetanus toxoid on the adsorption onto aluminium hydroxide at various pHs and 37 °C.

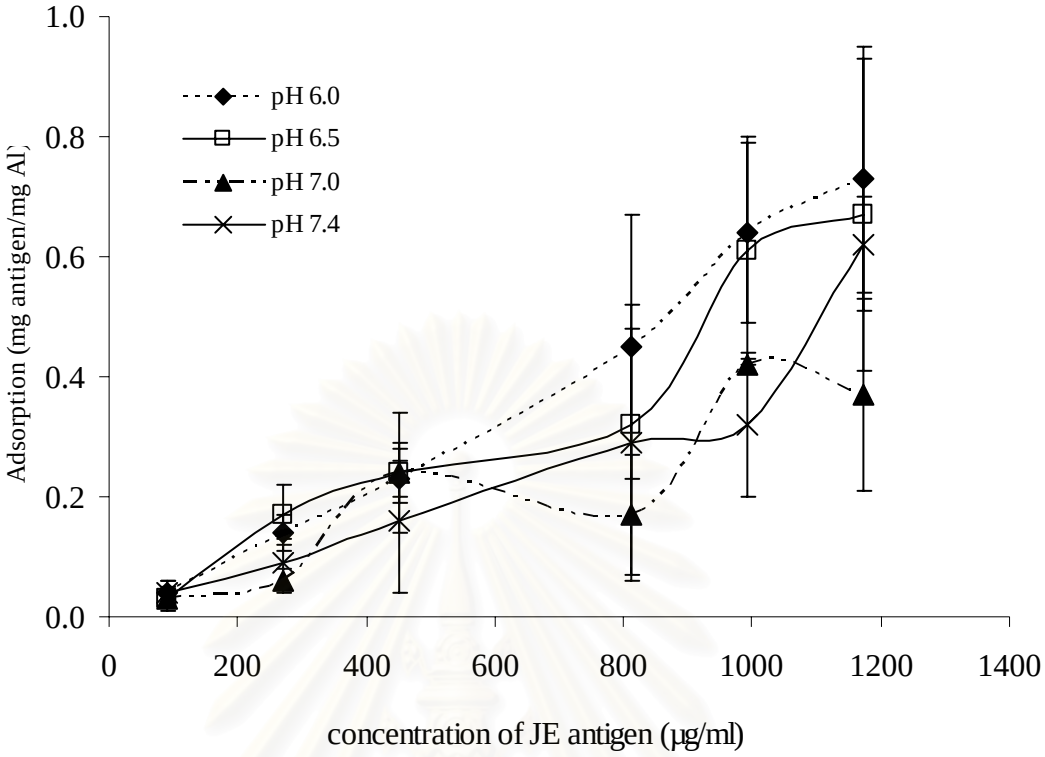


Figure 9 Effect of concentration of JE antigen on the adsorption onto aluminium hydroxide at various pHs and 37 °C

TT showed linear relation between concentration of antigen and adsorption. In addition, the adsorption at pH 6.0 was close to pH 6.5 and pH 7.0 was to pH 7.4. It suggested that the adsorption of TT depended on the pH. There were statistically significant differences between the adsorption of TT at pH 6.5 with pH 7.0-7.4 (p<0.05). JE displayed the patterns which were increased as the concentration was raised. There were no statistically significant differences between the adsorption of JE at concentration around 1200 µg/ml, pH 6.0 with pH 6.5, 7.0 and 7.4 (p>0.05).

Figures 10-12 illustrate the effect of concentration of antigens on AH at various pHs at 9 °C. All isotherms of DT, TT and JE were increased throughout the concentration range studied. The adsorption pattern of DT and TT was increased as the concentration was raised. JE at pH 7.0 and 7.4 were the same as DT and TT. There were no statistically significant differences between the adsorption of all antigens at pH 6.0 with other pH (p>0.05). The adsorption pattern of antigens on AH at 9 °C showed the similar pattern with at 37 °C. It indicated that temperature was not

a major factor which influenced the adsorption of antigens on AH. On the contrary, pH was the parameter which affected the adsorption.

The adsorption levels increased as the concentration increased. Due to at initial the extent of antigens did not cover over the surface of adjuvant and the adjuvants could adsorb more over antigens until the adjuvant was completely covered. The adsorption appeared constant when the surfaces of adjuvants were totally covered.

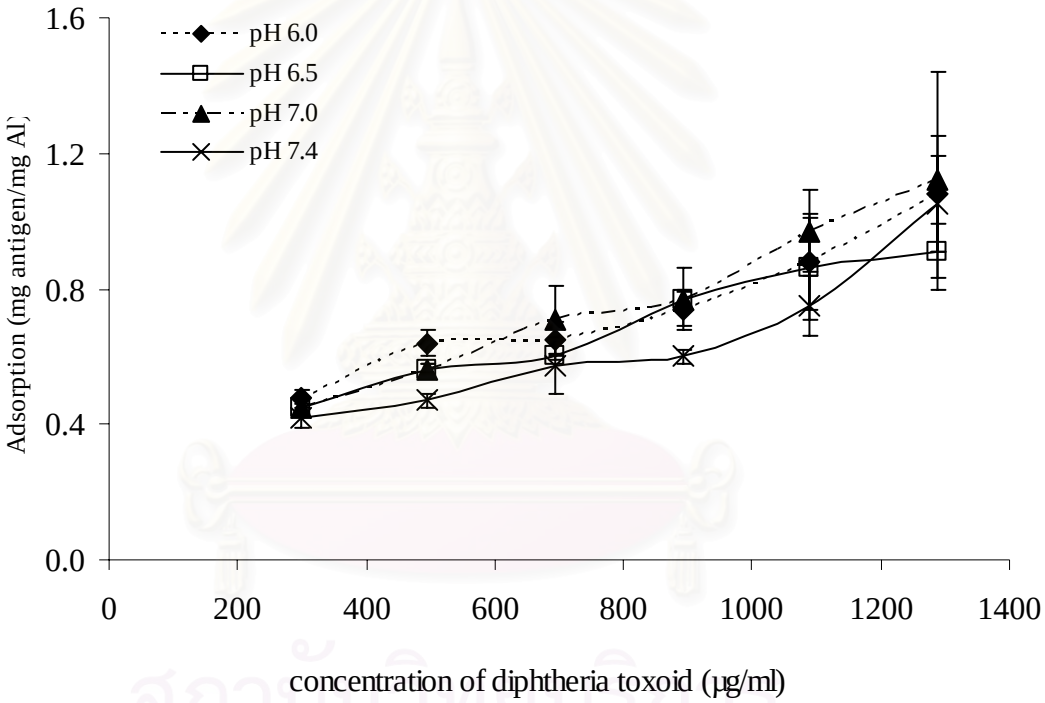


Figure 10 Effect of concentration of diphtheria toxoid on the adsorption onto aluminium hydroxide at various pHs and 9 °C

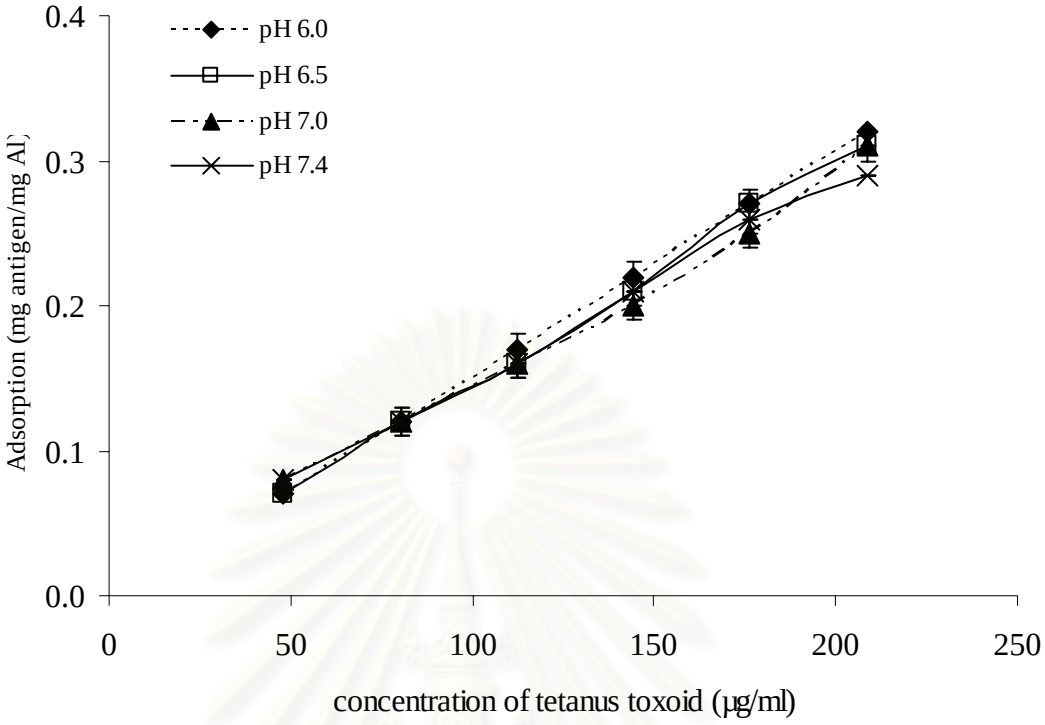


Figure 11 Effect of concentration of tetanus toxoid on the adsorption onto aluminium hydroxide at various pHs and 9 °C

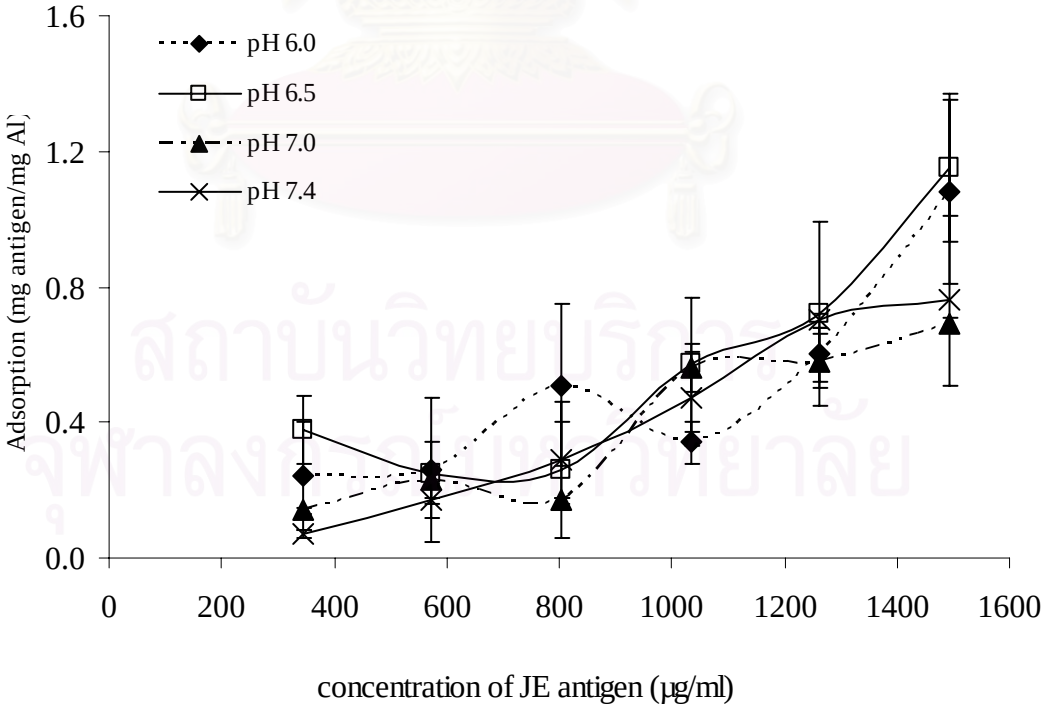


Figure 12 Effect of concentration of JE antigen on the adsorption onto aluminium hydroxide at various pHs and 9 °C

As seen in Table 33 (in Appendix B), the adsorption value of DT and JE on AH at 9 °C was more than at 37 °C for every pH but TT had the adsorption value at 9 °C less than at 37 °C for every pH. The results indicated that DT and JE preferred adsorption with AH at low temperature than at physiological temperature. On the other hand, TT had lower adsorption value at 9 °C than 37 °C. It might be concluded that DT and JE could be adsorbed on AH more than TT during formulation at low temperature. The study of Chaetanachan et al. (2001) reported that JE particles at 37 °C appeared to aggregate and deformed. Hence, JE was preferentially adsorbed at low temperature than at high temperature.

The effects of pH to the adsorption on AH are shown in Figure 13. DT showed the maximal adsorption value at pH 7.4 and 7.0 for 37 °C and 9 °C, respectively. TT showed the maximal adsorption value at pH 6.0 at both 37 °C and 9 °C. JE showed the maximal adsorption value at pH 6.0 and 6.5 for 37 °C and 9 °C, respectively. The adsorption pattern of JE at 37 °C was the same as at 9 °C. The adsorption of JE was gradually decreased from 672.8 to 418.6 µg and 1,148.1 to 690.8 µg at pH 6.5, 37 °C and 9 °C to pH 7.0, respectively. At pH 7.0, JE antigen had the minimal adsorption value.

The results suggested that at 9 °C, TT had the minimal adsorption value and the pH had a slight effect on the adsorption. DT and TT had the adsorption pattern similar to physiological temperature and there were statistically significant differences between each pH ($p<0.05$). The adsorption pattern of JE at 9 °C was the same that of 37 °C. It can be concluded that the adsorption values of DT, TT and JE antigen on AH adjuvant depended on pH at both temperature 37, and 9 °C.

In brief, the optimal condition for adsorbed DT, TT and JE antigens on AH was at pH 6.5; temperature 9 °C because it had the higher adsorption values than at 37 °C for DT and JE. In addition, TT adsorption at pH 6.5 was closed to pH 6.0, and pH 6.5 was the pH which closed to physiological pH.

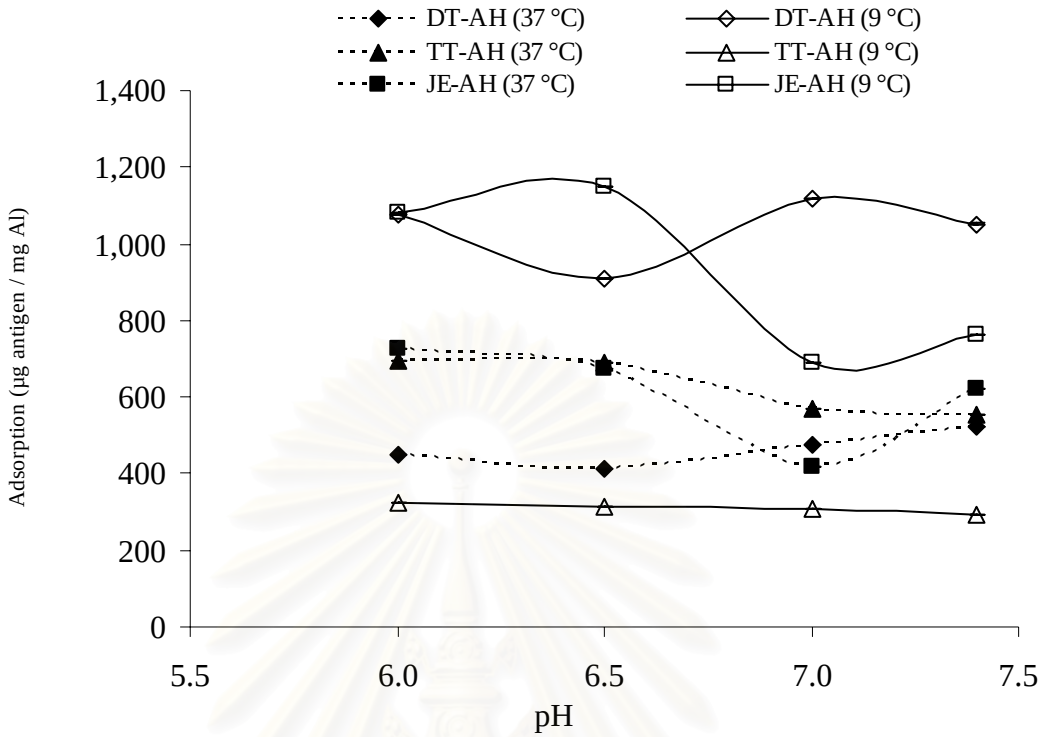


Figure 13 Effect of pH on adsorption of antigens on aluminium hydroxide at various temperatures.

3.2 Antigens adsorbed with aluminium phosphate adjuvant

The adsorption value of AP was calculated similarly to that of the AH adjuvant. Figures 14-16 present the effect of concentration of antigens on AP at various pHs and 37 °C. The extent of DT, TT and JE adsorption increased with increasing antigen concentration. There was statistically significant difference between the adsorption of DT at concentration around 300 µg/ml, pH 6.0 with other pH (p<0.05) and there was statistically significant difference between the adsorption of TT at concentration around 160 µg/ml, pH 7.4 with other pH (p<0.05). The adsorption pattern of JE seemed to be approximately constant above concentration of 900 µg/ml.

As seen in Figures 17-19, there were increases in adsorption levels of DT and TT throughout the increasing concentrations, while JE was gradually decreased as the concentration increased. It was due to the high amount of JE antigens caused completion between each other to adsorb on adjuvant and there was statistically significant difference between the adsorption at maximal concentration of pH 6.5 with pH 7.0 and pH 7.4 ($p<0.05$). There was statistically significant differences between the adsorption of DT at concentration around 900 μg/ml, pH 6.5 with pH 7.0 and pH 7.4 ($p<0.05$). TT displayed a linear pH dependence of adsorption, pH 6.0 showed the highest adsorption and pH 7.4 showed the lowest adsorption throughout the concentration studied. These results showed the same general trend with the adsorption at 37 °C. There was statistically significant difference between the adsorption of TT at pH 6.0 with other pH ($p<0.05$).

It indicated that temperature was not the major factor which influenced the adsorption of antigens on AP. In contrast, pH was the parameter which affected to the adsorption.

Table 40 (in Appendix B) shows the adsorption values at various pH. DT had less adsorption values at low temperature than at high temperature for every pH except pH 6.5. TT had the adsorption values at 9 °C lower than 37 °C for every pH, whereas JE antigen had the adsorption values at 9 °C higher than 37 °C for every pH. The adsorption values of TT on AP adjuvant were according to the adsorption on AH adjuvant that TT preferred to adsorb on AH and AP adjuvant at 37 °C than 9 °C. On the contrary, the adsorption values of DT on AP were conversely to that on AH adjuvant.

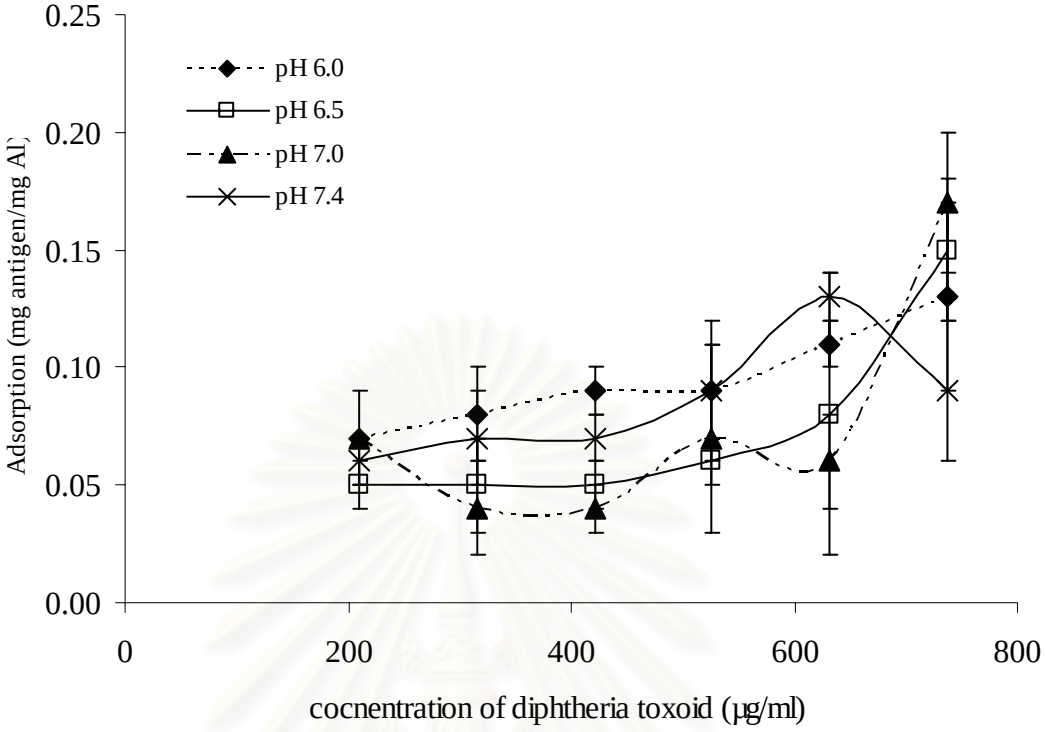


Figure 14 Effect on concentration of diphtheria toxoid on the adsorption onto aluminium phosphate at various pHs and 37 °C

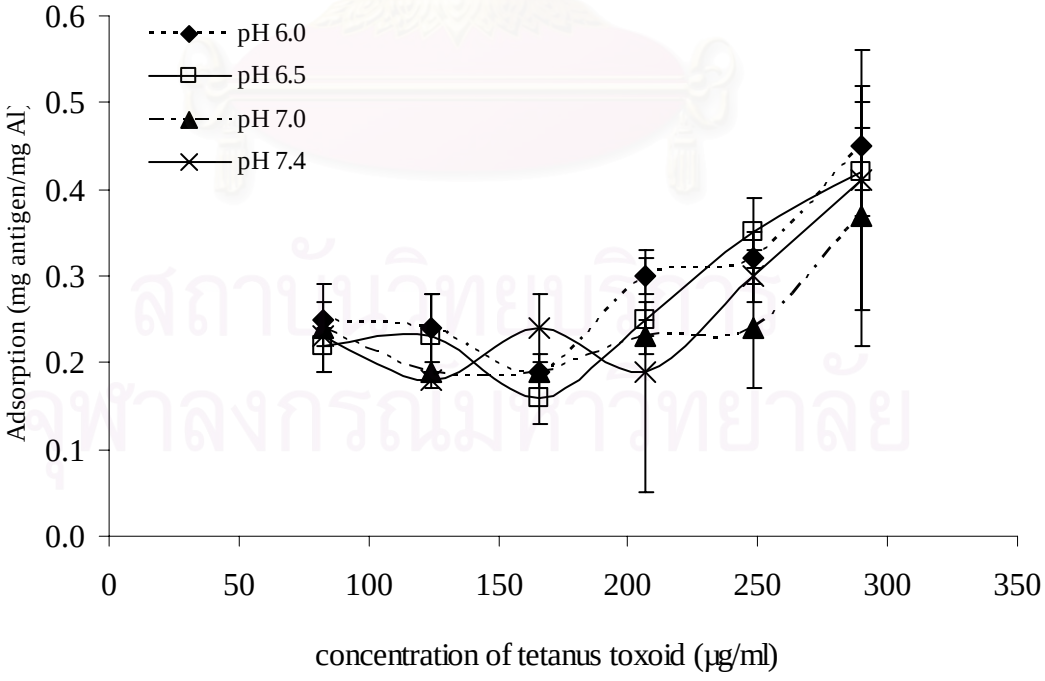


Figure 15 Effect of concentration of tetanus toxoid on the adsorption onto aluminium phosphate at various pHs and 37 °C

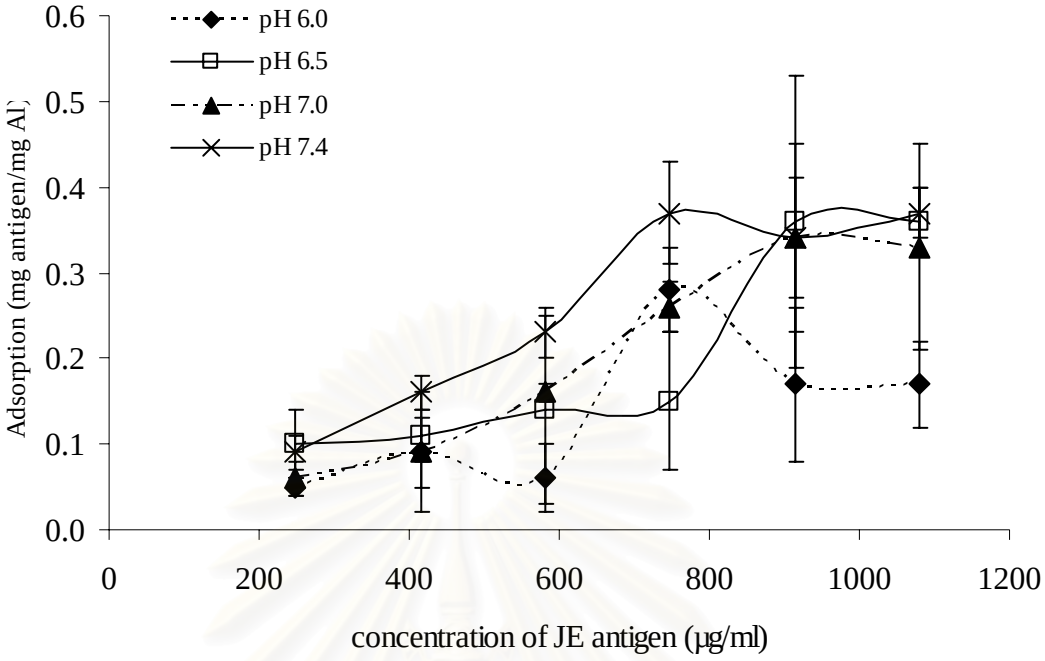


Figure 16 Effect of concentration of JE antigen on the adsorption onto aluminium phosphate at various pHs and 37 °C

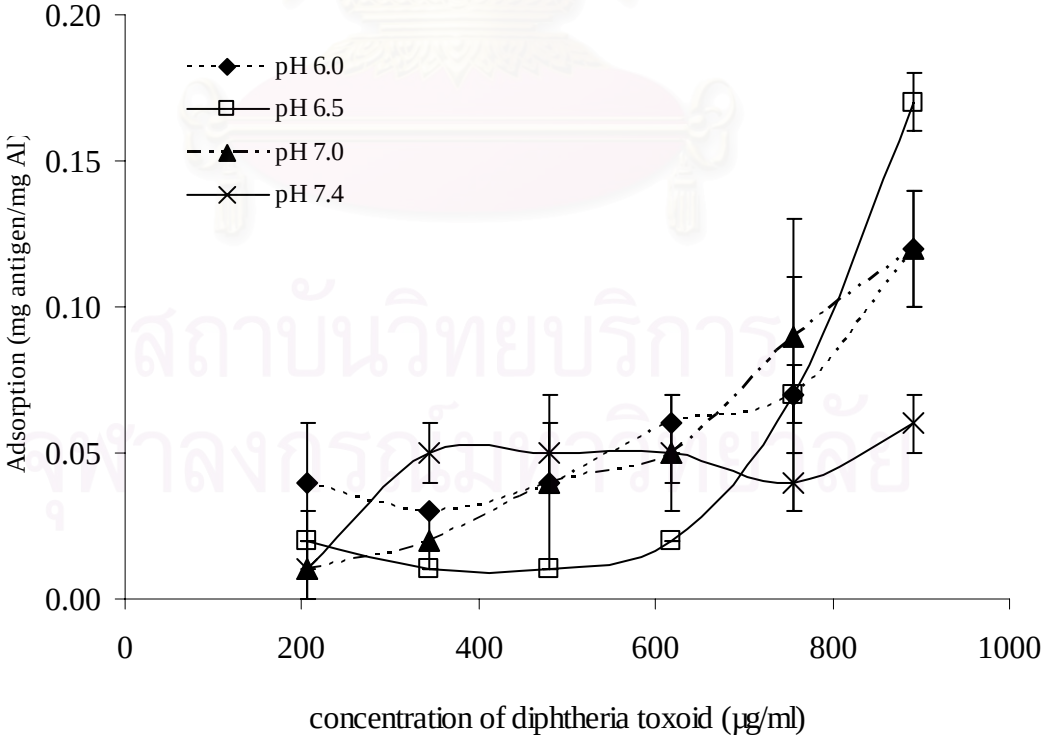


Figure 17 Effect of concentration of diphtheria toxoid on the adsorption onto aluminium phosphate at various pHs and 9 °C

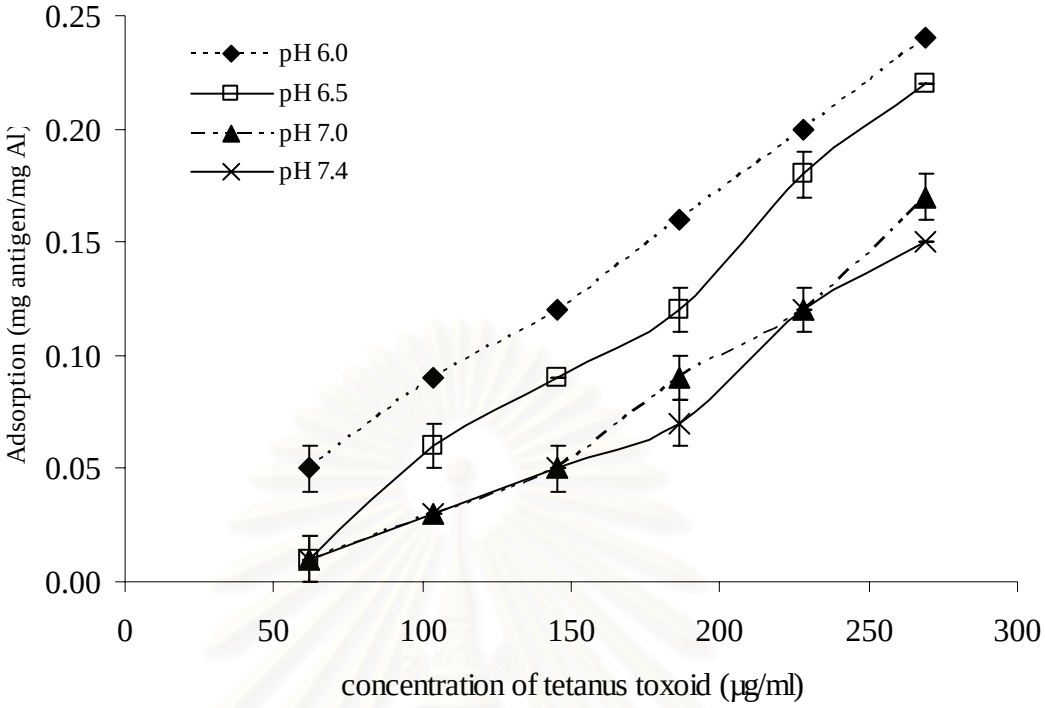


Figure 18 Effect of concentration of tetanus toxoid on the adsorption onto aluminium phosphate at various pHs and 9 °C

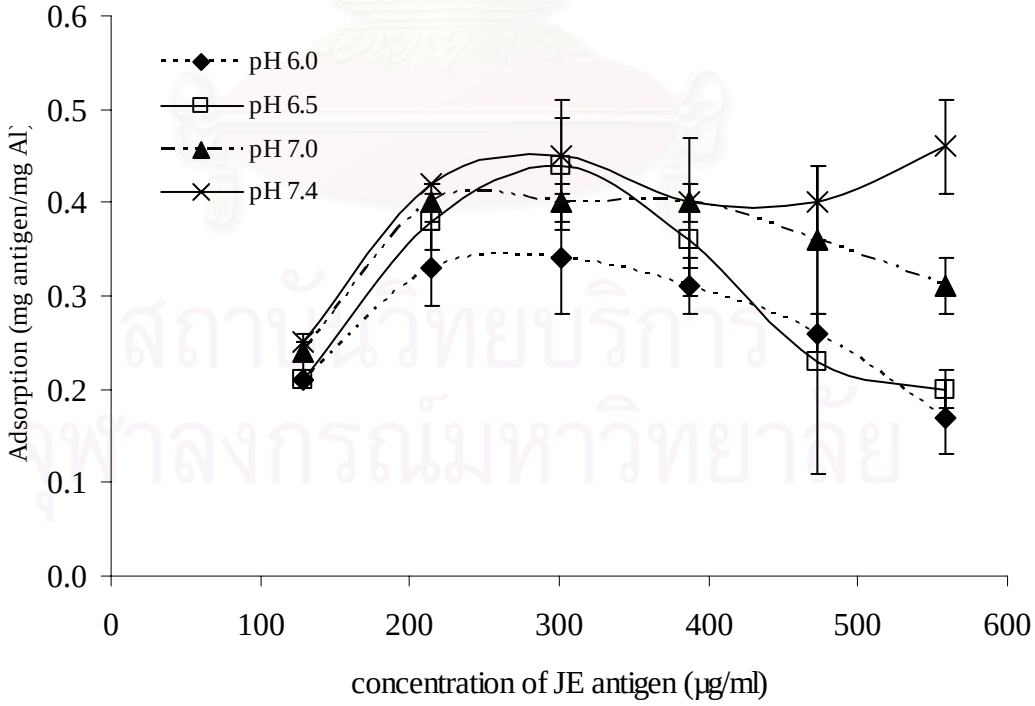


Figure 19 Effect of concentration of JE antigen on the adsorption onto aluminium phosphate at various pHs and 9 °C

The effects of pH for the adsorption on AP are shown in Figure 20. At high temperature, adsorption of DT was the lowest while that of TT was the highest in every pH. The adsorption values of DT at 37 °C were not significantly different between each other in every pH (p>0.05). TT had the adsorption values closed to JE antigen at pH 6.5, 7.0 and 7.4. At pH 6.0, adsorption values of every antigen were very different. The different adsorption of each antigen at initial pH might be affected from size, surface morphology or surface charge of the antigens, DT and TT were the toxoid solution whereas JE was virus particle. The morphology and surface charge were further studied.

At 9 °C, the adsorption values of DT were the lowest and JE antigen were the highest for every pH. Adsorption value of DT at pH 6.5 was the highest. There were statistically significant differences between the adsorption value of DT at pH 6.5 with other pHs (p<0.05). The adsorption value pattern of TT was decreased when the pH was increased. JE antigen had the pattern of adsorption at 9 °C similar to at 37 °C. It had the maximal value at pH 7.4 and minimal value at pH 6.0. Furthermore, the adsorption value was raised when the pH was changed from 7.0 to 7.4. It could be concluded that the adsorption values of DT, TT and JE antigen on AP adjuvant were depended on pH at both temperatures 37, and 9 °C.

Therefore, the optimal condition for adsorbed DT, TT and JE antigen on AP adjuvant was pH 6.5; temperature 9 °C because at this temperature it had maximum adsorption to DT and it had the higher adsorption values than 37 °C for DT and JE. Moreover, pH 6.5 was closed to physiological pH. According to the report of Gupta et al. (1998), the formulation of DTP vaccine with aluminium phosphate was usually done at pH close to 6.0 as to allow maximum adsorption of diphtheria toxoid.

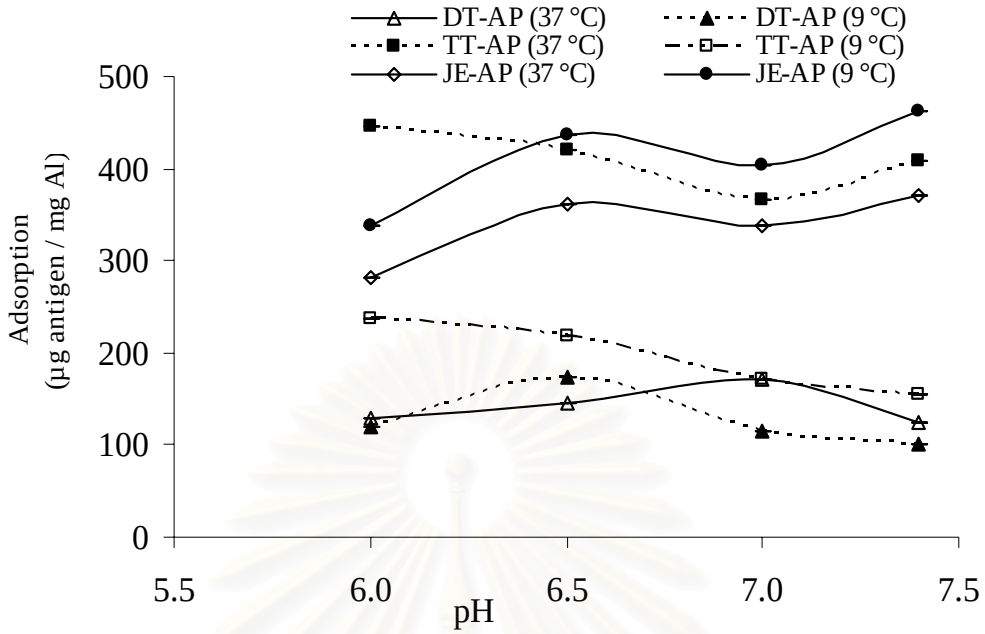


Figure 20 Effect of pH on adsorption of antigens on aluminium phosphate at various temperatures.

In summary, AH had greater adsorption capacity in every pH than AP both 37 and 9 °C. It conformed to the studies done by Gupta (1998), Shi et al. (2001), and Baylor et al. (2002) which had shown that AH has adsorption capacity more than AP. Sepelyak et al. (1984) and Callahan et al. (1991) contributed that AH displayed the porous nature, resulted in a large surface area and the adsorption on AH was similar to adsorption in a long narrow channel.

The quantities of AH and AP were calculated from the adsorption values at various conditions with the amount of antigens which were used in combined preparation. Consideration from the adsorption values, the optimal condition which was chosen for both aluminium adjuvants was pH 6.5 at temperature 9 °C. Total aluminium from Table 9 indicated that the amount of AP adjuvant which was used in combined preparation was more than AH adjuvant. Similar results were also obtained from Table 10. Total aluminium of AH in both Table 9 and Table 10 were not over the limit of regulation. It could be concluded that the optimal adjuvants was AH and the optimal condition was pH 6.5, phosphate buffer at temperature 9 °C.

Table 9 The calculated quantities of aluminium in adsorbed adjuvants at various pHs, 37°C.

pH	Quantities of aluminium at 37 ± 1 °C (mg)						Total aluminium (mg)	
	Diphtheria		Tetanus		JE			
	AH	AP	AH	AP	AH	AP	AH	AP
6.0	0.18	0.64	0.04	0.06	0.19	0.50	0.42	1.20
6.5	0.20	0.56	0.04	0.07	0.21	0.39	0.45	1.02
7.0	0.17	0.48	0.05	0.07	0.34	0.42	0.56	0.97
7.4	0.16	0.65	0.05	0.07	0.23	0.38	0.43	1.10

Table 10 The calculated quantities of aluminium in adsorbed adjuvants at various pHs, 9°C.

pH	Quantities of aluminium adjuvants at 9 ± 1 °C (mg)						Total aluminium (mg)	
	Diphtheria		Tetanus		JE			
	AH	AP	AH	AP	AH	AP	AH	AP
6.0	0.08	0.69	0.08	0.12	0.13	0.42	0.29	1.22
6.5	0.09	0.47	0.09	0.12	0.13	0.32	0.30	0.92
7.0	0.08	0.71	0.09	0.16	0.20	0.35	0.37	1.22
7.4	0.08	1.46	0.09	0.18	0.18	0.30	0.36	1.94

3.3 Evaluation of adsorbed preparations

3.3.1 Surface charge analysis

Surface charges of AH and AP when the supernatant was adjusted to the pH range of 5-12 and 3-9 are shown in Figure 21 and Table 11, respectively. As seen in Figure 21, the isoelectric point (pI) or point of zero charge (PZC) of AH and AP were estimated to be about 11.0 and 4.25, respectively. The net surface charge was positive charge when the pH was below the pI. Likewise, the net surface charge was negative charge when the pH was above the pI. Then, AH and AP were positive charge when the pH was below 11.0 and 4.25, respectively. These results were according to the previous studies of Al-Shakhshir et al. (1994), Rinella Jr. et al. (1995) and Rinella Jr. et al. (1998). Al-Shakhshir et al. (1994) examined the effect of protein adsorption on the surface charge of aluminium adjuvants. Their results showed that pI was determined to be 11.1 and 5.0 for AH and AP. Rinella Jr. et al.

(1995) determined the pI of aliquots diluted 1:80 with the supernatant of AH and AP. AH and AP showed pI around 11.4 and 4.7, respectively. Rinella Jr. et al. (1998) studied the pI of AH and AP. It showed that pI of AH and AP were 11.5 and 5.0, respectively. Hence, at pH 6.0-7.4 AH which had positive charge would adsorb negatively charged antigens and AP which had negative charge would adsorb positively charged antigens.

Although AH had strong positive charge at pH range 6.0-7.4 (Table 11) but in phosphate buffer it presented negative charge at the same pH range as shown in Table 12. This was due to the effect of phosphate anion. Adding phosphate anion, decreased the positive surface charge of the AH adjuvant to negative charge. At higher pH, the surface charge became more negative. AH adjuvant became more negatively charged from -2.22 to -15.45 as increasing of pH from 6.0 to 7.4. It was due to the monobasic phosphate ion from phosphate buffer, HPO_4^{2-}, interact covalently with the positively charged aluminium sites on the gel surface. These results were according to the studies of Al-Shakhshir et al. (1994); Rinella et al. (1995, 1996, 1998); Wittayanukulluk et al. (2004). The study of Rinella et al. (1996) had shown that at phosphate anion concentrations of >2 mM, the zeta potential of AH was negatively charge. The phosphate anion interacted covalently with the positively charged aluminium sites on the gel surface. The decreased antigen adsorption was caused by anionic attachment of phosphate to the aluminium surface sites (Callahan et al., 1991).

The results of mean surface charge of single material AH, AP, DT, TT, PT, JE and adsorbed adjuvants which dispersed in deionized water or buffer are presented in Table 12. AH gel had positive charge when was dispersed in deionized water, whereas it had negative charge in phosphate buffer pH 6.0-7.4. AP had negative charge both in deionized water and phosphate buffer pH 6.0-7.4. DT and TT expressed net negative charge in deionized water and every pH of phosphate buffer. PT had slightly negative charge or neutral charge in deionized water medium and all pH of phosphate buffer. JE antigen expressed slightly positive or neutral surface charge in deionized water medium but had negatively charged when dispersed in phosphate buffer pH 6.0-7.4.

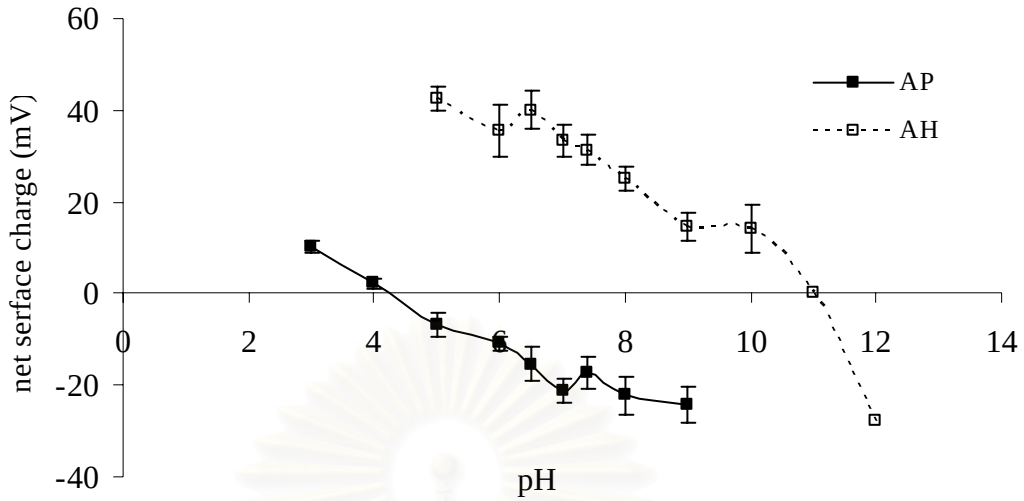


Figure 21 Net surface charge of aluminium adjuvant at various pH. (aliquot diluted 1:80 with supernatant).

Table 11 Surface charge of aluminium hydroxide and aluminium phosphate at different pH.

pH	mean surface charge (mV) (±SD)	
	aluminium hydroxide	aluminium phosphate
3	/	+ 10.12 (± 1.24)
4	/	+ 2.14 (± 0.97)
5	+ 42.72 (± 4.21)	- 6.81 (± 2.61)
6	+ 35.69 (± 3.68)	- 10.84 (± 1.48)
6.5	+ 40.11 (± 2.59)	- 15.43 (± 3.71)
7	+ 33.32 (± 5.64)	- 21.29 (± 2.66)
7.4	+ 31.35 (± 4.23)	- 17.40 (± 3.45)
8	+ 25.02 (± 3.65)	- 22.30 (± 4.11)
9	+ 14.61 (± 3.12)	- 24.28 (± 3.84)
10	+ 14.08 (± 2.64)	/
11	+ 0.23 (± 3.12)	/
12	- 27.83 (± 5.12)	/

/ not measured

Table 12 The surface charge of aluminium adjuvants, antigens, adsorbed adjuvants in various medium.

Adjuvants / Antigens	Mean surface charge in various medium (mV) (± SD)				
	Deionized water	0.01 M Phosphate buffer			
		pH 6.0	pH 6.5	pH 7.0	pH 7.4
AH	+ 39.43 (±6.67)	- 2.22 (±3.05)	- 8.23 (±4.21)	- 12.57 (±3.62)	- 15.45 (±3.00)
AP	- 32.62 (±5.48)	- 26.99 (±3.22)	- 30.85 (±3.22)	- 32.18 (±3.43)	- 38.71 (±3.75)
DT	- 18.74 (±4.28)	- 2.19 (±3.72)	- 0.14 (±4.03)	- 12.64 (±3.61)	- 18.11 (±3.14)
TT	- 6.78 (±5.64)	- 4.77 (±3.23)	- 1.48 (±3.58)	- 14.18 (±3.87)	- 13.59 (±3.14)
PT	- 0.02 (±3.50)	- 0.10 (±3.17)	- 0.17 (±3.58)	- 0.14 (±5.08)	- 0.06 (±3.50)
JE	+ 0.10 (±3.33)	- 11.36 (±8.14)	- 3.65 (±6.60)	- 6.12 (±12.20)	- 28.50 (±5.46)
* DT-AH	/	- 18.52 (±1.98)	- 25.05 (±0.25)	- 24.70 (±1.40)	- 27.16 (±1.53)
* TT-AH	/	- 15.61 (±0.66)	- 13.00 (±2.48)	- 15.62 (±3.12)	- 21.52 (±3.04)
* JE-AH	/	- 8.56 (±1.81)	- 9.42 (±0.69)	- 11.18 (±0.81)	- 13.55 (±1.71)
* DT-AP	/	- 25.88 (±0.29)	- 27.42 (±0.94)	- 31.34 (±1.04)	- 38.96 (±2.10)
* TT-AP	/	- 21.10 (±1.40)	- 24.68 (±0.45)	- 31.27 (±0.97)	- 32.59 (±2.73)
* JE-AP	/	- 15.85 (±0.43)	- 18.22 (±0.26)	- 17.65 (±0.34)	- 21.14 (±0.60)

*adsorbed at 9 °C, / no examined

From the pI results, AH showed positive charge and AP showed negative charge at pH range 6.0-7.4. Therefore, AH was a good adsorbent for negatively charged antigens (DT and TT) whereas AP which was not good adsorbent for DT and TT but it should be better for JE than DT and TT.

The preparations of adsorbed adjuvant, DT-AH, TT-AH, JE-AH, DT-AP, TT-AP and JE-AP, had all negative charge in every pH of phosphate buffer medium (Figure 23). The antigens could adsorb on AH in phosphate buffer due to any charge of antigen molecule. Diphtheria toxin composes of 2 fragments A (23 kDa) and B (38 kDa) and these fragments are hold together by disulfide bonds (Grage, 2002). The A chain showed NH_3^+ which was positively charge (Figure 47, in Appendix B). It could attach with the negative surface charge of AH by electrostatic attractive mechanism. TT and JE compose of many polypeptide chains which have many amino acids. The amino acids are amphoteric protein which can show the different charge in various medium. TT and JE possibly showed positive charge in this condition and could adsorb on AH by electrostatic attractive mechanism. The ligand exchange might be the adsorption mechanism for these antigens. The mechanism was the interaction between a phosphate on antigen and a surface hydroxyl of AH (Iyer et al., 2004). This mechanism was the strongest adsorption mechanism.

AP could adsorb DT, TT and JE by electrostatic mechanism the same as AH and the ligand exchange was possibly the mechanism as AH due to the chemical structure of AP was aluminium hydroxyphosphate (Gupta, 1998) which hydroxyl group of adjuvant could interact with antigen. Due to the alteration of the net surface charge of investigated antigens with both AH and AP and the combined preparations compared with plain adjuvants and plain antigens in each medium, it could be concluded that DT, TT and JE were adsorbed on AH and AP adjuvants.

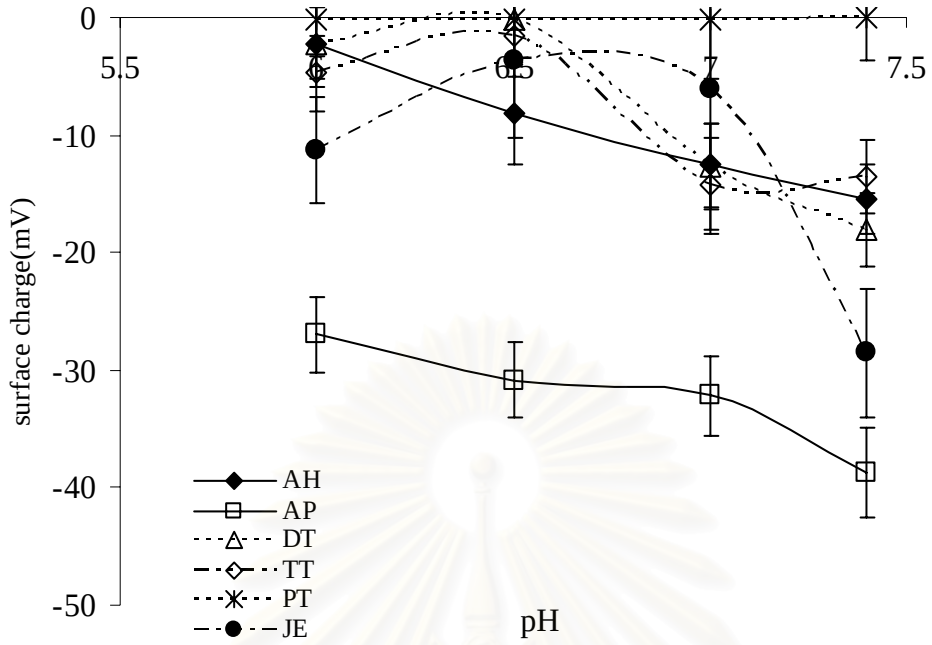


Figure 22 The net surface charge of pure adjuvants and antigens at various pH of phosphate buffer.

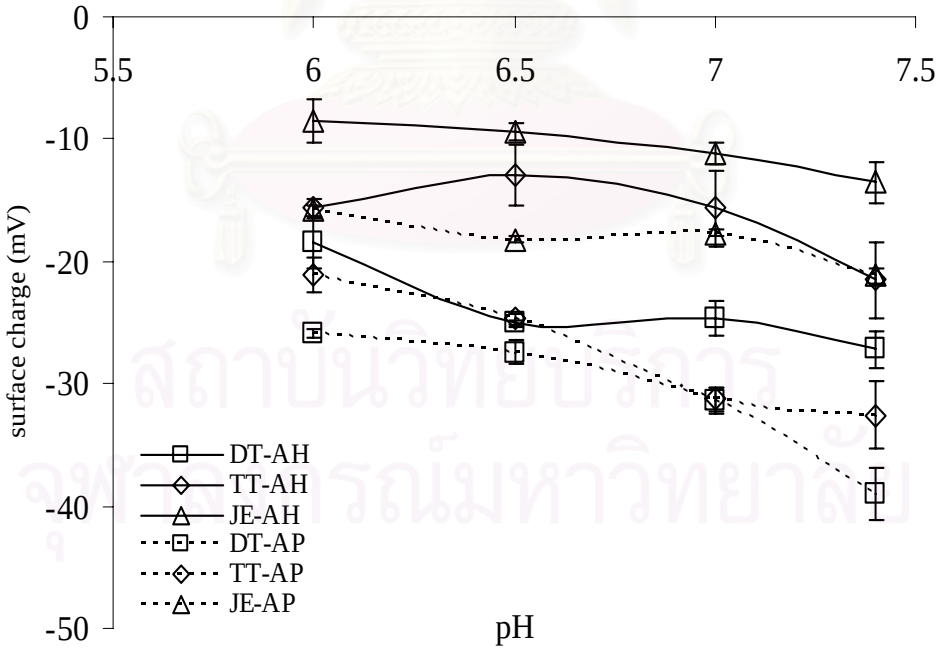


Figure 23 The net surface charge of adsorbed adjuvants at various pHs of phosphate buffer.

3.3.2 Particle size distribution

The particle sizes analyzed by laser diffractrometry (LD) are displayed in Tables 13-17. Data represented the mean of three determinations. The mean particle size of plain AH was 15.21 μm and processed AH, which was processed without antigens at 37 and 9 °C, were 15.08 and 15.11 μm, respectively. The particle size of processed adjuvant was smaller than plain adjuvant due to the processed adjuvant which was the complex fibrous particle (Sepelyak et al., 1984) was broken to small particle during the adsorption process.

As seen in Table 14 and Figure 24, the mean particle size of all adsorbed AH at 37 °C and 9 °C, in all pHs were larger than that of processed AH. JE-AH showed the largest size and TT-AH had the smallest size in every pH. The particle sizes of DT-AH, TT-AH and JE-AH at 37 and 9 °C were increased from processed AH about 17.28-28.71 %, 2.59-6.23% and 37.39-46.45% at 37 °C, and 10.97-16.18%, 8.93-14.38% and 33.74-37.55% at 9 °C, respectively.

Table 13 The particle size of plain and processed adjuvants determined by laser diffractrometry.

Adjuvants		Mean particle size (μm) ± SD			
		d(0.1)	d(0.5)	d(0.9)	uniformity
AH	plain	6.89 ± 0.07	22.72 ± 0.07	69.54 ± 0.09	0.84 ± 0.11
	processed (37 °C)	5.23 ± 0.07	15.08 ± 0.05	40.10 ± 0.05	0.72 ± 0.08
	processed(9 °C)	4.84 ± 0.12	13.19 ± 0.08	32.31 ± 0.10	0.66 ± 0.06
AP	plain	1.56 ± 0.10	2.93 ± 0.11	7.46 ± 0.21	3.29 ± 0.04
	processed (37 °C)	1.54 ± 0.00	2.68 ± 0.01	4.61 ± 0.01	0.36 ± 0.00
	processed (9 °C)	1.63 ± 0.08	2.70 ± 0.05	4.42 ± 0.04	0.33 ± 0.09

Table 14 The particle size of adsorbed aluminium hydroxide determined by laser diffractrometry.

Adsorbed antigen	pH	Mean particle size of adsorbed aluminium hydroxide (μm) (± SD)							
		37 ± 1 °C				9 ± 1 °C			
		d(0.1)	d(0.5)	d(0.9)	uniformity	d(0.1)	d(0.5)	d(0.9)	uniformity
DT	6.0	4.87 (±0.02)	17.69 (±0.18)	54.30 (±0.76)	0.86 (±0.01)	4.63 (±0.02)	16.74 (±0.15)	52.38 (±0.67)	0.88 (±0.01)
	6.5	4.85 (±0.01)	17.79 (±0.10)	54.40 (±0.51)	0.87 (±0.01)	4.63 (±0.01)	16.83 (±0.07)	53.39 (±0.49)	0.89 (±0.01)
	7.0	5.07 (±0.02)	18.76 (±0.17)	56.56 (±1.03)	0.85 (±0.01)	4.73 (±0.01)	17.53 (±0.09)	56.07 (±0.28)	0.91 (±0.01)
	7.4	5.23 (±0.00)	19.41 (±0.02)	58.05 (±0.64)	0.84 (±0.00)	4.75 (±0.01)	17.47 (±0.06)	54.44 (±0.22)	0.88 (±0.01)
TT	6.0	4.24 (±0.01)	15.47 (±0.06)	52.12 (±0.11)	0.96 (±0.01)	4.13 (±0.02)	16.59 (±0.23)	54.98 (±1.31)	0.94 (±0.01)
	6.5	4.26 (±0.02)	15.72 (±0.17)	52.04 (±0.68)	0.94 (±0.01)	4.15 (±0.01)	16.43 (±0.13)	55.20 (±1.08)	0.96 (±0.01)
	7.0	4.58 (±0.20)	15.90 (±0.30)	49.43 (±1.04)	0.87 (±0.01)	4.29 (±0.04)	16.93 (±0.04)	57.06 (±0.29)	0.96 (±0.00)
	7.4	4.45 (±0.02)	16.02 (±0.25)	51.43 (±1.85)	0.92 (±0.05)	4.43 (±0.02)	17.25 (±0.19)	58.64 (±1.59)	0.97 (±0.02)
JE	6.0	5.92 (±0.02)	22.09 (±0.23)	64.37 (±2.05)	0.82 (±0.02)	5.11 (±0.01)	20.71 (±0.06)	62.66 (±0.46)	0.86 (±0.01)
	6.5	5.77 (±0.06)	21.36 (±0.58)	62.74 (±2.01)	0.82 (±0.01)	5.03 (±0.02)	20.17 (±0.20)	62.23 (±0.66)	0.88 (±0.02)
	7.0	5.75 (±0.02)	20.72 (±0.22)	59.86 (±1.15)	0.81 (±0.02)	5.07 (±0.03)	20.62 (±0.26)	63.91 (±1.41)	0.90 (±0.03)
	7.4	5.87 (±0.04)	21.69 (±0.26)	63.59 (±0.52)	0.82 (±0.01)	5.17 (±0.01)	20.75 (±0.09)	62.81 (±0.86)	0.86 (±0.01)

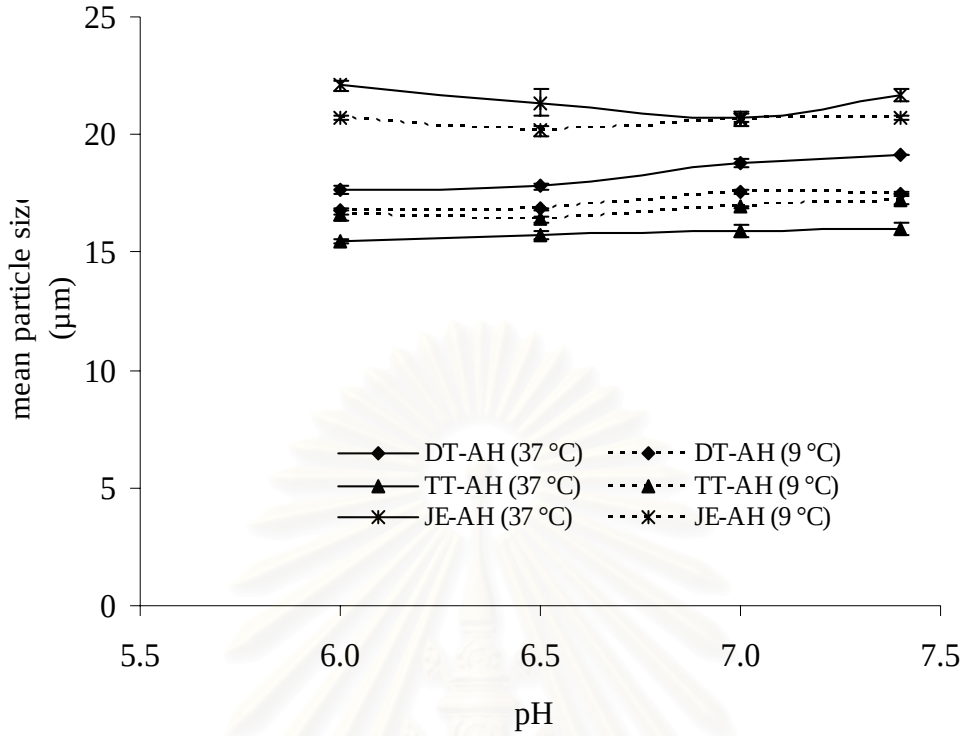


Figure 24 The particle size of adsorbed aluminium hydroxide at various pHs determined by laser diffractrometry.

JE-AH exhibited the highest increased which was due to the larger particle size of JE than DT and TT which were toxoid solutions. DT and JE showed the bigger size at 37 °C than at 9 °C unlike TT which had the larger size at 9 °C. Due to JE virus particle appeared to aggregate and deform at 37 °C, the obtained particle size might be the aggregated form of JE attached on adjuvants (Chetanachan et al., 2001). DT and TT could not be clearly concluded about the size distribution between 37 °C and 9 °C.

Table 13 shows the mean particle size distributions of plain AP and processed adjuvants at 37 °C and 9 °C analyzed by LD. The mean particle size of plain AP was 2.87 µm and processed AP at 37 °C and 9 °C were 2.68 and 2.74 µm, respectively. The processed adjuvant both temperatures were smaller than plain adjuvant because the morphology of AP which was aggregated irregular particle (Shirodkar et al., 1990; Burrell et al., 2001) was broken to small particle during the adsorption process.

Figure 25 and Table 15 show the mean particle size of adsorbed AP at 37 °C and 9 °C analyzed by LD. At 37 °C, DT-AP, TT-AP and JE-AP at 37 °C exhibited bigger particle size than processed AP except DT-AP pH 7.0 and 7.4, TT-AP pH 7.4. At 9 °C, the particle size of all pH of DT-AP and TT-AP pH 7.0, 7.4 were smaller than processed AP and the uniformity values were higher than 1. JE-AP showed the biggest particle size in every pH at both investigated temperatures. At 37 °C, the particle sizes of DT-AP, TT-AP and JE-AP were maximal increased to 29.07%, 35.90% and 66.64%, respectively, while the highest increased of particle size at 9 °C of TT-AP and JE-AP was 9.63% and 43.02%, respectively. All of the particle size of DT-AP at 9 °C was smaller than processed AP. The particle sizes of all adsorbed adjuvants at 37 °C were larger than at 9 °C; it might be the effect of aggregated antigens.

The smaller particle sizes of adsorbed adjuvants than processed adjuvants might due to the morphology of AP adjuvant which was an irregular shape (Shirodkar et al., 1990; Burrell et al., 2001) was broken during the measurement, while AH particle was the crystalline and composed of multilayer of six-membered ring (Nail et al., 1976) that was more rigid than AP.

The particle size of adsorbed AP both temperatures were decreased as the pH was increased. They related to the adsorption values of single antigen on AP which showed the less adsorption at pH 6.0 than pH 7.4. It was due to less adsorption which had low attached antigens on adjuvant would had the smaller size than higher adsorption.

Tables 16-17 show the mean particle size of adsorbed AH and adsorbed AP by photon correlation spectroscopy (PCS), respectively. Only one temperature at 9 °C was measured. Table 16 shows the mean particle size of plain and processed AH and plain and processed AP were 6.95, 6.85 μm and 2.01, 1.88 μm, respectively. As seen in Table 17, the mean particle sizes of all adsorbed AH were larger than processed AH except DT-AH, pH 6.5, pH7.4 and TT-AH, pH 6.5, pH 7.4. All of adsorbed AP had the particle size bigger than processed AP except TT-AP, pH 7.4. The particle sizes of JE-AP were the biggest size.

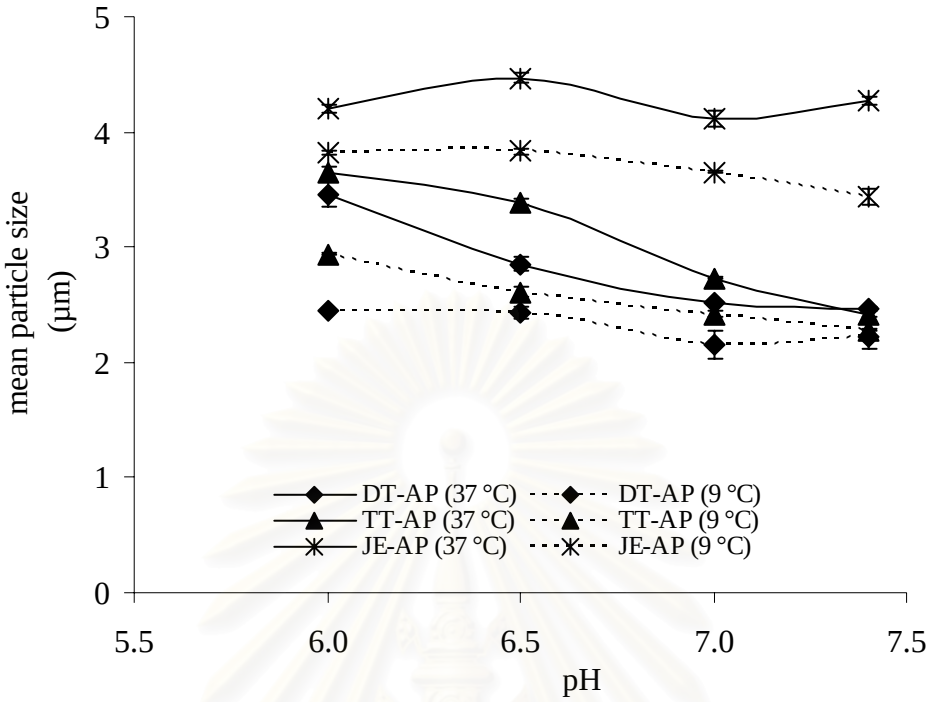


Figure 25 The particle size of adsorbed aluminium phosphate at various pHs determined by laser diffractrometry.

The particle sizes which were detected from the different method were different owing to the procedure and sensitivity of instrument. PCS is the optimal tool to proceed with smaller materials, while LD is appropriate for larger materials than PCS. Frantzen et al. (2003) reported that PCS was a widely used method for measuring submicron particles, and its range (from 5 nm up to 5 µm) made it especially convenient for measuring the size distribution of submicron particles. It is generally accepted that PCS analysis yields reliable results when monodisperse samples are measured and the micron-range contaminants in submicron particle dispersions were hardly detected by dynamic laser light scattering

Table 15 The particle size of adsorbed aluminium phosphate determined by laser diffractrometry.

Adsorbed antigen	pH	Mean particle size of adsorbed aluminium phosphate (μm) (± SD)							
		37 ± 1 °C				9 ± 1 °C			
		d(0.1)	d(0.5)	d(0.9)	uniformity	d(0.1)	d(0.5)	d(0.9)	uniformity
DT	6.0	1.91 (±0.09)	3.46 (±0.11)	6.25 (±0.41)	0.39 (±0.03)	1.53 (±0.00)	2.44 (±0.00)	3.91 (±0.06)	0.30 (±0.00)
	6.5	1.49 (±0.02)	2.85 (±0.06)	6.23 (±1.56)	1.36 (±0..81)	1.25 (±0.01)	2.43 (±0.05)	4.25 (±1.99)	5.18 (±2.22)
	7.0	1.39 (±0.01)	2.52 (±0.00)	4.55 (±0.01)	0.39 (±0.00)	1.21 (±0.07)	2.15 (±0.12)	4.22 (±3.34)	2.87 (±4.05)
	7.4	1.45 (±0.06)	2.47 (±0.02)	4.19 (±0.22)	0.35 (±0.03)	1.25 (±0.08)	2.23 (±0.11)	4.36 (±3.89)	2.96 (±4.23)
TT	6.0	1.99 (±0.05)	3.64 (±0.05)	6.58 (±0.19)	0.39 (±0.02)	1.62 (±0.01)	2.94 (±0.01)	5.30 (±0.02)	0.39 (±0.00)
	6.5	1.92 (±0.01)	3.39 (±0.03)	5.96 (±0.06)	0.37 (±0.00)	1.38 (±0.02)	2.61 (±0.04)	6.08 (±1.28)	2.04 (±1.42)
	7.0	1.61 (±0.03)	2.73 (±0.01)	4.64 (±0.13)	0.35 (±0.02)	1.25 (±0.02)	2.42 (±0.03)	20.22 (±3.18)	2.47 (±0.12)
	7.4	1.37 (±0.02)	2.42 (±0.02)	4.21 (±0.14)	0.36 (±0.02)	1.30 (±0.02)	2.28 (±0.01)	3.90 (±0.11)	0.36 (±0.01)
JE	6.0	2.25 (±0.01)	4.20 (±0.03)	7.79 (±0.37)	0.46 (±0.10)	1.96 (±0.00)	3.82 (±0.01)	7.44 (±0.03)	0.47 (±0.00)
	6.5	2.33 (±0.02)	4.47 (±0.04)	8.36 (±0.54)	0.92 (±0.89)	1.98 (±0.06)	3.83 (±0.02)	7.18 (±0.19)	0.42 (±0.02)
	7.0	2.21 (±0.04)	4.12 (±0.07)	7.40 (±0.10)	0.39 (±0.00)	1.92 (±0.00)	3.65 (±0.01)	7.00 (±0.06)	0.44 (±0.00)
	7.4	2.28 (±0.02)	4.27 (±0.03)	7.61 (±0.05)	0.39 (±0.00)	1.91 (±0.02)	3.43 (±0.07)	6.16 (±0.27)	0.39 (±0.02)

In conclusion, the results of particle sizes distribution which were measured by LD and PCS confirmed that the antigens were attached on the aluminium adjuvants at both investigated temperatures. The particle sizes of adsorbed adjuvants which were smaller than processed adjuvants might be the result of structure conformation. AP showed the aggregated particles that might be broken down after agitation (Shirodkar et al., 1990). The studies by Johnston et al. (2002) and Morefield et al. (2004) had shown that AH composed of very small primary particles. These particles formed irregularly shaped aggregates having diameters between 5 and 10 µm. They mixed AH adjuvant with labeled BSA and observed the label region with flow cytometry so the adjuvant aggregates underwent a de-aggregation and re-aggregation process during mixing. Therefore, the particle size of AH and AP could be broken after the adsorption process.

Table 16 The mean particle size of plain and processed adjuvants at 9 °C determined by photon correlation spectroscopy.

Adjuvants		Mean particle size (± SD)	
		Size (µm)	PI
AH	plain	6.95 ± 0.09	0.58 ± 0.12
	processed	6.85 ± 0.35	0.42 ± 0.35
AP	plain	2.01 ± 0.09	0.43 ± 0.11
	processed	1.88 ± 0.23	0.36 ± 0.21

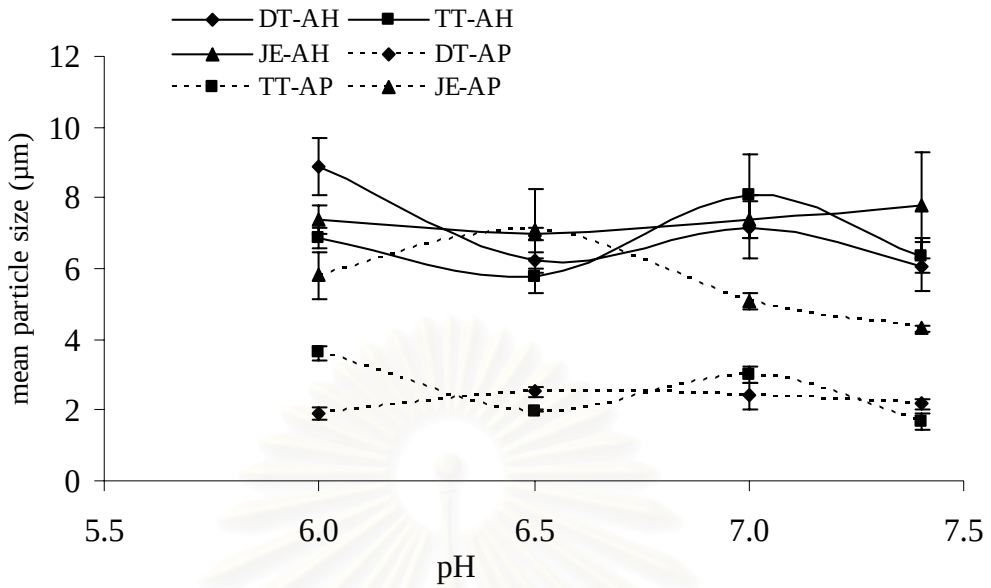


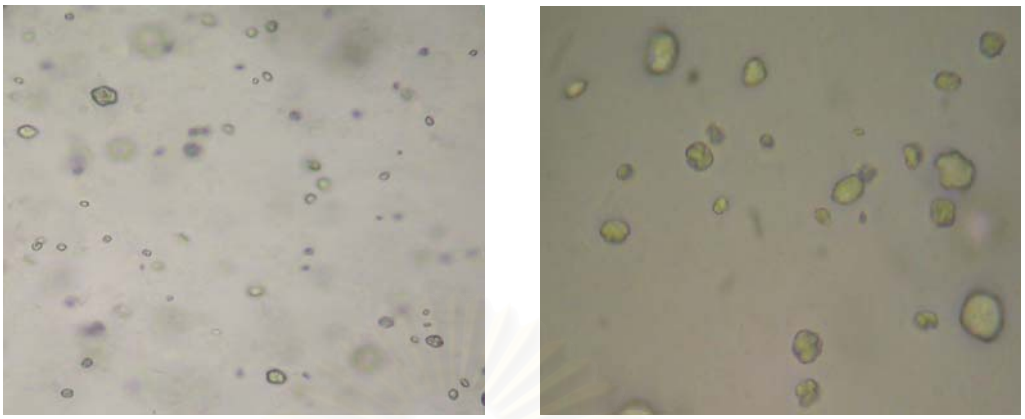
Figure 26 The particle size of adsorbed aluminium at various pHs, 9 °C determined by photon correlation spectroscopy.

3.3.3 Optical microscopy

Only AH and PT could be observed by optical microscope because the size of AP and other antigens were too small. Figure 27 illustrates the photomicrographs of AH from optical microscope. They were spherical shape with various sizes. PT are shown in Figure 28. They were aggregate particles with fragments from the cloudy sample. The particle shape was various. However, it seemed to be spherical. Those small fragments were likely parts to be of whole cell which was broken during the preparation of antigen.

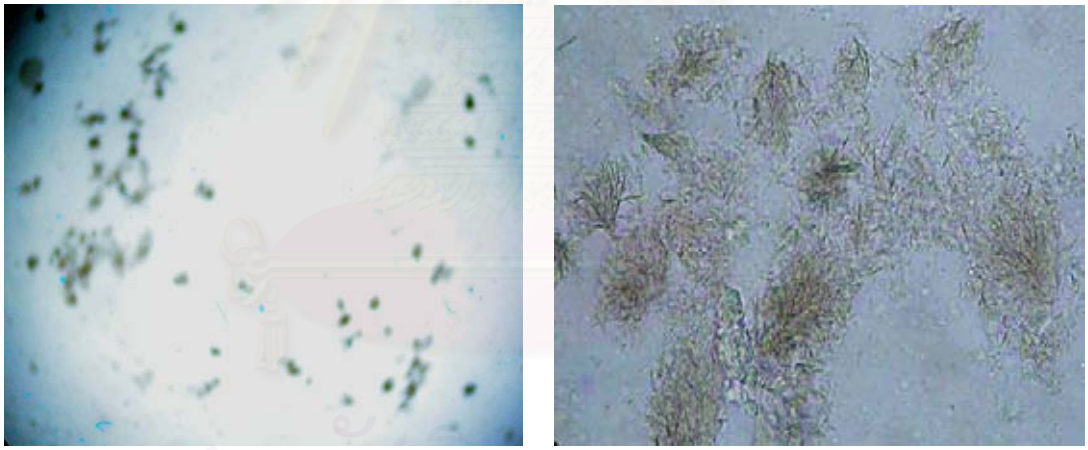
Table 17 The particle size of adsorbed aluminium hydroxide and adsorbed aluminium phosphate at 9 °C determined by photon correlation spectroscopy.

Adsorbed antigen	pH	Mean particle size (± SD) (μm)			
		Aluminium hydroxide		Aluminium phosphate	
		size	PI	size	PI
DT	6.0	8.87 (±0.81)	0.11 (±0.06)	1.92 (±0.17)	0.41 (±0.08)
	6.5	6.23 (±0.21)	0.23 (±0.17)	2.51 (±0.12)	0.35 (±0.07)
	7.0	7.17 (±0.89)	0.29 (±0.19)	2.41 (±0.37)	0.47 (±0.06)
	7.4	6.05 (±0.69)	0.27 (±0.09)	2.17 (±0.14)	0.36 (±0.07)
TT	6.0	6.88 (±0.28)	0.10 (±0.03)	3.61 (±0.19)	0.49 (±0.26)
	6.5	5.78 (±0.48)	0.39 (±0.11)	1.97 (±0.03)	0.45 (±0.04)
	7.0	8.05 (±1.20)	0.28 (±0.24)	3.01 (±0.22)	0.38 (±0.19)
	7.4	6.36 (±0.48)	0.69 (±0.21)	1.69 (±0.22)	0.54 (±0.09)
JE	6.0	7.38 (±0.40)	0.45 (±0.28)	5.81 (±0.68)	0.25 (±0.10)
	6.5	6.96 (±0.18)	0.32 (±0.11)	7.07 (±1.17)	0.43 (±0.10)
	7.0	7.39 (±0.51)	0.39 (±0.12)	5.07 (±0.22)	0.42 (±0.22)
	7.4	7.79 (±1.51)	0.38 (±0.09)	4.32 (±0.09)	0.98 (±0.04)



(A) (B)

Figure 27 The optical photomicrographs of aluminium hydroxide (A) 100x, (B) 1,000x.



(A) (B)

Figure 28 Optical photomicrographs of *Bordetella pertussis* (A) 100x, (B) 400x

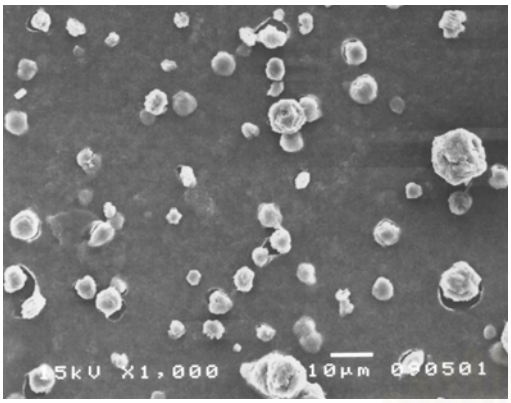
3.3.4 Scanning electron microscopy

The photomicrographs of AH, AP and the preparations of DT, TT and JE which were adsorbed on both adjuvants in 0.01 M phosphate buffer pH 6.5 at temperature 9 °C are shown in Figures 29-30. AH was spherical complex particle and AP was very small particles which aggregated to network, hence, the morphology of AP particles by SEM was irregular shape. Processed AH showed the morphology similar to plain adjuvants. As seen in Figure 30, the AH particles which were adsorbed with DT, TT and JE were different from plain and processed forms. Their morphology showed some small particles which were likely to be of antigens attached on the adjuvant. This result was contributed to the adsorption of DT, TT and JE on AH adjuvant in 0.01 M phosphate buffer pH 6.5.

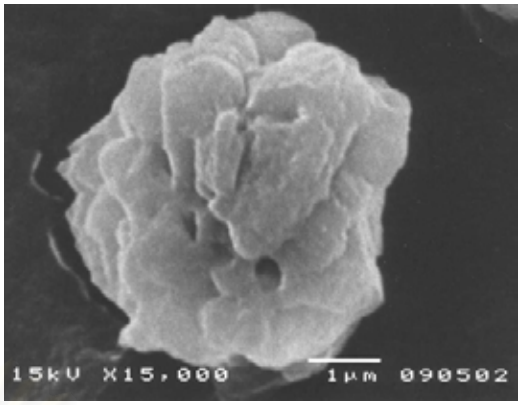
3.3.5 Transmission electron microscopy

The TEM negative staining photomicrographs of AH, AP, DT, TT, PT and JE are shown in Figure 31-32. The morphology of AH [Figure 31(A)] was fibrous whereas that of AP [Figure 31(B)] was aggregate particles. The results were similar to the previous observations (Shirodkar et al., 1990; Rinella et al., 1995; Burrell et al., 2001; and Johnston et al., 2002).

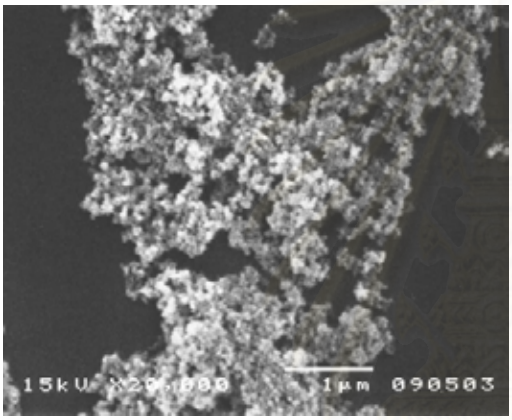
Figure 32 illustrates the morphology of antigens; DT, TT, PT and JE. The morphology of DT and TT were very small size and spherical shape. PT presented the oblong shape which was the general shape of this antigen. JE revealed the spherical particles. These results were similar to the studies of Heinz et al. (1993), Hockley, et al. (1999) and Chetanachan et al. (2001). In addition, Chetanachan et al. (2001) revealed that JE virus particles that were kept at 2-8 °C were distributed individually while which were kept at 37 °C appeared to become aggregated and deformed.



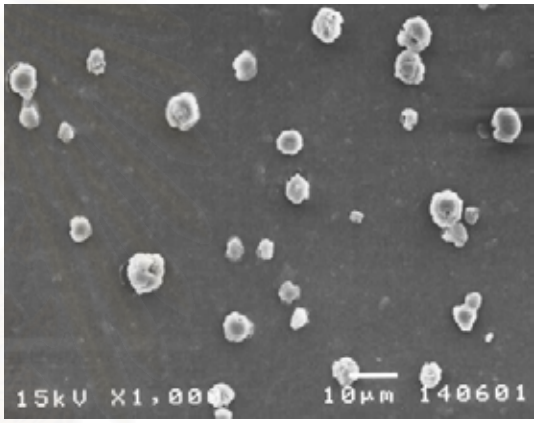
(A)



(B)



(C)



(D)

(E)

Figure 29 SEM photomicrographs of (A) AH (1,000x), (B) AH (15,000x), (C) AP (20,000x) (D) processed AH (1,000x), (E) processed AH (15,000x)

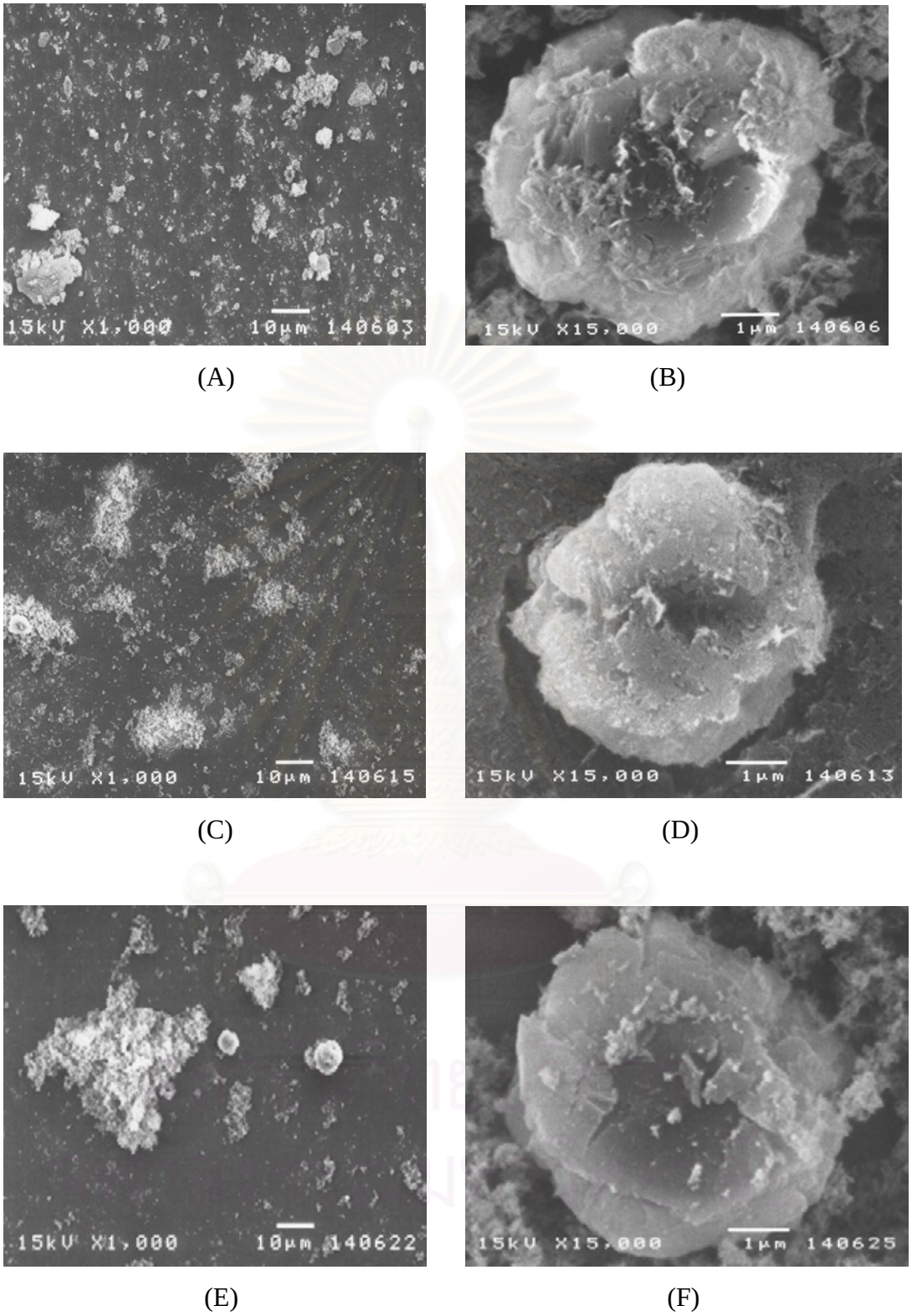


Figure 30 SEM photomicrographs of AH adsorbed with (A) DT (1,000x), (B) DT (15,000x), (C) TT (1,000x), (D) TT (15,000x), (E) JE (1,000x), (F) JE (15,000x).

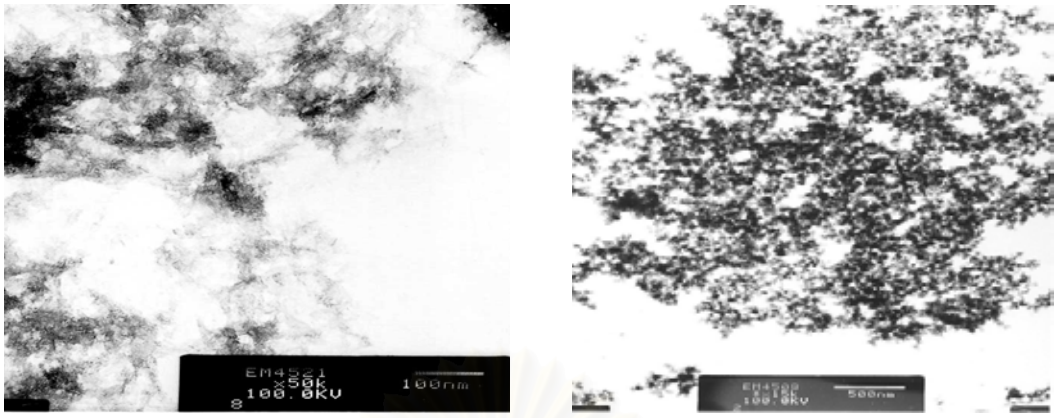
As seen in Figure 33, the TEM photomicrographs of the preparations of DT, TT and JE which were adsorbed on both adjuvants could not indicate the adsorption on AH and AP because the photomicrographs were not observed of any antigens on adjuvants. It was due to the very small particulate antigens compared to the larger sized adjuvants. Furthermore, the sensitivity of instrument was too low to observe. Therefore, TEM were not observed for adsorbed adjuvants.

3.3.6 Atomic force microscopy

The adsorbed preparations which were observed by AFM were the preparations that conducted on AP in 0.01 M phosphate buffer pH 6.5 at temperature 9 °C since SEM and TEM analysis could not observe the morphological difference between adsorbed adjuvants and plain adjuvants. Their true three-dimensional surface morphology compared with that of AP are shown in Figure 34.

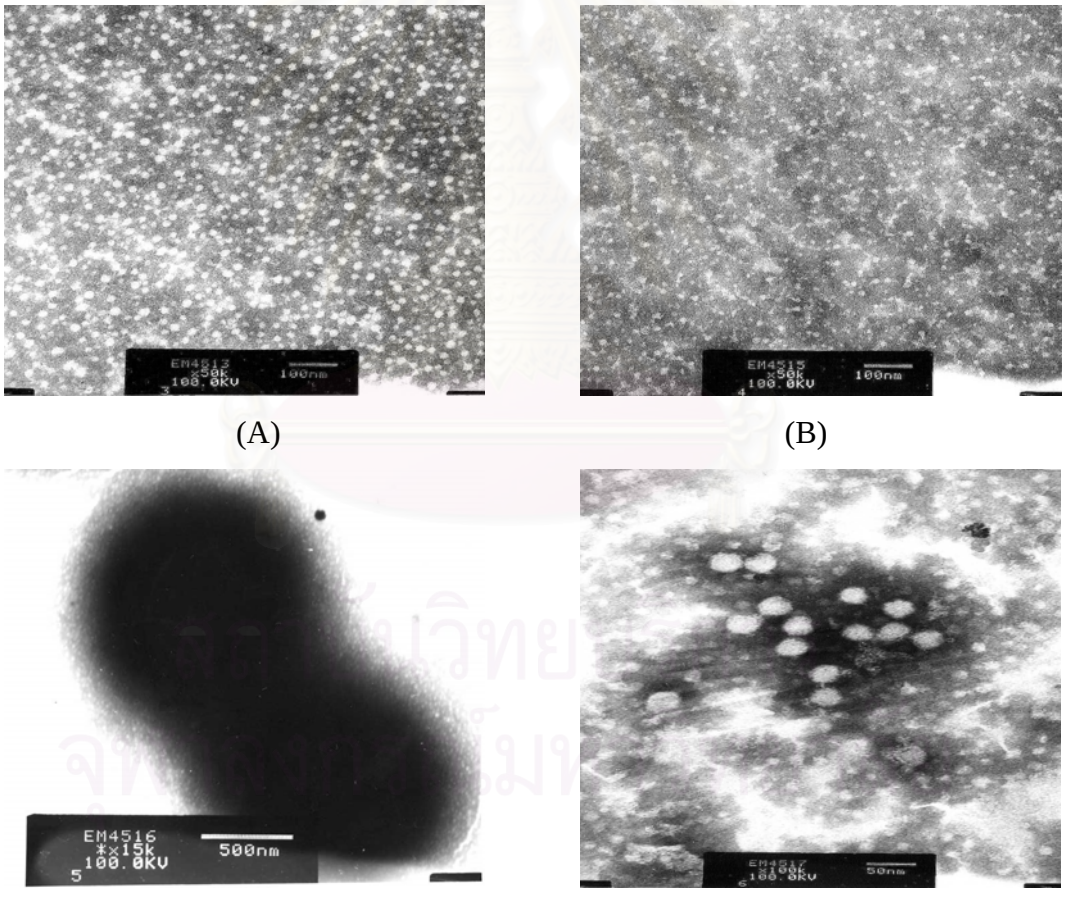
It could be seen that plain AP had different surface morphology from the adsorbed preparations. The latter had spherical particulate matters attached on the surface of AP. Figure 35 shows the top view surface morphology of AP and the adsorbed preparations. There were particles attached on the AP surface similarly to the three-dimensional surface morphology. From these results DT, TT and JE were evidently adsorbed on the surface of AP adjuvant.

The spherical particulate matters on adsorbed preparation were indicated as DT and TT and smaller size than plain AP. The size of JE particle in Figure 34(D) and Figure 35(D) was approximately equal to the size of plain AP and adsorbed preparation was more condensed than plain AP.



(A) (B)

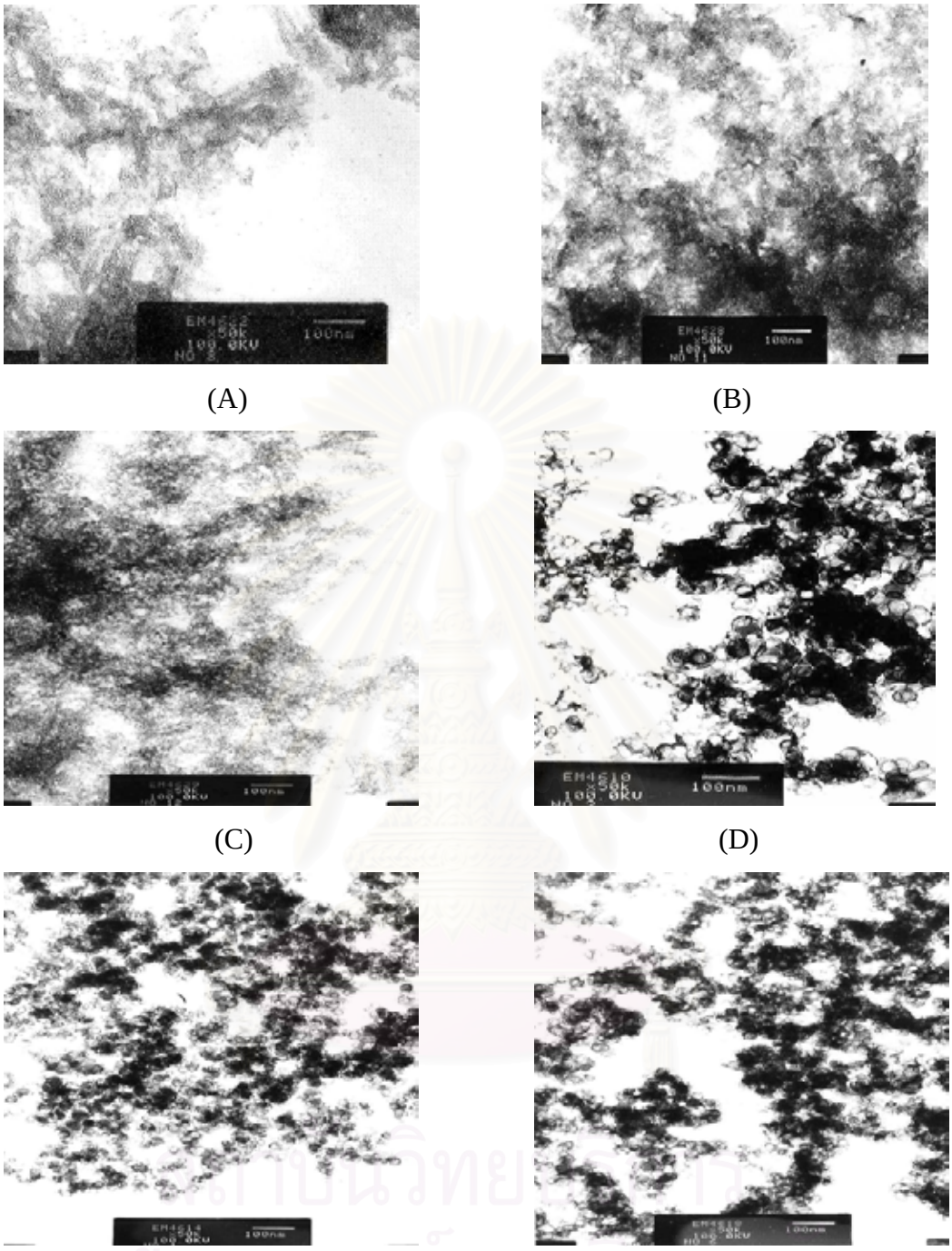
Figure 31 TEM photomicrographs of aluminium adjuvants (A) AH (110,000x), (B) AP (110,000x).



(A) (B)

(C) (D)

Figure 32 TEM photomicrographs of various antigens (A) DT (110,000x), (B) TT (110,000x), (C) PT (33,000x), (D) JE (110,000x).



(A) (B)

(C) (D)

(E) (F)

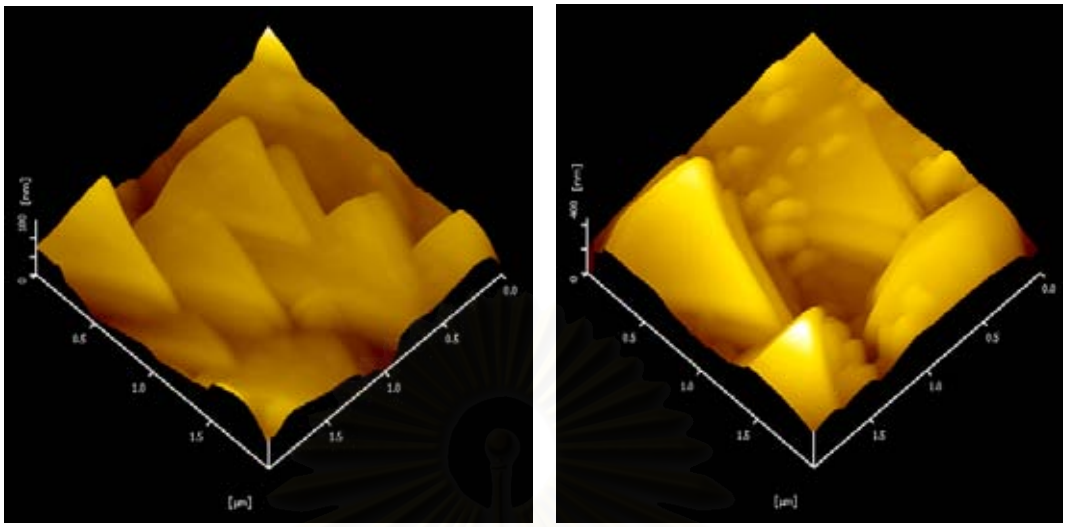
Figure 33 TEM photomicrographs of antigens adsorbed adjuvants (x110,000) (A) DT-AH, (B) TT-AH, (C) JE-AH, (D) DT-AP, (E) TT-AP, (F) JE-AP.

3.3.7 Fourier Transform Infrared spectrometry

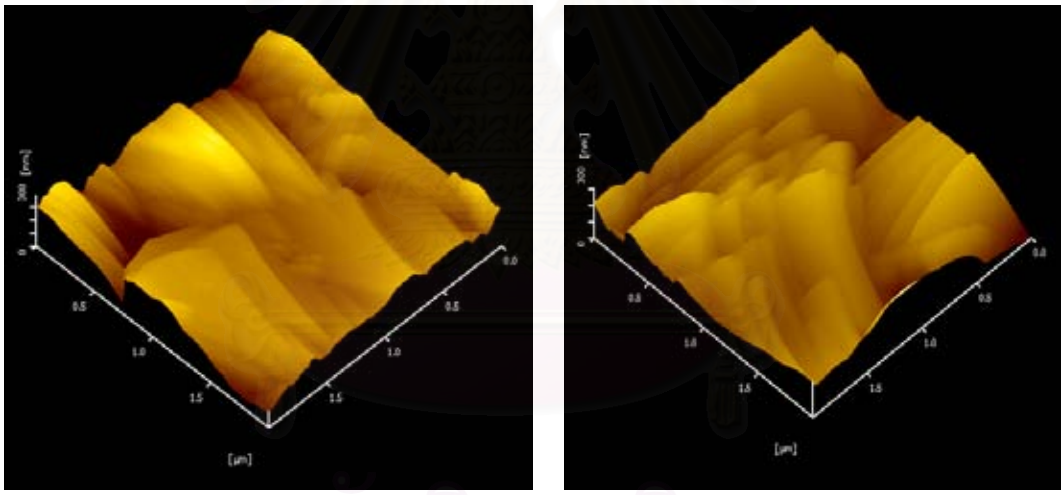
IR spectra of AH, DT-AH, TT-AH and JE-AH are shown in Figure 36. Their spectra showed peaks at around 1072.29, 1073.42, 1072.84, 1068.19 cm^{-1}, respectively which were O-H deformation region; 3097.81, 3092.15, 3094.38, 3098.78 cm^{-1}, respectively and 3412.73, 3421.95, 3413.85, 3397.99 cm^{-1}, respectively which were O-H stretching region. The results from O-H stretching were the broad peak between 3000-3500 cm^{-1} which indicated the existence of structural hydroxyl environments and the shoulder peak around 3100 cm^{-1}.

These results were conformed to the studies of Shirodkar et al. (1990) and Lindblad (2004) that the principle peaks of AH were observed at wave numbers of 1070 in the O-H deformation region and a shoulder at 3100 cm^{-1}. The strong shoulder at 3100 cm^{-1} is also unique for boehmite which is the mineralogical name of aluminium oxhydroxide.

No changes on absorption band position for the principle peaks were observed in the adsorbed adjuvants. This indicated no chemical changes of adsorbed AH due to the principle peaks were also represented. There were the minor differences between the absorption bands of JE-AH, DT-AH with AH at around 500-1000 cm^{-1}. AH showed the peak at 969.82 cm^{-1} but no peak at this position of DT-AH, while at the same region JE-AH showed the different peaks at 973.73 and 763.68 cm^{-1}. It could be concluded that interaction between DT with AH and JE with AH adjuvant induced some chemical changes of AH structure which was not the important function for adsorption. In other word, the adsorption had no effect on chemical structure of AH

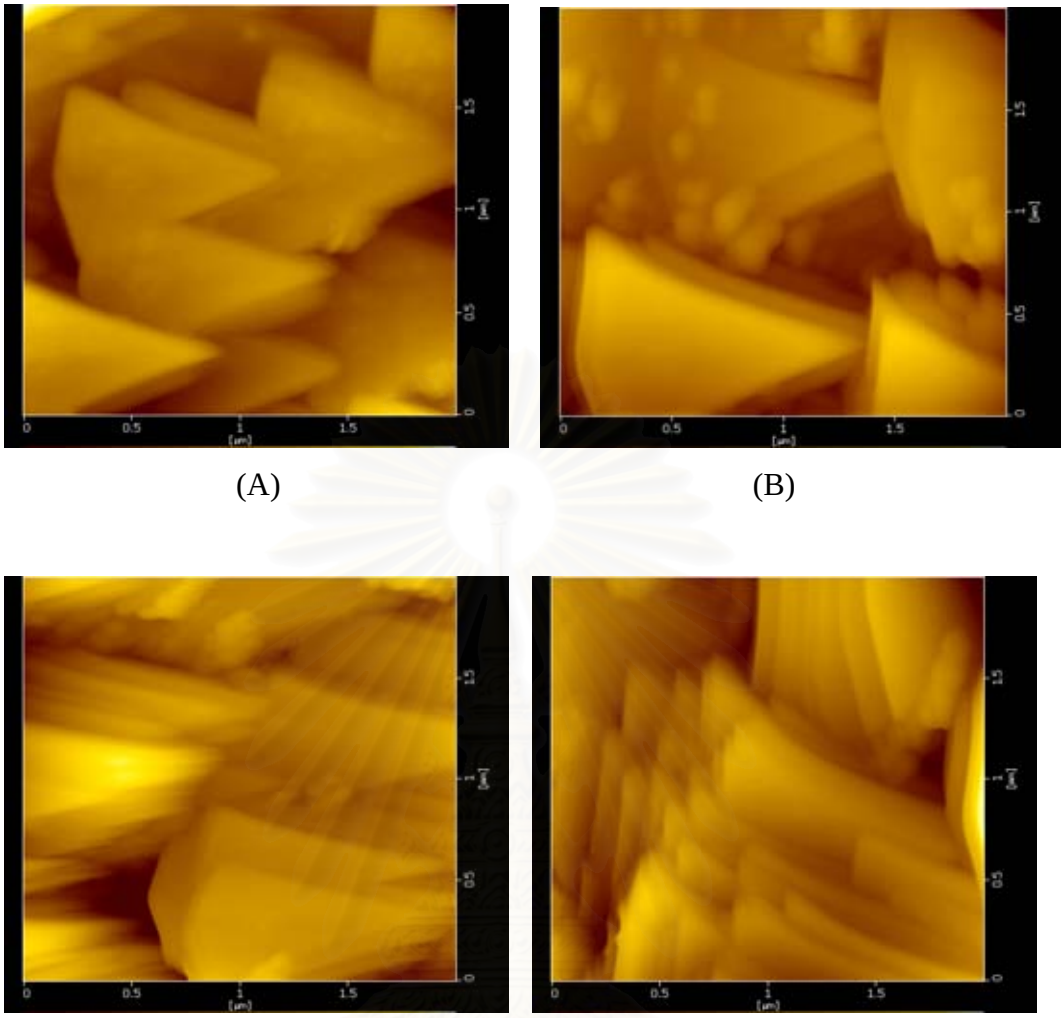


(A) (B)



(C) (D)

Figure 34 AFM three-dimensional surface morphology of (A) plain AP (B) DT-AP (C) TT-AP (D) JE-AP.



(A) (B)

(C) (D)

Figure 35 AFM top view surface morphology of (A) plain AP (B) DT-AP (C) TT-AP (D) JE-AP.

Figure 37 illustrates the IR spectra of AP, DT-AP, TT-AP and JE-AP. IR spectra of AP exhibited the major peaks at around 1087.25 and 3416.73 cm^{-1}. IR spectra of DT-AP, TT-AP and JE-AP showed peaks at around 1095.75, 1095.85, 1092.90 cm^{-1}, respectively and 3415.31, 3431.01, 3432.78 cm^{-1}, respectively. The adsorption band at around 1100 cm^{-1} is characteristic of phosphate (P-O stretching) and the broad band around 3400 cm^{-1} is O-H stretching. These results were similar to the results of Shirodkar et al. (1990) and Burrell et al. (2001) which showed the same position of the principle peaks.

There was a little difference in position of peaks. The different peak position between AP and DT-AP was 2345.56 cm^{-1}, it might be the interaction between phosphorus of AP and hydrogen of antigen because that peak is P-H stretch region. The results were concluded that no chemical changes of adsorbed AP. Therefore, the adsorption had no effect on chemical structure of AP adjuvant.

Therefore, the adsorption process did not affect to the chemical structure of both AH and AP adjuvants due to the adsorption between antigens and adjuvants was the physical mechanism as the electrostatic force which was the important role in this process (Swarbrick et al., 1988; Matheis et al., 2002).

3.3.8 Powder X-ray diffractrometry

Powder X-ray diffractrometry (PXRD) is a useful method for characterization aluminium adjuvants. The X-ray diffractograms of plain AH, DT-AH, TT-AH and JE-AH are shown in Table 18 and Figure 38.

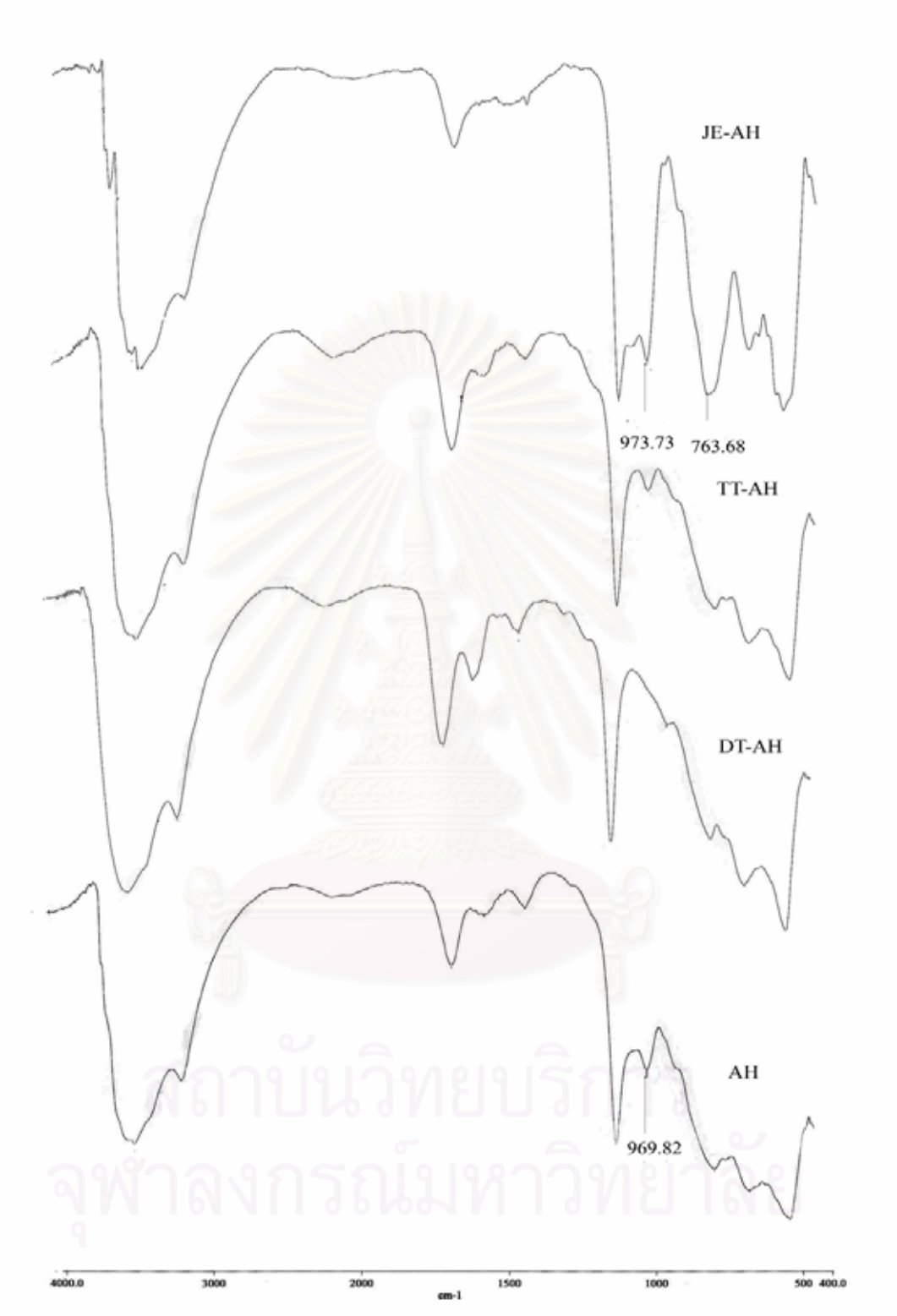


Figure 36 IR spectra of plain aluminium hydroxide, diphtheria adsorbed on aluminium hydroxide, tetanus toxoid adsorbed on aluminium hydroxide and JE antigen adsorbed on aluminium hydroxide.

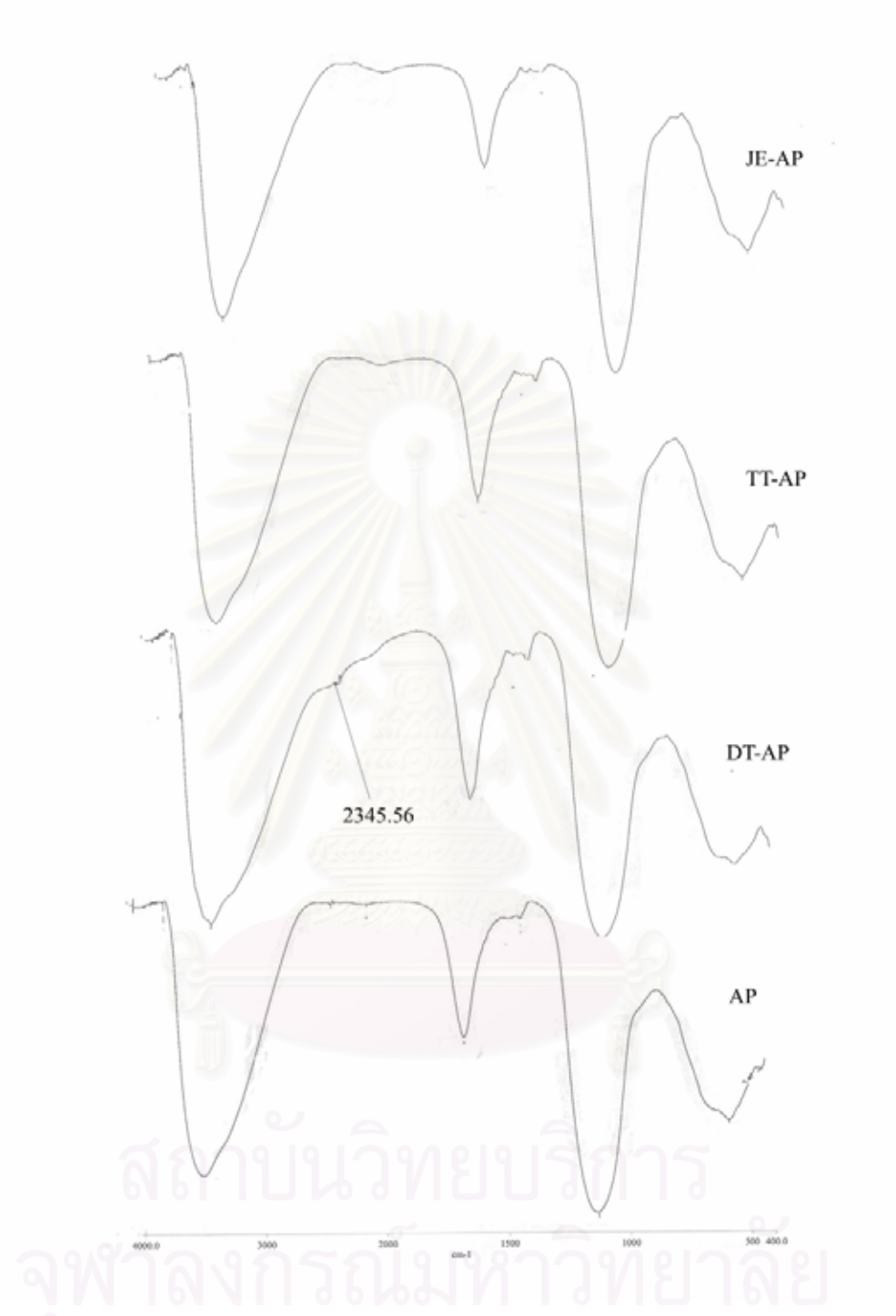


Figure 37 IR spectra of plain aluminium phosphate, diphtheria toxoid adsorbed on aluminium phosphate, tetanus toxoid adsorbed on aluminium phosphate and JE antigen adsorbed on aluminium phosphate.

Table 18 The X-ray diffraction bands of plain aluminium hydroxide and adsorbed preparations.

	small diffraction bands (°A)	sharp peaks (°A)
AH:	6.321, 3.188, 2.352, 1.863	4.716, 4.211 2.218, 1.719
DT-AH:	6.375, 3.211, 2.371, 1.855	4.756, 4.384, 2.829, 2.227, 1.998, 1.724, 1.631
TT-AH:	6.232, 3.193, 2.349, 1.849	4.726, 4.358, 2.815, 2.222, 1.991, 1.718, 1.626
JE-AH:	6.393, 3.202, 2.359, 1.860	4.736, 4.358, 2.819, 2.218, 1.993, 1.723, 1.627

All X-ray diffractograms (small diffraction bands) of adsorbed preparations and AH expressed the same principle bands which corresponded to the studies of Shirodkar et al. (1990) and Masood et al. (1994) that the diffraction bands which are characteristic of AH, crystalline aluminium oxyhydroxide, ranging from 6.27-6.48 °A for the d(020) spacing with additional bands at 3.18, 2.35 and 1.86 °A.

However, there were some differences in position of diffraction bands between pure AH with the adsorbed preparations. The different diffraction bands were 2.829, 1.998 and 1.631 °A for DT-AH; 2.815, 1.991 and 1.626 °A for TT-AH; 2.819, 1.993 and 1.627 °A for JE-AH. The results could not be clearly concluded about the effect of adsorption process on the polymorphism of AH. It could be assumed that the polymorphism of AH might be changed or it was salts from buffer.

Figure 39 presents the X-ray diffractrograms of plain AP and the adsorbed adjuvants. The X-ray patterns of AP and the adsorbed preparations exhibited the sharp diffraction bands. The sharp bands of AP, DT-AP, TT-AP and JE-AP were 3.262, 2.822, 1.994 and 1.629 °A; 3.257, 2.822, 1.994 and 1.627 °A; 3.257, 2.822, 1.994 and 1.628 °A; 3.252, 2.822, 1.994 and 1.627 °A, respectively. They revealed similar bands at the same positions. This result indicated that AP was in crystalline form and did not show any polymorphism. It could be assumed that the adsorption process between DT, TT and JE with AP adjuvant did not affect the polymorphism of

AP because the electrostatic force was not strong enough to change the polymorphism.

The results were in contrast to the previous studies of Shirodkar et al. (1990) and Burrell et al. (1999) which showed that AP did not exhibit any X-ray diffraction bands, indicating that it was amorphous. AP used in this experiment presented the crystalline form and might be due to the different source of material used.

4. Adsorption of combined antigens on adjuvants

4.1 The optimal condition of ELISA procedure

Table 19 shows correlation coefficient (R^2) of various conditions of DT and TT. The optimal condition of DT which gave the maximal R^2 (0.9996) was coating antigen concentration 10 µg/well, dilution of primary antibody (1°Ab) 1:500 and secondary antibody (2°Ab) 1:10,000. Moreover, the optimal condition of TT which gave the maximal R^2 (0.9967) was coating antigen concentration 10 µg/well, dilution of primary antibody (1°Ab) 1:10,000 and secondary antibody (2°Ab) 1:10,000.

The result of the effect of preparation method showed that the homogeneous suspension of PT sonicated for 30 minutes had the optical density (OD=2.181 $\pm$ 0.04) similar to that of the unsonicated suspension (OD=2.118 $\pm$ 0.05). Therefore, the suspension of PT antigen could be immediately coated on the plate without sonicated.

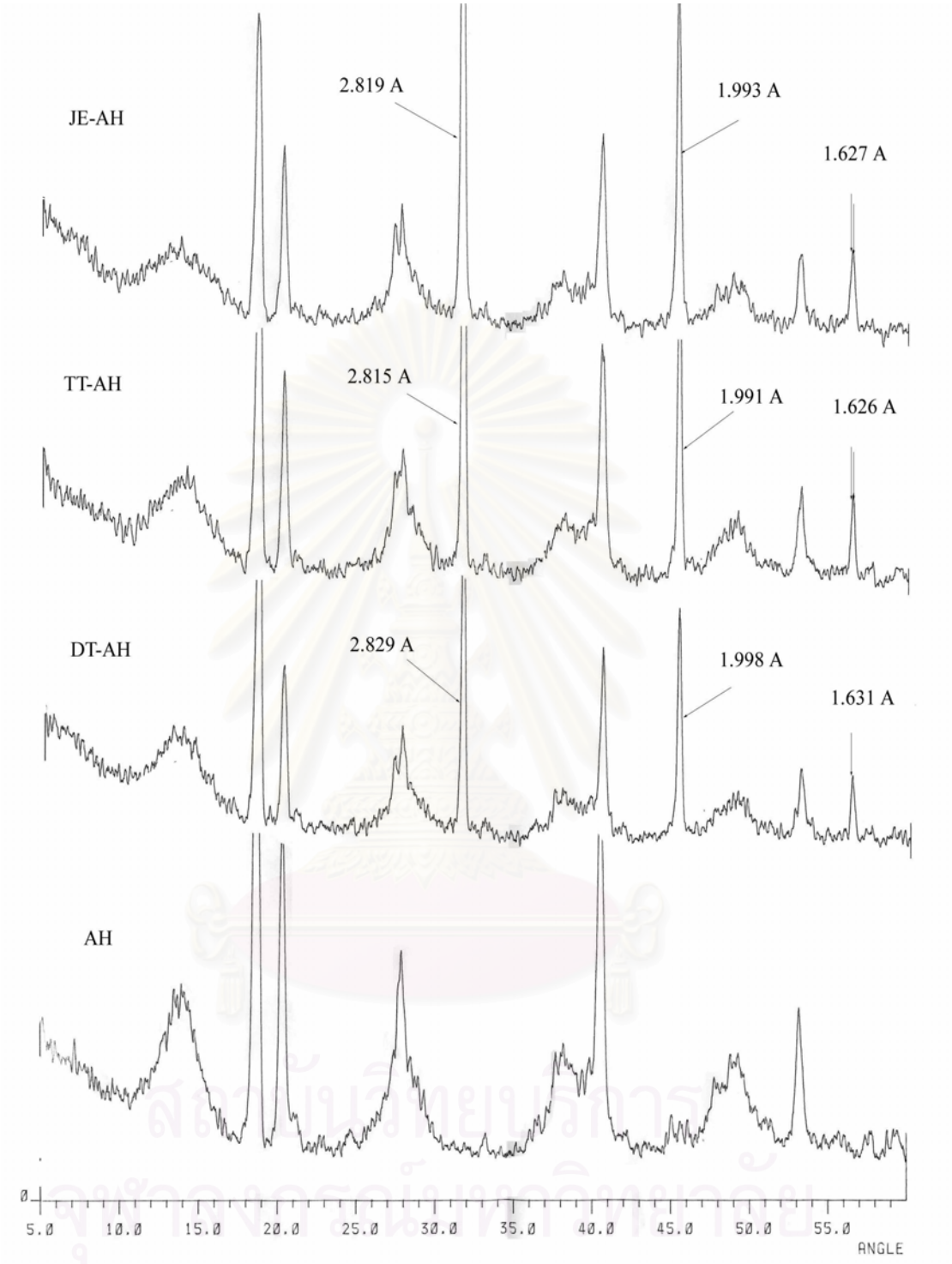


Figure 38 X-ray diffractograms of plain aluminium hydroxide, diphtheria toxoid adsorbed on aluminium hydroxide, tetanus toxoid adsorbed on aluminium hydroxide and JE antigen adsorbed on aluminium hydroxide.

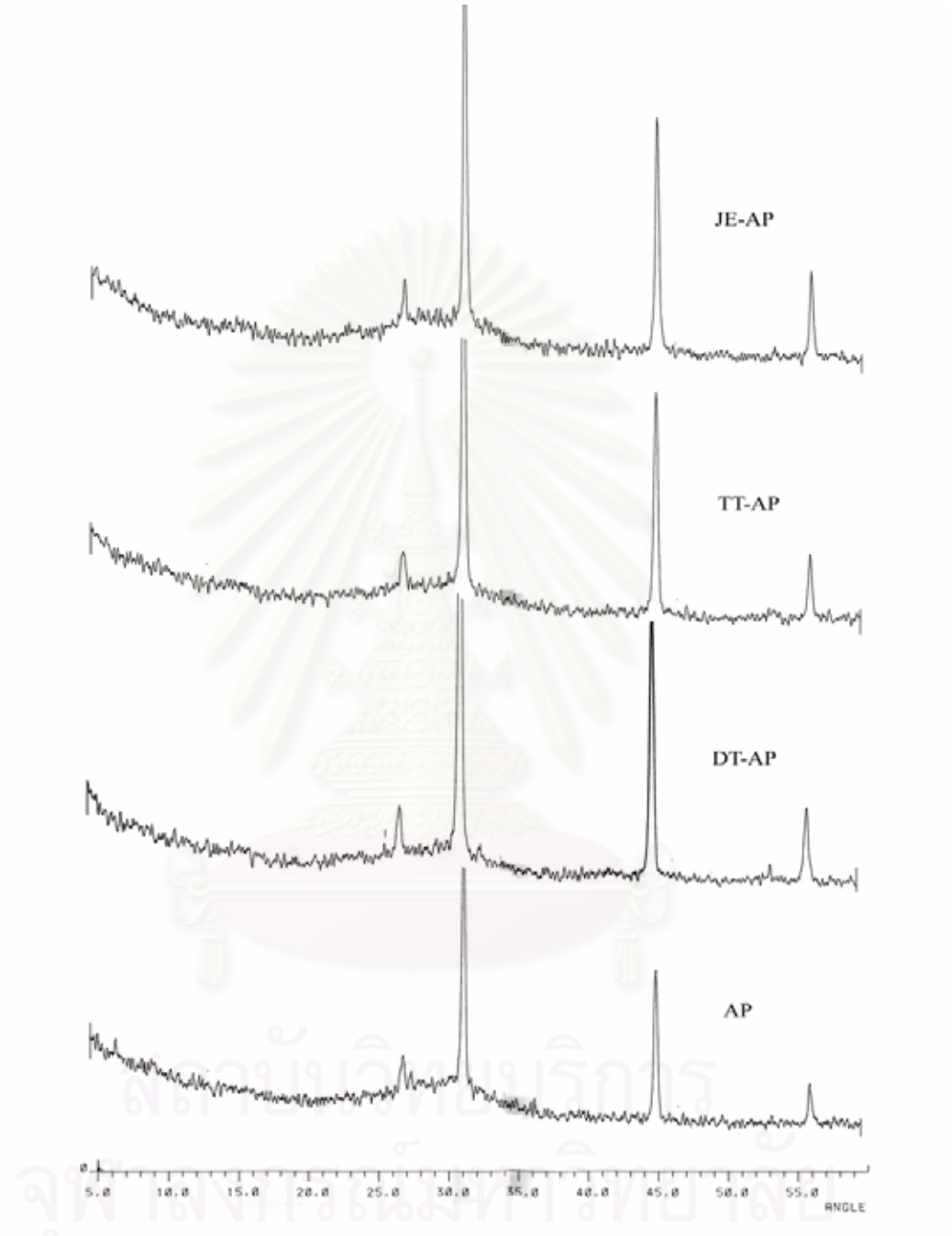


Figure 39 X-ray diffractograms of plain aluminium phosphate, diphtheria toxoid adsorbed on aluminium phosphate adjuvant, tetanus toxoid adsorbed on aluminium phosphate and JE antigen adsorbed on aluminium phosphate.

Table 20 shows OD and R^2 of various conditions of PT. The optimal condition of PT was only observed for the maximal OD at the initial due to insufficient supplies of PT primary antibody. After that, the two higher OD (1.585 and 1.139) was chosen to examine the maximal R^2.

The OD 1.585 gave the higher R^2 than OD 1.139 (R^2 as 0.9952 and 0.9874, respectively). Therefore, the condition of OD 1.585 was better than OD 1.139. The optimal condition of PT was coating antigen concentration as 10^{11} cell/well, dilution of 1°Ab as 1 : 4 and dilution of 2°Ab as 1 : 5,000.

4.2 Cross reaction analysis

All components in preparation were examined with individual antibody. The optical density of every sample showed similar optical density as that of control. The results showed that diphtheria primary antibody had no cross over reaction with TT, PT, JE, AH, AP, phosphate buffer and thimerosal. TT, PT and JE had the same results as DT. The results indicated that the other components in preparation had no effect to interfere the results of samples in ELISA analysis. In other words, if the sample had no reaction with primary antibody, the optical density of sample would be close to the optical density of control which was used as reference.

The control OD of DT, TT, PT and JE were 1.866, 2.254, 0.959 and 0.103, respectively.

Table 19 R^2 of various conditions of coating concentration, primary antibody and secondary antibody of diphtheria and tetanus toxoids.

	coating concentration (µg/well)	1 °Ab	2 °Ab	R^2
DT	10	1: 500	1:5,000	0.9814
	10	1: 500	1:10,000	0.9996
	10	1:1,000	1:5,000	0.9957
	10	1:1,000	1:10,000	0.9971
	1	1: 500	1:5,000	0.9774
	1	1: 500	1:10,000	0.9789
	1	1:1,000	1:5,000	0.9846
	1	1:1,000	1:10,000	0.9575
	0.1	1: 500	1:5,000	0.8962
	0.1	1: 500	1:10,000	0.8875
	0.1	1:1,000	1:5,000	0.8784
	0.1	1:1,000	1:10,000	0.8701
TT	10	1: 5,000	1:5,000	0.9895
	10	1: 5,000	1:10,000	0.9886
	10	1:10,000	1:5,000	0.9917
	10	1:10,000	1:10,000	0.9967
	1	1: 5,000	1:5,000	0.9801
	1	1: 5,000	1:10,000	0.9824
	1	1:10,000	1:5,000	0.9795
	1	1:10,000	1:10,000	0.9841
	0.1	1: 5,000	1:5,000	0.9748
	0.1	1: 5,000	1:10,000	0.9709
	0.1	1:10,000	1:5,000	0.9738
	0.1	1:10,000	1:10,000	0.9687

Table 20 OD and R^2 of various conditions of *Bordetella pertussis*

coating concentration (cell/well)	1 °Ab	2 °Ab	OD
10^{11}	1: 2	1:1,000	1.121
10^{11}	1: 2	1:5,000	1.104
10^{11}	1:4	1:1,000	1.139 (R^2 = 0.9874)
10^{11}	1:4	1:5,000	1.585 (R^2 = 0.9952)
10^{10}	1: 2	1:1,000	1.001
10^{10}	1: 2	1:5,000	1.034
10^{10}	1: 4	1:1,000	0.982
10^{10}	1: 4	1:5,000	0.997
10^{9}	1: 2	1:1,000	0.501
10^{9}	1: 2	1:5,000	0.539
10^{9}	1: 4	1:1,000	0.560
10^{9}	1: 4	1:5,000	0.599

Table 21 OD of cross reaction analysis between individual primary antibodies with various samples

Samples	Mean OD of individual antibody (± SD)			
	DT	TT	PT	JE
DT	-	2.306 (± 0.06)	0.944 (± 0.02)	0.126 (± 0.01)
TT	1.776 (± 0.01)	-	0.965 (± 0.01)	0.110 (± 0.01)
PT	1.625 (± 0.01)	2.232 (± 0.07)	-	0.086 (±0 .01)
JE	1.762 (±0.02)	2.233 (± 0.08)	1.029 (± 0.05)	-
AH	1.632 (± 0.03)	2.266 (±0 .04)	1.036 (± 0.05)	0.092 (± 0.01)
DT-AH	-	2.195 (± 0.03)	1.005 (± 0.01)	0.083 (± .01)
TT-AH	1.526 (± 0.01)	-	0.977 (± 0.01)	0.086 (± 0.00)
PT-AH	1.599 (± 0.03)	2.203 (± 0.02)	-	0.086 (± 0.01)
JE-AH	1.621 (± 0.01)	2.248 (± 0.04)	0.894 (± 0.02)	-
AP	1.696 (± 0.02)	2.197 (± 0.04)	0.946 (± 0.01)	0.090 (± 0.00)
DT-AP	-	2.229 (± 0.03)	0.844 (± 0.03)	0.121 (± 0.01)
TT-AP	1.620 (± 0.02)	-	0.794 (± 0.02)	0.10 (± 0.00)
PT-AP	1.583 (± 0.01)	2.181 (± 0.06)	-	0.078 (± 0.00)
JE-AP	1.576 (± 0.01)	2.168 (± 0.06)	0.741 (± 0.02)	-
buffer and thimerosal	1.784 (± 0.03)	2.176 (± 0.05)	0.782 (± 0.02)	0.078 (± 0.00)

5. Stability study

The combined preparations were formulated in three processes; F1-F3, F1 and F2 were formulated with separate adsorption. F1 was mixed at 30 minutes whereas F2 was mixed at 3 minutes. F3 was formulated with competitive adsorption process. The physical appearances of all preparations did not change throughout the storage periods of 3 months. The preparations were cloudy suspension after shaken and precipitated to clear supernate solution upon standing. The mean amounts of DT, TT, PT and JE antigen are presented in Table 22.

F1 had lower DT content than F2 and F3 throughout the storage periods. On the contrary, F2 had the antigen contents close to F3. There was no statistically significant difference between F2 and F3 throughout the storage periods ($p>0.05$). The DT contents of F1 were minimal during 1 to 3 months except at 1 month (not statistical significance from other formulations ($p>0.05$)). It due to F1 was mixed for a long time (60 minutes) than F2 and F3 (33 and 30 minutes, respectively). DT content might be lost when long mixing time was conducted. It could indicate that the mixing time influenced to DT content but the adsorption process had no effect to the content.

There was statistically significant difference in TT contents between F1, F2 and F3 at initial period ($p<0.05$), but not after that ($p>0.05$). F3 presented the lowest TT content at 3 months. F1 showed the maximal antigen contents throughout storage periods except initial time. The result showed that the different process affected to TT content. The competitive adsorption process (F3) was not a good process for TT antigen. It might be due to TT antigen had weak electrostatic attraction force than other antigens. Hence, TT which was competed by others could lose TT content.

Table 22 The content of antigens in adsorbed preparations during storage at 2-8 °C for 3 months

Antigens	Formulations	Mean concentration of remained antigens at various time [a] (± SD)			
		initial	1 month	2 months	3 months
DT (Lf)	F1	20.67 (± 4.44)	18.47 (± 0.84)	16.52 (± 0.46)	14.99 (± 0.53)
	F2	24.78 (± 1.84)	17.94 (± 0.94)	18.30 (± 3.64)	18.87 (± 1.11)
	F3	25.48 (± 1.88)	18.44 (± 0.17)	22.78 (± 4.00)	18.15 (± 0.58)
TT (Lf)	F1	5.77 (± 0.16)	7.70 (± 0.20)	6.45 (± 0.61)	6.03 (± 0.26)
	F2	6.22 (± 0.05)	7.44 (± 0.21)	5.84 (± 0.15)	5.55 (± 0.13)
	F3	6.61 (± 0.10)	7.55 (± 0.16)	5.88 (± 0.39)	5.44 (± 0.31)
PT (cell)	F1	3.81×10^{10} (± 4.16)	2.34×10^{9} (± 0.47)	1.77×10^{9} (± 0.57)	1.60×10^{9} (± 0.76)
	F2	1.45×10^{10} (± 3.76)	2.66×10^{9} (± 0.51)	2.54×10^{9} (± 0.78)	1.46×10^{9} (± 0.22)
	F3	1.93×10^{10} (± 5.75)	2.78×10^{9} (± 0.86)	2.23×10^{9} (± 0.50)	1.48×10^{9} (± 0.20)
JE (antigen unit)	F1	0.29 (± 0.02)	0.15 (± 0.00)	0.17 (± 0.03)	0.03 (± 0.00)
	F2	0.29 (± 0.03)	0.19 (± 0.00)	0.17 (± 0.00)	0.09 (± 0.00)
	F3	0.27 (± 0.00)	0.18 (± 0.00)	0.18 (± 0.00)	0.09 (± 0.00)

[a]remaining amounts of DT and TT were presented as Lf, PT as cell/ml and JE as antigen unit.

During 1 month to 3 months, the PT contents of F1, F2 and F3 were similarly and there was no statistically significant difference between F1, F2 and F3 (p>0.05). It concluded that the adsorption process and the mixing time had no influence to PT. This was similar to the report of Callahan et al. (1991) that vaccines composed of killed whole virus or bacteria could be self-adjuvant.

The contents of JE antigen at initial time were very low in every formulation. There was no statistically significant difference between F1, F2 and F3 at initial time (p>0.05). After 1 month, F1 had the antigen content lower than F2 and F3, while F2 had the maximal antigen content and same as initial period. At 2 and 3 months, the antigen contents of F1 were the lowest, whereas F2 had the antigen contents close to F3. F1 presented the minimal antigen contents throughout the storage periods and F2 showed the maximal JE content except at 2 months which was slightly lower than F3. The result suggested that the longer mixing time of F1 could interfere the JE content. Moreover, the adsorption method had low effect to antigen content.

Therefore, the adsorption process had the effect to TT content while mixing time affected to DT and JE content. Furthermore, PT was not be interfered from both factors and it could be self-adjuvant.

Figure 40 shows the stability pattern of DT during storage periods. The pattern of F1-F3 showed that the antigen content was rapidly decreased. The antigen content of F1was gradually decreased in every interval period. F2 rapidly decreased between the initial with 1 month, after that the content was slightly increased. F3 showed the fluctuation on amount of antigen during the storage period of 3 months, so one of the causes might be the error from analyst. The results suggested that the long mixing time (F1) could decrease DT content by desorbed the antigen from adjuvant and the separate adsorption (F2) was better than competitive adsorption (F3). It indicated that F2 showed higher stability than others. There was no statistically significant difference at 1 month, 2 months and 3 months (p>0.05).

The antigen contents of TT are shown in Figure 41. The stability patterns of F1, F2 and F3 were all the same. The antigen content of F1 was higher than F2 and F3 interval 1 month to 3 months. There was no significant difference in the antigen content of F1 between at initial and at 3 months (p>0.05). After 3 months, F1 showed the maximal antigen content. The results indicated that TT which was separated adsorption could attach on adjuvant and showed more remained antigen than competitive adsorption. It was concluded that F1 showed higher stability than other formula.

As seen in Figure 42, the stability pattern of PT in F1 was the same as in F2 and F3. F1 presented the maximal antigen content at initial and F2 had the minimal value. The PT contents of all formulations were rapidly dropped from initial period at 1 month. There were no significant difference of the antigen contents of F1, F2 and F3 during 1 month to 3 months (p>0.05). From these results, PT contents were stabilized for all formulations. It suggested that the mixing time and the process of adsorption showed no effect on PT.

Figure 43 shows the antigen contents of JE antigen during stability study. The antigen content of F1, F2 and F3 were gradually decreased in every interval periods. The antigen content values of every formulation were close to each other. F1 expressed the minimal value at 3 months, while F2 showed the maximal content. The results indicated that formulation which had the short mixing time (F2 and F3) had more JE content than that of the long time (F1). Moreover, the separate adsorption process (F2) showed the higher antigen content than competitive method after storage. It was concluded that F2 was the highest stable formulation.

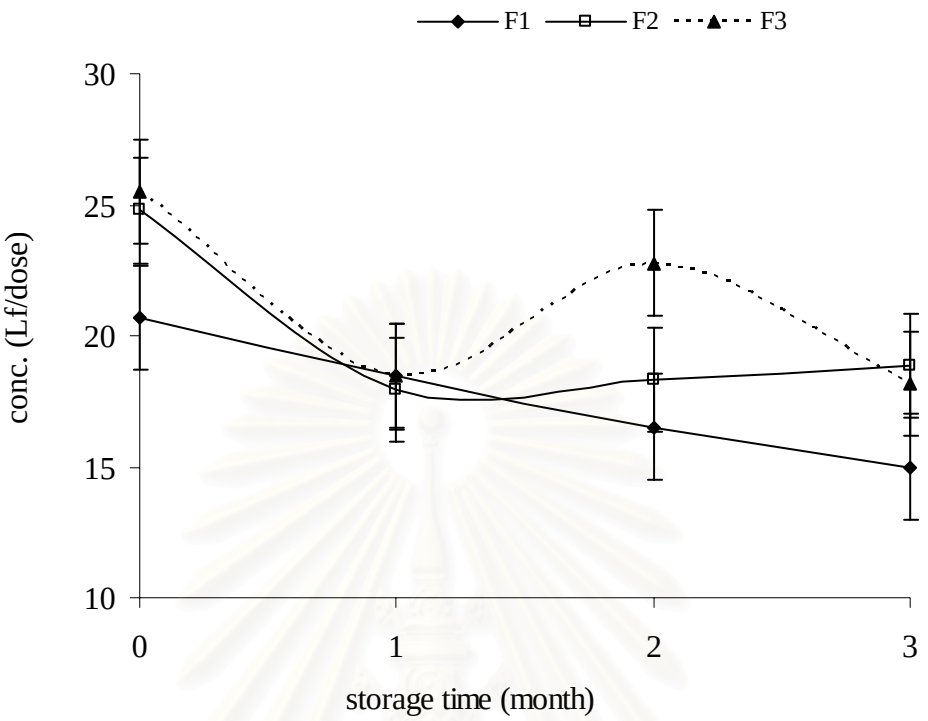


Figure 40 The amounts of diphtheria toxoid in combined formulations by different process during storage periods at 2-8 °C.

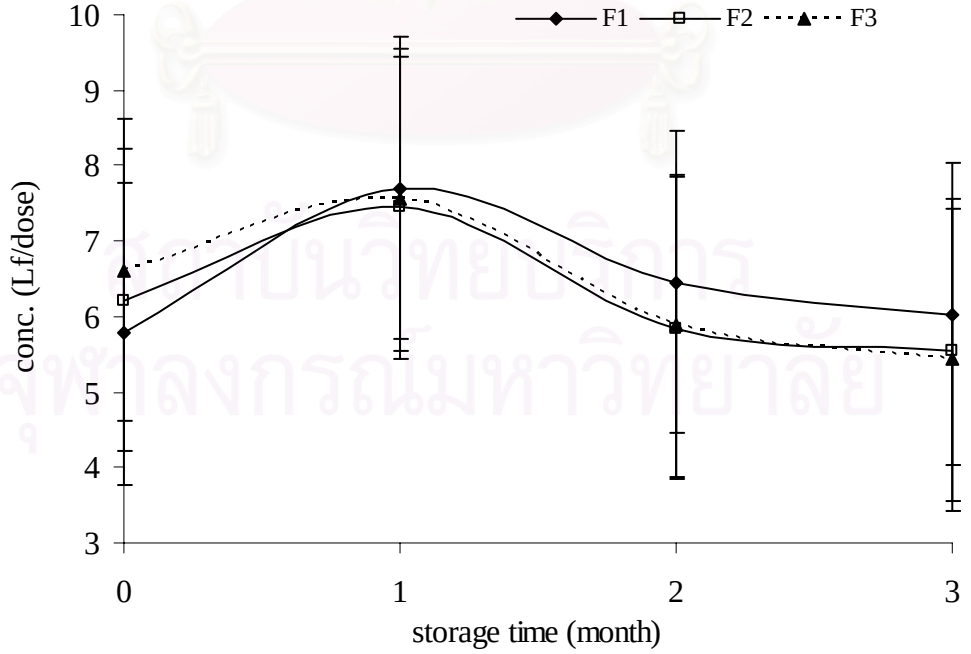


Figure 41 The amounts of tetanus toxoid in combined formulations by different process during storage periods at 2-8 °C.

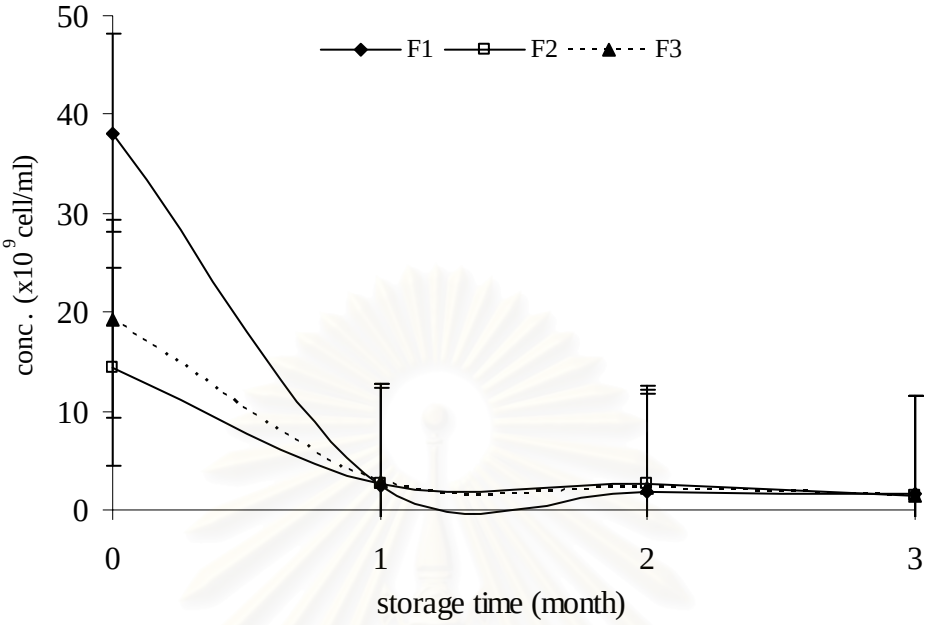


Figure 42 The amounts of *Bordetella pertussis* in combined formulations by different process during storage periods at 2-8 °C.

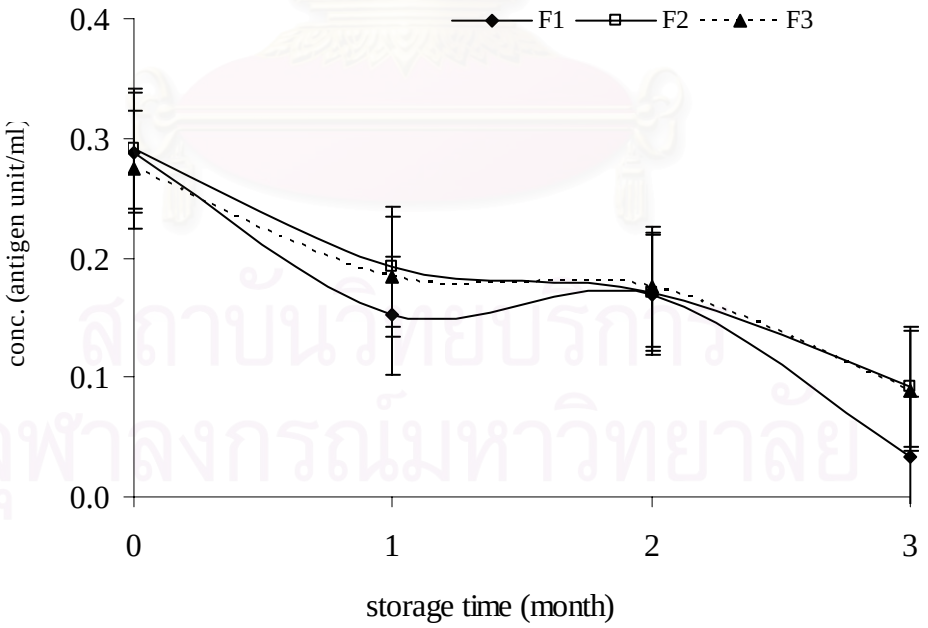


Figure 43 The amounts of JE antigen in combined formulations by different process during storage periods at 2-8 °C.

The results of Table 22 and Figure 40-43 indicated that after 3 months at 2-8 °C, F1 had the maximal TT and PT contents and the minimal DT and JE contents, whereas F2 had the maximal DT and JE contents and the minimal PT content. F3 showed the minimal TT content and close to the contents of F2 for every antigen. The results could be concluded that F2 which was formulated by separate adsorption and was mixed with total mixing time 33 minutes was the optimal formulation. Due to the fact that the total mixing time of F1 was 60 minutes and F2 was 33 minutes. Long mixing time might desorb some less stable antigens. The separate adsorption was individually mixed antigen with adjuvant and the antigen could be completely adsorbed under controlled conditions. Other adding ions or components were less likely to interfere the adsorption process. On the other hand, competitive adsorption was the process which all antigens were simultaneously mixed with adjuvant and other components in preparation at the same time. The antigen which the first adsorbed on the adjuvant might be partly or completely adsorbed and the later antigen might not be adsorbed completely. In addition, the antigen which adsorbed already might be desorbed from the adjuvant. The weakly adsorbed antigens might be desorbed during mixing and storage. The other ions or other components could interfere the adsorption process (Matheis et al., 2002).

Figure 44-46 present the antigen contents of combined formulations during storage period. DT showed the different pattern among F1, F2 and F3. The DT content in F1 was decreased throughout storage time, F2 showed an approximately constant concentration during 1 to 3 months while F3 was the fluctuation pattern. It showed that the formulation process was the controlling factor in the antigen content. In other words, the long mixing time could decrease DT content and the separate adsorption could keep the constant quantity of antigen better than competitive adsorption.

The antigen content pattern of TT in F1 was similar to F2 and F3, while F2 and F3 had less TT content than F1. It indicated that the mixing time and the adsorption process had the effect on TT content.

The stability pattern of PT in F1 was the same as in F2 and F3. PT content was gradually decreased during first month period and kept to approximately constant during 1 month to the third months. It could be concluded that the mixing time and the adsorption method were not factor which affected to the PT content.

As seen in Figure 44-46, the antigen content of JE was very low. F1, F2 and F3 showed the similar pattern of JE content that were constant or slightly decreased during storage period. They were not changed or slightly decreased from initial to 3 months if compared with other antigens. It could not observe the change of antigen content from these Figures.

Therefore, the antigen content of DT, TT and JE were affected by the adsorption process and the mixing time while PT content was not interfered from these factors.

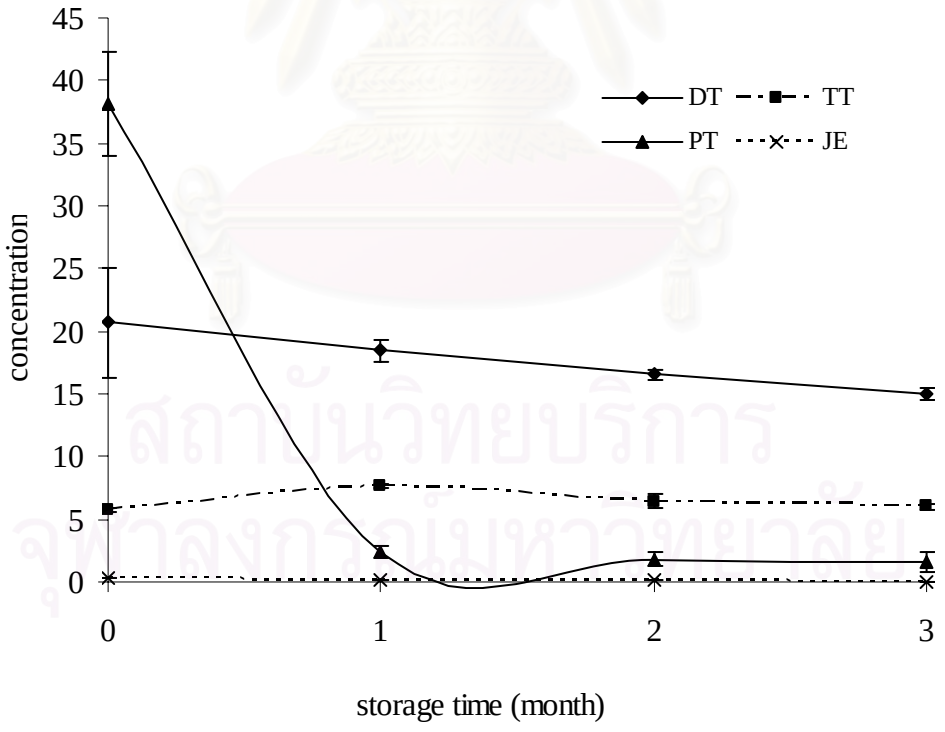


Figure 44 The antigen content of combined formulation (F1) during storage periods at 2-8 °C.

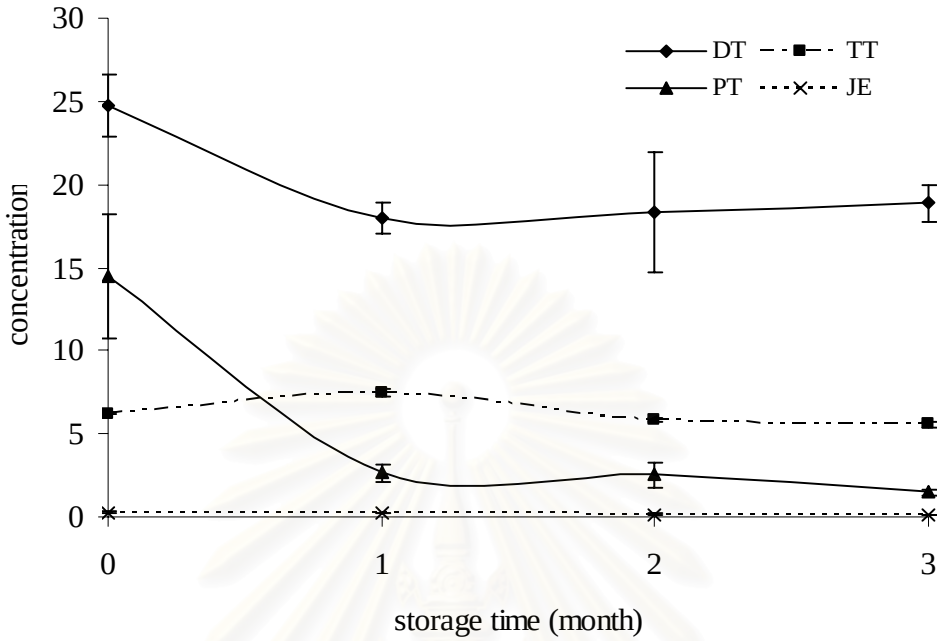


Figure 45 The antigen content of combined formulation (F2) during storage periods at 2-8 °C.

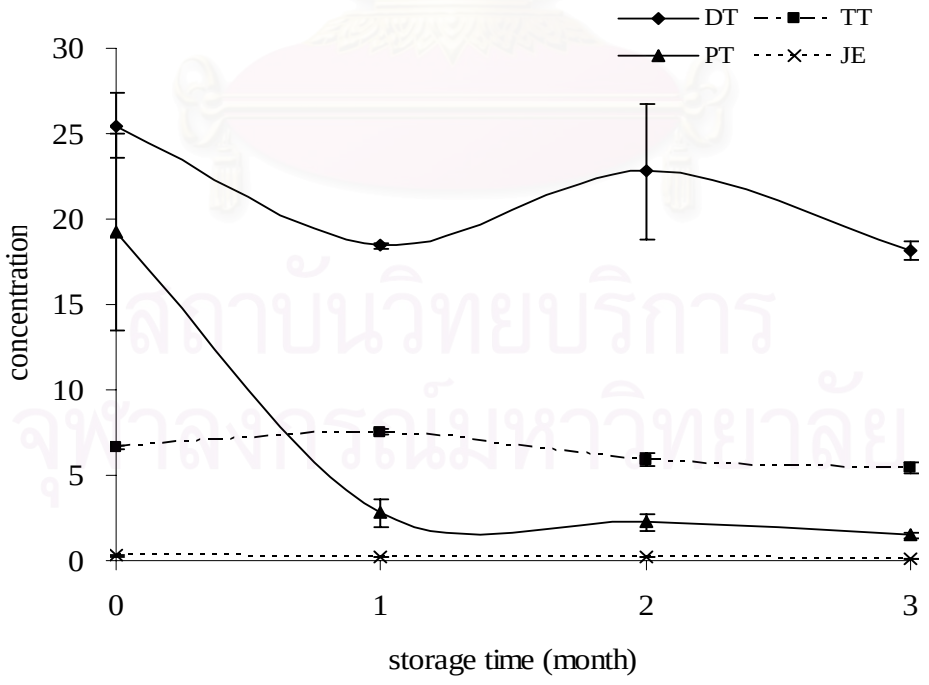


Figure 46 The antigen content of combined formulation (F3) during storage periods at 2-8 °C.

CHAPTER V
CONCLUSIONS

The results of this study were concluded as followed:

1. The percentage of aluminium content in aluminium hydroxide gel and aluminium phosphate gel were 1.94 $\pm$ 0.03 and 2.25 $\pm$ 0.02 %Al, respectively.

2. The rate of centrifugation which was used to test the adsorption of single antigen on aluminium adjuvants was 3,000 rpm due to the supernatant was mostly clear and the remained aluminium content was lower than 1% (0.99 %Al and < 0.12 %Al for AH and AP, respectively).

3. The results from the adsorption of single antigen on aluminium adjuvants indicated that type of adjuvants, temperature and pH hence influence on the adsorption process. Aluminium hydroxide adjuvant had greater capacity than aluminium phosphate, so aluminium hydroxide was used to prepare the combined preparations. The optimal condition of adsorption process was at pH 6.5, 9 $\pm$ 1 °C.

4. Evaluation data of adsorbed single antigen preparations showed that

   4.1 AH and AP had pI around 11.0 and 4.25, respectively. Therefore, AH and AP showed positive charge and negative charge at pH range 6.0-7.4 in dilution 1:80. On the other hand, AH and AP expressed negative charge when dispersed in phosphate buffer. The electrostatic force and ligand exchange were possibly the adsorption mechanism on AH and AP.

   4.2 Sizes of all adsorbed adjuvants were larger than processed adjuvants determined by both LD and PCS method.

   4.3 The morphology by TEM showed that AH was fibrous particles and AP was the network platy particles.

4.4 Optical microscopy could observe only AH and PT. The adsorbed AH preparations could be observed by SEM, while TEM could not detect the adsorbed antigens on adjuvants. Furthermore, the adsorbed AP preparations observed by AFM showed the true three-dimensional surface morphology of adjuvants that were attached with spherical particulate matters.

4.5 The adsorbed preparations showed no change in the chemical structure of AH and AP due to the presented principle peaks in IR spectra.

4.6 The X-ray diffractrograms of adsorbed adjuvants both AH and AP were also presented the principle diffraction bands but adsorbed AH preparations had some different bands. It could be assumed that the polymorphism of AH might be changed or the different bands were the X-ray diffraction bands of salt from buffer. However, the diffraction bands of adsorbed AP did not change, hence, adsorption had no effect on the crystallinity of AP.

5. The results of the adsorption of combined antigens showed that

5.1 The optimal coating antigen concentration of DT and TT were 10 µg/well, PT was 10^{11} cell/well. The coating suspension of PT was prepared by unsonicated and incubated at 4 $\pm$ 1 °C overnight. The 1Ab dilution of DT was 1:500, TT was 1:10,000 and PT was 1:4. The 2Ab dilution of DT and TT were 1:10,000 and PT antibody was 1:5,000.

5.2 The cross reaction analysis indicated that other components in preparation had no effect to interfere the results of samples in ELISA analysis.

6. F2 which was prepared by separate adsorption with total mixing time 33 minutes was the best formulation for stability study of combined preparation under storage condition at 2-8°C for 3 months. The adsorption process and mixing time may interfere with DT, TT and JE content, however not on PT.

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APPENDICES

APPENDICES

APPENDIX A

Aluminium content assay

Table 23 The percentage of aluminium content in aluminium hydroxide and aluminium phosphate.

Adjuvants	% Aluminium	Av. % aluminium (± SD)
Aluminium hydroxide gel	1.92 1.97 1.92	1.94 ± 0.03
Aluminium phosphate gel	2.27 2.23 2.26	2.25 ± 0.02

Separation of unadsorbed antigens by centrifugation

Table 24 The percentage of aluminium content in supernatant of aluminium hydroxide gel after centrifugation.

rpm	% Al in supernatant			Av. Al in supernatant(%)	SD
	A1	A2	A3		
200	81.98	82.25	82.09	82.10	0.14
600	3.49	3.54	3.85	3.63	0.19
1,000	2.69	2.72	2.95	2.78	0.14
1,400	2.15	2.42	1.95	2.17	0.24
1,800	2.02	1.49	2.05	1.85	0.31
2,200	1.46	1.60	1.77	1.61	0.16
2,600	1.36	1.29	1.47	1.37	0.09
3,000	0.85	1.01	0.98	0.94	0.08
3,400	0.61	0.72	0.80	0.71	0.09

Table 25 The percentage of aluminium content in supernatant of aluminium phosphate gel after centrifugation.

rpm	% Al in supernatant			Av. Al in supernatant(%)	SD
	A1	A2	A3		
200	1.08	1.09	1.08	1.08	0.01
500	0.71	0.65	0.66	0.67	0.03
800	0.35	0.37	0.36	0.36	0.01
1,100	0.37	0.31	0.33	0.34	0.03
1,400	0.34	0.34	0.30	0.33	0.02
1,700	0.18	0.25	0.21	0.21	0.04
2,000	0.18	0.17	0.19	0.18	0.01
2,300	0.16	0.16	0.15	0.16	0.01
2,600	0.14	0.13	0.17	0.15	0.02
2,900	0.13	0.12	0.12	0.12	0.01
3,200	0.07	0.12	0.06	0.08	0.03

APPENDIX B

The concentration unit of DT, TT and JE

Table 26 The calculation of concentration unit between µg/ml with Lf/ml or antigen unit/ml of diphtheria toxoid, tetanus toxoid and JE antigen.

Antigen	conc. (µg/ml) from BCA					Av. Conc. (µg/ml)	%CV	conc. (Lf/ml)	content / Lf
DT	720.42	845.38	737.93	800.52	941.83	818.60	9.37	300.00	2.73
	862.63	908.33	729.06	855.44	784.50				
TT	296.43	282.67	240.57	238.43	274.61	273.09	10.18	60.00	4.55
	261.16	237.33	282.19	307.11	310.38				

Antigen	conc. (µg/ml) from BCA					Av. Conc. (µg/ml)	%CV	conc. (antigen unit/ml)	content/ antigen unit
JE	1845.37	1650.10	2006.07	1664.30	1617.80	1768.87	7.28	8.80	201.01
	1683.44	1926.24	1695.99	1790.64	1808.75				

Adsorption of single antigen on adjuvants

Antigens adsorbed with aluminium hydroxide adjuvants

Table 27 Adsorption of diphtheria toxoid on aluminium hydroxide at 37 °C.

conc. DT (µg/ml)	Average adsorption (mg antigen / mg Al)							
	pH 6.0	SD	pH 6.5	SD	pH 7.0	SD	pH 7.4	SD
256.63	0.35	0.01	0.25	0.01	0.36	0.05	0.32	0.05
342.18	0.41	0.12	0.45	0.05	0.35	0.14	0.40	0.05
427.72	0.31	0.03	0.44	0.04	0.36	0.09	0.37	0.12
513.27	0.35	0.15	0.29	0.03	0.41	0.12	0.42	0.12
598.81	0.34	0.10	0.26	0.10	0.40	0.06	0.52	0.03
684.36	0.37	0.04	0.30	0.04	0.47	0.05	0.45	0.07

Table 28 Adsorption of diphtheria toxoid on aluminium hydroxide at 9 °C.

conc. DT (µg/ml)	Average adsorption (mg antigen / mg Al)							
	pH 6.0	SD	pH 6.5	SD	pH 7.0	SD	pH 7.4	SD
297.29	0.48	0.02	0.45	0.02	0.45	0.02	0.42	0.03
495.48	0.64	0.04	0.56	0.02	0.56	0.02	0.47	0.02
693.67	0.65	0.05	0.60	0.01	0.71	0.10	0.57	0.08
891.87	0.74	0.05	0.77	0.03	0.77	0.09	0.60	0.02
1090.06	0.88	0.14	0.86	0.15	0.97	0.12	0.75	0.09
1288.25	1.08	0.17	0.91	0.08	1.12	0.32	1.05	0.14

Table 29 Adsorption of tetanus toxoid on aluminium hydroxide at 37 °C.

conc. TT (µg/ml)	Average adsorption (mg antigen / mg Al)							
	pH 6.0	SD	pH 6.5	SD	pH 7.0	SD	pH 7.4	SD
84.46	0.16	0.00	0.16	0.00	0.13	0.00	0.13	0.00
140.77	0.26	0.00	0.25	0.01	0.21	0.00	0.21	0.00
197.08	0.37	0.00	0.36	0.01	0.30	0.01	0.29	0.01
253.38	0.48	0.00	0.47	0.00	0.39	0.00	0.38	0.00
309.69	0.59	0.00	0.58	0.01	0.47	0.00	0.47	0.00
366.00	0.69	0.00	0.69	0.00	0.57	0.00	0.55	0.01

Table 30 Adsorption of tetanus toxoid on aluminium hydroxide at 9 °C.

conc. TT (µg/ml)	Average adsorption (mg antigen / mg Al)							
	pH 6.0	SD	pH 6.5	SD	pH 7.0	SD	pH 7.4	SD
48.11	0.07	0.00	0.07	0.00	0.08	0.00	0.08	0.00
80.19	0.12	0.01	0.12	0.00	0.12	0.00	0.12	0.01
112.26	0.17	0.01	0.16	0.00	0.16	0.01	0.16	0.01
144.34	0.22	0.01	0.21	0.00	0.20	0.01	0.21	0.01
176.41	0.27	0.00	0.27	0.01	0.25	0.01	0.26	0.01
208.49	0.32	0.00	0.31	0.00	0.31	0.01	0.29	0.00

Table 31 Adsorption of JE antigen on aluminium hydroxide at 37 °C.

conc. JE (µg/ml)	Average adsorption (mg antigen / mg Al)							
	pH 6.0	SD	pH 6.5	SD	pH 7.0	SD	pH 7.4	SD
90.13	0.04	0.02	0.03	0.00	0.03	0.02	0.04	0.02
270.38	0.14	0.03	0.17	0.05	0.06	0.02	0.09	0.04
450.64	0.23	0.03	0.24	0.10	0.24	0.05	0.16	0.12
811.15	0.45	0.22	0.32	0.16	0.17	0.10	0.29	0.23
991.41	0.64	0.15	0.61	0.19	0.42	0.01	0.32	0.12
1171.67	0.73	0.22	0.67	0.26	0.37	0.16	0.62	0.08

Table 32 Adsorption of JE antigen on aluminium hydroxide at 9 °C.

conc. JE (µg/ml)	Average adsorption (mg antigen / mg Al)							
	pH 6.0	SD	pH 6.5	SD	pH 7.0	SD	pH 7.4	SD
344.42	0.24	0.16	0.38	0.10	0.14	0.01	0.07	0.01
574.03	0.26	0.21	0.25	0.09	0.23	0.05	0.17	0.05
803.64	0.51	0.24	0.26	0.20	0.17	0.01	0.29	0.11
1033.25	0.34	0.06	0.57	0.20	0.56	0.07	0.47	0.14
1262.86	0.60	0.08	0.72	0.27	0.58	0.08	0.70	0.02
1492.47	1.08	0.27	1.15	0.22	0.69	0.02	0.76	0.25

Table 33 Adsorption of antigens onto aluminium hydroxide adjuvant.

pH	Mean adsorption (µg antigen/mg Al) (± SD)					
	Diphtheria		Tetanus		JE	
	37 ± 1 °C	9 ± 1 °C	37 ± 1 °C	9 ± 1 °C	37 ± 1 °C	9 ± 1 °C
6.0	450.00 ± 0.05	1,076.50 ± 0.17	694.10 ± 0.00	322.70 ± 0.00	726.90 ± 0.22	1,081.30 ± 0.27
6.5	413.50 ± 0.12	908.50 ± 0.08	687.70 ± 0.00	313.00 ± 0.00	672.80 ± 0.26	1,148.10 ± 0.22
7.0	473.00 ± 0.05	1,119.50 ± 0.32	567.10 ± 0.00	309.30 ± 0.01	418.60 ± 0.01	690.80 ± 0.02
7.4	520.90 ± 0.03	1,052.50 ± 0.14	553.30 ± 0.01	290.30 ± 0.00	619.30 ± 0.08	760.50 ± 0.25

Antigens adsorbed with aluminium phosphate adjuvant

Table 34 Adsorption of diphtheria toxoid on aluminium phosphate at 37 °C.

conc. DT (µg/ml)	Average adsorption (mg antigen / mg Al)							
	pH 6.0	SD	pH 6.5	SD	pH 7.0	SD	pH 7.4	SD
210.44	0.07	0.00	0.05	0.01	0.07	0.02	0.06	0.01
315.67	0.08	0.02	0.05	0.02	0.04	0.02	0.07	0.02
420.89	0.09	0.01	0.05	0.01	0.04	0.01	0.07	0.01
526.11	0.09	0.02	0.06	0.01	0.07	0.04	0.09	0.03
631.33	0.11	0.01	0.08	0.06	0.06	0.02	0.13	0.01
736.56	0.13	0.04	0.15	0.03	0.17	0.03	0.09	0.03

Table 35 Adsorption of diphtheria toxoid on aluminium phosphate at 9 °C.

conc. DT (µg/ml)	Average adsorption (mg antigen / mg Al)							
	pH 6.0	SD	pH 6.5	SD	pH 7.0	SD	pH 7.4	SD
205.81	0.04	0.02	0.02	0.01	0.01	0.01	0.01	0.01
343.01	0.03	0.00	0.01	0.00	0.02	0.01	0.05	0.01
480.22	0.04	0.00	0.01	0.00	0.04	0.03	0.05	0.01
617.42	0.06	0.01	0.02	0.00	0.05	0.01	0.05	0.02
754.62	0.07	0.01	0.07	0.04	0.09	0.04	0.04	0.01
891.83	0.12	0.02	0.17	0.01	0.12	0.02	0.06	0.01

Table 36 Adsorption of tetanus toxoid on aluminium phosphate at 37 °C.

conc. TT (µg/ml)	Average adsorption (mg antigen / mg Al)							
	pH 6.0	SD	pH 6.5	SD	pH 7.0	SD	pH 7.4	SD
82.77	0.25	0.02	0.22	0.03	0.24	0.05	0.23	0.01
124.50	0.24	0.04	0.23	0.05	0.19	0.00	0.18	0.01
165.53	0.19	0.00	0.16	0.03	0.19	0.02	0.24	0.04
206.92	0.30	0.02	0.25	0.02	0.23	0.02	0.19	0.14
248.30	0.32	0.03	0.35	0.04	0.24	0.07	0.30	0.03
289.68	0.45	0.05	0.42	0.05	0.37	0.15	0.41	0.15

Table 37 Adsorption of tetanus toxoid on aluminium phosphate at 9 °C.

conc. TT (µg/ml)	Average adsorption (mg antigen / mg Al)							
	pH 6.0	SD	pH 6.5	SD	pH 7.0	SD	pH 7.4	SD
62.16	0.05	0.01	0.01	0.01	0.01	0.00	0.01	0.01
103.60	0.09	0.00	0.06	0.01	0.03	0.00	0.03	0.00
145.04	0.12	0.00	0.09	0.00	0.05	0.01	0.05	0.00
186.48	0.16	0.00	0.12	0.01	0.09	0.01	0.07	0.01
227.92	0.20	0.00	0.18	0.01	0.12	0.00	0.12	0.01
269.36	0.24	0.00	0.22	0.00	0.17	0.01	0.15	0.00

Table 38 Adsorption of JE antigen on aluminium phosphate at 37 °C.

conc. JE	Average adsorption (mg antigen / mg Al)							
(µg/ml)	pH 6.0	SD	pH 6.5	SD	pH 7.0	SD	pH 7.4	SD
249.12	0.05	0.01	0.10	0.04	0.06	0.02	0.09	0.02
415.20	0.09	0.04	0.11	0.03	0.09	0.07	0.16	0.02
581.27	0.06	0.04	0.14	0.11	0.16	0.01	0.23	0.03
747.35	0.28	0.05	0.15	0.08	0.26	0.03	0.37	0.06
913.43	0.17	0.09	0.36	0.17	0.34	0.07	0.34	0.11
1079.51	0.17	0.05	0.36	0.04	0.33	0.12	0.37	0.03

Table 39 Adsorption of JE antigen on aluminium phosphate at 9 °C.

conc. JE	Average adsorption (mg antigen / mg Al)							
(µg/ml)	pH 6.0	SD	pH 6.5	SD	pH 7.0	SD	pH 7.4	SD
128.95	0.21	0.00	0.21	0.00	0.24	0.02	0.25	0.00
214.92	0.33	0.04	0.38	0.03	0.40	0.02	0.42	0.00
300.89	0.34	0.06	0.44	0.07	0.40	0.02	0.45	0.04
386.86	0.31	0.03	0.36	0.06	0.40	0.07	0.40	0.02
472.83	0.26	0.02	0.23	0.12	0.36	0.08	0.40	0.04
558.80	0.17	0.04	0.20	0.02	0.31	0.03	0.46	0.05

Table 40 Adsorption of antigens onto aluminium phosphate adjuvant.

pH	Mean adsorption values (µg antigen/mg Al) (± SD)					
	Diphtheria		Tetanus		JE	
	37 ± 1 °C	9 ± 1 °C	37 ± 1 °C	9 ± 1 °C	37 ± 1 °C	9 ± 1 °C
6.0	128.60 ± 0.04	119.40 ± 0.02	445.80 ± 0.05	236.50 ± 0.00	280.90 ± 0.05	337.30 ± 0.06
6.5	146.10 ± 0.03	174.30 ± 0.01	420.80 ± 0.05	219.30 ± 0.00	360.60 ± 0.04	436.40 ± 0.07
7.0	170.30 ± 0.03	115.40 ± 0.02	367.10 ± 0.15	171.50 ± 0.01	338.60 ± 0.07	404.60 ± 0.07
7.4	125.30 ± 0.01	101.60 ± 0.00	408.60 ± 0.15	153.80 ± 0.00	371.40 ± 0.03	462.50 ± 0.05

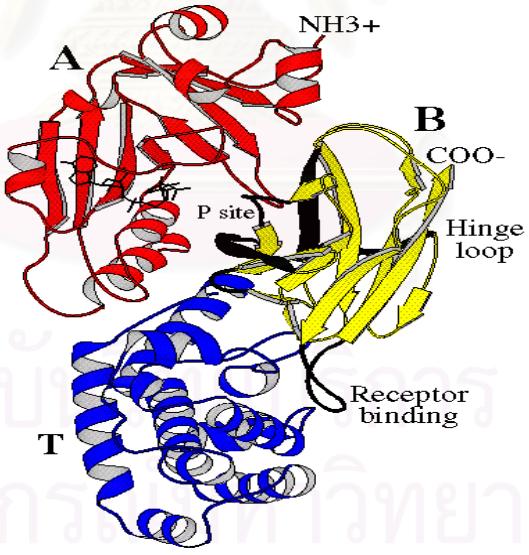

Figure 47 The diphtheria toxin monomer (Grage, 2002).

APPENDIX C

Particle size distribution

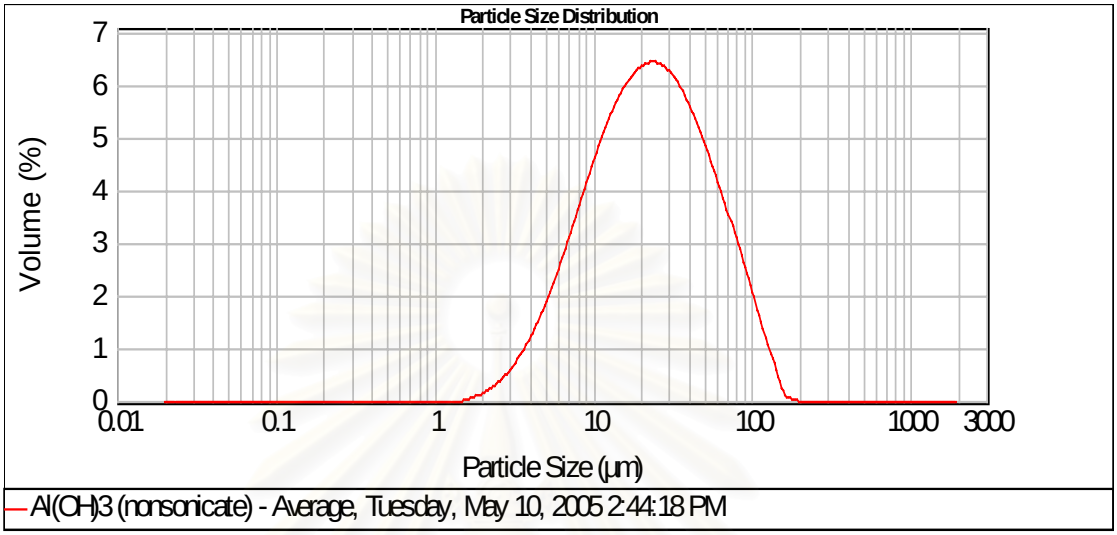

Figure 48 The particle size distribution of plain aluminium hydroxide adjuvant

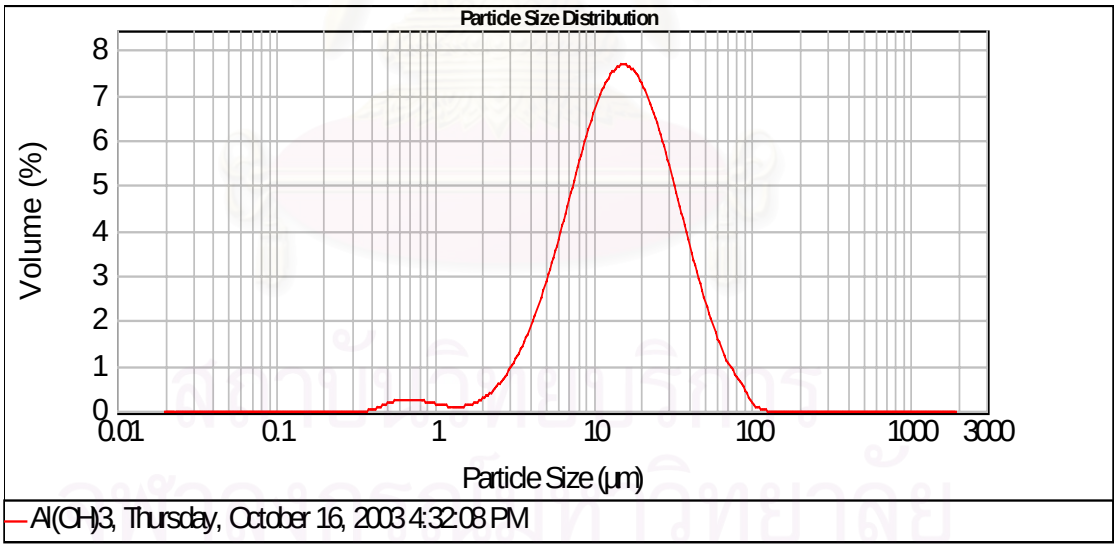

Figure 49 The particle size distribution of processed aluminium hydroxide at 37 °C

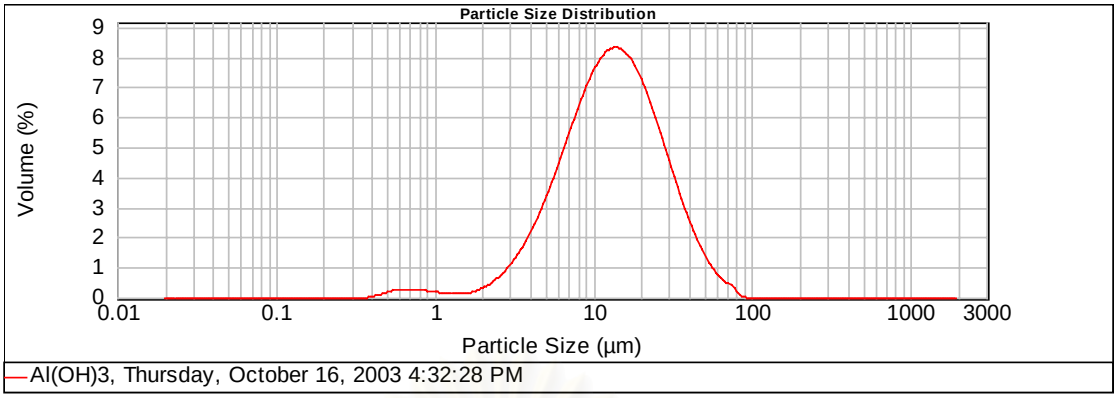

Figure 50 The particle size distribution of processed aluminium hydroxide at 9 °C

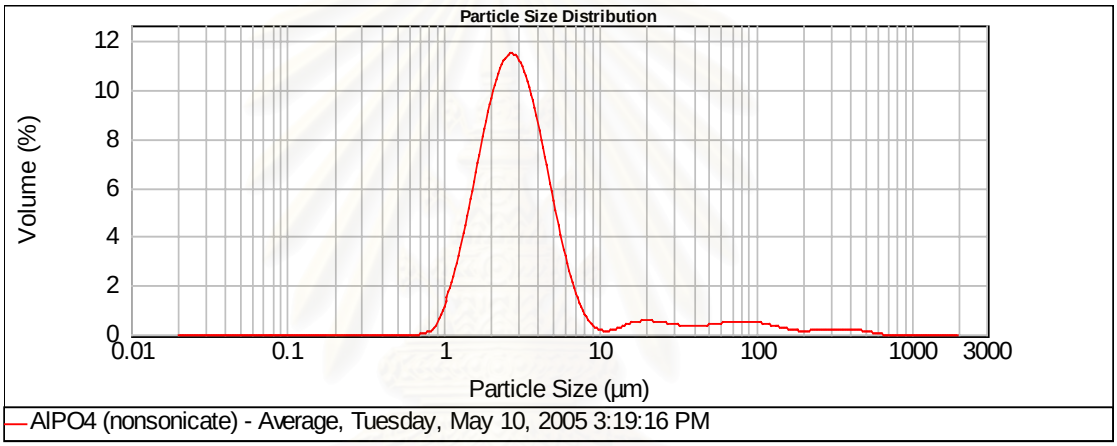

Figure 51 The particle size distribution of plain aluminium phosphate adjuvant

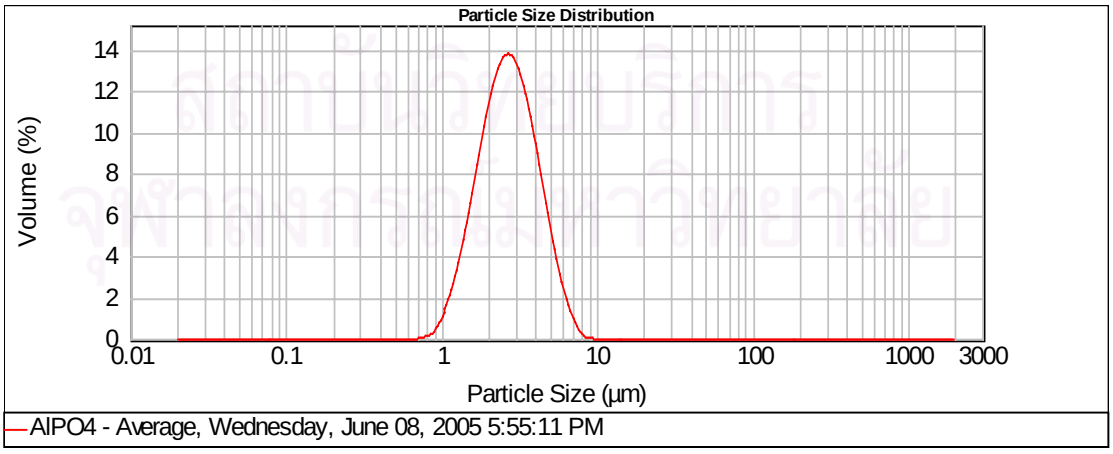

Figure 52 The particle size distribution of processed aluminium phosphate at 37 °C

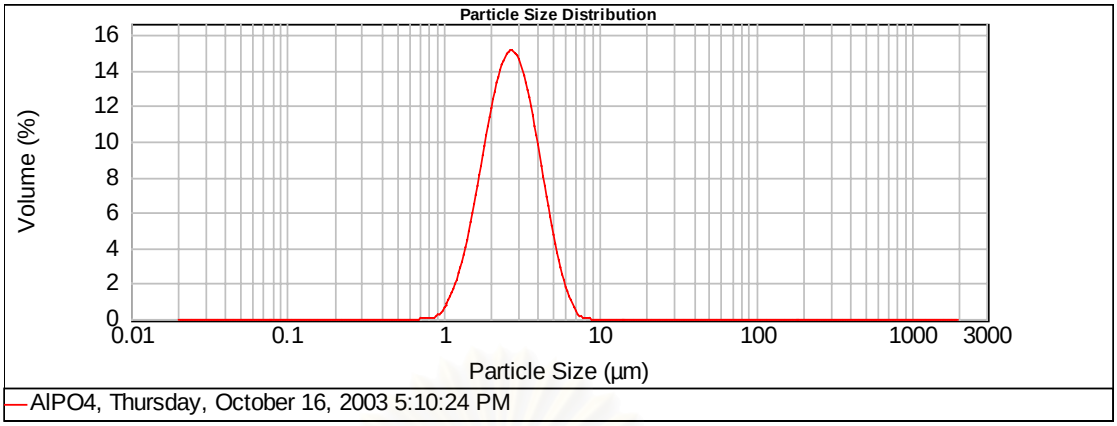

Figure 53 The particle size distribution of processed aluminium phosphate at 9 °C

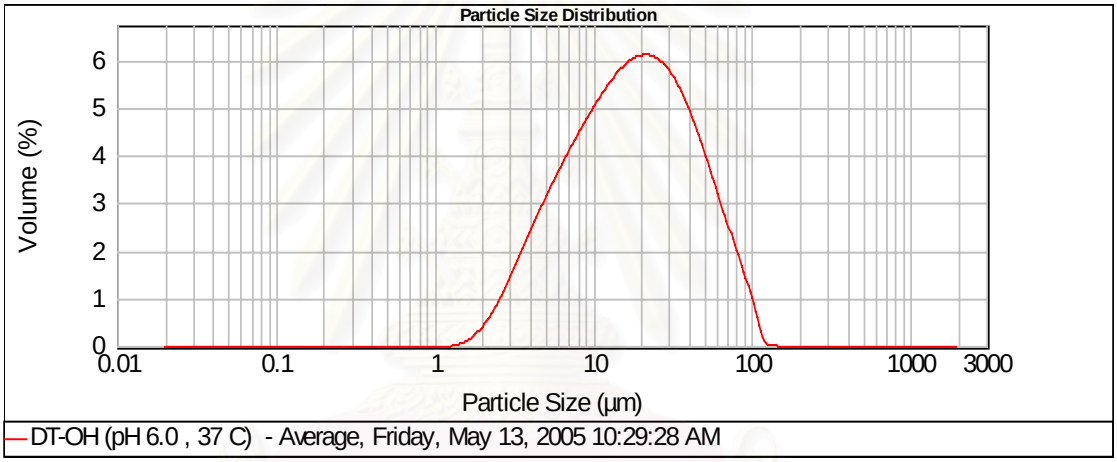

Figure 54 The particle size distribution of DT-AH at pH 6.0 and 37 °C

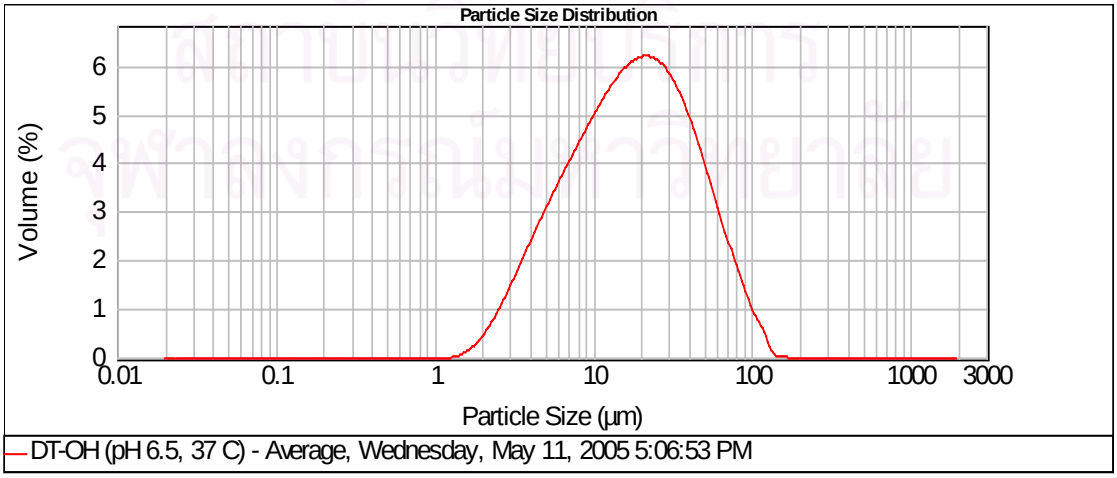

Figure 55 The particle size distribution of DT-AH at pH 6.5 and 37 °C

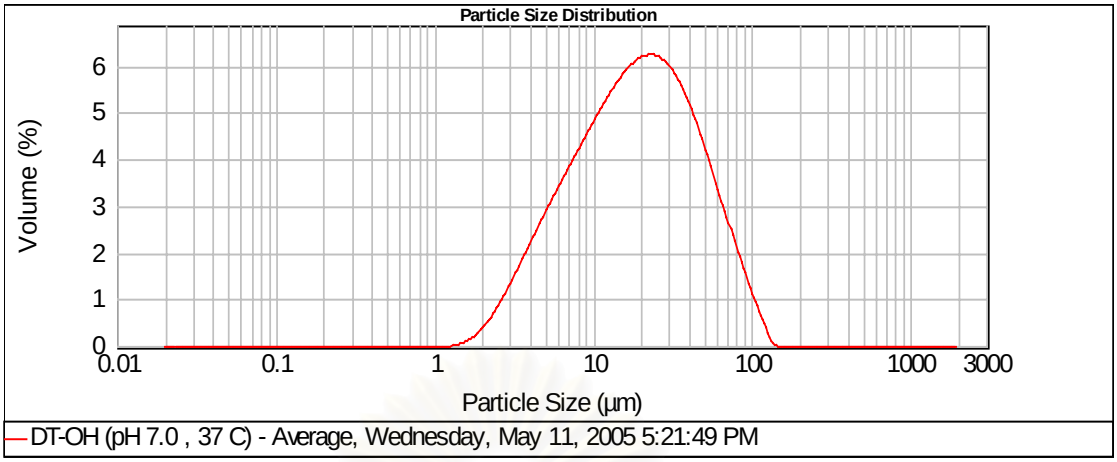

Figure 56 The particle size distribution of DT-AH at pH 7.0 and 37 °C

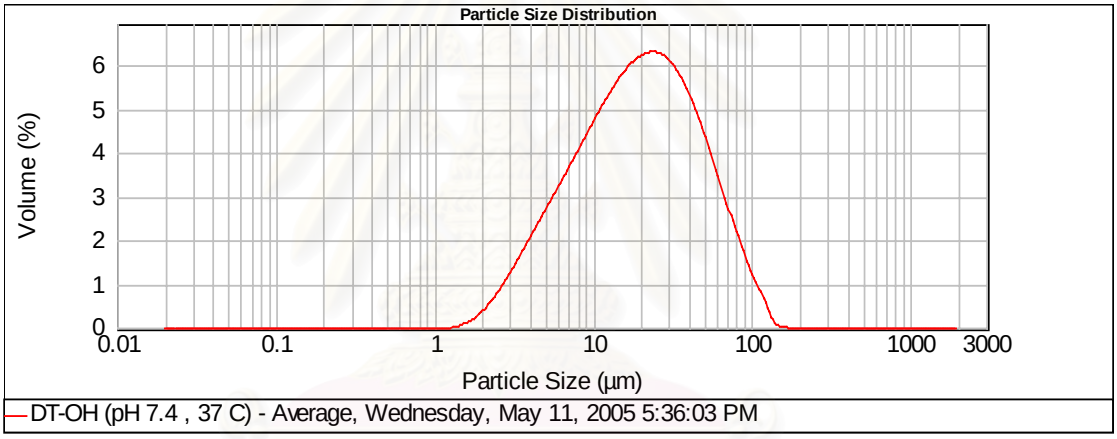

Figure 57 The particle size distribution of DT-AH at pH 7.4 and 37 °C

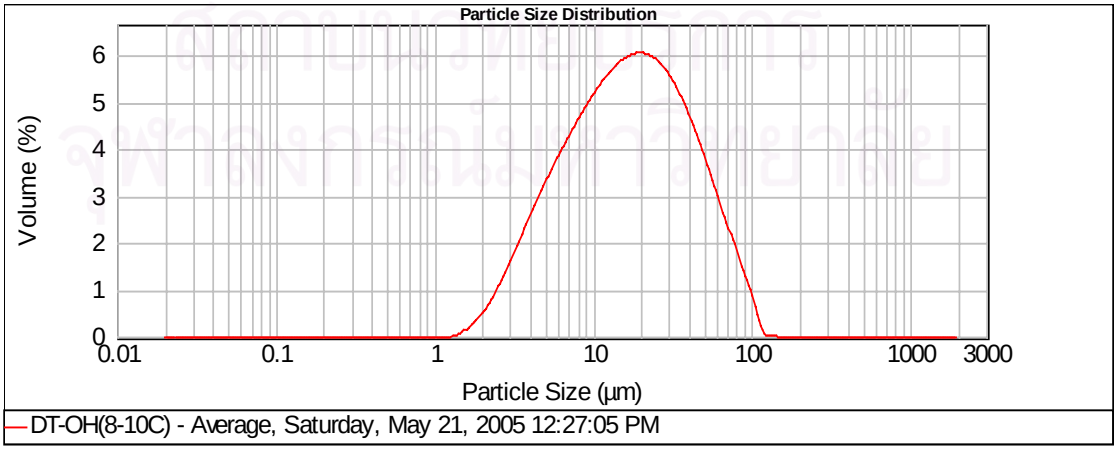

Figure 58 The particle size distribution of DT-AH at pH 6.0 and 9 °C

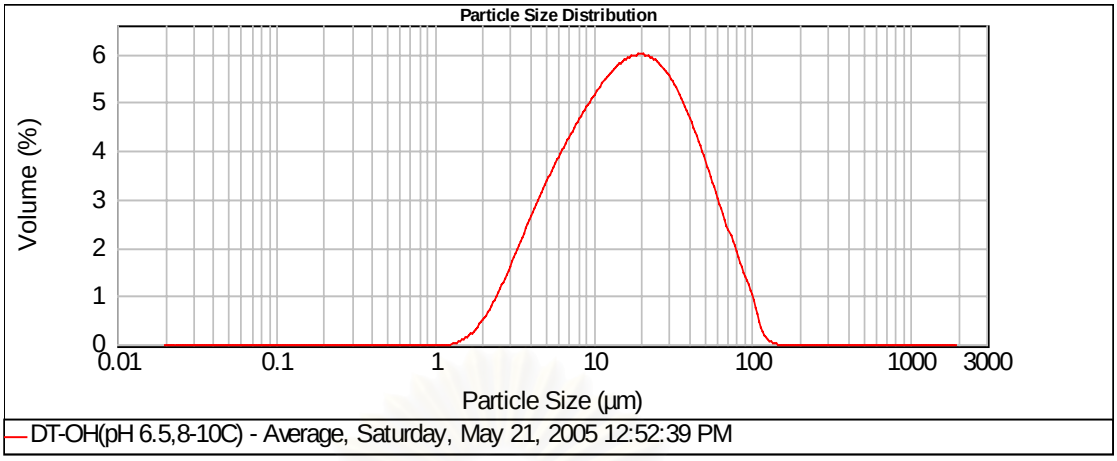

Figure 59 The particle size distribution of DT-AH at pH 6.5 and 9 °C

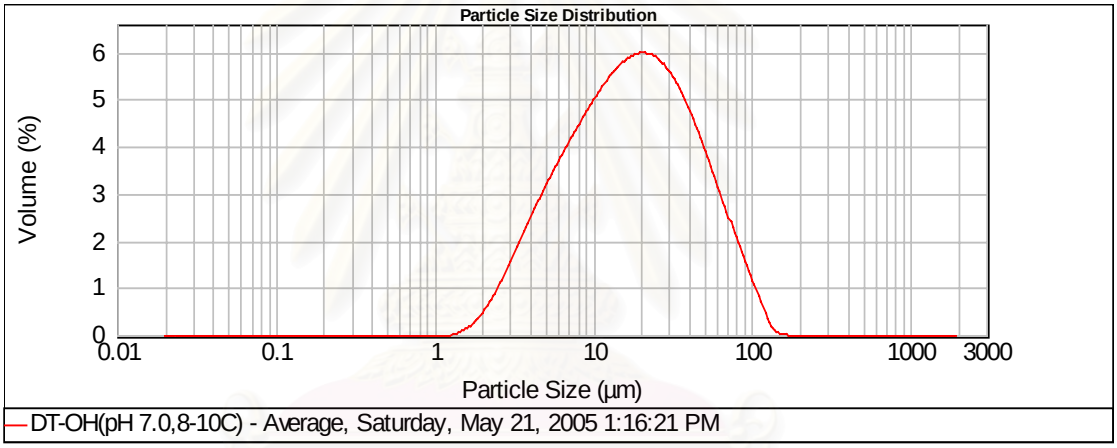

Figure 60 The particle size distribution of DT-AH at pH 7.0 and 9 °C

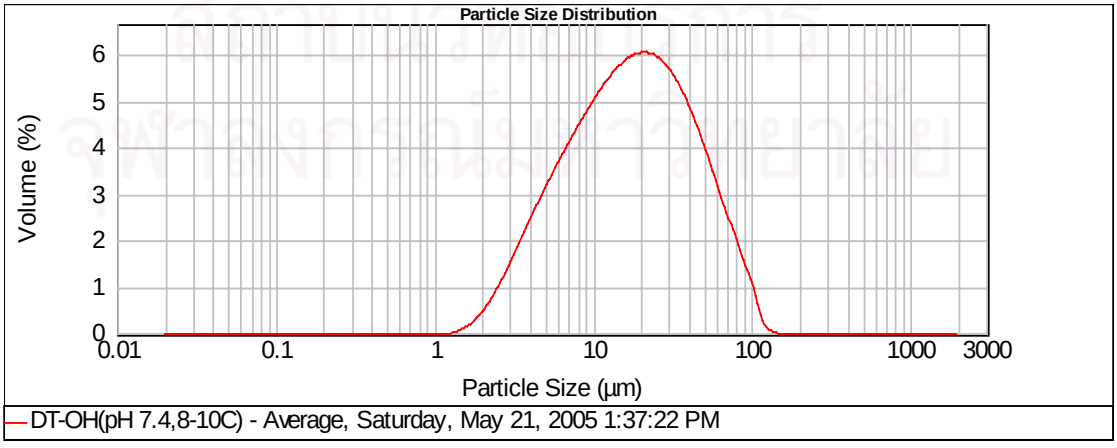

Figure 61 The particle size distribution of DT-AH at pH 7.4 and 9 °C

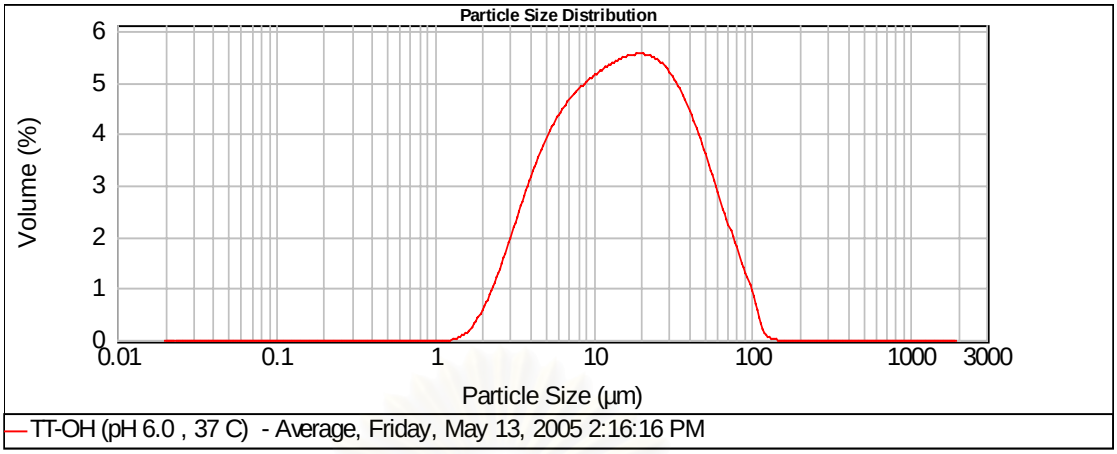

Figure 62 The particle size distribution of TT-AH at pH 6.0 and 37 °C

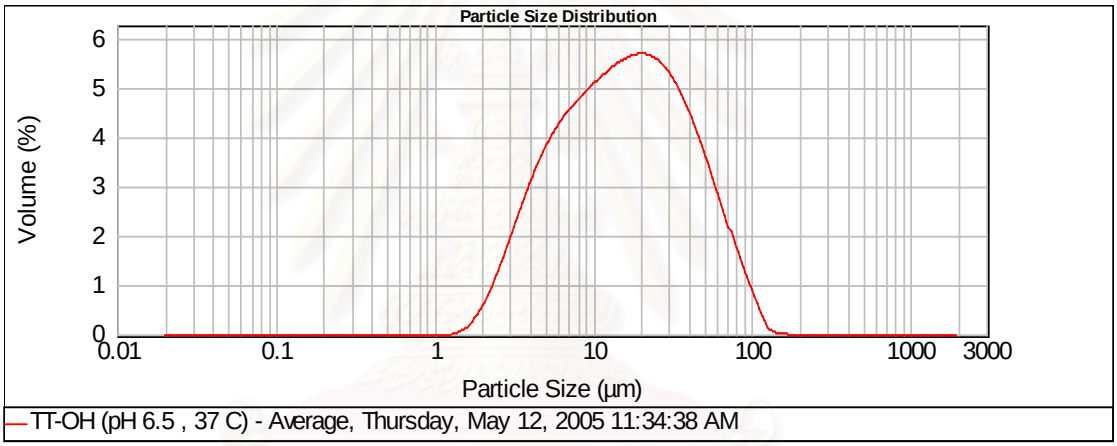

Figure 63 The particle size distribution of TT-AH at pH 6.5 and 37 °C

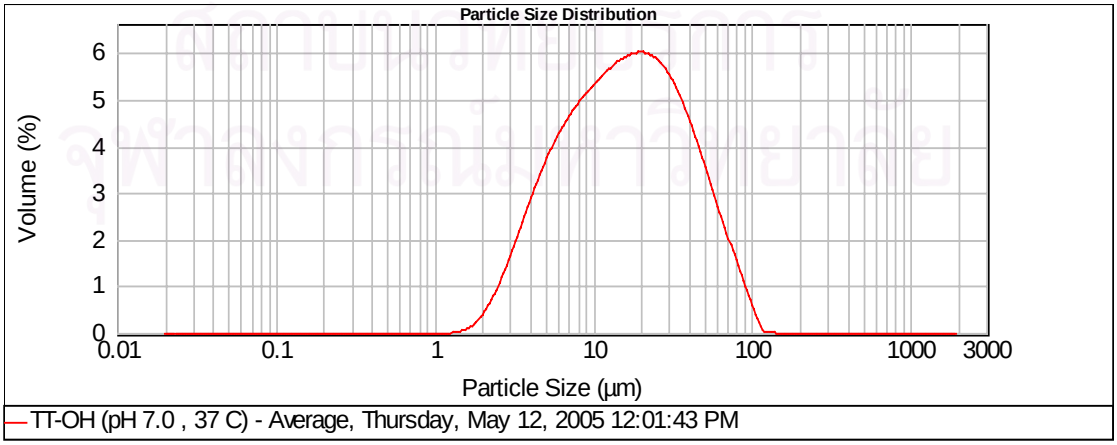

Figure 64 The particle size distribution of TT-AH at pH 7.0 and 37 °C

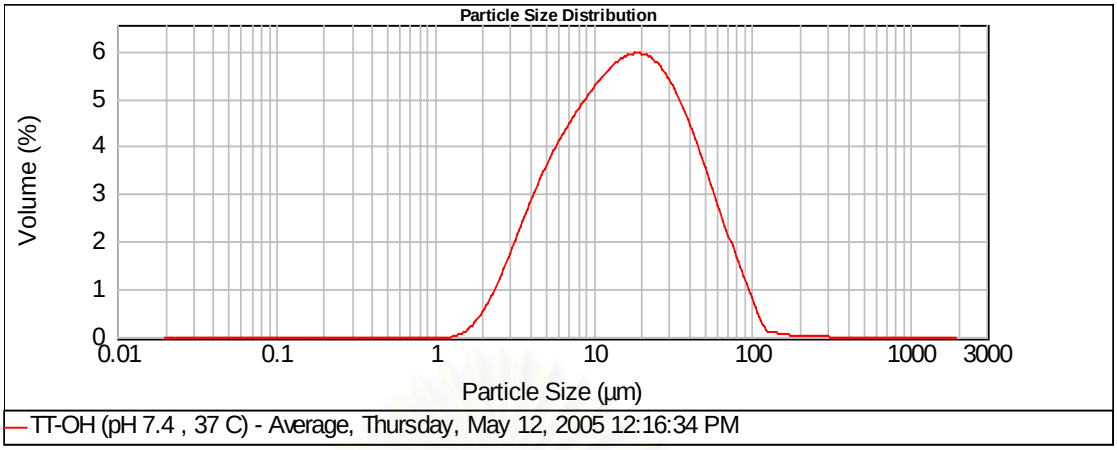

Figure 65 The particle size distribution of TT-AH at pH 7.4 and 37 °C

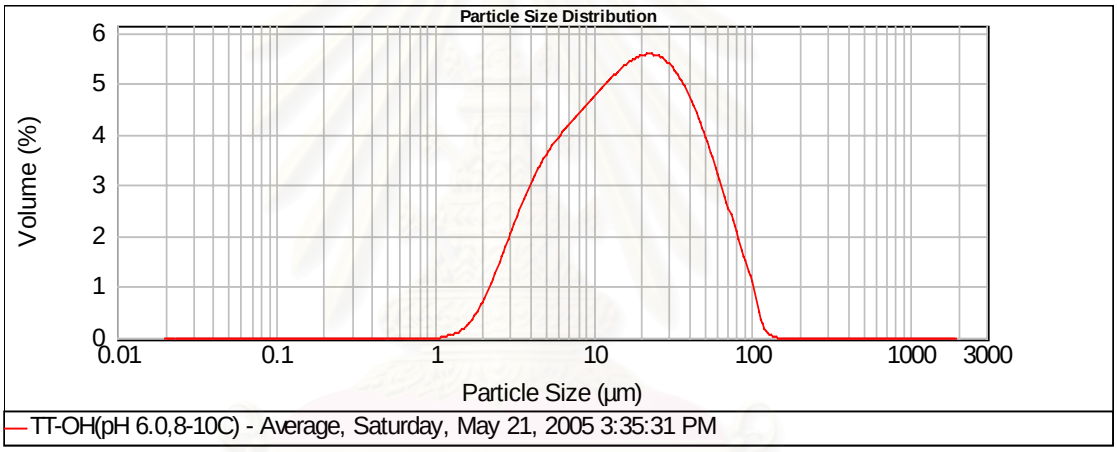

Figure 66 The particle size distribution of TT-AH at pH 6.0 and 9 °C

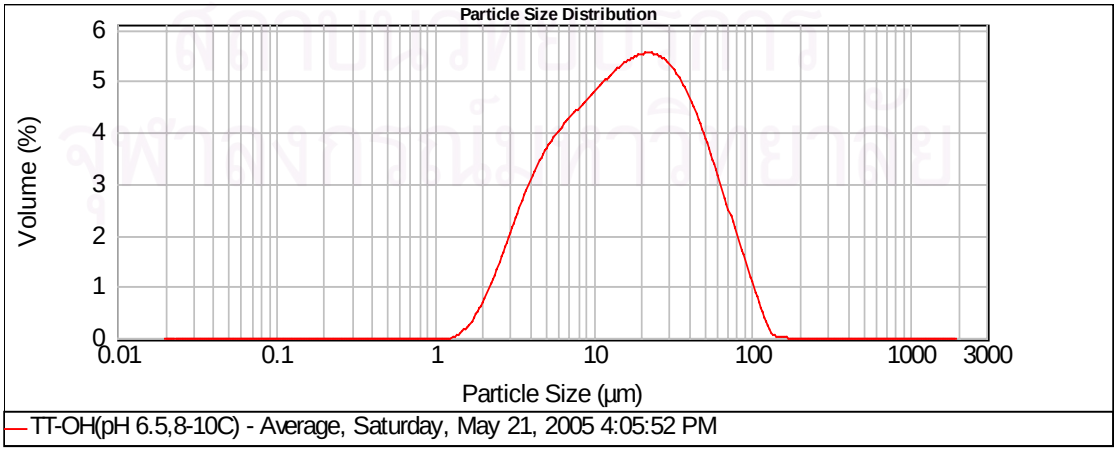

Figure 67 The particle size distribution of TT-AH at pH 6.5 and 9 °C

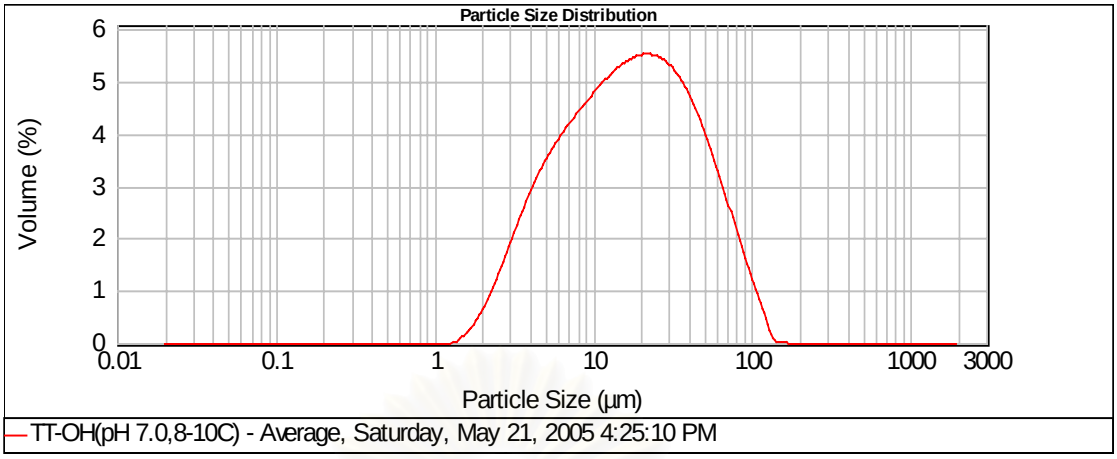

Figure 68 The particle size distribution of TT-AH at pH 7.0 and 9 °C

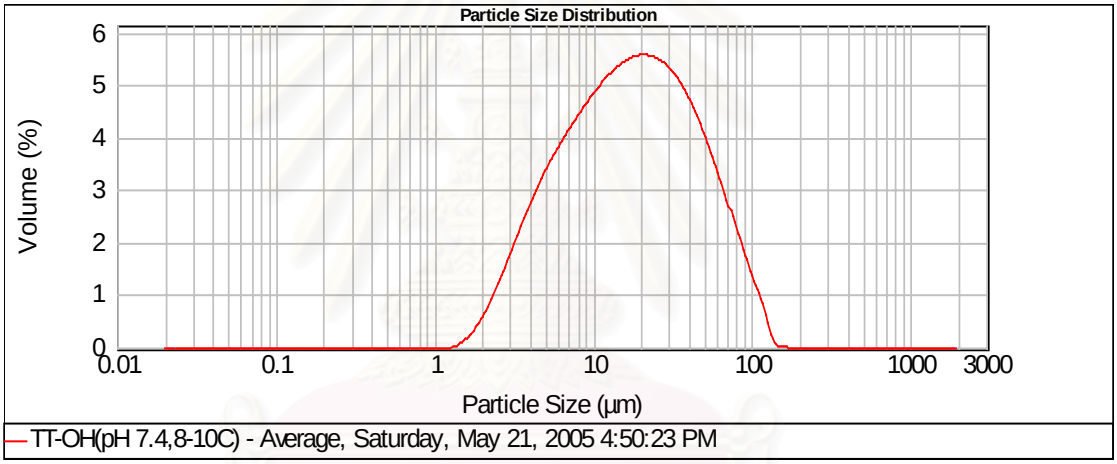

Figure 69 The particle size distribution of TT-AH at pH 7.4 and 9 °C

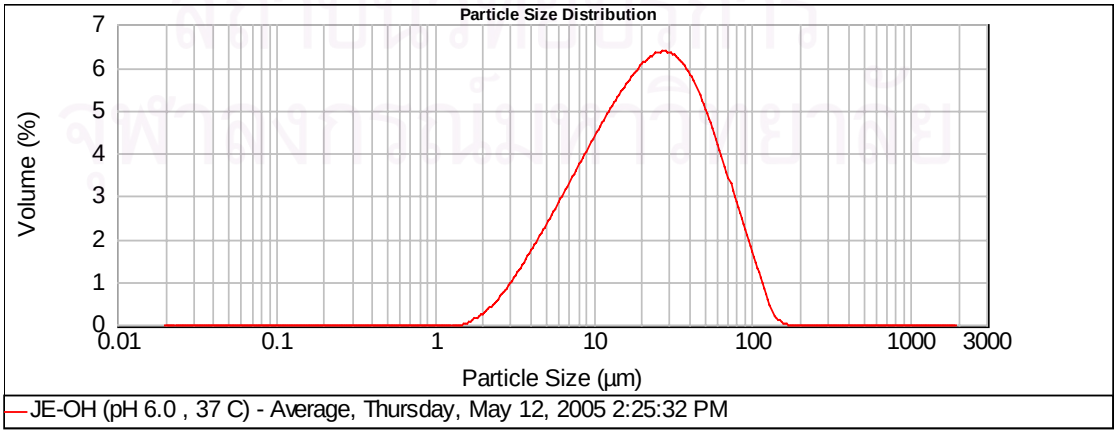

Figure 70 The particle size distribution of JE-AH at pH 6.0 and 37 °C

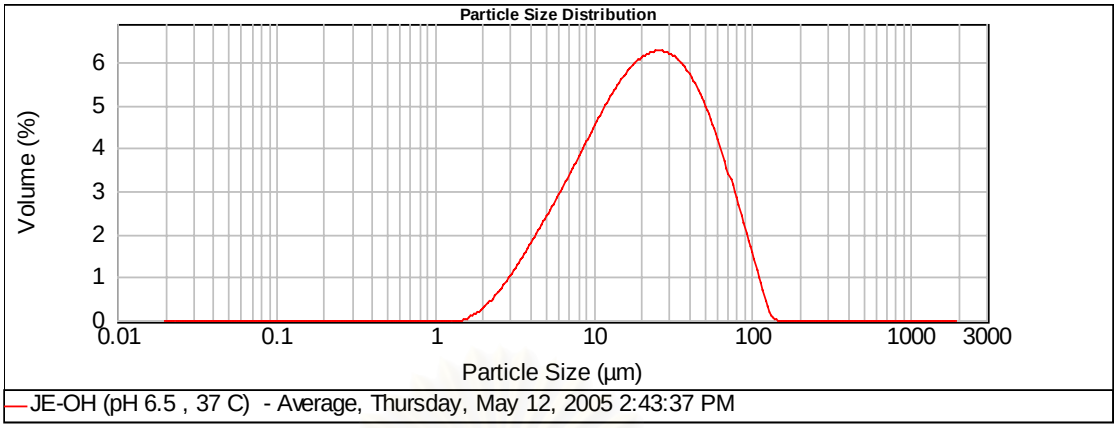

Figure 71 The particle size distribution of JE-AH at pH 6.5 and 37 °C

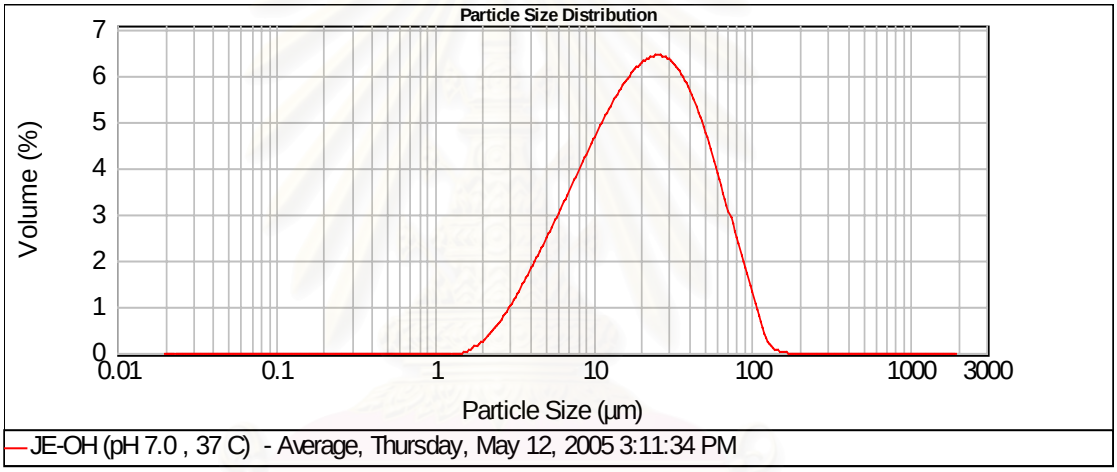

Figure 72 The particle size distribution of JE-AH at pH 7.0 and 37 °C

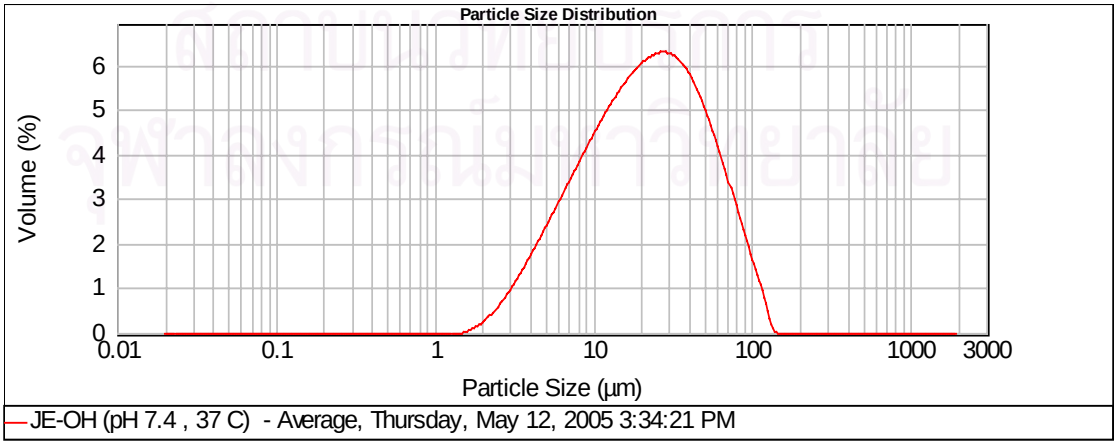

Figure 73 The particle size distribution of JE-AH at pH 7.4 and 37 °C

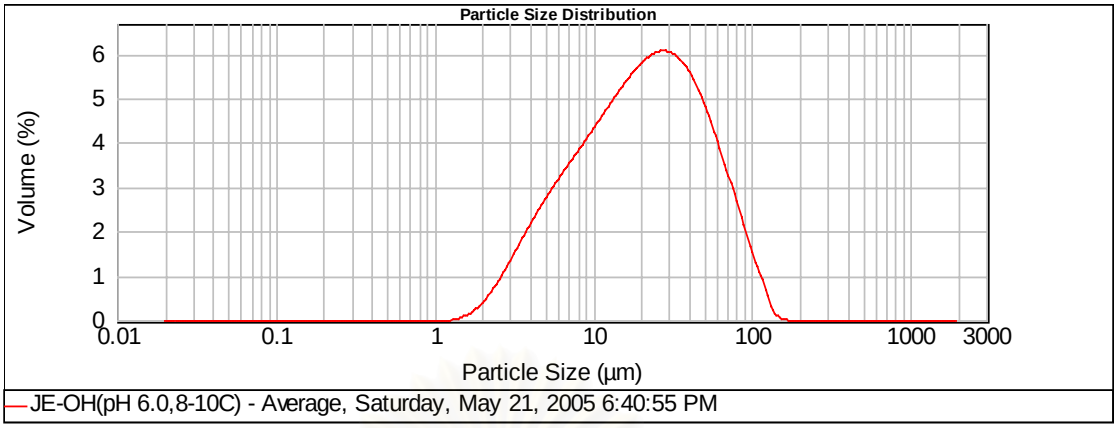

Figure 74 The particle size distribution of JE-AH at pH 6.0 and 9 °C

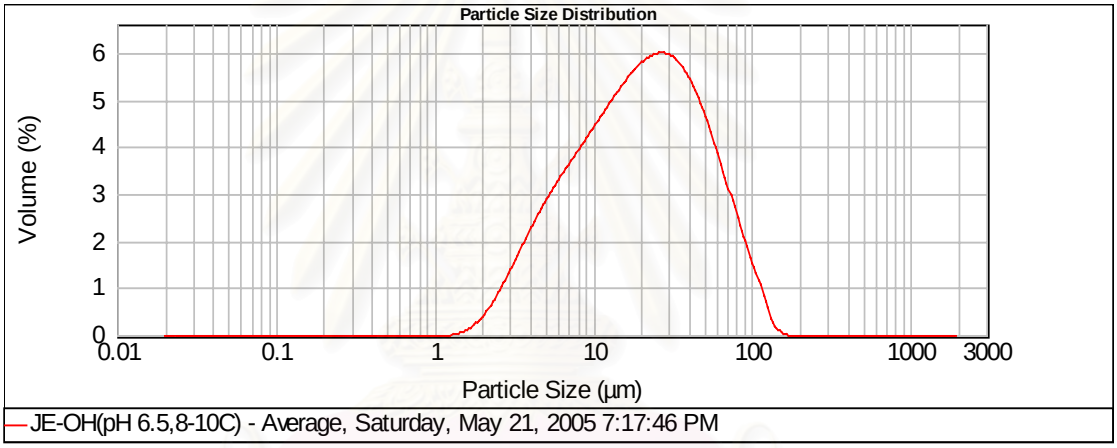

Figure 75 The particle size distribution of JE-AH at pH 6.5 and 9 °C

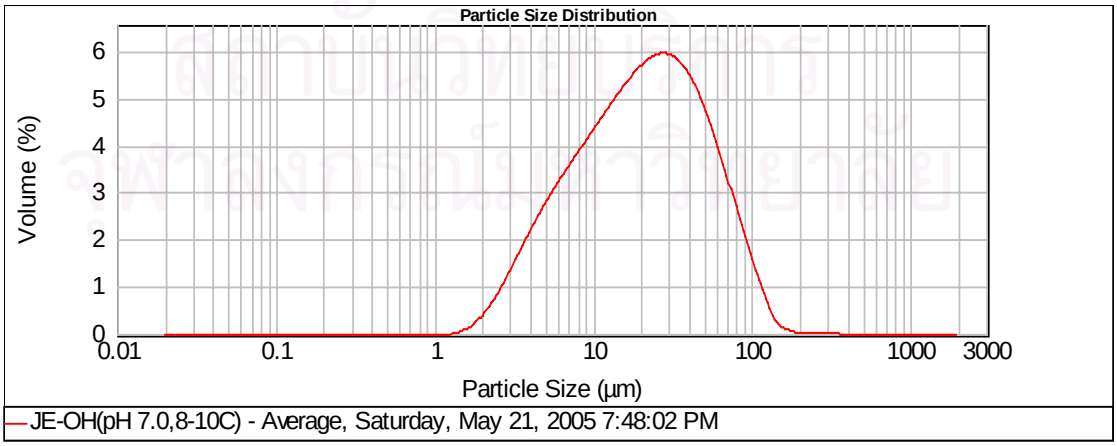

Figure 76 The particle size distribution of JE-AH at pH 7.0 and 9 °C

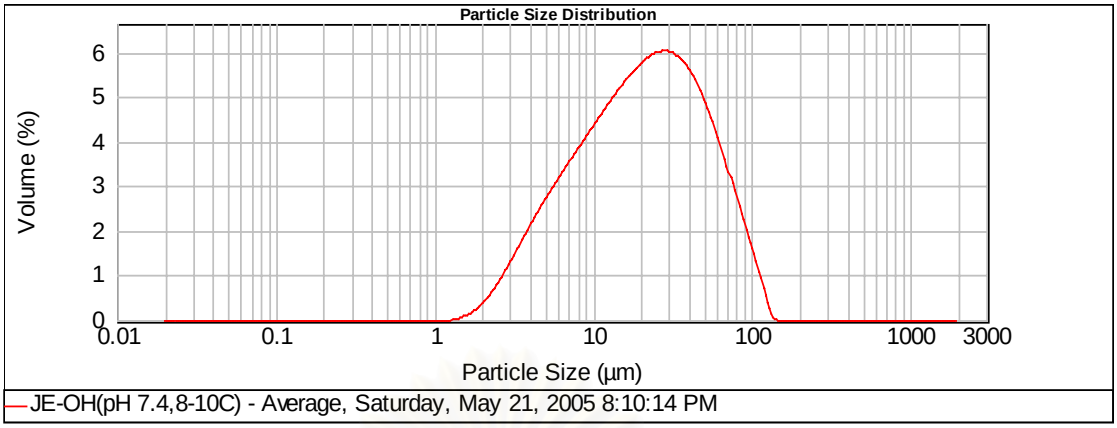

Figure 77 The particle size distribution of JE-AH at pH 7.4 and 9 °C

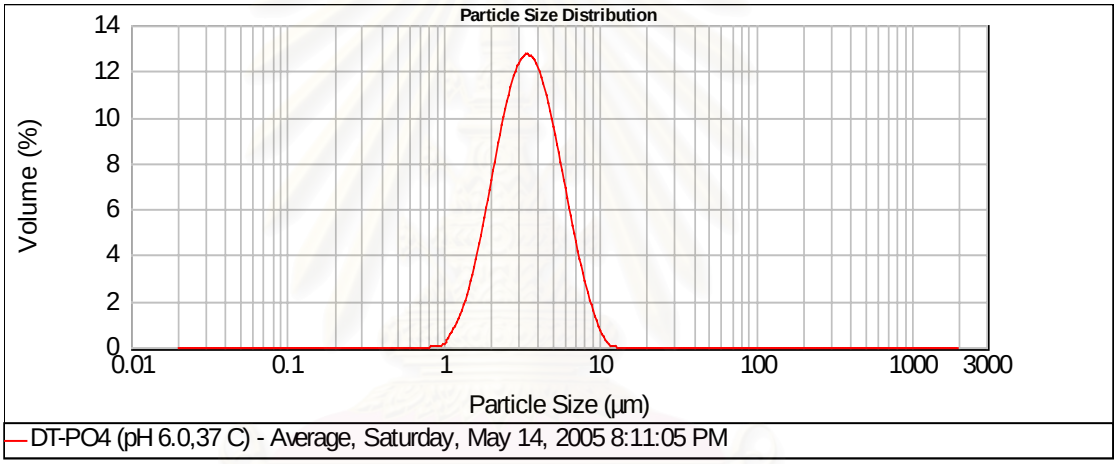

Figure 78 The particle size distribution of DT-AP at pH 6.0 and 37 °C

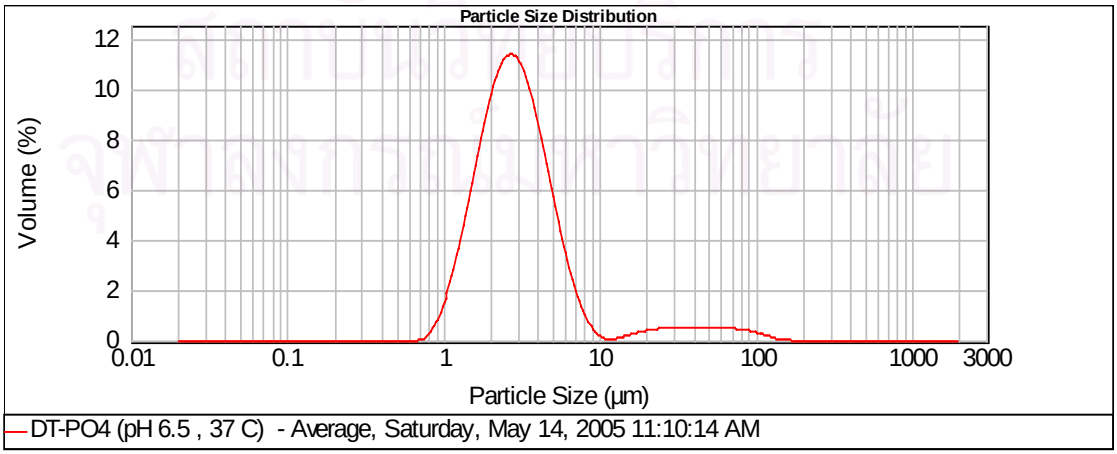

Figure 79 The particle size distribution of DT-AP at pH 6.5 and 37 °C

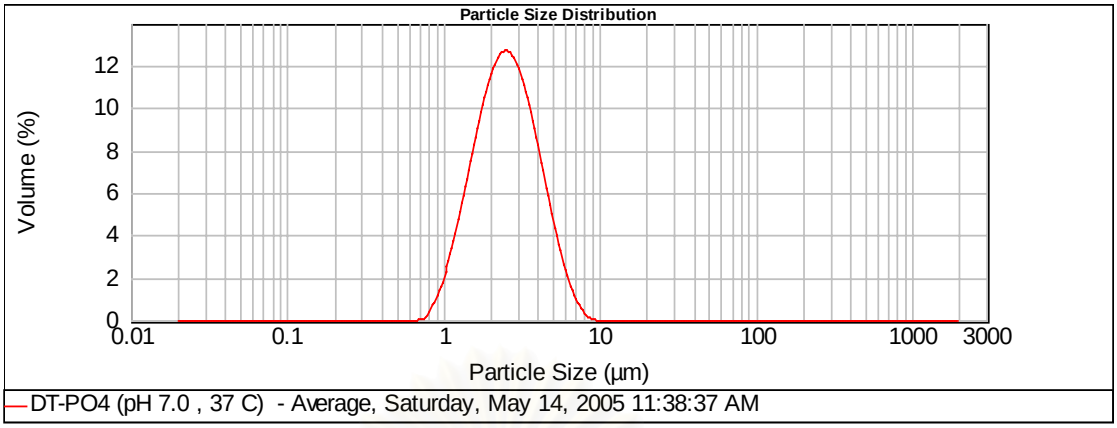

Figure 80 The particle size distribution of DT-AP at pH 7.0 and 37 °C

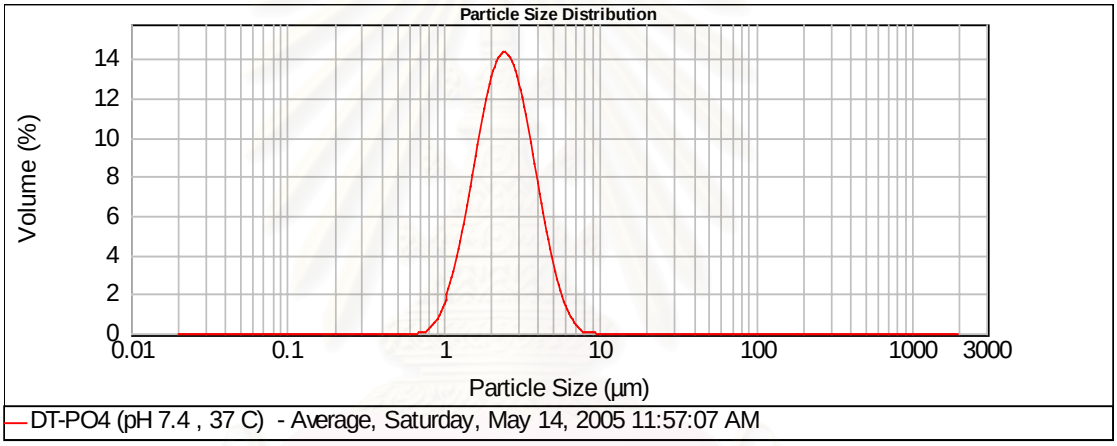

Figure 81 The particle size distribution of DT-AP at pH 7.4 and 37 °C

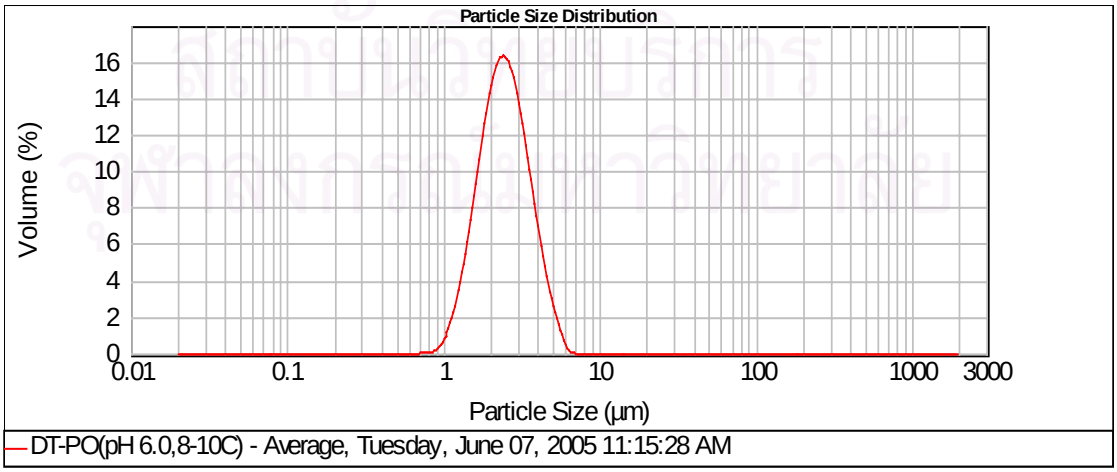

Figure 82 The particle size distribution of DT-AP at pH 6.0 and 9 °C

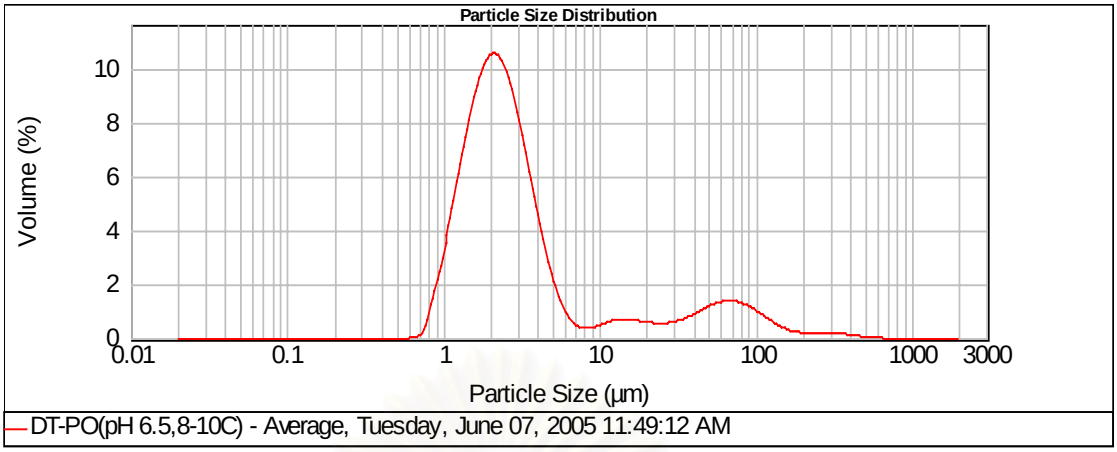

Figure 83 The particle size distribution of DT-AP at pH 6.5 and 9 °C

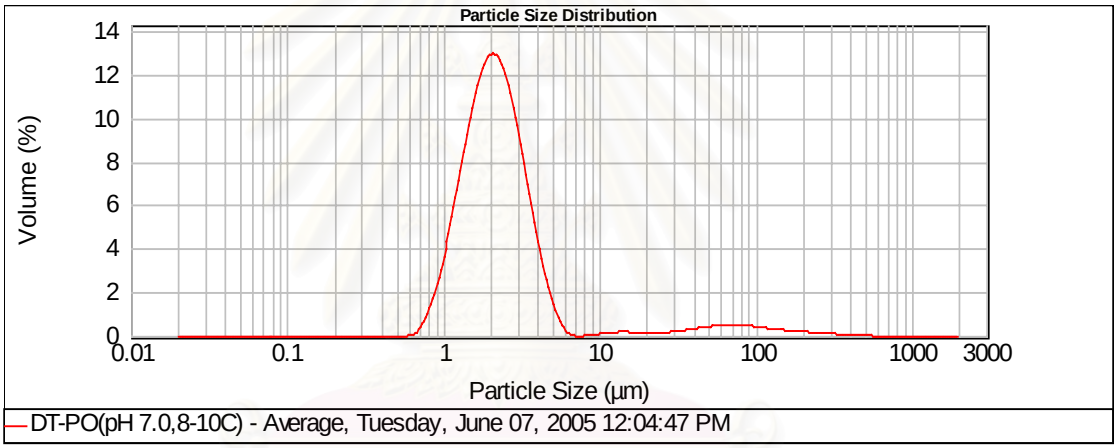

Figure 84 The particle size distribution of DT-AP at pH 7.0 and 9 °C

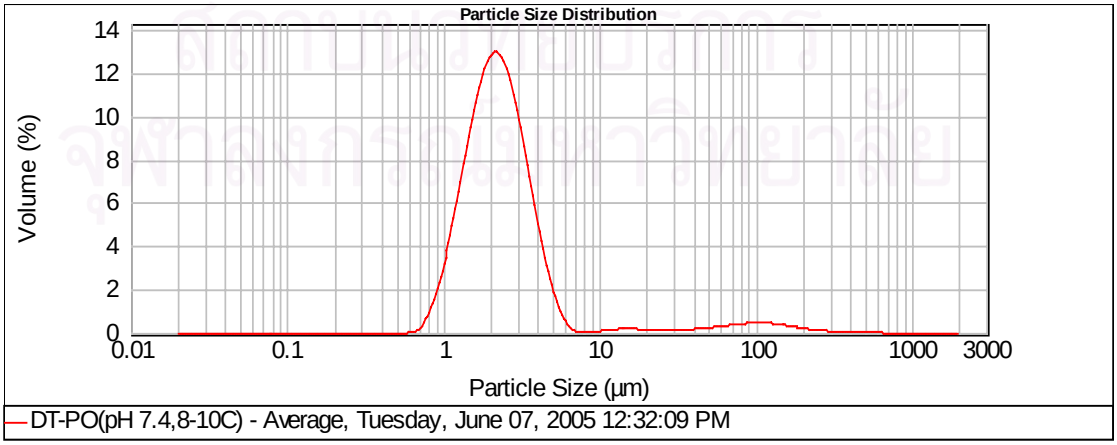

Figure 85 The particle size distribution of DT-AP at pH 7.4 and 9 °C

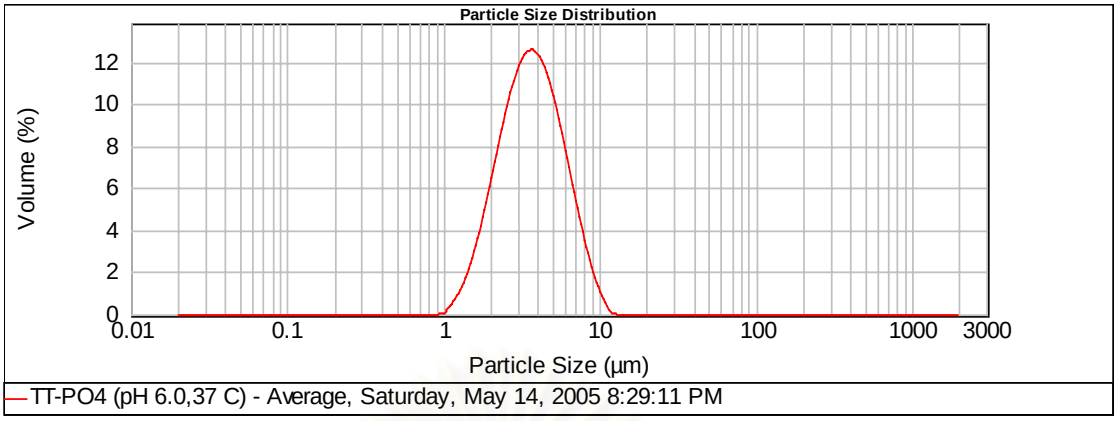

Figure 86 The particle size distribution of TT-AP at pH 6.0 and 37 °C

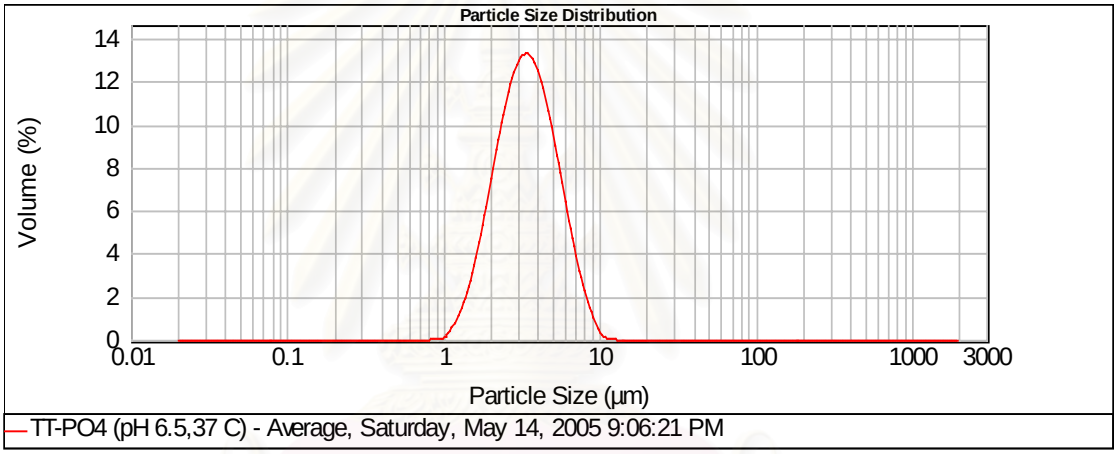

Figure 87 The particle size distribution of TT-AP at pH 6.5 and 37 °C

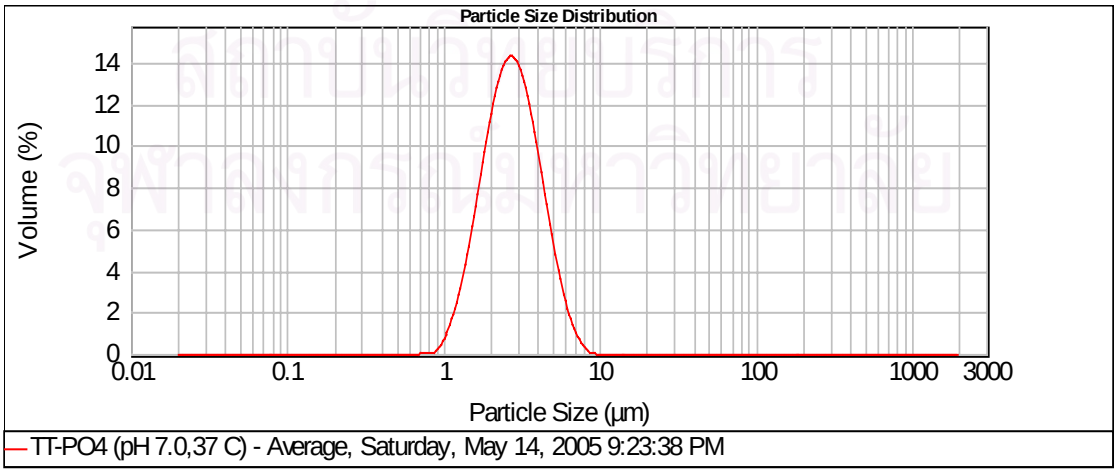

Figure 88 The particle size distribution of TT-AP at pH 7.0 and 37 °C

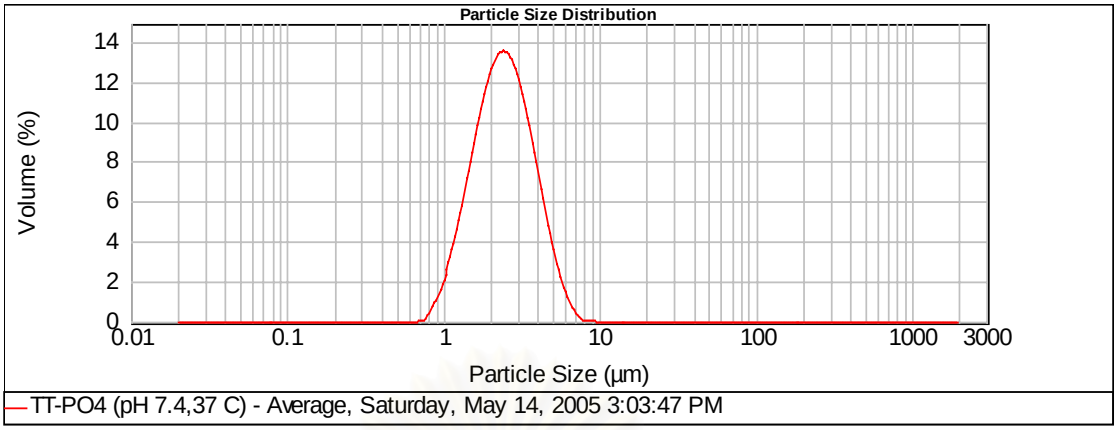

Figure 89 The particle size distribution of TT-AP at pH 7.4 and 37 °C

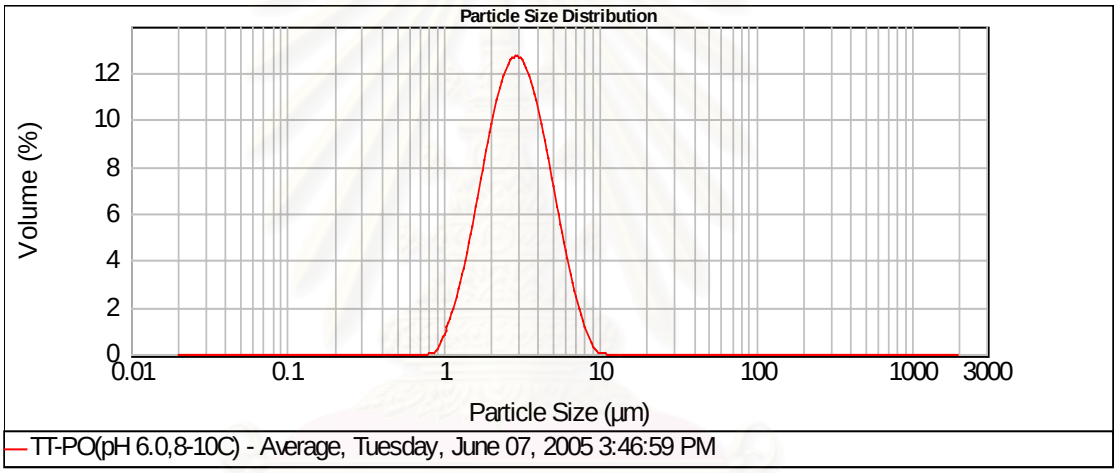

Figure 90 The particle size distribution of TT-AP at pH 6.0 and 9 °C

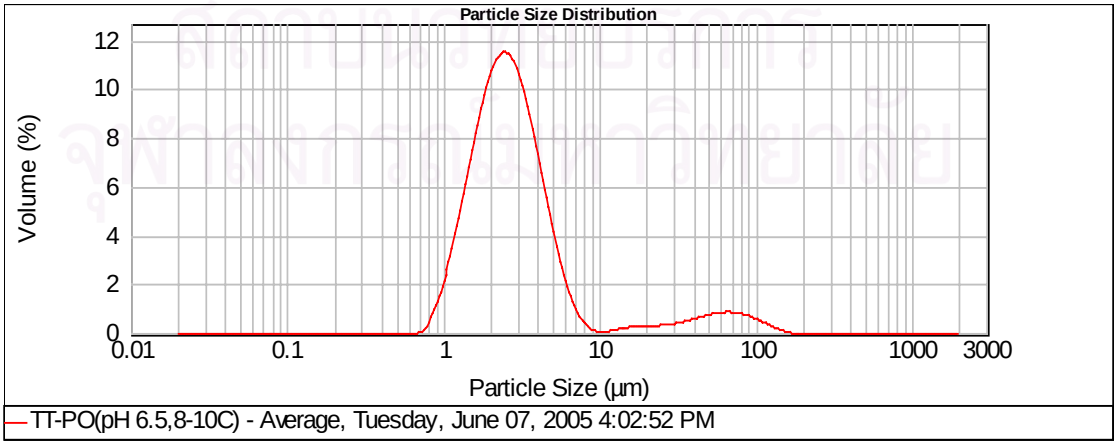

Figure 91 The particle size distribution of TT-AP at pH 6.5 and 9 °C

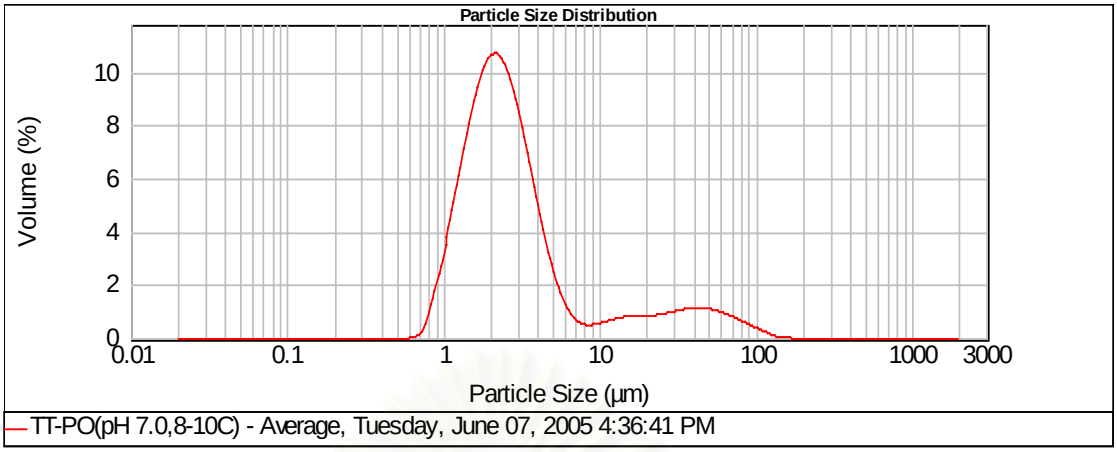

Figure 92 The particle size distribution of TT-AP at pH 7.0 and 9 °C

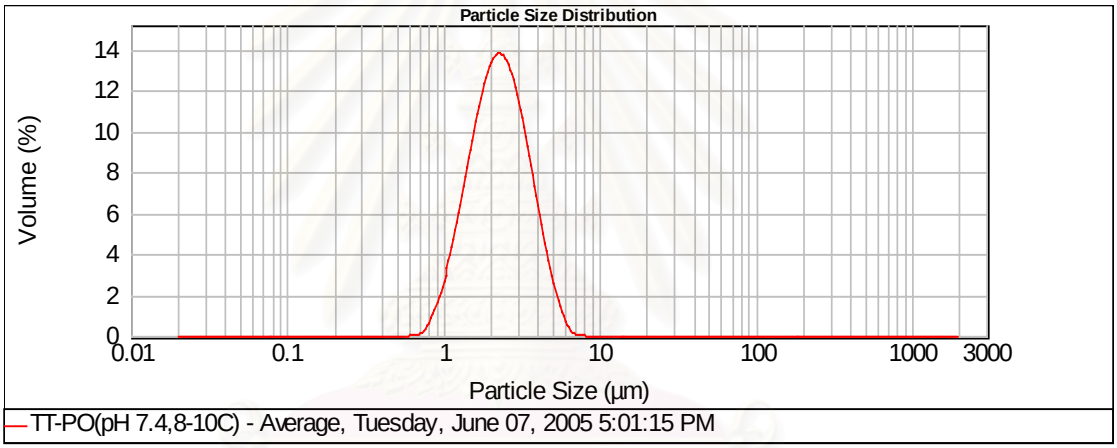

Figure 93 The particle size distribution of TT-AP at pH 7.4 and 9 °C

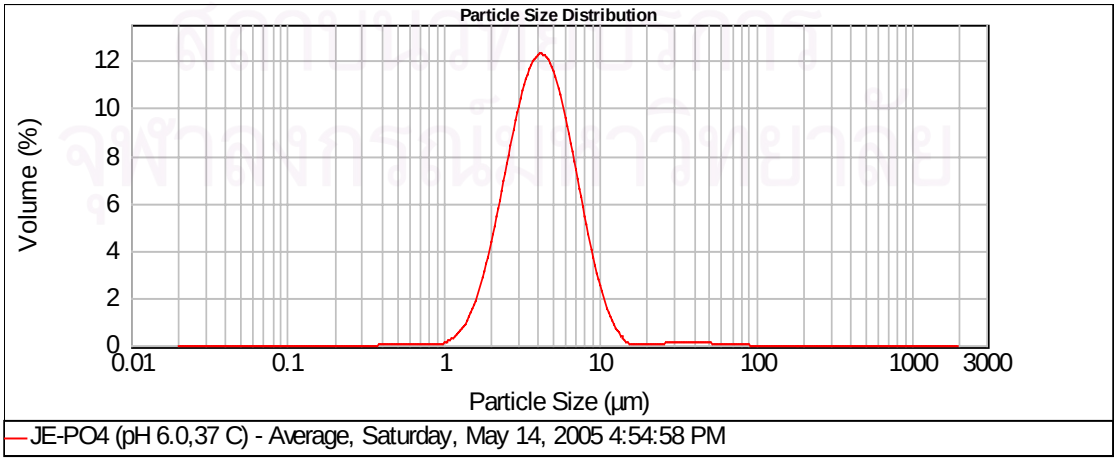

Figure 94 The particle size distribution of JE-AP at pH 6.0 and 37 °C

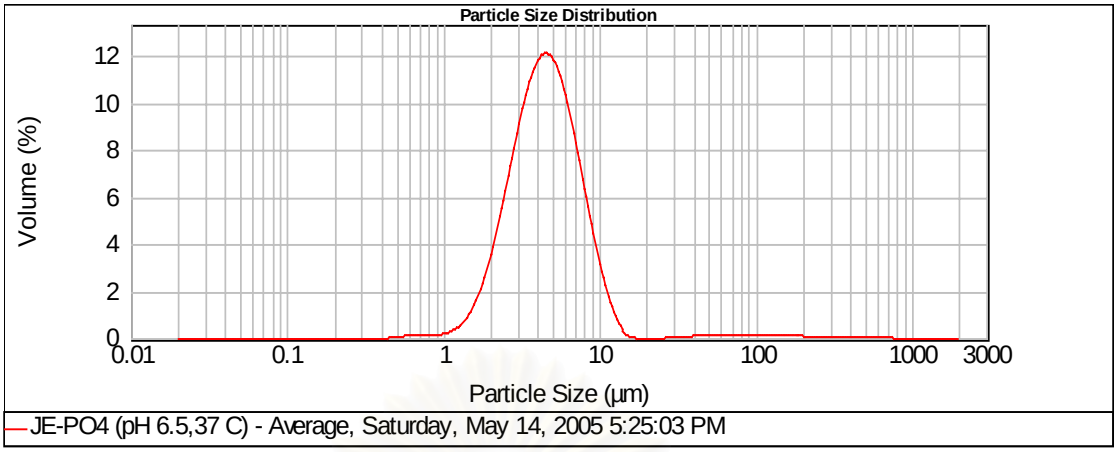

Figure 95 The particle size distribution of JE-AP at pH 6.5 and 37 °C

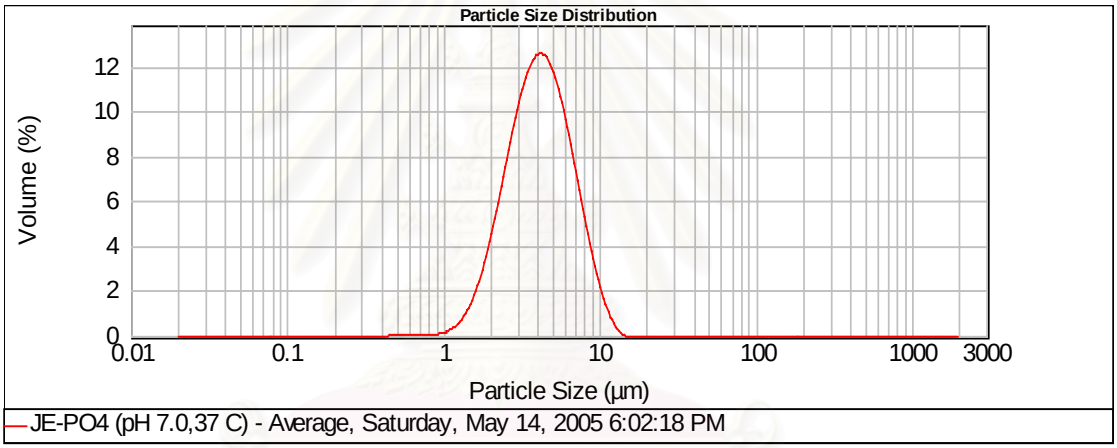

Figure 96 The particle size distribution of JE-AP at pH 7.0 and 37 °C

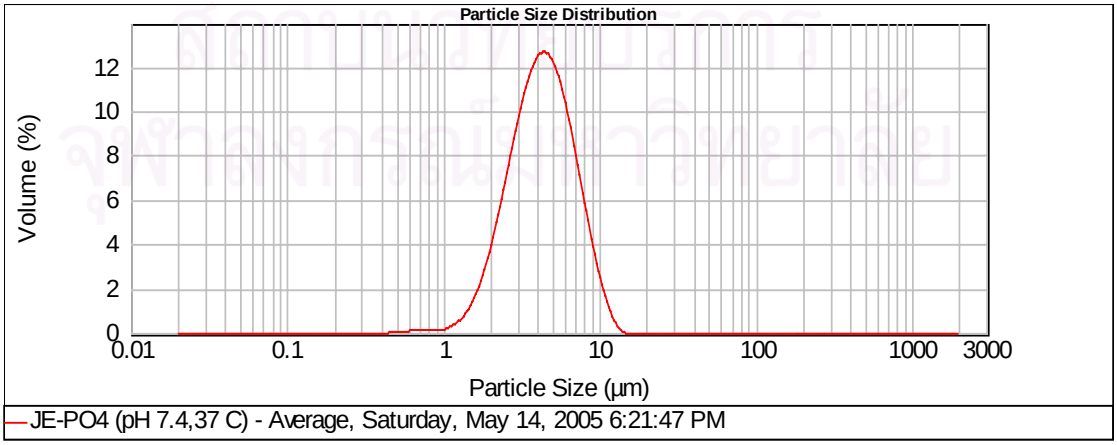

Figure 97 The particle size distribution of JE-AP at pH 7.4 and 37 °C

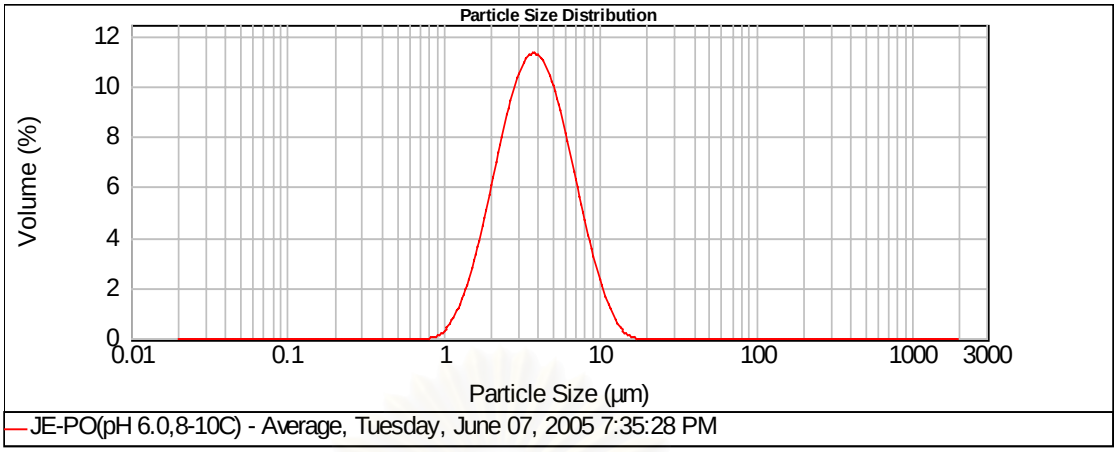

Figure 98 The particle size distribution of JE-AP at pH 6.0 and 9 °C

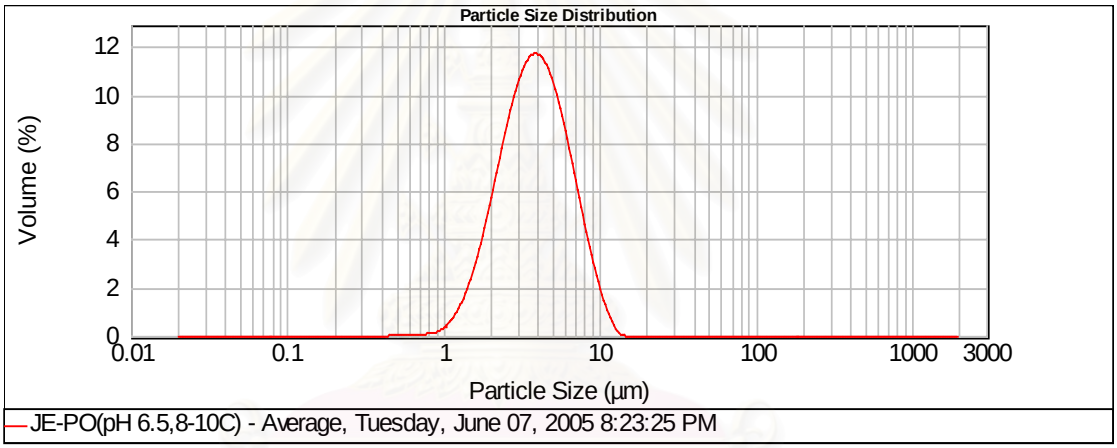

Figure 99 The particle size distribution of JE-AP at pH 6.5 and 9 °C

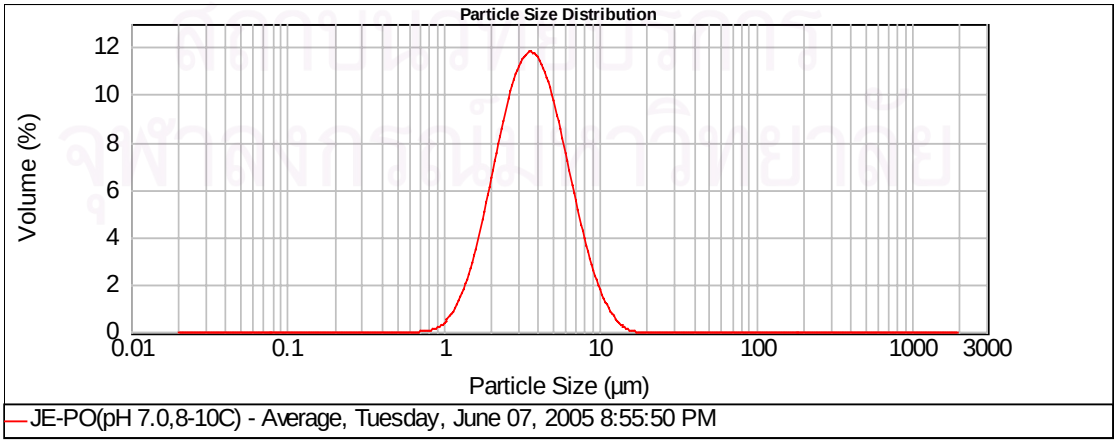

Figure 100 The particle size distribution of JE-AP at pH 7.0 and 9 °C

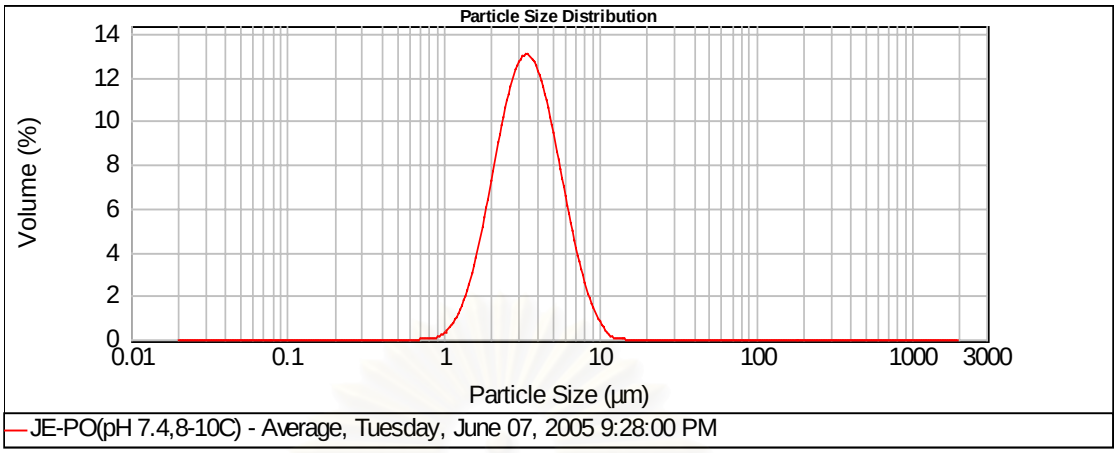

Figure 101 The particle size distribution of JE-AP at pH 7.4 and 9 °C

APPENDIX D

ELISA reagent

1. 0.05 M Carbonate-bicarbonate buffer (pH 9.6) (coating buffer)

Sodium carbonate	0.8	g.
Sodium hydrogen carbonate	1.5	g.
Distrilled water to	500	ml.

(adjust pH to 9.6 before bringing to volume)

2. Phosphate buffer saline (PBS) pH 7.4 with 0.05% Tween 20 (PBS-T , washing buffer)

Sodium chloride	8.0	g.
Potassium dihydrogen phosphate	0.2	g.
Disodium hydrogen phosphate	2.9	g.
Potassium chloride	0.2	g.
Thimerosol	0.1	g.
Tween 20	0.5	ml.
Distrilled water to	1,000	ml.

(adjust pH to 7.4 before bringing to volume)

3. 3% gelatin in PBS-T (blocking solution)

gelatin	3.0	g.
PBS-T to	100.0	ml.

4. Citrate – phosphate buffer pH 5.0 (substrate buffer)

Citric acid (monohydrate)	10.30	g.
Sodium hydrogen phosphate ($Na_2HPO_4 .2H_2O$)	18.16	g.
30% Hydrogen peroxide	1.0	ml.
Distrilled water to	1,000	ml.

5. 1% gelatin in PBS-T (diluent)

gelatin	1.0	g.
PBS-T to	100.0	ml.

6. 4 N Sulfuric acid (stop solution)

98% Sulfuric acid	54.4	ml.
Distrilled water to	500.0	ml.

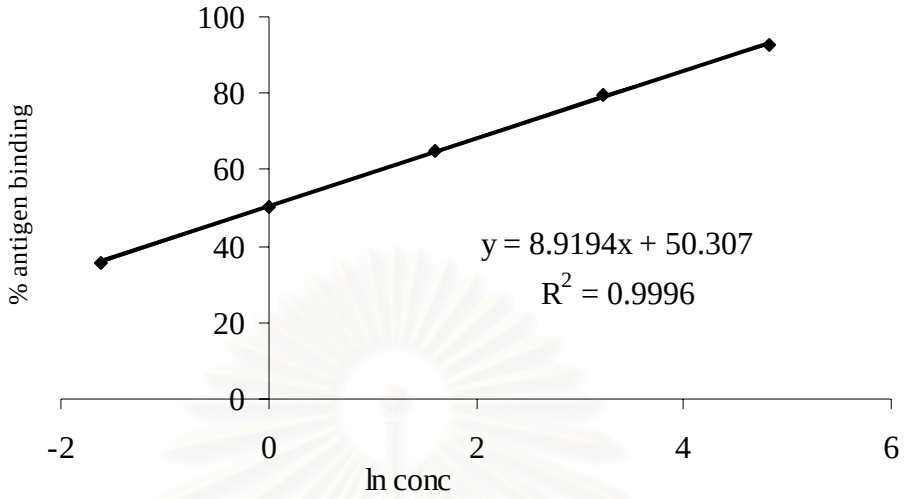

Figure 102 Standard curve of the optimal condition of diphtheria toxoid

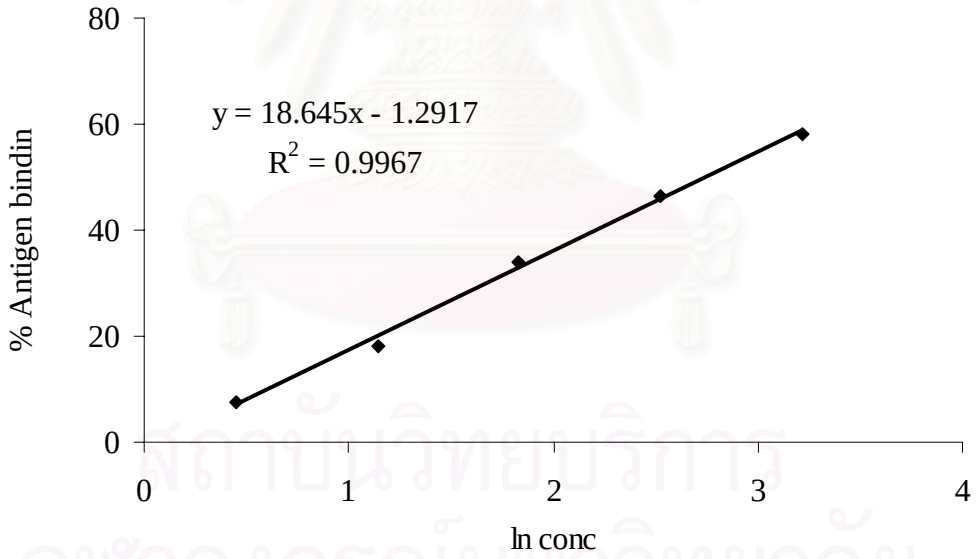

Figure 103 Standard curve of the optimal condition of tetanus toxoid

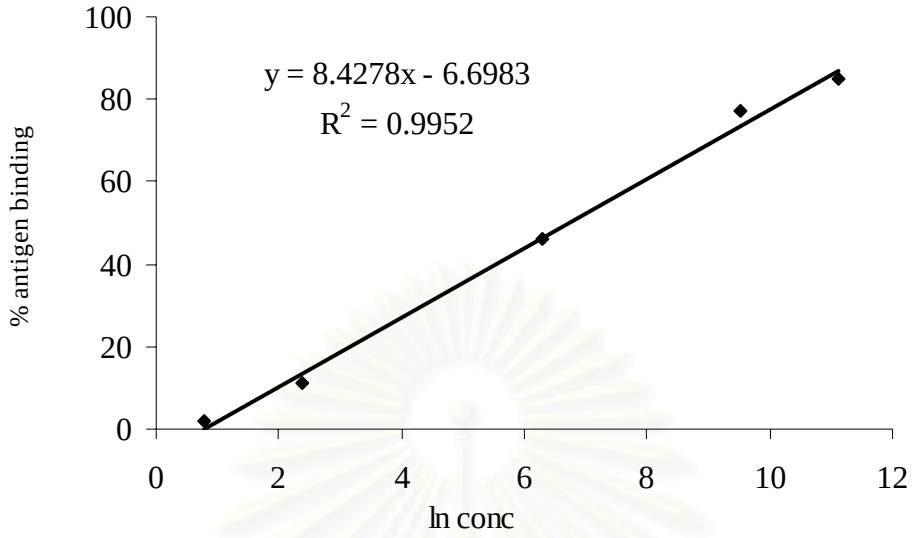

Figure 104 Standard curve of *Bordetella pertussis* at OD 1.585

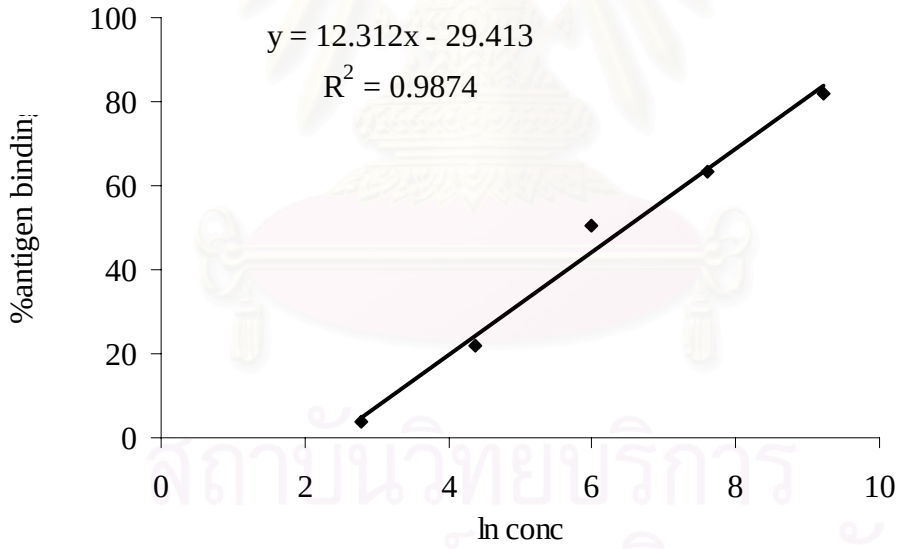

Figure 105 Standard curve of *Bordetella pertussis* at OD 1.139

APPENDIX E

Table 41 Statistical test for adsorption capacity of adsorbed aluminium hydroxide at various pHs.

Analysis of variance (ANOVA) and LSD test for post hoc comparisons

Dependent variable: adsorption value

LSD

(I) pH	(J) pH	DT		TT		JE	
		37 ± 1 °C	9 ± 1 °C	37 ± 1 °C	9 ± 1 °C	37 ± 1 °C	9 ± 1 °C
		Sig.	Sig.	Sig.	Sig.	Sig.	Sig.
6.0	6.5	.543	.024*	.250	.170	.717	.713
6.0	7.0	.699	.795	.000*	.071	.065	.057
6.0	7.4	.252	.884	.000*	.001*	.477	.105
6.5	7.0	.030*	.023*	.000*	.582	.116	.031*
6.5	7.4	.098	.034*	.000*	.008*	.720	.058
7.0	7.4	.429	.686	.027*	.019*	.201	.701

* The mean difference is significant at the .05 level.

Table 42 Statistical test for adsorption capacity of adsorbed aluminium phosphate at various pHs.

Analysis of variance (ANOVA) and LSD test for post hoc comparisons

Dependent variable: adsorption value

LSD

(I) pH	(J) pH	DT		TT		JE	
		37 ± 1 °C	9 ± 1 °C	37 ± 1 °C	9 ± 1 °C	37 ± 1 °C	9 ± 1 °C
		Sig.	Sig.	Sig.	Sig.	Sig.	Sig.
6.0	6.5	.478	.001*	.791	.034*	.092	.095
6.0	7.0	.115	.712	.413	.000*	.203	.234
6.0	7.4	.893	.130	.694	.000*	.062	.044*
6.5	7.0	.336	.001*	.572	.000*	.610	.560
6.5	7.4	.403	.000*	.897	.000*	.804	.632
7.0	7.4	.093	.229	.661	.031*	.454	.301

* The mean difference is significant at the .05 level.

Table 43 Statistical test for adsorption at different concentration of adsorbed aluminium hydroxide at various pHs.

Analysis of variance (ANOVA) and LSD test for post hoc comparisons
Dependent variable: adsorption value

LSD: Conc1

(I) pH	(J) pH	DT		TT		JE	
		37 ± 1 °C	9 ± 1 °C	37 ± 1 °C	9 ± 1 °C	37 ± 1 °C	9 ± 1 °C
		Sig.	Sig.	Sig.	Sig.	Sig.	Sig.
6.0	6.5	.084	.095	.059	.083	.289	.115
6.0	7.0	.785	.157	.000*	.079	.014*	.218
6.0	7.4	.792	.009*	.000*	.077	.002*	.057
6.5	7.0	.129	.751	.000*	.004*	.003*	.015*
6.5	7.4	.127	.162	.000*	.004*	.001*	.004*
7.0	7.4	.993	.098	.823	.988	.251	.402

* The mean difference is significant at the .05 level.

Dependent variable: adsorption value

LSD: Conc2

(I) pH	(J) pH	DT		TT		JE	
		37 ± 1 °C	9 ± 1 °C	37 ± 1 °C	9 ± 1 °C	37 ± 1 °C	9 ± 1 °C
		Sig.	Sig.	Sig.	Sig.	Sig.	Sig.
6.0	6.5	.663	.007*	.118	.389	.885	.944
6.0	7.0	.253	.009*	.000*	.953	.767	.808
6.0	7.4	.549	.000*	.000*	.635	.023*	.421
6.5	7.0	.458	.882	.000*	.420	.878	.862
6.5	7.4	.868	.004*	.000*	.197	.018*	.460
7.0	7.4	.561	.004*	.945	.594	.014*	.567

* The mean difference is significant at the .05 level.

Dependent variable: adsorption value

LSD: Conc3

(I) pH	(J) pH	DT		TT		JE	
		37 ± 1 °C	9 ± 1 °C	37 ± 1 °C	9 ± 1 °C	37 ± 1 °C	9 ± 1 °C
		Sig.	Sig.	Sig.	Sig.	Sig.	Sig.
6.0	6.5	.080	.421	.143	.790	.652	.097
6.0	7.0	.264	.330	.000*	.968	.329	.033*
6.0	7.4	.320	.211	.000*	.732	.302	.139
6.5	7.0	.444	.096	.000*	.822	.170	.512
6.5	7.4	.371	.623	.000*	.939	.155	.815
7.0	7.4	.891	.043*	.392	.763	.951	.381

* The mean difference is significant at the .05 level.

Dependent variable: adsorption value

LSD: Conc4

(I) pH	(J) pH	DT		TT		JE	
		37 ± 1 °C	9 ± 1 °C	37 ± 1 °C	9 ± 1 °C	37 ± 1 °C	9 ± 1 °C
		Sig.	Sig.	Sig.	Sig.	Sig.	Sig.
6.0	6.5	.523	.578	.108	.249	.397	.067
6.0	7.0	.231	.627	.000*	.073	.100	.073
6.0	7.4	.197	.012*	.000*	.093	.314	.260
6.5	7.0	.546	.942	.000*	.435	.362	.957
6.5	7.4	.480	.005*	.000*	.525	.862	.392
7.0	7.4	.915	.006*	.067	.878	.454	.421

* The mean difference is significant at the .05 level.

Dependent variable: adsorption value

LSD: Conc5

(I) pH	(J) pH	DT		TT		JE	
		37 ± 1 °C	9 ± 1 °C	37 ± 1 °C	9 ± 1 °C	37 ± 1 °C	9 ± 1 °C
		Sig.	Sig.	Sig.	Sig.	Sig.	Sig.
6.0	6.5	.200	.790	.065	.463	.799	.348
6.0	7.0	.043*	.459	.000*	.020*	.079	.856
6.0	7.4	.003*	.228	.000*	.043*	.021*	.415
6.5	7.0	.343	.323	.000*	.066	.118	.270
6.5	7.4	.019*	.333	.000*	.142	.031*	.894
7.0	7.4	.092	.071	.362	.631	.413	.326

* The mean difference is significant at the .05 level.

Dependent variable: adsorption value

LSD: Conc6

(I) pH	(J) pH	DT		TT		JE	
		37 ± 1 °C	9 ± 1 °C	37 ± 1 °C	9 ± 1 °C	37 ± 1 °C	9 ± 1 °C
		Sig.	Sig.	Sig.	Sig.	Sig.	Sig.
6.0	6.5	.132	.324	.250	.170	.740	.713
6.0	7.0	.003*	.795	.000*	.071	.053	.057
6.0	7.4	.009*	.884	.000*	.001*	.513	.105
6.5	7.0	.039*	.223	.000*	.582	.090	.031*
6.5	7.4	.112	.394	.000*	.008*	.742	.058
7.0	7.4	.519	.686	.027*	.019*	.151	.701

* The mean difference is significant at the .05 level

Table 44 Statistical test for adsorption at different concentration of adsorbed aluminium phosphate at various pHs.

Analysis of variance (ANOVA) and LSD test for post hoc comparisons
Dependent variable: adsorption value

LSD: Conc1

(I) pH	(J) pH	DT		TT		JE	
		37 ± 1 °C	9 ± 1 °C	37 ± 1 °C	9 ± 1 °C	37 ± 1 °C	9 ± 1 °C
		Sig.	Sig.	Sig.	Sig.	Sig.	Sig.
6.0	6.5	.053	.057	.213	.000*	.031*	1.000
6.0	7.0	.456	.030*	.415	.000*	.516	.003*
6.0	7.4	.326	.022*	.324	.000*	.154	.001*
6.5	7.0	.178	.689	.635	.819	.088	.003*
6.5	7.4	.258	.564	.770	.895	.326	.001*
7.0	7.4	.800	.856	.854	.719	.398	.191

* The mean difference is significant at the .05 level.

Dependent variable: adsorption value

LSD: Conc2

(I) pH	(J) pH	DT		TT		JE	
		37 ± 1 °C	9 ± 1 °C	37 ± 1 °C	9 ± 1 °C	37 ± 1 °C	9 ± 1 °C
		Sig.	Sig.	Sig.	Sig.	Sig.	Sig.
6.0	6.5	.114	.060	.721	.000*	.440	.109
6.0	7.0	.060	.116	.132	.000*	.156	.022*
6.0	7.4	.731	.006*	.048*	.000*	.021*	.007*
6.5	7.0	.685	.681	.227	.000*	.045*	.330
6.5	7.4	.194	.000*	.085	.000*	.073	.113
7.0	7.4	.104	.001*	.528	.797	.002*	.479

* The mean difference is significant at the .05 level.

Dependent variable: adsorption value

LSD: Conc3

(I) pH	(J) pH	DT		TT		JE	
		37 ± 1 °C	9 ± 1 °C	37 ± 1 °C	9 ± 1 °C	37 ± 1 °C	9 ± 1 °C
		Sig.	Sig.	Sig.	Sig.	Sig.	Sig.
6.0	6.5	.006*	.059	.226	.000*	.815	.050*
6.0	7.0	.001*	.942	.913	.000*	.005*	.163
6.0	7.4	.043*	.802	.042*	.000*	.002*	.031*
6.5	7.0	.245	.052	.265	.000*	.007*	.460
6.5	7.4	.219	.039*	.006*	.000*	.002*	.770
7.0	7.4	.032*	.859	.035*	.363	.384	.312

* The mean difference is significant at the .05 level.

Dependent variable: adsorption value

LSD: Conc4

(I) pH	(J) pH	DT		TT		JE	
		37 ± 1 °C	9 ± 1 °C	37 ± 1 °C	9 ± 1 °C	37 ± 1 °C	9 ± 1 °C
		Sig.	Sig.	Sig.	Sig.	Sig.	Sig.
6.0	6.5	.201	.007*	.420	.000*	.537	.272
6.0	7.0	.360	.340	.254	.000*	.389	.048*
6.0	7.4	.814	.694	.089	.000*	.043*	.049*
6.5	7.0	.683	.030*	.713	.002*	.158	.283
6.5	7.4	.283	.012*	.311	.000*	.016*	.288
7.0	7.4	.488	.562	.503	.035*	.172	.990

* The mean difference is significant at the .05 level.

Dependent variable: adsorption value

LSD: Conc5

(I) pH	(J) pH	DT		TT		JE	
		37 ± 1 °C	9 ± 1 °C	37 ± 1 °C	9 ± 1 °C	37 ± 1 °C	9 ± 1 °C
		Sig.	Sig.	Sig.	Sig.	Sig.	Sig.
6.0	6.5	.217	.816	.444	.000*	.094	.621
6.0	7.0	.050*	.451	.060	.000*	.122	.146
6.0	7.4	.640	.249	.537	.000*	.112	.062
6.5	7.0	.360	.332	.017*	.000*	.868	.066
6.5	7.4	.105	.345	.185	.000*	.915	.028
7.0	7.4	.023*	.076	.160	.234	.953	.590

* The mean difference is significant at the .05 level.

Dependent variable: adsorption value

LSD: Conc6

(I) pH	(J) pH	DT		TT		JE	
		37 ± 1 °C	9 ± 1 °C	37 ± 1 °C	9 ± 1 °C	37 ± 1 °C	9 ± 1 °C
		Sig.	Sig.	Sig.	Sig.	Sig.	Sig.
6.0	6.5	.532	.001*	.791	.034*	.009*	.268
6.0	7.0	.159	.733	.413	.000*	.021*	.002*
6.0	7.4	.237	.001*	.694	.000*	.007*	.000*
6.5	7.0	.395	.001*	.572	.000*	.596	.011*
6.5	7.4	.089	.000*	.897	.000*	.852	.000*
7.0	7.4	.022*	.001*	.661	.031*	.478	.001*

* The mean difference is significant at the .05 level

Table 45 Statistical test for particle size distribution (LD) of adsorbed aluminium hydroxide.

Paired sample t-test

Pairs	DT		TT		JE	
	37 ± 1 °C	9 ± 1 °C	37 ± 1 °C	9 ± 1 °C	37 ± 1 °C	9 ± 1 °C
	Sig.	Sig.	Sig.	Sig.	Sig.	Sig.
blank - pH 6.0	.004*	.006*	.020*	.012*	.020*	.002*
blank - pH 6.5	.000*	.001*	.039*	.007*	.045*	.001*
blank - pH 7.0	.002*	.002*	.035*	.002*	.055	.000*
blank - pH 7.4	.000*	.001*	.033*	.005*	.023*	.000*

* The mean difference is significant at the .05 level.

Table 46 Statistical test for particle size distribution (LD) of adsorbed aluminium phosphate.

Paired sample t-test

Pairs	DT		TT		JE	
	37 ± 1 °C	9 ± 1 °C	37 ± 1 °C	9 ± 1 °C	37 ± 1 °C	9 ± 1 °C
	Sig.	Sig.	Sig.	Sig.	Sig.	Sig.
blank - pH 6.0	.006*	.000*	.001*	.000*	.000*	.000*
blank - pH 6.5	.046*	.014*	.000*	.116*	.000*	.000*
blank - pH 7.0	.000*	.018*	.013*	.005*	.001*	.000*
blank -pH 7.4	.002*	.020*	.001*	.000*	.000*	.003*

* The mean difference is significant at the .05 level.

Table 47 Statistical test for particle size distribution (PCS) of adsorbed adjuvant.

Paired sample t-test

Pairs	DT		TT		JE	
	AH	AP	AH	AP	AH	AP
	Sig.	Sig.	Sig.	Sig.	Sig.	Sig.
blank - pH 6.0	.089	.838	.951	.019*	.300	.050*
blank - pH 6.5	.123	.081	.117	.570	.764	.016*
blank - pH 7.0	.622	.150	.240	.000*	.092	.006*
blank -pH 7.4	.193	.151	.366	.113	.425	.001*

* The mean difference is significant at the .05 level.

Table 48 Statistical test for the antigen content among F1, F2 and F3.

Analysis of variance (ANOVA) and LSD test for post hoc comparisons

Dependent variable: adsorption value

LSD

(I) formula	(J) formula	DT				TT			
		t_0	t_1	t_2	t_3	t_0	t_1	t_2	t_3
		Sig.	Sig.	Sig.	Sig.	Sig.	Sig.	Sig.	Sig.
F1	F2	.143	.414	.513	.001*	.003*	.148	.183	.055
F1	F3	.096	.957	.050*	.003*	.000*	.377	.404	.026*
F2	F3	.783	.443	.130	.300	.005*	.507	.565	.599

* The mean difference is significant at the .05 level.

(I) formula	(J) formula	PT				JE			
		t_0	t_1	t_2	t_3	t_0	t_1	t_2	t_3
		Sig.	Sig.	Sig.	Sig.	Sig.	Sig.	Sig.	Sig.
F1	F2	.001*	.569	.183	.732	.864	.000*	.868	.610
F1	F3	.003*	.428	.404	.766	.439	.000*	.677	.588
F2	F3	.251	.819	.565	.963	.353	.010*	.800	.974

* The mean difference is significant at the .05 level.

Table 49 Statistical test for the antigen content among initial, 1 month, 2 months and 3 months.

(I) months	(J) months	DT			TT		
		F1	F2	F3	F1	F2	F3
		Sig.	Sig.	Sig.	Sig.	Sig.	Sig.
Initial	1 month	.272	.005*	.005*	.000*	.000*	.003*
Initial	2 months	.057	.006*	.177	.047*	.014*	.011*
Initial	3 months	.016*	.010*	.004*	.392	.001*	.001*
1 month	2 months	.326	.846	.044*	.003*	.000*	.000*
1 month	3 months	.099	.613	.878	.000*	.000*	.000*
2 months	3 months	.437	.753	.035*	.188	.038*	.076

* The mean difference is significant at the .05 level.

(I) months	(J) months	PT			JE		
		F1	F2	F3	F1	F2	F3
		Sig.	Sig.	Sig.	Sig.	Sig.	Sig.
Initial	1 month	.000*	.000*	.000*	.000*	.000*	.000*
Initial	2 months	.000*	.000*	.000*	.000*	.000*	.000*
Initial	3 months	.000*	.000*	.000*	.000*	.000*	.000*
1 month	2 months	.752	.942	.822	.344	.114	.038*
1 month	3 months	.682	.471	.599	.000*	.000*	.000*
2 months	3 months	.924	.515	.761	.000*	.000*	.000*

* The mean difference is significant at the .05 level.

All statistic analysis were calculated using SPSS version 11.0.

VITA

Miss Woranuch Sripongsarn was born on August 10th 1976, in Bangkok, Thailand. She received her Bachelor of Science in Pharmacy in 1999 from the Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand. She worked at Siam Bheasach Co., Ltd. from March 15th 1999 to May 31st 2002 as Chief of Penicillin Department, Production Division. She continued studying in the Master's Degree in Industrial Pharmacy Program in the Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand.