รายงานผลการวิจัย
ทุนวิจัยรัชดาภิเษกสมโภช

เรื่อง

การศึกษาประสิทธิภาพและกลไกของไคโตแซน ในการเป็นสารเพิ่มการดูดซึมทางจมูกของยาเปปไทด์

โดย

ภาคภูมิ เต็งอำนวย
อัจฉริยา ไทละสูต
กาญจน์พิมล ฤทธิเดช

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CHULALONGKORN UNIVERSITY

RESEARCH REPORT

EFFICACY AND MECHANISTIC STUDIES OF CHITOSAN AS NASAL ABSORPTION ENHANCER OF PEPTIDE DRUGS

BY

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January, 1998

Acknowledgments

The investigators would like to thank the Committee of the Ratchadapisek Sompoj Research Funds, Chulalongkorn University, for providing financial support of this project. Without their funding, this research would not have been accomplished. Profound appreciation is also given to the Department of Pharmacy, Faculty of Pharmaceutical Sciences of Chulalongkorn University for providing research facilities. Finally, special recognition is given to all the research assistants who have contributed to the completion of this project, particularly Miss Amorn Sahamethapat and Miss Maytinee Limsiriwongs.

ชื่อโครงการวิจัย การศึกษาประสิทธิภาพและกลไกของไคโตแซนในการเป็นสารเพิ่มการดูดซึมทางจมูกของยาเป็ปไทด์

ชื่อผู้วิจัย ภาคภูมิ เต็งอำนวย, อัจฉริยา ไศละสูต และ กาญจน์พิมล ฤทธิเดช

เดือนและปีที่ทำวิจัยเสร็จ มกราคม 2541

บทคัดย่อ

วัตถุประสงค์ ศึกษาถึงประสิทธิภาพและความปลอดภัยของไคโตแซนในการเป็นสารเพิ่มการดูดซึมทางจมูกของยาเป็ปไทด์ **วิธีการ** ประเมินประสิทธิภาพของไคโตแซนในการเพิ่มการดูดซึมทางจมูกของยาเป็ปไทด์ตัวอย่าง L-Tyr-D-Arg ([D-Arg^2]-Kyotorphin) ซึ่งเป็นไดเป็ปไทด์ที่ทนต่อการสลายตัวโดยเอนไซม์ในเนื้อเยื่อบุโพรงจมูก โดยศึกษาไคโตแซนสองชนิดคือไคโตแซนในรูปด่างอิสระ CS J และรูปเกลือกลูตาเมท CS G โดยใช้เทคนิคการเพอร์ฟิวส์โพรงจมูกหนูขาว จากนั้นจึงศึกษาถึงฤทธิ์ในการทำลายเยื่อบุโพรงจมูกโดยดูจากปริมาณสารต่าง ๆที่ถูกปลดปล่อยออกมาจากเนื้อเยื่อระหว่างการเพอร์ฟิวส์โพรงจมูก และการตรวจดูความผิดปกติของเนื้อเยื่อภายใต้กล้องจุลทรรศน์ **ผลการทดลอง** ไคโตแซนทั้งสองชนิด ที่ความเข้มข้น 0.5% สามารถเพิ่มการดูดซึมทางจมูกของ [D-Arg^2]-Kyotorphin ได้ โดยพบว่าฤทธิ์เพิ่มการดูดซึมของ CS J ที่ pH 4.0 จะมากกว่าที่ pH 5.0 และ 6.0 ($p < 0.05$) ซึ่งสอดคล้องกับธรรมชาติของไคโตแซนในรูปด่างอิสระที่จะพองตัวและละลายได้ดีในสภาวะ pH ที่เป็นกรด ขณะที่ฤทธิ์เพิ่มการดูดซึมของ CS G ซึ่งเป็นรูปเกลือที่ละลายน้ำได้ดี จะไม่แตกต่างกันในช่วง pH 4.0 ถึง 6.0 ($p > 0.05$) การทดลองพบว่า pH ที่เหมาะสมสำหรับ CS J คือ pH 4.0 และสำหรับ CS G คือ pH 6.0 จึงได้นำสภาวะนี้มาใช้ในการทดลองขั้นต่อไป ที่ความเข้มข้นที่ต่ำเพียง 0.02% พบว่าไคโตแซนทั้งสองชนิด สามารถออกฤทธิ์เพิ่มการดูดซึมได้ทัดเทียมกับ 5% ไฮดรอกซีพรอพิลเบต้าไซโคลเด็กซ-ตริน (HP-β-CD) ที่ความเข้มข้น 0.1% CS J และ CS G มีฤทธิ์เพิ่มการดูดซึมที่มากขึ้น แต่ฤทธิ์ในการทำลายเนื้อเยื่อเมื่อพิจารณาจากปริมาณโปรตีนและฟอสฟอรัสที่ถูกปลดปล่อยออกมากลับไม่แตกต่างกันอย่างมีนัยสำคัญเมื่อเทียบกับ 5% HP-β-CD ($p > 0.05$) นอกจากนี้เมื่อเปรียบเทียบปริมาณการปลดปล่อยโปรตีนและฟอสฟอรัสกับค่าที่รายงานก่อนหน้านี้สำหรับไดเมธิลเบต้าไซโคลเด็กซตริน (DM-β-CD) ซึ่งเป็นสารเพิ่มการดูดซึมที่มีประสิทธิภาพสูงตัวหนึ่งที่ศึกษากันมาก พบว่าที่ความเข้มข้น 5% DM-β-CD ให้การปลดปล่อยสารเหล่านี้มากกว่าไคโตแซนถึง 4-7 เท่า เมื่อศึกษาถึงสภาพการเปลี่ยนแปลงของเนื้อเยื่อบุโพรงจมูกภายใต้กล้องจุลทรรศน์ หลังจากการให้ไคโตแซนทั้งสองชนิดที่ความเข้มข้นที่สูงถึง 1% ทางจมูกหนูขาวติดต่อกันเป็นเวลา 14 วัน เปรียบเทียบกับ 5% HP-β-CD พบว่าสารเพิ่มการดูดซึมทั้งสามชนิดมีผลระคายเคืองต่อเนื้อเยื่อบุโพรงจมูกหนูในระดับต่ำถึงปานกลาง ความผิดปกติที่พบส่วนใหญ่คือการบวมของเซลล์ก็อบเบล็ทและการหลั่งมิวคัสที่เพิ่มมากขึ้น นอกจากนี้ยังพบว่าเนื้อเยื่อบุโพรงจมูกสามารถกลับคืนสู่สภาพเดิมได้ โดยดูจากการลดลงของปริมาณเอนไซม์แลคเตทดีเฮโดรจีเนสที่ถูกปลดปล่อยออกมาระหว่างการเพอร์ฟิวส์ภายหลังจากที่ชะล้างเอาสารช่วยเพิ่มการดูดซึมออกจากโพรงจมูกหนูแล้ว **สรุป** ทั้ง CS J และ CS G สามารถเพิ่มการดูดซึมทางจมูกของยาตัวอย่าง [D-Arg^2]-Kyotorphin ได้อย่างมีประสิทธิภาพ โดยผลการศึกษาความปลอดภัยเบื้องต้นบ่งชี้ว่า ไคโตแซนเป็นสารจากธรรมชาติที่มีศักยภาพสูงยิ่ง ในการนำมาศึกษาเพิ่มเติมสำหรับการพัฒนาเป็นสารเพิ่มการดูดซึมของยาเป็ปไทด์ทางจมูก

Project Title Efficacy and Mechanistic Studies of Chitosan as Nasal Absorption Enhancer of Peptide Drugs

Name of Investigators Parkpoom Tengamnuay, Achariya Sailasuta, and Garnpimol C. Ritthidej

Year January, 1998

Abstract

Objective. To evaluate the safety and efficacy of chitosans (CS) as nasal absorption enhancers of peptides. **Methods.** Two types of chitosans, i.e., CS J (free amine chitosan) and CS G (water-soluble glutamate salt), were evaluated for their nasal absorption enhancing effects on L-Tyr-D-Arg ([D-Arg^2]-Kyotorphin), an enzymatically stable dipeptide, using an *in situ* rat nasal perfusion technique. The two chitosans were subsequently studied for their possible membrane damaging effects based on measurements of released mucosal components and histological evaluation. **Results.** At 0.5 % w/v, both CS J and CS G were effective in enhancing the nasal absorption of [D-Arg^2]-Kyotorphin. The enhancing effect of CS J was significantly greater at pH 4.0 than at pH 5.0 and 6.0 ($p < 0.05$) in accordance with the nature of the free amine chitosan to swell and dissolve better in the more acidic conditions. However, there were no significant differences in the adjuvant activity of the soluble acid salt CS G at pH 4.0, 5.0 and 6.0 ($p > 0.05$). CS J and G were subsequently selected for further studies at their optimum pH (4.0 for CS J and 6.0 for CS G). At only 0.02 % w/v, their enhancing effects were already significant and similar to that of 5 % w/v hydroxypropyl-β-cyclodextrin (HP-β-CD). Determination of the protein and phosphorus content in the nasal perfusates indicated that the two chitosans, at 0.1 % w/v, caused minimal release of these substances similar to that of HP-β-CD ($p > 0.05$). However, they were much smaller than the previously reported values for dimethyl-β-cyclodextrin, an effective enhancer which, at 5 % w/v, gave the protein and total phosphorus release rates about 4-7 folds higher than chitosans. Morphological evaluation of the rat nasal mucosa following daily administration of 1 % w/v CS J and G and 5 % w/v HP-β-CD for 14 days indicated that the three enhancers produced only mild to moderate irritation, the most common signs being mucus hypersecretion and goblet cell distention. The effects of the two chitosans on the rat nasal epithelial integrity were also reversible as judged from the reduction in the extent of lactate dehydrogenase release, a cytosolic enzyme marker, following removal of chitosans from the nasal mucosa. **Conclusions.** Both CS J and CS G were effective in enhancing nasal absorption of [D-Arg^2]-Kyotorphin. Results from the mucosal component release, morphological and reversibility studies indicated that chitosans may have a potential for further studies as a safe and effective nasal absorption enhancer.

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LIST OF ABBREVIATIONS

A-B buffer	=	Isotonic acetate-borate buffer
ANOVA	=	Analysis of variance
°C	=	Degree Celcius
cm	=	Centimeter
CS J	=	Chitosan free amine
CS G	=	Chitosan glutamate
DM-β-CD	=	Dimethyl-β-cyclodextrin
Fig	=	Figure
gm	=	Gram
HE stain	=	Hematoxylin and Eosin stain
HPLC	=	High pressure liquid chromatography
HP-β-CD	=	Hydroxypropyl-β-cyclodextrin
hr	=	Hour
i.p.	=	Intraperitoneal
IPB	=	Isotonic phosphate buffer
IU	=	International Unit
kg	=	Kilogram
k_{obs}	=	Apparent first order rate constant
LPC	=	Lysophosphatidylcholine
M	=	Molar (mole/liter)
mM	=	Millimolar (mmole/liter)
ml	=	Milliliter
μg	=	Microgram
μl	=	Microliter
mg	=	Milligram

min	=	Minute
N	=	Normality
PHR	=	Peak height ratio
rpm	=	Revolutions per minute
r^2	=	Correlation coefficient
SD	=	Standard deviation
SE	=	Standard error
sec	=	Second
STDHF	=	Sodium taurodihydrofusidate
T_{120}	=	perfusion time at 120 min
UV	=	Ultraviolet
U/ml	=	Units/ml

CHAPTER I
INTRODUCTION

As the research in the field of biotechnology utilizing genetic recombination and cell fusion technology progresses, greater interest has been focused on the use of physiologically active peptides and proteins as medicines. For example, recombinant human insulin for the treatment of diabetes mellitus is one of the most prescribed drugs derived from this technology. However, most of these products are currently administered by injection. Oral administration of peptides and proteins is often limited by their instability in the gastrointestinal environment and poor absorption from the gut (Su, 1991). Nevertheless, frequent parenteral administration subjects the patient to considerable pain and many side effects which lead to a rather poor compliance. Therefore, a strongly desirable goal is to develop an administration method for peptides and proteins that can serve as an alternative to the oral and parenteral administrations (Yamamoto et al., 1993).

The nasal pathway has been pursued as an alternative to parenteral route. The nasal mucosa has been shown to be a site from which some peptides and proteins are absorbed systematically. The nasal mucosa is composed primarily of pseudostratified columnar epithelium which has a large surface area available for absorption due to the presence of microvilli structure at the apical membrane. The tissue is highly vascularized and provides ready access to the circulatory system by avoiding first-pass metabolism at the liver (Donovan et al., 1990). Furthermore, peptides and proteins usually have short duration of action. Nasal administration thus offers a clear advantage over the injection route with regard to patient compliance.

Nasal bioavailability of peptides is generally much higher than oral bioavailability. However, it it usually less than 100 %. In many cases, it is very low because of the metabolism by the nasal mucosal peptidases, poor membrane

penetration, or both (Hussain et al.,1992). In order to improve the nasal bioavailability of such peptides, various groups have explored the possibility of enhancing the absorption across the epithelial membrane by means of so-called absorption enhancers (Illum et al.,1989). However, many effective absorption enhancers like surfactants, bile salts and fatty acids increase membrane permeability by aggressive mechanisms which often cause severe or permanent damages to the nasal mucosa. Consequently, these materials are unacceptable for chronic use in humans and novel classes of nasal absorption enhancers which are effective without potential topical or systemic toxicity need to be discovered and evaluated.

Chitosan is a cationic polysaccharide derived from naturally occurring chitin in crab and shrimp shells by deacetylation. Chitosan has been shown to have mucoadhesive properties, a mechanism believed to be important for nasal absorption enhancement. Mucoadhesion of the drug dispersed or dissolved in chitosan solution can decrease the rate of drug clearance from the nasal cavity and results in a longer contact time with the absorptive epithelium (Illum et al.,1994). However, the safety and effectiveness of various chitosans have not been extensively investigated, particularly the damaging effects of chitosans on nasal membrane components like protein and phosphorus as well as their effects on the reversibility of the nasal membrane permeability. Also, the morphological integrity of the nasal mucosa following frequent administration of chitosan has not been evaluated. These questions remain to be answered before a suitable guideline for clinical application of using chitosan as nasal absorption enhancer can be established.

Therefore, the objectives of this research were as follows:

1. to evaluate the efficacy of chitosan as a nasal absorption enhancer of a model dipeptide, [D-Arg^2]-Kyotorphin, by using the rat *in situ* nasal perfusion technique.

2. to study the effects of some physicochemical factors, e.g. chemical form (free amine versus salt form), pH, and concentration on the nasal absorption enhancing activity of chitosan in order to determine the optimal enhancing condition for each type of chitosan.
3. to study the possible mechanisms of absorption enhancement and membrane damaging effects of chitosan by measuring the rate and extent of protein and phosphorus releases from the rat nasal mucosa in comparison with a reference enhancer (hydroxypropyl-β-cyclodextrin) .
4. to study subacute local toxicity of chitosan by histological evaluation of the rat nasal epithelium after daily nasal administration of chitosan solutions for two weeks and compare the results with hydroxypropyl-β-cyclodextrin.
5. to study the possible reversibility of the nasal membrane permeability following removal of chitosan from the nasal mucosa in comparison with hydroxypropyl-β-cyclodextrin and dimethyl-β-cyclodextrin.

CHAPTER II
LITERATURE REVIEW

Recently, biologically active macromolecules have become increasingly important as a new class of therapeutic agents. Most of them are peptides and their derivatives such as insulin, calcitonin, growth hormone and its analogs, adrenocorticotropic hormone (ACTH) and its analogs, interferons, vasopressin, luteinizing hormone releasing hormone (LHRH) and its analogs etc. These peptides have clinical implications in the treatment of several chronic, hormone-deficiency diseases such as diabetes, paget's disease, dwarfism, cancer etc. (Chien and Chang, 1985). Since these peptides are mostly destroyed by the acids and enzymes in the gastrointestinal tract, they cannot be administered orally. Currently, the only effective route of administration of these drugs is by parenteral administration, e.g. intravenous, subcutaneous or intradermal injection. However, the parenteral route suffers several serious drawbacks such as patient compliance, high risk of overdose, infections and local thrombophlebitis as a result of its invasive nature of administration.

Non-oral, non-parenteral routes of administration such as nasal, sublingual, rectal, pulmonary, and transdermal routes have received greater attention as alternative means for systemic delivery of peptide drugs. Among these routes, nasal administration offers several promising advantages. Although limited in the surface area, the nasal mucosa is rich in blood supply and its capillary permeability is greater than the intestinal mucosa (Watanabe et al.,1980). Drug which is absorbed across the nasal epithelium enters the systemic circulation directly without hepatic first pass metabolism (Colaizzi, 1985). In addition, there are no pancreatic enzymes and strongly acidic conditions in the nasal cavity, rendering the environment of the nasal cavity less rigorous for peptide drugs than in the GI-tract. The volume of the nasal secretion is also much less than the GI-secretion, leading to smaller dilution of the

administered drug and better contact with the absorptive epithelium (Stratford and Lee, 1986). Nasal administration is also considered to be non-invasive as opposed to the parenteral route, thereby encouraging self-administration and increasing patient compliance. Another advantage of nasal administration of peptides is that the drugs can be given in a pattern which simulates the release cycle of the endogenous peptides, thereby preserving their biological rhythms and reducing unwanted side effects (Banga and Chien, 1988).

The mucosal membranes of the mammalian nasal cavity are the moist lining epithelium, including several types of epithelia. A small portion extending into the nasal cavity from the nares is a stratified squamous epithelium. The remainder of the nasal membrane is made up of respiratory epithelium, which is composed of goblet cells, ciliated cuboidal and columnar cells, and the olfactory epithelium, which is a pseudostratified neuroepithelium. Another connective tissue layer called the submucosa usually connects the mucosa to the underlying structures. On the surface of the mucosal epithelium, there exists a layer of mucus composed of muco-polysaccharides secreted from the goblet cells of the mucosa (Hsieh, 1994).

The nasal vasculature consists of a rich capillary network found in the subepithelium and around the nasal glands and cavernous plexuses. It is characterized by fenestrated endothelium. Thus, the nasal mucosa is obviously well suited for heat exchange and for potential drug absorption. In general, drugs absorbed via the nasal mucosa enter the right side of the heart for direct distribution to the systemic arterial circulation prior to traversing the liver (Su, 1991).

Barriers to nasal delivery of protein/peptide drugs

Since protein and peptide drugs are relatively large hydrophilic molecules, the mammalian mucosa possesses several extremely efficient barriers to restrict their entry to the systemic circulation. Although nasal administration of peptide drugs generally

results in much greater absorption than the oral administration, the absolute bioavailability as compared to the intravenous injection is still low. For example, nasal administration of insulin (Tengamnuay and Mitra, 1990b) and calcitonin (O'hagan and Illum, 1990) resulted in only 1% absorption. It thus appears that several absorption barriers may be present in the nasal mucosa which are responsible for their lower-than-expected nasal bioavailabilities. These barriers include the presence of many mucosal proteolytic enzymes which are responsible for biochemical degradation of peptides before and during absorption as well as the epithelium itself which acts as an efficient barrier against membrane penetration.

The proteolytic enzyme barrier is perhaps the most important barrier against the absorption of peptide and protein drugs since these molecules are highly susceptible to hydrolysis by many enzymes present in the nasal cavity. The enzymes that may be present in the mucosal tissues include exopeptidases (aminopeptidase and carboxypeptidase), endopeptidases, angiotensin-converting enzymes, dipeptidases, aminotripeptidases, prolidases, prolinases and carnosinases (Zhou,1994). Furthermore, these enzymes are widely distributed in the mammalian body, especially between the entry point into the systemic circulation and the target site, thereby making them a very efficient system to decrease the systemic bioavailability of many peptide drugs.

Other types of barriers include various clearance systems at the administration sites and the structural barriers of the epithelium which lines the mucosal surface. In order to penetrate the epithelium, a drug molecule may have to diffuse either through the epithelial cell membrane (transcellular transport), or alternatively, through the tight junctions between cells (paracellular pathway). Thus, diffusion of a drug molecule across an epithelial barrier is dependent on several factors such as pKa, molecular weight, structural conformation and hydrophilic characteristics of the drug as well as pH of the environment (Zhou, 1994).

In general, only the relatively small compounds with adequate lipophilicity can partition into the membrane lipids of the epithelial cells to be absorbed by the transcellular route. The absorption of large, hydrophilic drugs like peptides, on the other hand, appears to be limited to the paracellular pathway (McMartin et al.,1987). However, the paracellular pathway occupies only a small portion of the total surface area of the nasal epithelium. This, together with the molecular weight-restricted permeation through the tight junctions, further limits the nasal absorption of most peptide drugs (Anderberg et al.,1993).

General approaches to bypassing enzymatic and epithelial barriers

In order to bypass the enzymatic and epithelial barriers for the purpose of increasing the nasal bioavailability of high molecular weight protein and peptide drugs, several approaches are available: (i) inhibition of their enzymatic degradation; (ii) improving their resistance to breakdown or their permeability across the membrane by structural modification; (iii) by special pharmaceutical formulations which prolong their retention time with mucus at the administration site; and (iv) increasing their permeability across the relevant membrane by using chemical absorption enhancers (Zhou, 1994).

Inhibition of proteolytic enzymes

For several years it has been known that protease inhibitors increase the absorption of protein drugs. Compounds such as boroleucine and phosphinic acid dipeptide have been suggested as having good potential in enhancing the bioavailability of peptide and protein drugs due to their appropriate molecular sizes and potent inhibitory effects on various proteolytic enzymes (Hussain et al.,1989). However, the safety of these inhibitors after nasal administration is doubtful and must be further tested (Hussain et al.,1992). Bestatin and puromycin are another class of inhibitors

that have been studied. Nevertheless, their effects on peptidases were much less effective than boroleucine (Hussain et al.,1989).

Chemical modification

Hydrogen bonding potential or lipophilicity of peptides also can be altered by chemical modification. This often leads to conformational changes of the peptides and thereby may increase their permeability and/or stability during transport across the cell membrane. For example, when four methyl groups were added to the peptide, acetamido-D-Phe-D-Phe-D-Phe-carboxamide by methylation, it was found that the penetration rate of this peptide through Caco-2 cell membrane was significantly enhanced (Conradi et al.,1992). Tengamnuay and Mitra (1990a) also reported that by substituting L-Arg with D-Arg in the L-Tyr-L-Arg structure resulted in a dipeptide analogue which was highly resistant to hydrolysis by the nasal mucosal enzymes.

Formulation approach

The formulation approach has been employed to develop an effective nasal delivery system for peptide and protein drugs for many years. The most popular dosage form is a powder or microspheres system since the nasal solutions and sprays tend to provide lower peptide drug availability. In a recent study by Bjork and Edman (1988), insulin (0.75 and 1.7 IU/kg) which was dispersed in degradable starch microspheres (DSM) and administered nasally as a drug powder resulted in a dose-dependent decrease in blood glucose in rats. The bioavailability of the nasal insulin was found to be 30 %, whereas the administration of DSM alone or soluble insulin alone produced no effect. The effectiveness of the nasally delivered peptide in this dosage form was due to the uptake of water by DSM and subsequent swelling which produced dehydration of the epithelial cells, leading to a widening of the tight junctions and thereby facilitating paracellular transport of large hydrophilic molecules (Bjork et al., 1990). When DSM was combined with an enhancer, lysophosphatidylcholine (LPC), the extent of insulin nasal absorption was improved even further (Farraj et al.,

1992). This improved formulation also has been used for enhanced nasal absorption of human growth hormone (Illum et al.,1990).

Use of nasal absorption enhancers

Many researchers in nasal drug delivery have included certain adjuvants in the nasal peptide formulations in an attempt to increase their systemic bioavailability. The compounds that have been studied as nasal absorption enhancers are, for example, anionic and cationic surfactants (Hirai et al., 1981a), bile salts (Gordon et al., 1985; Pontiroli, 1985), bile salt-fatty acid mixed micelles (Tengamnuay and Mitra, 1990a and 1990b), fusidic acid derivatives (Longenecker et al., 1987; Baldwin et al., 1990), medium chain fatty acids (Mishima et al., 1987) and cyclodextrins (Merkus et al., 1991).

The mechanisms of action of these absorption enhancers are not clearly known, but several possibilities have been postulated. The first is the increased solubility of the drugs brought about by the surfactant-type enhancers. Because proteins and peptides usually form aggregates in aqueous solutions, their dissociation into more readily soluble monomers by these enhancers may facilitate their paracellular transport (Brange et al., 1992). Secondly, surfactant-type enhancers like hydrophobic bile salts may also facilitate transcellular transport of insulin probably by reverse micelle formation (Gordon et al., 1985). A third mechanism of the enhancer is to inhibit the activity of proteolytic enzymes. Some enhancers such as bile salts, fusidic acid derivatives and cyclodextrins have been shown to inhibit mucosal proteolytic activity. However, these compounds are not specific inhibitors or substrate analogues of the proteinases or peptidases (Zhou et al., 1991a). It is postulated that binding of the peptide drug with the enhancer may prevent the formation of the enzyme-substrate (enzyme-protein drug) complexes which are intermediates of the degradation process. Fourthly, the positively charged enhancers may react with the negatively charged membrane surface, and thereby reduce the peptide drug-mucus interactions (Chandler et al., 1991a) resulting in an increase of the drug bioavailability. The fifth possibility is

that the enhancers can lower the barrier function of the mucosal membrane and associated mucus layer. For example, bile salts, including sodium taurodihydrofusidate (STDHF), have been shown to reduce the viscosity of the mucus layer adhering to all mucosal surfaces and increase the pore size within the cell membrane, thereby allowing diffusion of insulin through the cells (Longenecker et al.,1987). The barrier function of the mucosal membrane could also be lowered by a more severe mechanism, e.g., the removal of certain membrane components like membrane proteins and phospholipids. Several enhancers, especially the surfactant-type compounds, have strong solubilizing capacity which may not only dissolve or dissociate the peptide aggregates but also may effectively cause leaching of these membrane components, leading to a substantial increase in membrane permeability (Tengamnuay, 1989).

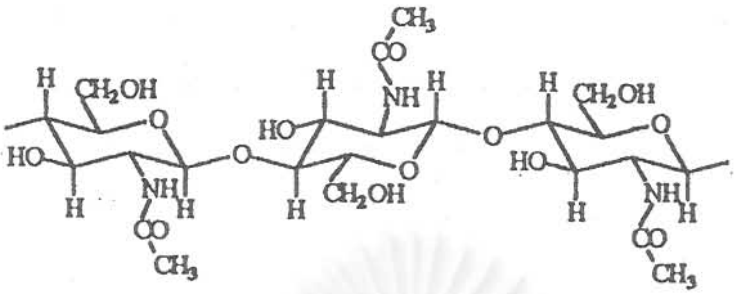
Although these enhancers are able to significantly increase nasal absorption of peptides, many studies have later revealed that their use can cause damages to the membrane. This has caused great concerns and prevented them from potential application in clinical setting, particularly during chronic administration. For example, many enhancers can cause cellular changes in the mucosa which include loss of nasal membrane components (Shao et al., 1992a; Shao and Mitra, 1992b), ciliotoxicity (Hermens et al.,1990), and severe alterations in the morphology of the nasal mucosa such as necrosis and even complete loss of epithelium (Tengamnuay and Mitra, 1990b; Donovan et al.,1990; Ennis et al.,1990). The membrane damaging properties of these enhancers, especially the surfactant type, pose problems in their prospective clinical applications, especially in long term therapy of many hormone-deficient diseases. As a result, more efforts have been pushed toward finding novel absorption enhancers which can give better safety and efficacy.

Recently, cyclodextrins, one class of cyclic polysaccharides, have been suggested as potential enhancers for non-parenteral peptide absorption. Merkus et al. (1991) reported that 109 % of nasal bioavailability (2.0 IU/ kg) could be obtained

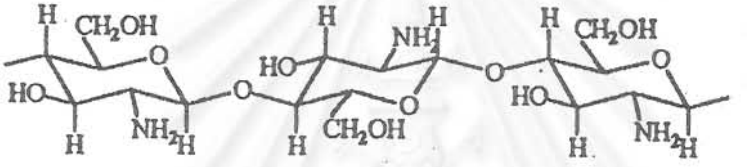
when insulin was co-administered with 5 % dimethyl-β-cyclodextrin (DM-β-CD), a highly soluble cyclodextrin derivative. A recent report by Irie et al. (1992) also provided similarly promising results for this compound. However, it was found later that DM-β-CD caused the release of many membrane and cellular components such as phospholipids, enzymes and proteins (Shao et al., 1992a). These researchers also found that hydroxypropyl-β-cyclodextrin (HP-β-CD), another derivative, was the least membrane damaging. However, its absorption enhancing effect was also minimal (Verhoef et al., 1994). DM-β-CD also shows, to some extent depending on concentration (1-5%), damages to the ciliary system of chicken embryo trachea, although its ciliostatic potency is much less than that found for STDHF (0.5%), LPC (0.5%) and bile salts such as deoxycholate (0.2%), glycocholate (1.5%) and taurocholate (1.3%) (Merkus et al., 1993). Therefore, the long term clinical use of DM-β-CD as a potent absorption enhancer in nasal peptide formulations appears to be questionable and more studies are needed to verify its safety profiles.

Chitosan as potential nasal absorption enhancer of peptides

Chitosan is a polymer obtained from deacetylation of chitin, a naturally-occurring structural polymer abundant in crab and shrimp shells. Chitosan, or partially N-deacetylated chitin, is a cationic polysaccharide with linear chain consisting of two monosaccharides, i.e. N-acetyl-D-glucosamine and D-glucosamine, joining together by β-(1,4)-glycosidic linkage. The greater is the extent of deacetylation, the smaller is the proportion of N-acetyl-D-glucosamine in the polymer chain. Figure 1 shows the chemical structures of chitin and chitosan. Since chitin and chitosan are obtained from crab and shrimp shells which are the waste products of Thailand's marine food industry and can be manufactured and purified in large scale, any research attempt to increase the applicability of chitosan is always highly attractive.



Chitin



Chitosan

Figure 1 Chemical structures of chitin and chitosan

There are several pharmaceutical applications of chitosan. For example, it has been used as a pharmaceutical excipient to increase water solubility of several oral drug formulations (Imai et al., 1991). It has also been used as polymer matrix in the sustained release drug preparations (Miyazaki et al., 1988). Being a substance of natural origin with biocompatibility, chitosan has found many applications in other areas such as food and cosmetic industries. Recently, Artursson et al. (1994) have studied the effects of chitosan on the transport of water-soluble molecules across the cultured monolayer of intestinal epithelial cancer cells (Caco-2 cells) grown in vitro. They found that chitosan significantly increased the permeability of these cells. They also postulated that chitosan may react with the protein of the cellular tight junctions, leading to the opening of the tight junction and subsequent passage of hydrophilic

macromolecules through the paracellular pathway. In the same year, Illum et al.(1994) studied the nasal administration of insulin in sheep and rats with and without chitosan. They found that inclusion of chitosan at concentrations from 0.1 to 1.0 % w/v in the nasal insulin solutions could significantly enhance the nasal absorption of this peptide over the control group (nasal insulin without chitosan). These preliminary results indicated the potential application of chitosan as nasal absorption enhancer of poorly absorbed drugs like peptides. However, very few information is available with respect to its safety and efficacy. It is interesting to know if chitosan could enhance nasal absorption of other peptides apart from insulin. Moreover, its safety profiles with respect to the membrane damaging effects need to be established as well as its possible mechanisms of absorption enhancement. The primary purpose of this research project is to further characterize chitosan in terms of its safety and efficacy, including some important physicochemical factors which may affect its adjuvant activities.

CHAPTER III
EXPERIMENTAL METHODOLOGY

This annual report covers the first four parts of the experiments (parts 5.1, 5.2, 5.3 and 5.4 as described in the full proposal). The first part involved the investigation of the effects of chitosan type, pH and concentration on their nasal absorption enhancing activity using the *in situ* rat nasal perfusion model and the results were subsequently compared with that of a reference enhancer, hydroxypropyl-β-cyclodextrin (HP-β-CD). The second part explored the possible effects of chitosans on the nasal membrane integrity by measuring the rate and extent of protein and phosphorus releases from the rat nasal mucosa following nasal perfusion of the chitosan solutions. The third part was the histological evaluation of the effects of chitosans on the morphological integrity of the rat nasal mucosa following two-week daily nasal administration of chitosan solutions to intact rats. The fourth part involved a study to determine if chitosans had a reversible effect on the nasal membrane integrity following removal of the enhancer from the nasal cavity. The results of parts II, III and IV were also compared with that of HP-β-CD. All the experiments of these parts were carried out and completed within the timeframe (months 4 - 11) as specified in the proposal's schedule.

Materials

Chitosan J (CS J) = Chitosan free amine, Kyowa Technos Co., Ltd., Japan.

Chitosan G (CS G) = Chitosan glutamate salt (Seacure G210+), Lot no. 206-409-03, Pronova Biopolymer, Drammen, Norway.

[D-Arg^2]-Kyotorphin = L-Tyr-D-Arg (acetate salt), Lot no. 25H0567, Sigma Chemicals Co., St. Louis, MO, USA.

L-Phenylalanine, Lot no. 104H2605, Sigma Chemicals Co., St. Louis, MO, USA.

Hydroxypropyl-β-cyclodextrin (HP-β-CD), Sigma Chemicals Co., St. Louis, MO, USA.

Dimethyl-β-cyclodextrin (DM-β-CD), Sigma Chemicals Co., St. Louis, MO, USA.

Acetonitrile HPLC grade, Farmitalia Carlo Erba, Milan, Italy.

Albumin, Bovine Fraction V (96-99 % Albumin), Sigma Chemicals Co., St. Louis, MO, USA.

Fiske & Subbarow Reducer, Sigma Diagnostics Co., St. Louis, MO, USA.

Folin-Ciocalteus Phenol reagent, E. Merck, Darmstadt, Germany.

Lactic acid 90 %, E. Merck, Darmstadt, Germany.

Trichloroacetic acid GR grade, E. Merck, Darmstadt, Germany.

Cupric sulphate anhydrous AR grade, BDH Laboratories Supplies, Poole, England.

Phosphoric acid 85 % GR grade, E. Merck, Darmstadt, Germany.

Formic acid (Ammonium salt), Sigma Chemicals Co., St. Louis, MO, USA.

Lactate dehydrogenase enzymatic assay kit, Sigma Diagnostics, St. Louis, MO, USA.

All other reagents were of analytical grade and used as received.

Equipment

UV Spectrophotometer, Model 7800, Jasco Corporation, Tokyo, Japan.

pH Meter, Model 420A, Orion Research Corporation, Boston, MA, USA.

Analytical Balance, Sartorius 1615 MP, Gottingen, Germany.

Thermostatted Circulating Water Bath, Heto InterMed, Heto Birkerod, Denmark.

Vortex Mixer, Model K-550-GE, Scientific Industries Inc., New York, USA.

Ultrasonic Bath, Model 3210, Branson Ultrasonic Corporation, Danbury, CT, USA.

Peristaltic Pump, Minipuls 2 Gilson, Villier Le Bel, France.

Osmometer, Osmomat 030-D, Gonotec, Berlin, Germany.

Cone and Plate Viscometer, Model RV TDCP, Serial A 03969, Brookfield Engineering Laboratories Inc., USA.

High Performance Liquid Chromatograph (HPLC), Waters and Associates, Millipore, MA, USA, consisting of:

- Tunable UV detector, Model M 484
- Autoinjector, Model WISP 712
- Constant Flow Pump, Model 510
- Integrator, Model 745 B Data Module

HPLC column (stationary phase) = μBondapak C18, 10 μm, stainless steel column, 300 x 3.9 mm, Waters, MA, USA.

Part I. Studies to Evaluate Nasal Absorption Enhancing Activity of Chitosan: Effects of Chitosan Type, pH and Concentration (Experiments 5.1 in the Proposal)

These preliminary studies were divided into two parts:

1. Effects of Chitosan Type and pH on Its Nasal Absorption Enhancing Activity (Experiment 5.1.1 in the proposal)
2. Effects of Chitosan Concentration on Its Nasal Absorption Enhancing Activity (Experiment 5.1.2 in the proposal)

Investigational Technique: *In Situ* Rat Nasal Perfusion

The *in situ* nasal perfusion in rat model was used as a primary method of investigation due to its simple experimental setup and ease of sample handling. It was first developed by Hirai et al. (1981a) and successfully used by many other researchers to screen for drugs with potential nasal absorption. With slight

modification, Tengamnuay and Mitra (1990a) have found this technique to be very useful in evaluating the effect of nasal absorption enhancers such as bile salt and its mixed micelles with various fatty acids. The following is the detailed description of the perfusion procedure.

Male Sprague-Dawley rats weighing 250-300g were obtained from National Laboratory Animal Centre, Mahidol University, Nakorn Pathom. After anesthetization by intraperitoneal injection of 45 mg/kg sodium pentobarbital, an incision was made at the neck of the animal to expose the trachea. A 4 cm-polyethylene tube was inserted about 1.5 cm deep into the trachea toward the lungs to maintain respiration during the experiment. The esophagus was also cannulated with another similar polyethylene tube which was inserted toward the posterior part of the nasal cavity. This cannula served to introduce the perfusion solution into the nasal cavity. The nasopalatine was sealed with an adhesive agent (Elephant Glue) to prevent drainage of the drug solution from the nasal cavity into the mouth. A funnel was then placed between the nose and reservoir. Respective diagrams of the surgery and setup of the in situ perfusion experiment are illustrated in Figures 2 and 3.

Drug solution was placed in a reservoir beaker, which was water jacketed at 37 ± 0.5 ^{o}C via a circulating water bath. The solution was recirculated through the rat nasal cavity by means of a peristaltic pump. The rate of perfusion and the perfusate volume were set at 2.0 ml/min and 5.0 ml, respectively in all experiments. These are the values which gave optimal results during previous perfusion studies (Huang et al.,1985; Tengamnuay and Mitra, 1990a). The perfusate was constantly pumped from the reservoir through a larger polyethylene tubing the other end of which was connected to the esophageal cannula, flushed through the nasal cavity, and then returned to the reservoir via the nostrils. The concentration of the drug

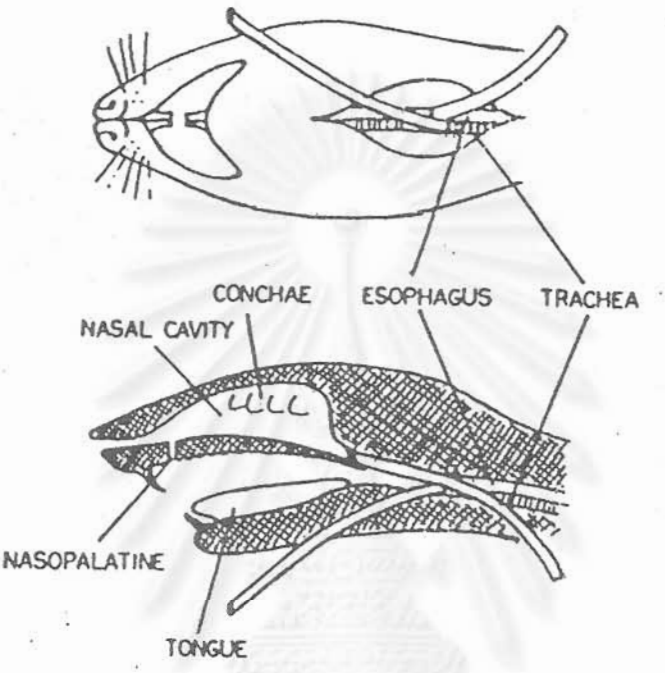


Figure 2 Diagram of the surgical procedure used in the in situ and in vivo nasal absorption studies

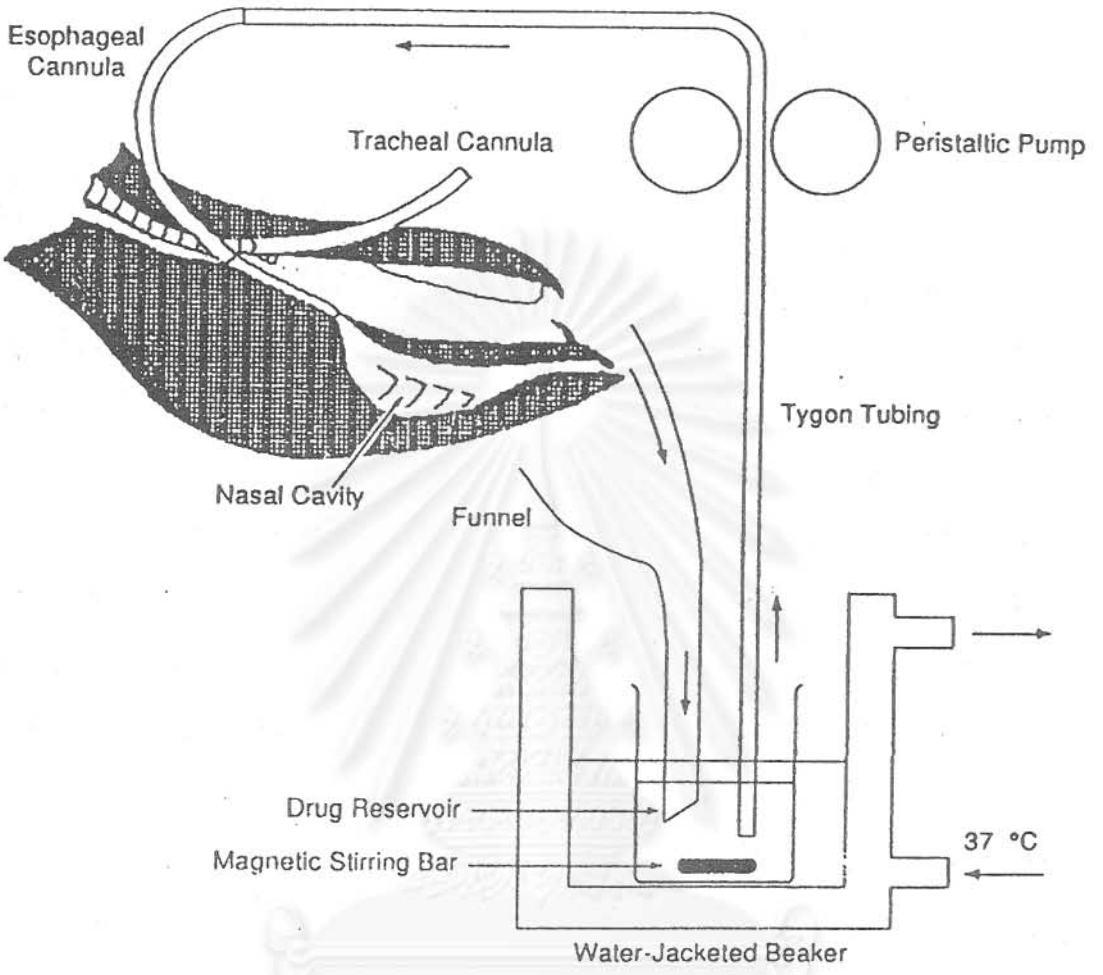


Figure 3 Schematic diagram of the in situ perfusion of the rat nasal cavity

remaining in the perfusate was analyzed as a function of time to determine the extent of nasal absorption.

Preparation of Perfusion Solutions

L-Tyr-D-Arg ([D-Arg^2]-Kyotorphin) was selected as a model peptide in this study. It is an analog of L-Tyr-L-Arg or Kyotorphin, a neural dipeptide which possesses opioid activity by stimulating the release of endogenous enkephalin. The reasons for choosing this dipeptide are due to its good stability in the nasal mucosa. It is not hydrolyzed by the nasal mucosal enzymes and at the same time is poorly absorbed from the perfusate (Tengamnuay and Mitra, 1990a). It thus serves as a suitable model dipeptide to study the effect of nasal absorption enhancers since the loss of the compound from the perfusate would indicate the occurrence of nasal absorption.

1 mM of [D-Arg^2]-Kyotorphin was prepared by separately dissolving the dipeptide in isotonic phosphate buffers (IPB), pH 3.0, 4.0, 5.0, and 6.0. The two chitosans (CS J and CS G) were prepared at 1 % w/v concentration by dissolving each one in 1 % v/v acetic acid solution and allowing them to swell overnight. The pH was subsequently adjusted to the same as the dipeptide solution (pH 3.0, 4.0, 5.0, or 6.0) by dropwise addition of either 1 N hydrochloric acid or 1 N sodium hydroxide solution. The tonicity was then adjusted by stepwise addition of sodium chloride to obtain an isotonic solution which was checked by osmometer (290-310 mosmole/Kg). The sample solution containing 0.5 % w/v chitosan and 0.5 mM [D-Arg^2]-Kyotorphin was finally prepared by mixing the same pH solutions of 1 mM [D-Arg^2]-Kyotorphin and 1 % w/v chitosan in an equal portion. The concentration of [D-Arg^2]-Kyotorphin (0.5 mM) was fixed throughout the entire experiments whereas the concentration of CS J and G was also prepared at 0.02 % and 0.1 % w/v. Similar method of preparation was applied by mixing chitosan

solutions (0.04 % or 0.2 % w/v) with 1 mM [D-Arg^2]-Kyotorphin solution at a 1:1 ratio. All the solutions were freshly prepared prior to each perfusion experiment. Preparations of isotonic phosphate buffers (IPB) are given in Appendix II.

Analytical Method

Drug concentration in the perfusate was quantitated by a reversed-phase HPLC using a solvent delivery pump equipped with a variable wavelength UV detector set at 274 nm. Acetonitrile 1 % v/v in 0.01 M sodium acetate pH 4.0 was used as a mobile phase with a flow rate of 1.5 ml/min. Values of the peak areas or peak heights were obtained with an integrator. Chart speed was set at 0.1 cm/min. An aliquot (25 µl each) of the perfusate was removed from the reservoir at 0, 30, 60, 90, and 120 min, immediately mixed with an equal volume of the internal standard solution (5 mM of L-Phenylalanine in the same buffer as the sample solution), and then injected to the HPLC. Standard curve was prepared by dissolving [D-Arg^2]-Kyotorphin in the same buffer at the concentrations of 0.1, 0.2, 0.3, 0.4, and 0.5 mM. Each of the standard curves also contained respective type of chitosan except for the control groups (peptide alone without enhancer). Standard solutions were also mixed with the internal standard in a similar manner prior to HPLC analysis. Each calibration curve was constructed by plotting the peak height ratios (or peak area ratios) of [D-Arg^2]-Kyotorphin to L-Phenylalanine versus the concentration of [D-Arg^2]-Kyotorphin in each buffer and the straight line was obtained by linear regression.

The rate and extent of nasal absorption was determined from the semilogarithmic plots of percent [D-Arg^2]-Kyotorphin remaining in the perfusate versus time. The apparent first order absorption rate constants after nasal perfusion were calculated from the slopes of these plots whereas the percent [D-Arg^2]-Kyotorphin remaining at 120 min was used as a parameter indicating the extent of nasal absorption. The effect of pH of the perfusion solutions was first

investigated by varying the pH from 3.0 to 6.0 for each type of chitosan (concentration fixed at 0.5 % w/v). After determining the pH of optimal enhancing activity for each chitosan, the effect of chitosan concentration was subsequently studied at 0.02 and 0.1 % w/v. The results were then compared to that of 5 % w/v hydroxypropyl-β-cyclodextrin (HP-β-CD), another novel enhancer reported to cause only mild irritation to the nasal mucosa (Shao et al.,1992a).

Statistical Analysis

Statistical evaluation of the data was made by Student's t-test, analysis of variance (ANOVA) and by multiple comparison of the means using Duncan's test at 5 % significance level where appropriate. The computation was performed using a statistical software package (SAS Inc.).

Part II. Studies of the Effects of Chitosans on the Release of Protein and Phosphorus from the Rat Nasal Mucosa (Experiments 5.2 in the Proposal)

Investigational Technique

Two chitosans with optimum conditions selected from part I and 5% w/v HP-β-CD were used in this part to investigate their effects on the nasal membrane integrity as characterized by the release of protein and phosphorus which are major components of the mucosal cells. *In situ* nasal perfusion was also used as an investigational technique. Details of the surgical procedures and perfusion setup have been described in part I. The rate of the perfusion and the perfusate volume were always set at 2.0 ml/min and 5.0 ml. The perfusion was carried out in each rat for at least 120 min with aliquots of the perfusate (200 μl each) taken at 0, 30, 60, 90 and 120 min. After each sampling, the same volume of the fresh sample solution was replaced at the same experimental temperature (37 °C). Each of 200 μl perfusate was divided into 2 parts of 100 μl each for separate protein and total

phosphorus analysis. The perfusate after 120 min perfusion was also quantitated for phospholipid content.

Preparation of Sample Solutions

Three perfusing solutions were used in this part, i.e. two selected chitosan solutions and one 5 % w/v HP-β-CD solution in buffer pH 7.4. The pH and concentration of each chitosan solution were the optimum values which have been determined from part I. Tonicity was adjusted with stepwise addition of sodium chloride. Phosphate buffers (IPB) were not allowed to use in this part because phosphorus from the buffers would interfere with that released from the nasal membrane. So the lactate and the borate-acetate buffers were used instead (See preparation of buffers in appendix II). The reasons for selecting these buffers are described in the next chapter. All of the sample solutions contained no [D-Arg^2]-Kyotorphin, with only an enhancer in each sample solution. The buffer solution, at its respective pH for each enhancer, was also perfused as a control group. Care was taken during the entire experiments to prevent phosphorus contamination from water and containers. Each group was performed using 3 rats.

Analytical Method

Protein Determination: The protein contents in the perfusate at various sampling points were measured by the modified method of Hartree (1972) based on the method described by Lowry et al.(1951). Bovine serum albumin (BSA) was used as the standard. Standard curve was prepared at the concentrations of 5-200 μg/ml by dissolving BSA in the same buffer as the enhancer solution. Aliquots of perfusate samples (50 μl each) were diluted with distilled water to 1.0 ml and mixed with 0.9 ml solution A. Standard BSA solutions (1.0 ml each) were similarly treated with solution A. Distilled water (1.0 ml) was used as a blank. After

mixing, all the tubes (samples, standards and blank) were placed in a water bath at 50 °C for 10 min, cooled to room temperature (21-25 °C), and then treated with 0.1 ml solution B. The solutions were left at room temperature for at least 10 min, then 3 ml of solution C was forced in rapidly to ensure mixing within 1 sec. The tubes were again heated at 50 °C for 10 min and cooled to room temperature. Absorbances were measured in a 1 cm cuvette at 650 nm with UV/visible spectrophotometer against the blank. The presence of HP-β-CD and chitosans did not interfere with the assay procedure for protein content. Preparations of solutions A, B and C are described in Appendix II.

Total Phosphorus Determination: The assay procedure was based on the method described by Bartlett (1959) and modified by Feldman et al. (1973). The perfusate samples (100 µl each) were diluted with distilled water to 1.0 ml and placed in 18 ml conical centrifuge tubes, and 0.5 ml of 10 N sulfuric acid was added to each tube. The tubes were placed in an oven at 160 °C for a 3 hr digestion. After the initial 3-hr heating period, 3 drops of 30 % hydrogen peroxide were added to each tube and the tubes were returned to the oven for another 3 hr at 160 °C. After the samples were allowed to cool to room temperature, 4.4 ml of distilled water, 0.2 ml of 5 % ammonium molybdate, and 0.2 ml of Fiske-Subbarow reagent were added and the contents were vortexed for 20 sec. The samples were then heated in boiling water bath for an additional 10 min. The developed color was subsequently read at 830 nm. A standard curve in the same buffer was prepared using potassium phosphate monobasic as the standard and linearity was obtained in the range of 0 to 4 µg of phosphorus content. The presence of HP-β-CD and chitosans did not interfere with the assay procedure. Preparations of reagents used in this assay are described in Appendix II.

Phospholipid Phosphorus Determination: Phospholipid contents of the perfusate samples were measured according to Zilversmit and Davis (1950).

Proteins and phospholipids in the perfusate sample (1.0 ml) were first precipitated with 3 ml of 10 % trichloroacetic acid (TCA), which was added in a dropwise manner. The sample was then centrifuged at 2,000 rpm for 5 min. The supernatant was decanted and the pellets were washed with 3 ml of 10 % TCA and centrifuged at 2,000 rpm for 5 min. Subsequently, 1.0 ml of distilled water and 0.5 ml of 10 N sulfuric acid were added to the pellets in each tube and the entire phosphorus assay procedure described previously was repeated.

Part III. Effects of Chitosans on the Morphological Integrity of the Rat Nasal Mucosa (Experiment 5.3 in the Proposal)

Histological studies were performed to evaluate morphological integrity of the rat nasal mucosa upon exposure to a relatively high concentration of chitosans under a repeated nasal administration for two weeks (subacute toxicity). The results obtained would partly address the feasibility of employing chitosans as a nasal absorption enhancer in the formulation of nasal drug delivery systems. The procedure described in this part was modified from that of Chandler et al. (1991a).

Male Sprague-Dawley rats weighing 250-350 gm were anesthetized by intraperitoneal injection of a 45 mg/kg sodium pentobarbital. The sedated rat was laid with the dorsal site down and the test solution was delivered to the right nostril only, using a Hamilton microsyringe with a blunt needle. The needle tip was inserted about 0.5 cm deep into the nostril. The dose volume was 30 µl each. Rats were dosed nasally everyday for two weeks with the same procedure. Pentobarbital was used at a lowest possible dose just to facilitate the nasal administration. The rats were allowed to recover and resume their normal conscious state after each daily administration. ,There were 7 groups of 3 rats each in this study: two groups separately received a daily dose (30 µl) of 1 % w/v solutions of the two previously selected chitosans; one group received 5 % w/v

HP-β-CD; three groups received only the buffers; and the last group was an undosed control group. Rats were recorded for their weight and sleeping time everyday in order to adjust the dose of sodium pentobarbital for optimum sedation in each rat.

After dosing for 2 weeks, rats were anesthesized, surgically treated and the *in situ* nasal perfusion was set up as previously described. Nasal tissues were fixed by perfusing the nasal cavity of each rat with a mixture of Bouin's fixative and IPB pH 7.4 (1:1) for approximately 15 min. After that, the rats were sacrificed by an overdose of sodium pentobarbital and decapitated. The mandible, brain and excess soft tissues were subsequently removed. The specimens were placed in fresh fixative baths overnight. After fixing overnight, the specimens were washed in several changes of 50 % alcohol for 4-6 hr, agitating constantly, to ensure proper removal of picric acid from the tissues. Specimens were then decalcified in formic acid-sodium citrate solution for 4-5 days by which time it was possible to divide each specimen into 5 regions using a razor blade (Figure 3/1). The preparation of reagents used in this experiment are described in Appendix II. All regions were processed through paraffin wax blocks using routine histological method. Each region was orientated in the wax so that complete transverse cross-sections of the nasal cavity were produced, with the anterior face presented for cutting first. Sections were cut serially at 7-8 μm thickness with a microtome, mounted and stained with hematoxylin and eosin (HE stain). Acidic mucopolysaccharides were demonstrated with alcian blue staining (Preece, 1972). Cross-sections of the nasal cavity were examined under light microscope. The appearance and distribution of the normal rat nasal epithelium were identified in sections from the completely undosed control rats. Comparisons were also made between the dosed and the undosed sides of the same sections of the nasal cavity and also between the

undosed side of these sections and corresponding sections from undosed control rats.

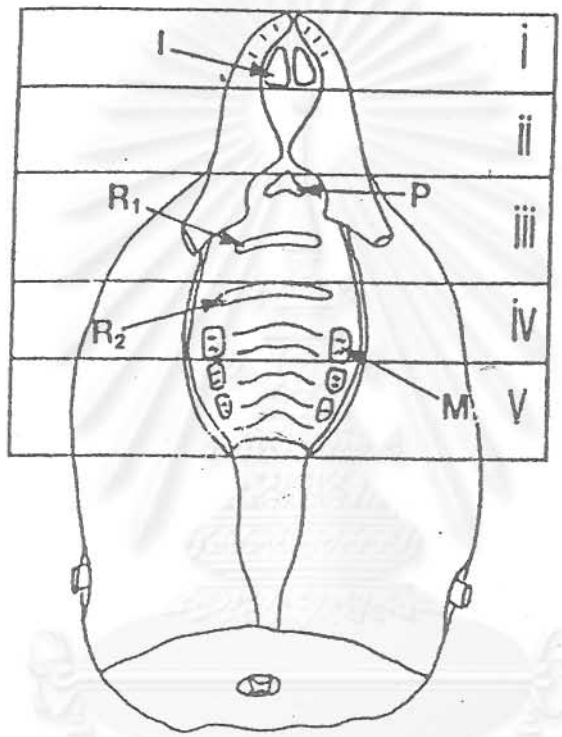


Figure 3/1 Diagram illustrating the surface features of the roof of the oral cavity in the rat. Those landmarks used to define cutting positions for the division of the nasal cavity into regions (i)-(v) are indicated: upper incisor root (I); incisive papilla (P); first palatal ridge (R_1); second palatal ridge (R_2); first upper molar (M).

Part IV. Study to Determine the Reversibility of the Nasal Membrane Integrity following Removal of Chitosans from the Rat Nasal Mucosa (Experiment 5.4 in the Proposal)

The purpose of this part was to determine whether chitosans could have a transient effect on the rat nasal mucosa. The same *in situ* nasal perfusion technique was employed as in Parts I and II. The perfusing solutions contained only the enhancer which was dissolved in isotonic saline (0.85 % NaCl solution with pH adjusted to 6.0 with 1 N HCl). The enhancers employed in this part were the same as those used in previous experiments, i.e., CS J, CS G, and HP-β-CD. In addition, dimethyl-β-cyclodextrin (DM-β-CD) was also studied for comparative purposes (See explanation in the next chapter). The concentration of each enhancer in the perfusing solution was set at 0.5 % for CS J and CS G, 5.0 % for HP-β-CD, and 1.25 % for DM-β-CD. Reasons for selecting these concentrations are given in Chapter IV.

5.0 ml of each enhancer solution was recirculated at 37 ^{o}C through the rat nasal cavity for 60 min at a flow rate of 2.0 ml/min. Aliquots (80 μl each) were taken at 15, 30, 45 and 60 min for subsequent analyses. At the end of perfusion, the nasal cavity was flushed for 10 min with isotonic saline at the same flow rate to remove the enhancer. After that, the nasal mucosa was reperfused for further 60 min with 5.0 ml of fresh isotonic saline (no enhancer) and aliquots were similarly taken at 15 min intervals.

Aliquots of the perfusates were analyzed for the content of lactate dehydrogenase (LDH), an intracellular enzyme which may have been released from the nasal epithelium due to changes in the membrane integrity caused by the enhancer. Therefore, any leakage of this enzyme into the nasal perfusates could be used as a specific biochemical indicator of the extent of membrane-irritating effect (Pujara et al., 1995). Furthermore, if an enhancer had only a transient effect, its

removal from the nasal mucosa would be able to restore, in whole or in part, the membrane integrity to its original state. Comparison of the extent of LDH release during the first hour of perfusion (in the presence of an enhancer) with that of the second hour (perfusion after removal of the enhancer) would therefore provide some ideas as to the reversible effects of that particular enhancer.

Determination of lactate dehydrogenase activity in the nasal perfusates

Analyses were made immediately after completion of each perfusion experiment. The procedure was based on the spectrophotometric method of Wroblewski and LaDue (1955). The activity of LDH was measured by monitoring the rate at which the substrate, pyruvate, was reduced to lactate. The reduction was coupled with the oxidation of nicotinamide adenine dinucleotide, reduced form (NADH), which was followed spectrophotometrically in terms of reduced absorbance at 340 nm.

Pyruvate + NADH <----------> Lactate + NAD
(High A_{340}) (Low A_{340})

Since NADH has a high absorbance at 340 nm compared to NAD, the reaction was measured in terms of the rate of decrease in absorbance at this wavelength.

Procedure

The LDH assay kit (Sigma Chemicals Co.) contained 20 preweighed vials of dried NADH (0.2 mg/vial), one bottle (100 ml) of 0.1 M potassium phosphate buffer, pH 7.5, and one bottle (100 ml) of 22.7 mM sodium pyruvate solution in 0.1 M phosphate buffer, pH 7.5.

1. Maintain cuvet compartment at 37 ^{o}C
2. Pipet directly into an NADH vial 2.85 ml phosphate buffer and 0.05 ml (50 µl) perfusate sample. Cap and mix well.
3. Leave the vial in a temperature-controlled water bath at 37 ^{o}C for 20 min
4. Add 0.1 ml sodium pyruvate solution. Cap and mix well by inversion and transfer to cuvet of 1-cm lightpath.
5. Read and record absorbance (A) at 340 nm at 30-second intervals for 3 minutes vs water as reference.

Calculations

Select a period when the decrease in absorbance is linear with time. Calculate the ΔA per minute for this period.

$$\text{LDH activity in nasal perfusate (Units/ml)} = \frac{\Delta A \text{ per min} \times TCF}{0.001 \times 0.05 \times \text{Lightpath (cm)}}$$

Where 0.001 = ΔA equivalent to 1 unit of LDH activity in a 3-ml volume with 1-cm lightpath at 37 °C

0.05 = Perfusate volume (ml) in cuvet

TCF = Temperature correction factor (0.51 at 37 °C)

Thus, if a 1-cm lightpath is used, the above equation reduces to :

LDH activity = ΔA per min x 20,000 x TCF

Calibration

Values obtained by the procedure were reported in LDH units as described by Wroblewski and LaDue (1955), based on the absorptivity of NADH at 340 nm. The rate of decrease of the absolute absorbance was measured at this wavelength in a narrow-bandwidth spectrophotometer. The activity of LDH in the nasal perfusate was then expressed as units/ml/min, where 1 unit equals an absorbance decrease of 0.001 under the specified test conditions.

The maximum LDH activity that may be measured by this procedure is approximately 700 units/ml. Higher activity samples may be assayed by pre-dilution with phosphate buffer and multiply the results by the appropriate dilution factor.

The reaction can be set at any constant temperature between 20 - 39 °C. If the reaction is run at 25 °C, no correction factor is needed (TCF = 1). Otherwise, the result must be multiplied by an appropriate TCF value at a particular temperature.

Unit definitions

One unit of LDH activity will cause a decrease in A_{340} of 0.001 per minute at 25 °C in a 3-ml reaction mixture in a cuvet of 1-cm lightpath. One international Unit (IU) of an enzyme is defined as that amount which will convert 1 μmol of substrate per minute under the specified conditions of the procedure. The conventional units of LDH, as used in the described method, may be converted to IU by multiplying by 0.48. For example, 100 LD units per ml equals 48 IU per liter.

CHAPTER IV
RESULTS AND DISCUSSION

Part I. Studies to Evaluate Nasal Absorption Enhancing Activity of Chitosans Using the *In Situ* Nasal Perfusion Technique

High Pressure Liquid Chromatographic Analysis

Figure 4 A is a representative chromatogram of isotonic phosphate buffer (IPB) pH 4.0 alone (no enhancers and [D-Arg^2]-Kyotorphin) whereas Figure 4 B is a chromatogram of [D-Arg^2]-Kyotorphin and L-Phenylalanine, the internal standard, in the same buffer. The retention time of [D-Arg^2]-Kyotorphin and L-Phenylalanine are about 9.0 and 6.5 min, respectively. The two compounds were well separated from each other with completely resolved baseline. In addition, their peaks did not overlap with any of the solvent peaks and the addition of chitosan (CS J and CS G) or HP-β-CD did not interfere with their chromatograms. The chromatograms of the two compounds in IPB at other pH values (3.0, 5.0, 6.0 and 7.4) gave similar results (Figures not shown).

Standard Curve

Standard [D-Arg^2]-Kyotorphin solutions were prepared at the final concentrations of 0.1, 0.2, 0.3, 0.4, and 0.5 mM in IPB at various pH. After adding the internal standard, each solution was injected to the HPLC. Figure 5 shows the representative chromatograms of standard [D-Arg^2]-Kyotorphin and L-Phenylalanine solutions dissolved in IPB pH 4.0. The chromatograms of standard [D-Arg^2]-Kyotorphin at other pH values were also similar (Figures not shown).

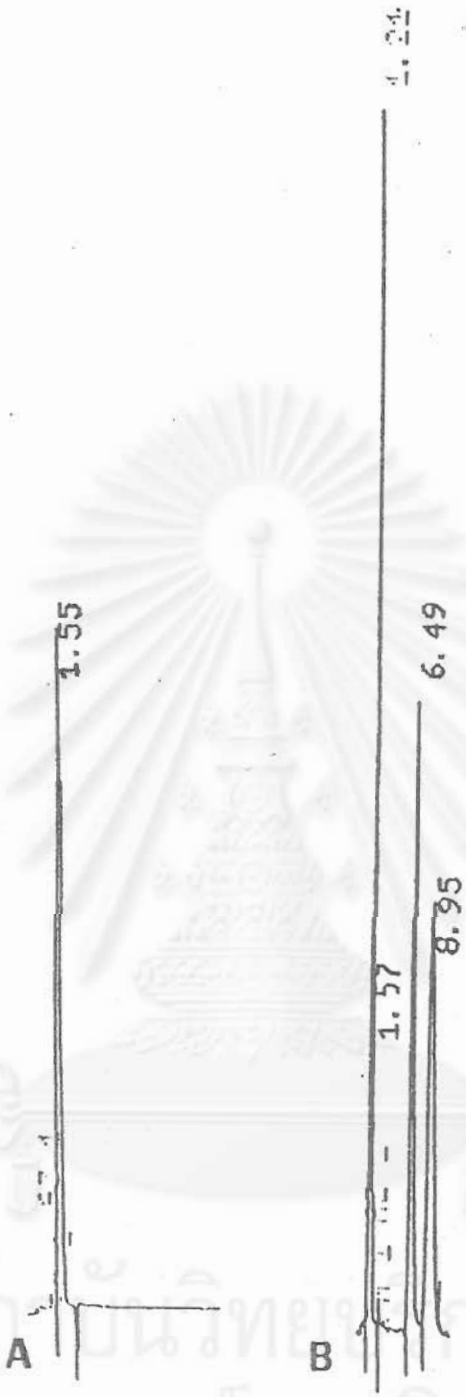


Figure 4 High pressure liquid chromatogram of (A): buffer (IPB) pH 4.0 alone and (B): [D-Arg^2]-Kyotorphin (8.95 min) and L-Phenylalanine (6.49 min) in the same buffer (IPB) pH 4.0

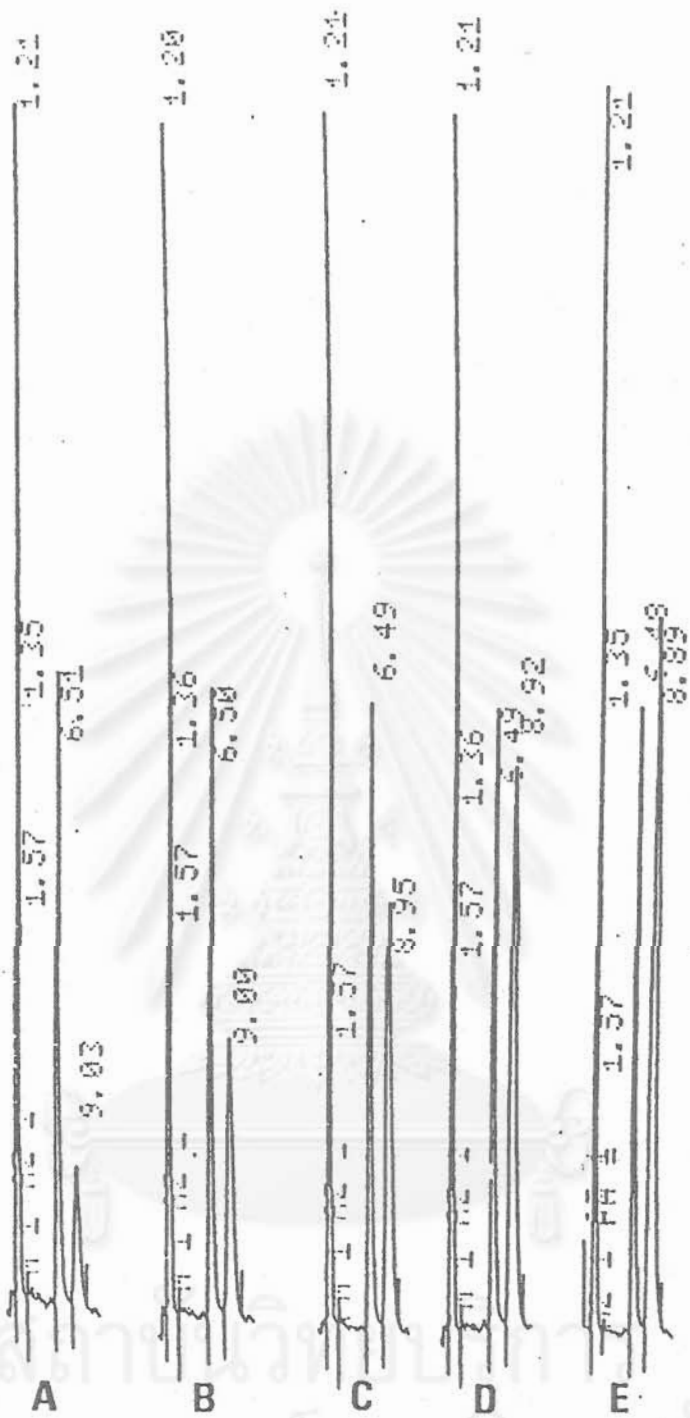


Figure 5 High pressure liquid chromatogram of the calibration curve of [D-Arg^2]-Kyotorphin (9 min) at the concentration of 0.1 (A), 0.2 (B), 0.3 (C), 0.4 (D) and 0.5 mM (E) with 5 mM L-Phenylalanine (6.5 min) as an internal standard, in IPB pH 4.0 ·

Testing of Tubing and Cannula for Possible Adsorption of [D-Arg^2]-Kyotorphin

To confirm that loss of [D-Arg^2]-Kyotorphin from the perfusion solution was not due to adsorption of the dipeptide onto the tubing and esophageal cannula during the nasal perfusion, 0.5 mM solution of [D-Arg^2]-Kyotorphin in IPB pH 4.0 was recirculated through the perfusion system without the rat. Analyses of [D-Arg^2]-Kyotorphin in the perfusion solution at 0 and 120 min revealed that there was no change in the chromatograms of the dipeptide during this period, indicating its good physicochemical stability in the solution without any apparent adsorption onto the tubing and cannula during perfusion.

Testing of Interferences from the Nasal Mucosa during Perfusion

These studies were conducted to ensure that there were no interferences with the analyses of [D-Arg^2]-Kyotorphin as a result of any endogenous substances (e.g. mucus protein) which may have been released from the nasal mucosa into the perfusate during perfusion. The results are shown in Figures 6, 7, and 8. Figure 6 demonstrates the chromatograms of the nasal perfusate containing only 0.5 % w/v CS J in IPB pH 4.0 at 0 and 120 min (no [D-Arg^2]-Kyotorphin and L-Phenylalanine). Figure 7 is the chromatograms of the perfusate at 0 and 120 min which contained only [D-Arg^2]-Kyotorphin and L-Phenylalanine in IPB pH 4.0 (no chitosan). Figure 8 shows the chromatogram of the perfusate containing [D-Arg^2]-Kyotorphin in the presence of 0.5 % w/v CS J in IPB pH 4.0 at 0 and 120 min. It can be seen from these figures that, even at 120 min perfusion, the peaks of [D-Arg^2]-Kyotorphin and the internal standard were not interfered by the endogenous substances of the nasal mucosa. All the endogenous peaks eluted early during the first five minutes. Similarly, perfusion with CS G and HP-β-CD

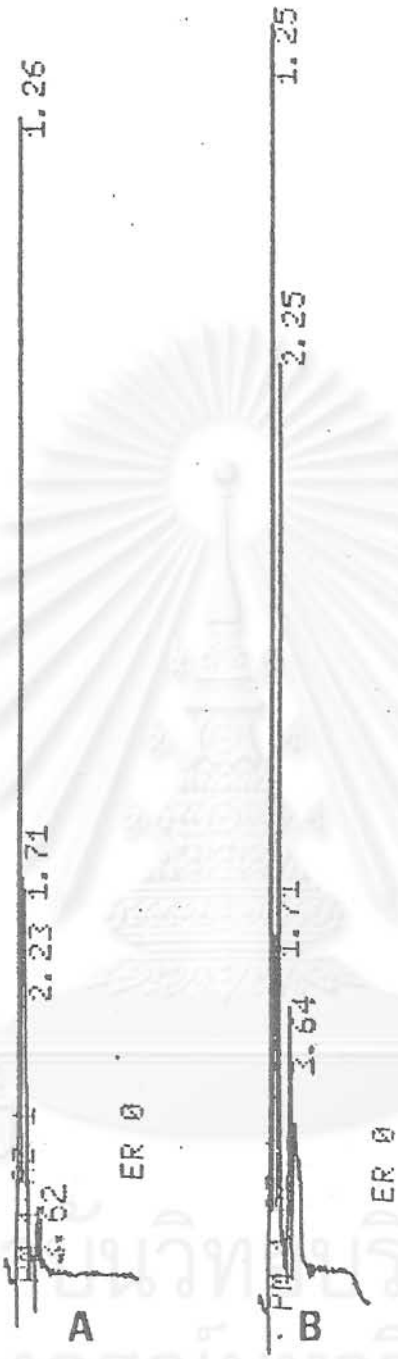


Figure 6 High pressure liquid chromatogram of CS J in buffer (IPB) pH 4.0 at 0 (A) and 120 min (B) of the perfusion (no peptide and internal standard)

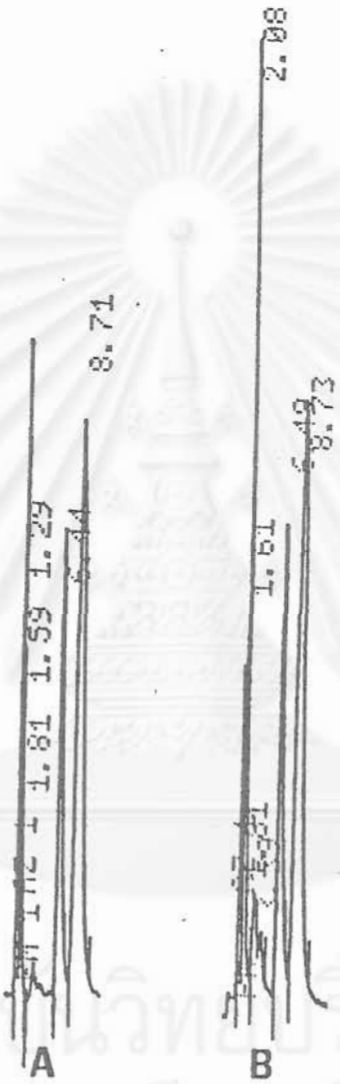


Figure 7 High pressure liquid chromatogram of [D-Arg^2]-Kyotorphin (8.7 min) and L-Phenylalanine (6.4 min) in buffer (IPB) pH 4.0 without CS J at 0 (A) and 120 min (B) of the perfusion

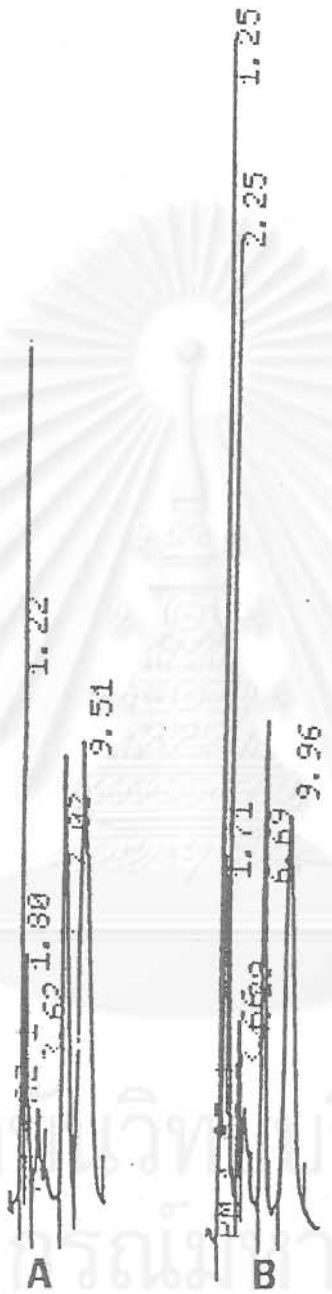


Figure 8 High pressure liquid chromatogram of [D-Arg^2]-Kyotorphin (about 9.5-9.9 min) and L-Phenylalanine (about 7 min) with 0.5%w/v CS J in buffer (IPB) pH 4.0 at 0 (A) and 120 min (B) of the perfusion

did not interfere with the chromatograms of the dipeptide and the internal standard regardless of the pH of the medium (chromatograms not shown).

Results in Figure 7 also confirmed our previous report (Tengamnuay and Mitra, 1990a) that [D-Arg^2]-Kyotorphin was enzymatically stable in the nasal perfusate without hydrolysis into amino acids L-Tyr and D-Arg. If enzymatic hydrolysis were to occur, a sharp peak of L-Tyr should have been observed at about 3 min. (D-Arg, on the other hand, did not absorb UV light at 274 nm and therefore did not give peak under this HPLC condition). Consequently, loss of the dipeptide from the perfusate should be caused by its absorption across the rat nasal mucosa and not by local metabolism.

1.1 *In Situ* Nasal Perfusion of [D-Arg^2]-Kyotorphin with Chitosan: Effect of Chitosan Type and pH

Four pH values (3.0, 4.0, 5.0, and 6.0) were selected in order to determine the pH of optimal enhancing activity for each type of chitosan. These values were chosen as a result of the ability of CS J and CS G to dissolve in this pH range. Generally, chitosan can be dissolved in an acidic solution which has pH not higher than 6.5 (Stanford et al, 1991). We found that the experiment could not be set at the physiological pH (pH 7.4) due to precipitation of both CS J and G when the pH of their solutions was raised above 6.5. However, HP-β-CD was able to dissolve in physiological pH. Therefore, perfusion of [D-Arg^2]-Kyotorphin with HP-β-CD was carried out using IPB pH 7.4 as a perfusion medium.

The first set of experiments involved nasal perfusion of [D-Arg^2]-Kyotorphin alone without any enhancer (control groups). Table 1 shows the average percent [D-Arg^2]-Kyotorphin remaining in the perfusates at various times (mean $\pm$ SD) after nasal perfusion of the dipeptide alone in IPB with different pH values. From this table, it can be seen that there was hardly any absorption of [D-

Arg^2]-Kyotorphin from the perfusate, particularly in the pH range of 4.0 to 7.4. The concentration of [D-Arg^2]-Kyotorphin remained relatively unchanged over the 120-min perfusion period, indicating a poor intrinsic nasal permeability of this dipeptide. However, perfusion at pH 3.0 resulted in some absorption, as evidenced by loss of the dipeptide from the perfusate. The average percent of [D-Arg^2]-Kyotorphin remaining at 120 min was 80.1 $\pm$ 4.2 % which was equivalent to about 20 % absorption. The high acidity of the buffer at this pH could be too irritating and may have caused some injuries to the nasal mucosa, leading to its increased permeability. On the other hand, perfusion of IPB at other pH conditions showed very little absorption (only about 1-7 % absorbed at 120 min).

Perfusion experiments were subsequently carried out in the presence of the two chitosans. First, CS J was studied at 0.5 % w/v in IPB with pH varying from 3.0 to 6.0. Figure 9 illustrates the representative calibration curve of [D-Arg^2]-Kyotorphin in IPB pH 4.0 with 0.5 % w/v CS J as an enhancer. The curve was constructed by plotting the peak height ratio (PHR) as a function of standard [D-Arg^2]-Kyotorphin concentration. The plot was linear with the regression coefficient (r^2) of 0.9992. All other standard curves gave similarly good linearity with the r^2 values greater than 0.99 regardless of the pH and type of the enhancer present in the standard solutions.

Table 2 shows percent of [D-Arg^2]-Kyotorphin remaining in the perfusates at various times during the 120-min perfusion in the presence of 0.5 % w/v CS J (values = mean $\pm$ SD). The data in this table indicated that CS J was absorbed better in the more acidic conditions (pH 3.0 and 4.0) than at pH 5.0 and 6.0. The percent [D-Arg^2]-Kyotorphin remaining at 120 min was only 66.9 $\pm$ 3.9 % at pH 4.0 and 70.1 $\pm$ 9.0 % at pH 3.0. This is equivalent to about 30 - 33 % absorption. At pH 5.0 and 6.0, the percent remaining at 120 min was 76.4 $\pm$ 2.2 % and 77.3 $\pm$ 3.4 %, respectively, equivalent to about 23 % absorption.

Table 1 % [D-Arg^2]-Kyotorphin remaining in the perfusate without enhancer (Control groups)

Perfusion medium	% [D-Arg^2]-Kyotorphin remaining in the perfusate					Apparent first order rate constant, k (min^{-1})	n
	0 min	30 min	60 min	90 min	120 min		
IPB pH 3.0	100	94.5 ± 0.9	89.8 ± 1.7	85.8 ± 3.3	80.1 ± 4.2	0.00181 ± 0.00045	4
IPB pH 4.0	100	98.9 ± 4.0	101.7 ± 5.1	100.0 ± 3.2	99.0 ± 2.7	- *	4
IPB pH 5.0	100	100.4 ± 1.7	100.7 ± 1.7	96.3 ± 3.6	93.0 ± 2.5	- *	4
IPB pH 6.0	100	99.8 ± 3.5	100.7 ± 1.2	100.1 ± 1.6	98.8 ± 3.6	- *	4
IPB pH 7.4	100	100.6 ± 1.1	101.2 ± 1.7	99.7 ± 0.8	98.5 ± 2.0	- *	4

The data show mean ± SD

IPB = Isotonic phosphate buffer

* unable to determine due to very poor absorption

Figure 9 Representative calibration curve of [D-Arg^2]-Kyotorphin with 0.5%w/v CS J in IPB pH 4.0

$y = -0.013 + 1.907x$ $r^2 = 0.9992$

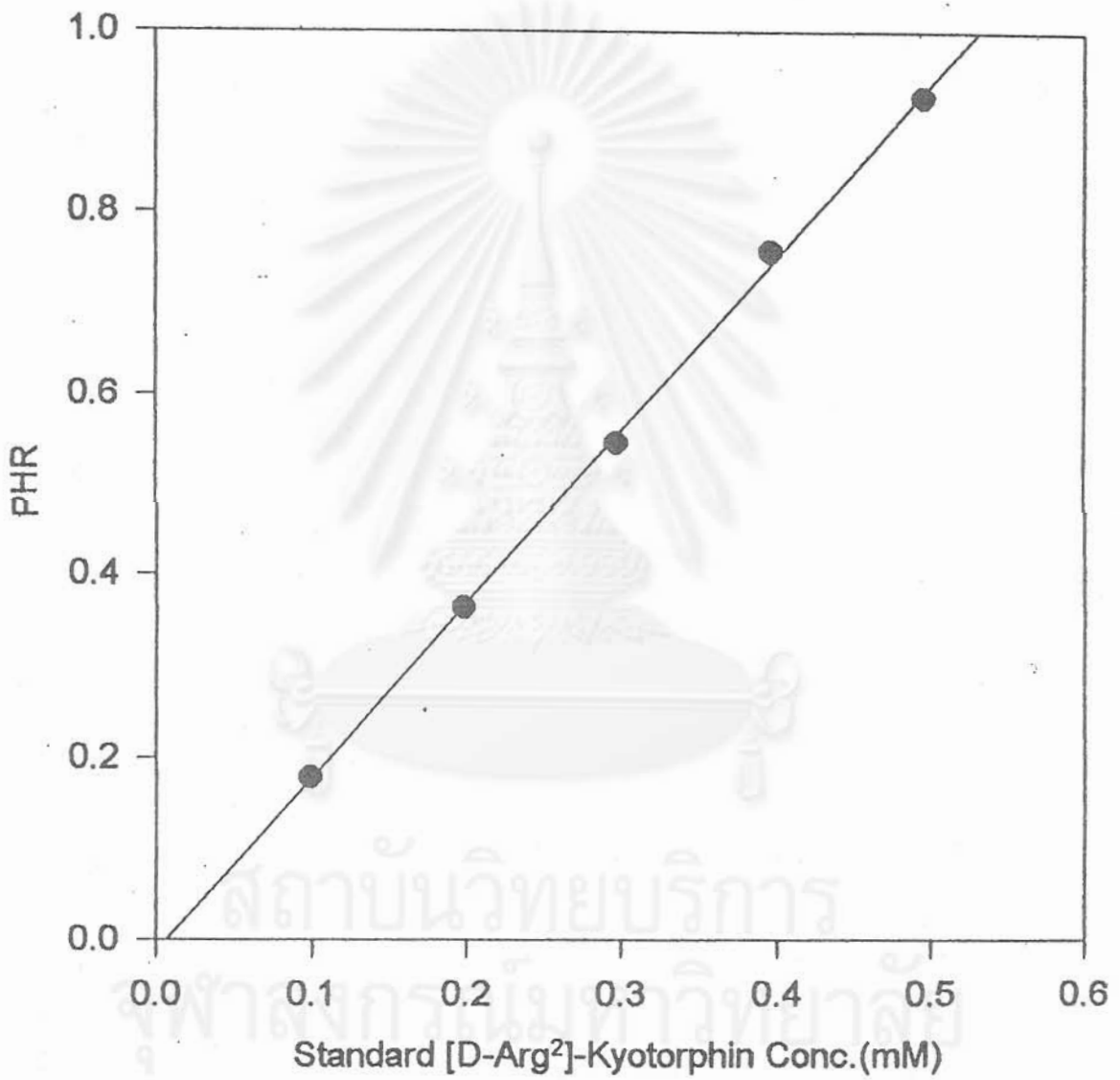


Table 2 % [D-Arg^2]-Kyotorphin remaining in the perfusate containing 0.5% w/v CS J at various pH

Chitosan CS J	% [D-Arg^2]-Kyotorphin remaining in the perfusate					Apparent first order rate constant, k (min^{-1})	n
	0 min	30 min	60 min	90 min	120 min		
pH 3.0	100	93.1 ± 5.0	82.3 ± 5.2	75.2 ± 7.6	70.1 ± 9.0	0.00315 ± 0.00109	5
pH 4.0	100	89.1 ± 7.2	82.4 ± 5.6	76.7 ± 7.0	66.9 ± 3.9	0.00294 ± 0.00061	4
pH 5.0	100	94.7 ± 3.4	89.6 ± 3.5	81.8 ± 2.6	76.4 ± 2.2	0.00229 ± 0.00016	4
pH 6.0	100	91.5 ± 3.4	88.0 ± 3.4	82.4 ± 3.8	77.3 ± 3.4	0.00207 ± 0.00032	4

The data show mean ± SD

Figure 10 shows semilogarithmic plots of percent [D-Arg^2]-Kyotorphin remaining as a function of time in the presence of 0.5 % w/v CS J at various pH. Derivation of the equation describing these plots was first characterized by Huang et al (1985) and was based on the first order transport kinetics, i.e. passive diffusion of drug across the absorptive membrane.

If loss of the drug from the perfusate appears to follow first order kinetics, the Fick's first law of diffusion may be applied:

$$dm/dt = - DA.(dc/dx) \quad \text{----------(1)}$$

Where m = amount of solute present at time t

dm/dt = rate of change of the amount of solute in the perfusate

D = diffusion coefficient of the drug, cm^2/time

A = surface area of the nasal mucosa (absorptive membrane), cm^2

dC/dx = concentration gradient across the nasal membrane barrier

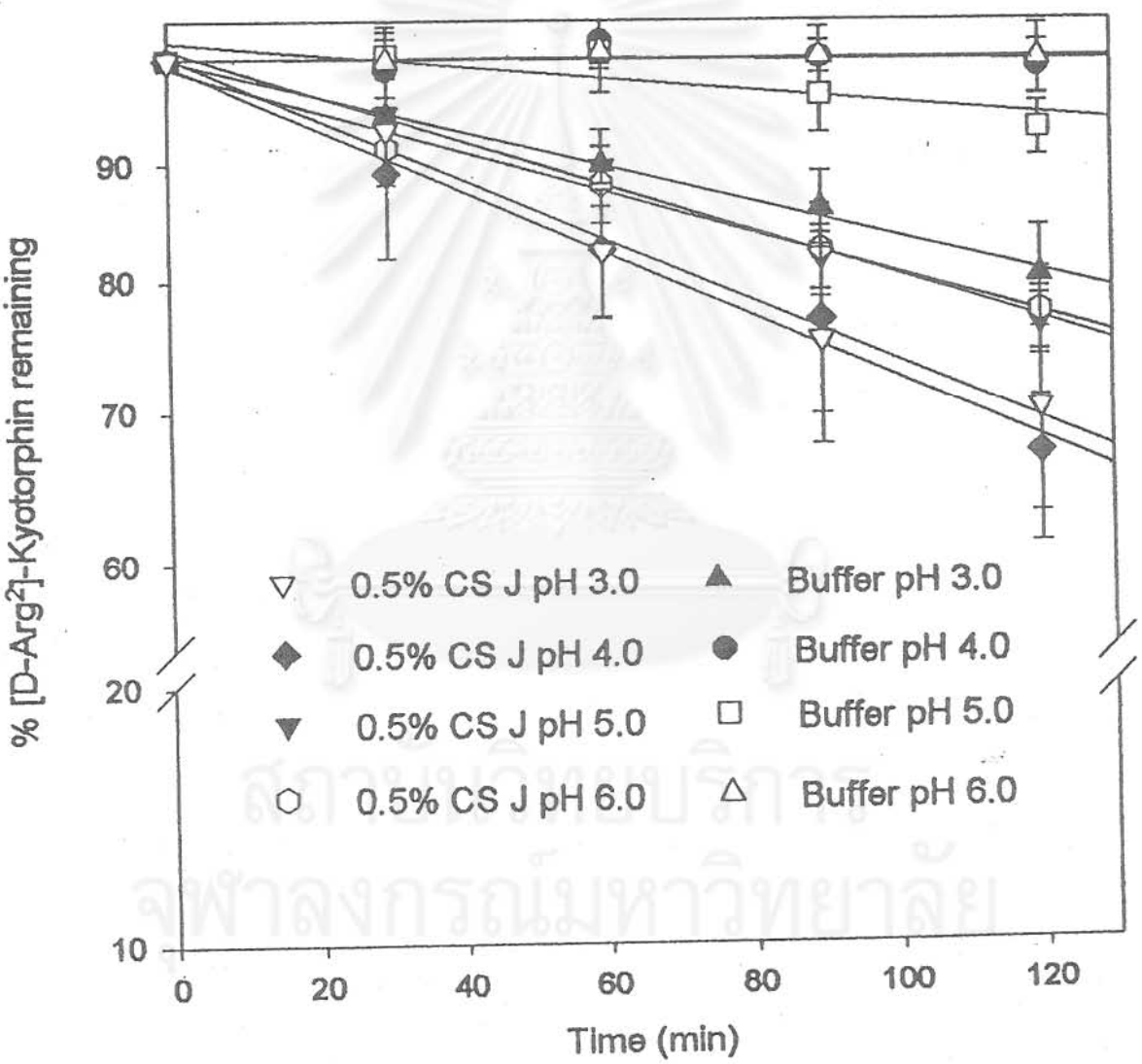
The conventional units for the amount and time in equation (1) are gm and sec, respectively. However, it was more convenient to express the time in term of a larger unit such as minute since the length of time involved in each experiment was 120 min. In addition, since all the solutions were prepared on a millimolar basis, the respective amount and concentration of the drug were thus expressed as mmole and mmole/l (mM).

$$\text{Since } dm/dt = V.(dC/dt) \quad \text{----------(2)}$$

Where dC/dt = rate of change of drug concentration in the perfusate, mM/min

Figure 10 Semilogarithmic plots of percent [D-Arg^2]-Kyotorphin remaining in the perfusate versus time after nasal perfusion with 0.5 % w/v CS J at various pH. The control groups were perfused with only [D-Arg^2]-Kyotorphin in the same buffers. The perfusion media were isotonic phosphate buffers (IPB) pH 3.0, 4.0, 5.0, and 6.0.

Value = mean ± SD (n = 4-5 rat/group)



and V = volume of the perfusate, l

It is therefore possible to rearrange equation (1) in term of dC/dt using the following approximation :

$$dC/dx = DC/Dx = (Cd - Cr)/h \quad \text{----------(3)}$$

Where h = thickness of the nasal mucosa and associated unstirred aqueous layer, cm.

Cd = concentration of drug in the donor side, mM

= concentration of drug in the perfusate

Cr = concentration of drug in the receptor side, mM

= concentration of drug in blood

By substituting equations (2) and (3) in equation (1), equation (4) is obtained

$$dC/dt = - DA(Cd - Cr)/hV \quad \text{----------(4)}$$

If sink conditions are assumed (Cd >> Cr), equation (5) is obtained

$$dC/dt = - DA.Cd/hV$$

$$= - k_{obs}.Cd \quad \text{----------(5)}$$

Where $k_{obs} = DA/hV$

k_{obs} is called the apparent in situ first order absorption rate constant and has a unit of min^{-1}. It is a constant which combined several parameters associated with the experimental conditions such as D, A, h, and V. These parameters can be considered unchanged during perfusion of the individual rat, i.e. the type of drug and buffer as well as the perfusate volume have been preselected and fixed in each perfusion experiment.

Equation (5) is in the form of first order differential equation which characterizes the drug transport after passive diffusion. When it is integrated between the limits of Co and C, equation (6) is achieved

$$\ln C = \ln Co - k_{obs}.t \quad \text{----------(6)}$$

or $$\log C = \log Co - k_{obs}.t/2.303 \quad \text{----------(7)}$$

By making a plot of logC as a function of time (or semilogarithmic plot), a straight line would be obtained with a slope and an intercept of $- k_{obs}/2.303$ and logCo, respectively. Table 2 also shows the mean and SD of k_{obs} which was calculated from the slope of the individual plot. The perfusion data of the individual rat as well as its k_{obs} value are provided in Appendix I. It can be seen from these data and Figure 10 that the perfusion profiles fairly followed the first order kinetics. All the semilogarithmic plots showed good linearity with the regression coefficient in the range of 0.9 - 0.99 in most cases. The value of k_{obs} reflects the rate of nasal drug absorption. This parameter, together with the percent [D-Arg^2]-Kyotorphin remaining at 120 min, were used for statistical comparison in order to determine the pH of optimal enhancing activity for each chitosan.

Although the data in Table 2 reveals that CS J in buffer pH 3.0 gave the highest value of absorption rate constant ($3.15 \pm 1.09 \times 10^{-3}$ min^{-1}), perfusion of [D-Arg^2]-Kyotorphin in this buffer alone also showed some absorption, with the calculated k_{obs} being $1.81 \pm 0.45 \times 10^{-3}$ min^{-1} (Table 1). It thus appears that the increased rate of [D-Arg^2]-Kyotorphin absorption by CS J at pH 3.0 was not caused solely by the enhancer, but also a result of some contribution from the high acidity of this buffer. On the other hand, perfusion data at pH 4.0 and above revealed that the absorption enhancement was primarily due to the effect of CS J since perfusion of the buffers alone (IPB pH 4.0, 5.0, and 6.0) had minimal effect on the absorption of [D-Arg^2]-Kyotorphin (Table 1 and Figure 10). It is possible that the IPB pH 3.0 could be too acidic for the nasal mucosa, which normally has pH of its secretion in the range of 5.5-6.5, and therefore may not be appropriate for use as the perfusion medium. As a result, the perfusion data at pH 3.0 was excluded from the statistical comparison in order to avoid the confounding effect from this buffer.

Statistical comparison using Student's t-test at 5 % significance level revealed that the extent of [D-Arg^2]-Kyotorphin absorption, as indicated by the percent drug remaining in the perfusate at 120 min (% T_{120}), was significantly greater in the CS J-treated group than in its corresponding control buffer group regardless of the pH condition ($p < 0.05$). One-way analysis of variance (ANOVA) was also applied to the values of k_{obs} and % T_{120} at the same significance level so as to compare the effect of varying pH on the enhancing activity of CS J. Significant difference was found in the values of % T_{120} when 0.5 % w/v CS J was added to the peptide solution at pH 4.0, 5.0, and 6.0 ($p < 0.05$). Duncan's New Multiple Range test was further applied in order to rank this difference. The ranking result, in an increasing order, was

pH 4.0 (66.9 %) < <u>pH 5.0 (76.4 %) < pH 6.0 (77.3 %)</u>.

The line underneath pH 5.0 and 6.0 implies that there was no significant difference in the average values of % T_{120} between the two pH ($p > 0.05$). However, the value at pH 4.0 was significantly smaller than at pH 5.0 and 6.0 ($p < 0.05$), indicating that the extent of absorption was greater in the more acidic condition.

ANOVA was also applied to the values of k_{obs}. Although there was no significant difference in this parameter among pH 4.0, 5.0, and 6.0 ($p > 0.05$). The average value of the absorption rate constant at pH 4.0 (2.94×10^{-3} min^{-1}) was still greater than at pH 5.0 (2.29×10^{-3} min^{-1}) and pH 6.0 (2.07×10^{-3} min^{-1}). The general tendency is that the free amine CS J showed increasing adjuvant activity when the pH of the solution was decreased. Thus, CS J appeared to be most effective at pH 4.0 and its enhancing activity tended to decrease at higher pH with increasing % T_{120} and decreasing k_{obs} values.

These results are in agreement with those of Artursson et al (1994) who explained that, at lower pH, chitosan is more protonated and becomes uncoiled, a configuration which facilitates intimate contact with the membrane, leading to greater enhancing activity. Chitosan has an apparent pKa of about 5.6. At higher pH, the chitosan molecule exists in a more coiled configuration. But as the pH decreases, the molecule becomes more ionized and assumes a more elongated shape with greater extent of hydration. Illum et al (1994) proposed that the cationic nature of chitosan could have a transient effect on the gating function of the tight junctions. Because of their positive charge, cationic macromolecules such as protamine, polylysine and chitosan can interact with the anionic components (sialic acid) of the glycoproteins on the surface of the epithelial cells (Artursson et al, 1994). These researchers have proposed that chitosan might be able to displace

cations from electronegative sites (such as tight junctions) on a membrane which require coordination with cations (such as calcium) for dimentional stability. Removal of these "pivot" ions could result in a loosening or opening of the tight junction. However, this hypothesis of absorption enhancement needs further proof of evidence. In addition, other unknown mechanisms of absorption enhancement may exist for chitosan such as inhibition of the proteolytic enzymes in the nasal cavity and its possible direct effects on the transcellular permeability.

Perfusion studies were subsequently conducted on CS G which is the soluble glutamate salt of chitosan. The average values of percent [D-Arg^2]-Kyotorphin remaining at various times and k_{obs} are given in Table 3. The semilogarithmic plots are shown in Figure 11. Since the buffer at pH 3.0 alone caused some absorption of the dipeptide, perfusion with CS G at this pH was not performed. Figure 11 shows that addition of CS G at 0.5 % w/v resulted in a significant increase in the nasal absorption of [D-Arg^2]-Kyotorphin over its corresponding buffer group at all pH studied ($p < 0.05$, Student's t-test on $\%T_{120}$ between the CS G-treated group and the control buffer group at the same pH). However, when ANOVA was applied to test for possible pH effect on the enhancing activity of CS G, no significant differences were found in the values of $\%\ T_{120}$ and k_{obs} among the three pH conditions ($p > 0.05$). The $\%\ T_{120}$ for CS G was found to be 80.1 ± 3.4, 80.0 ± 7.1, and 73.2 ± 2.8 % at pH 4.0, 5.0, and 6.0, respectively, whereas the respective values for k_{obs} were $1.86 \pm 0.44 \times 10^{-3}$, $1.92 \pm 0.83 \times 10^{-3}$, and $2.65 \pm 0.42 \times 10^{-3}$ min^{-1}. ANOVA results thus indicated that the enhancing activity of CS G was not affected by pH in the range of 4.0 to 6.0.

The reason for difference in the pH effect between CS J and CS G could be due to the difference in the chemical form of the two chitosans. CS J exists in a free amine form which normally requires an acidic condition for ionization,

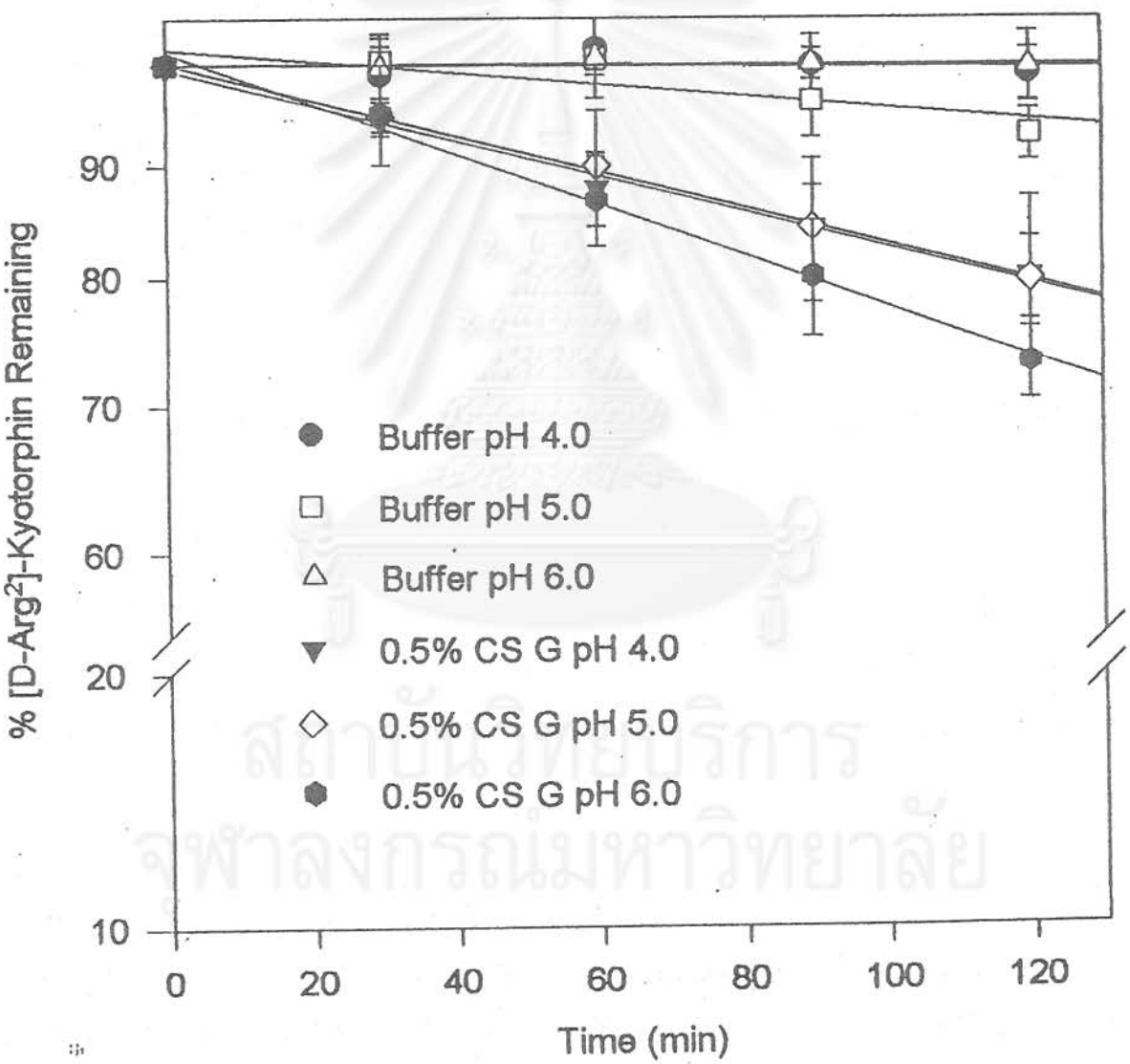
Table 3 % [D-Arg^2]-Kyotorphin remaining in the perfusate containg 0.5% w/v CS G at various pH

Chitosan CS G	% [D-Arg^2]-Kyotorphin remaining in the perfusate					Apparent first order rate constant, k (min^{-1})	n
	0 min	30 min	60 min	90 min	120 min		
pH 4.0	100	94.5 ± 1.2	87.8 ± 3.4	84.4 ± 3.8	80.1 ± 3.4	0.00186 ± 0.00044	6
pH 5.0	100	94.8 ± 1.9	90.0 ± 5.4	84.4 ± 6.3	80.0 ± 7.1	0.00192 ± 0.00083	6
pH 6.0	100	95.1 ± 5.0	86.8 ± 4.0	80.1 ± 4.8	73.2 ± 2.8	0.00265 ± 0.00042	6

The data show mean ± SD

Figure 11 Semilogarithmic plots of percent [D-Arg^2]-Kyotorphin remaining in the perfusate versus time after nasal perfusion with 0.5 % w/v CS G at various pH. The control groups were perfused with only [D-Arg^2]-Kyotorphin in the same buffers. The perfusion media were isotonic phosphate buffers (IPB) pH 4.0, 5.0, and 6.0.

Value = mean ± SD (n = 4-6 rat/group)



hydration and dissolution to occur in order to be able to interact with the nasal mucosa. CS G, on the other hand, is already in a soluble salt form. It may not need that much acidity to hydrate or dissolve. Lehr et al. (1992) found that the glutamate salt of chitosan was the most readily soluble of all chitosans studied. It could be dissolved in water whereas the other chitosans must be dissolved in acid to obtain a solution. It thus appears that the absorption enhancing activity of CS G may be less dependent on pH as opposed to the free amine CS J. Our previous results with chitosan hydrochoride (CS HCl), another soluble salt, also gave similar results in that the rate and extent of [D-Arg^2]-Kyotorphin nasal absorption were not affected by changes in pH of the perfusates from pH 4.0 to 6.0. It is possible that both CS G and CS HCl are still able to assume the highly ionized, elongated shape which helps maintain their adjuvant activities at higher pH values such as pH 6.0.

It is interesting to note that the order of absorption enhancement for CS G was opposite to that of CS J. Although not significant ($p > 0.05$), the values of $\%T_{120}$ and k_{obs} suggested that CS G tended to be more effective at higher pH, with the maximum enhancing activity observed at pH 6.0. At present, no explanations could be made regarding the reverse order of the pH effect observed with CS G. Apparently, more studies such as the in vivo absorption experiments are needed to clarify this observation.

1.2 Effect of Chitosan Concentration

The next part of experiments was to evaluate the effect of varying chitosan concentration on its absorption enhancing activity. Since CS J was shown to be most effective at pH 4.0, this pH was selected as the optimal pH for subsequent studies with CS J. On the other hand, CS G gave the highest absorption enhancement at pH 6.0. This value was thus considered to be the optimal pH

condition for CS G and used in all subsequent experiments. After selecting the optimal pH condition, the concentration of CS J and G was varied at 0.1 and 0.02 % w/v so as to determine the concentration that produced optimum enhancing results. It was not possible to perfuse solutions of CS J and G at concentrations higher than 0.5 % w/v due to the increase in viscosity of the solutions which led to excessive mucus discharge and precipitation of chitosan during nasal perfusion. The perfusion results were then compared to HP-β-CD, a cyclodextrin-type enhancer which, at 5 % w/v, was reported to have minimal deleterious effects on the rat nasal mucosa (Shao et al.,1992a).

Table 4 shows the results of the experiments using CS J as an enhancer at various concentrations at pH 4.0. The perfusion profiles are also shown in Figure 12 which includes the profile of 5 % w/v HP-β-CD in IPB pH 7.4 for comparison. Data in Table 4 and Figure 12 indicated that the enhancing activity of CS J was concentration-dependent. As the concentration was increased from 0.02 to 0.5 % w/v, the values of $\%T_{120}$ decreased from 84.1 $\pm$ 1.8 % to 66.9 $\pm$ 3.9 % and k_{obs} increased from 1.47 $\pm$ 0.28 x 10^{-3} to 2.94 $\pm$ 0.61 x 10^{-3} min^{-1}. It is also interesting to note that CS J was already effective even at a concentration as low as 0.02 % ($p < 0.05$, Student's t-test on $\%T_{120}$ when compared between the control buffer group (0 % CS J) and the 0.02 % CS J-treated group).

Similar results were obtained with CS G after perfusion at its optimal pH. Data in Table 5 and Figure 13 revealed that the enhancing activity of CS G at pH 6.0 was also concentration-dependent. As the concentration was increased from 0.02 to 0.5 % w/v, the values of $\%T_{120}$ decreased from 80.4 $\pm$ 3.9 % to 73.2 $\pm$ 2.8 % and k_{obs} increased from 1.90 $\pm$ 0.55 x 10^{-3} to 2.65 $\pm$ 0.42 x 10^{-3} min^{-1}. The enhancing effect was also significant at 0.02 % w/v, the lowest concentration studied ($p < 0.05$, Student's t-test on $\%T_{120}$ when compared between the control buffer group (0 % CS G) and the 0.02 % CS G-treated group). Thus, the

enhancing effect of both CS J and G was concentration-dependent, with the maximum activity observed at 0.5 % w/v, the highest concentration under study.

The results of CS J and G were compared to that of 5 % w/v HP-β-CD. Perfusion of HP-β-CD was conducted at physiological pH (7.4) due to its good solubility, safety and enhancing activity at this pH. The data of HP-β-CD and its control buffer group are given in Table 6. Their perfusion profiles are also illustrated in Figures 12 and 13 for direct comparison with CS J and G. As expected, perfusion with only pH 7.4 buffer hardly induced any absorption of [D-Arg^2]-Kyotorphin whereas addition of 5 % w/v HP-β-CD resulted in significant absorption ($p < 0.05$, Student's t-test on $\%T_{120}$ when compared between the control buffer group and the enhancer-treated group). The average values of $\%T_{120}$ and k_{obs} for HP-β-CD were 82.4 ± 2.3 % and $1.57 \pm 0.17 \times 10^{-3}$ min^{-1}, respectively. Interestingly, these values were in the same range as those for 0.02 % w/v CS J and G (Tables 4-6).

Figure 14 is the overall histogram showing the average percent absorption of [D-Arg^2]-Kyotorphin after 120-min nasal perfusion with various concentrations of CS J and G in comparison with 5 % w/v HP-β-CD at their respective optimal pH conditions. The percent absorbed at 120 min was calculated as the difference between the initial concentration of [D-Arg^2]-Kyotorphin (100 %) and percent dipeptide remaining in the perfusate at 120 min ($\%T_{120}$). From this Figure it can be noticed that the enhancing effect of 0.5 % w/v CS J and G, at their respective optimal pH, was superior to 5 % w/v HP-β-CD in IPB pH 7.4. At only 0.02 % w/v, the enhancing activity of both chitosans was already equivalent to that of 5 % w/v HP-β-CD. ANOVA on either the % drug absorbed or k_{obs} revealed that there was no significant difference in these parameters among 0.02 % CS J pH 4.0, 0.02 % CS G pH 6.0, and 5 % HP-β-CD pH 7.4 ($p > 0.05$).

Table 4 [D-Arg^2] Kyotorphin remaining in the perfusate when using various concentrations of chitosan J as an enhancer at pH 4.0

Concentration of Chitosans J (%w/v)	% [D-Arg^2] Kyotorphin remaining in the perfusate					Apparent first order rate constant, k (min^{-1})	n
	0 min	30 min	60 min	90 min	120 min		
Control (no CS)	100.0	98.9 ±4.0	101.7 ±5.1	100.0 ±3.2	99.0 ±2.7	-	4
0.5	100.0	89.1 ±7.2	82.4 ±5.6	76.7 ±7.0	66.9 ±3.9	0.00294 ± 0.00061	4
0.1	100.0	97.4 ±1.9	89.9 ±2.0	83.0 ±4.2	76.5 ±5.9	0.00234 ±0.00061	4
0.02	100.0	97.2 ±3.2	92.6 ±0.6	89.0 ±0.7	84.1 ±1.8	0.00147 ±0.00028	4

The data show mean ± SD

Figure 12 Semilogarithmic plots of percent [D-Arg^2]-Kyotorphin remaining versus time after nasal perfusion with various concentrations of CS J in IPB pH 4.0. The profiles are compared with that of 5 %w/v HP-β-CD.

Value = mean ± SD (n = 4 rat/group)

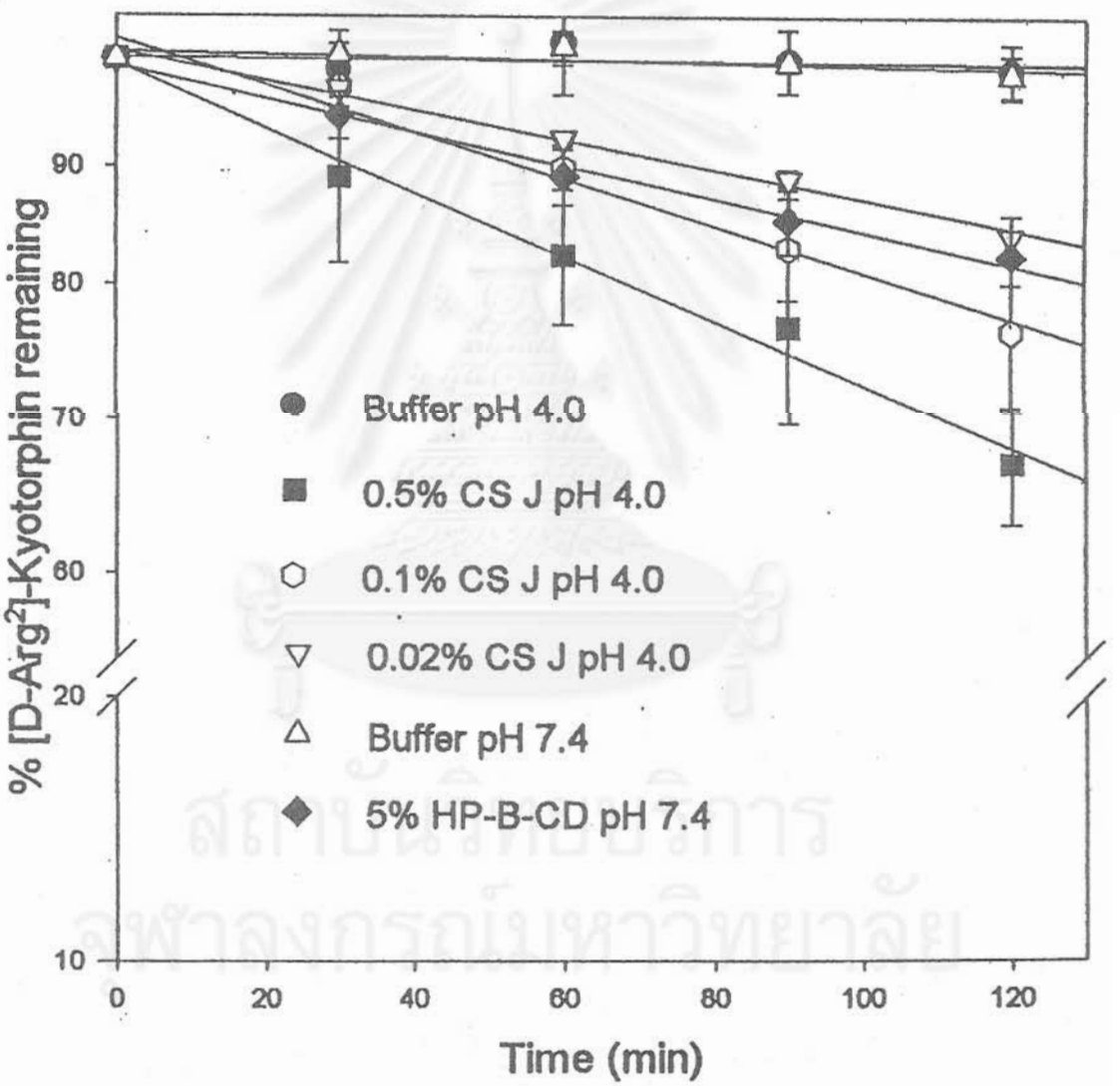


Table 5 % [D-Arg^2] Kyotorphin remaining in the perfusate when using various concentrations of chitosan G as an enhancer at pH 6.0

Concentration of Chitosan G (%w/v)	% [D-Arg^2] Kyotorphin remaining in the perfusate					Apparent first order rate constant, k (min^{-1})	n
	0 min	30 min	60 min	90 min	120 min		
Control (no CS)	100.0	99.8 ±3.5	100.7 ±1.2	100.1 ±1.6	98.8 ±3.6	-	4
0.5	100.0	95.1 ±5.0	86.8 ±4.0	80.1 ±4.8	73.2 ±2.8	0.00265 ±0.00042	6
0.1	100.0	95.8 ± 0.7	88.1 ± 3.0	85.2 ± 1.9	80.6 ± 1.5	0.00183 ± 0.00011	4
0.02	100.0	94.4 ± 3.2	85.7 ± 5.7	82.0 ± 5.6	80.4 ± 3.9	0.00190 ± 0.00055	4

The data show mean ± SD

Figure 13 Semilogarithmic plots of percent [D-Arg^2]-Kyotorphin remaining versus time after nasal perfusion with various concentrations of CS G in IPB pH 6.0. The profiles are compared with that of 5 %w/v HP-β-CD.

Value = mean ± SD (n = 4-6 rat/group)

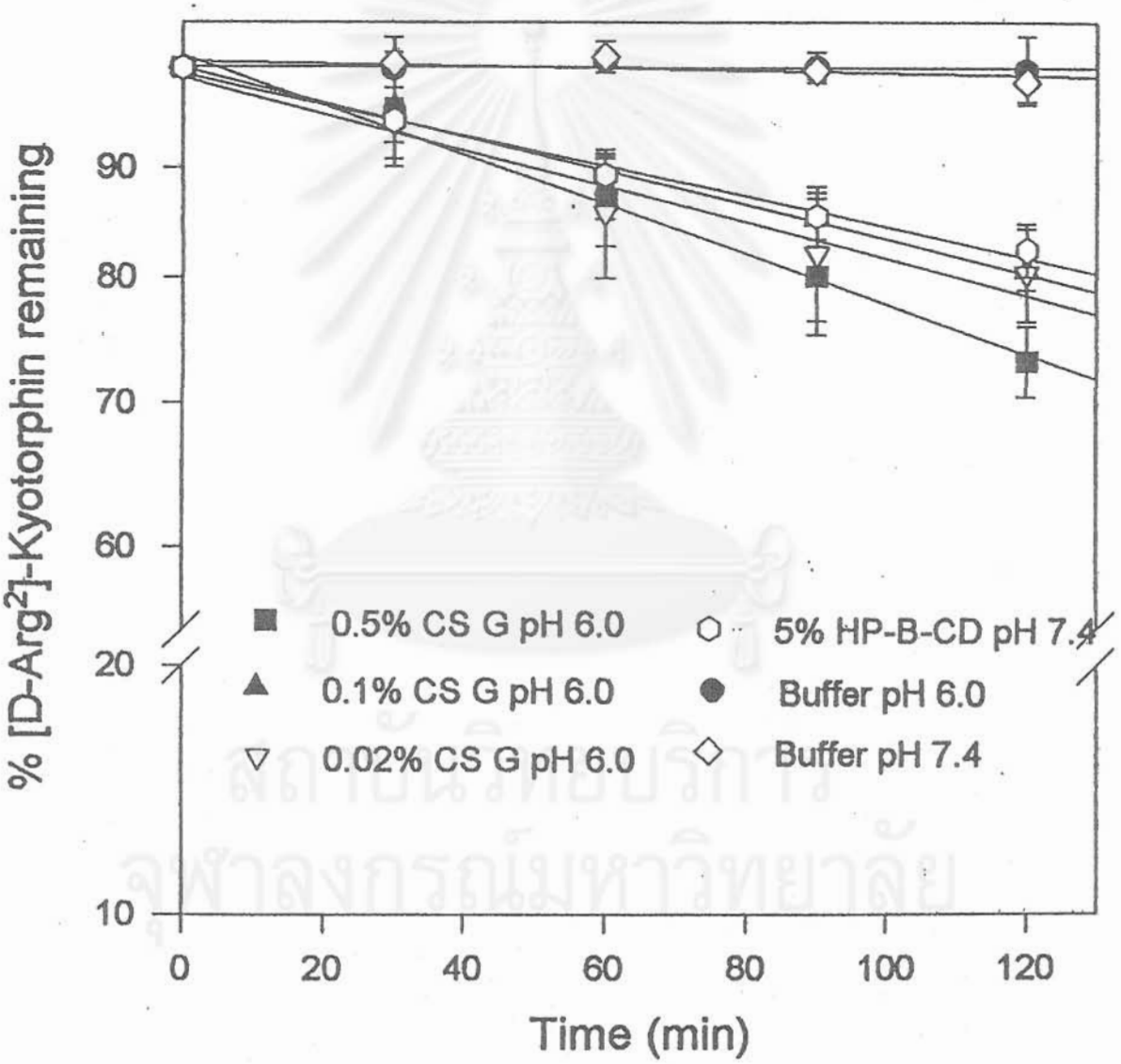
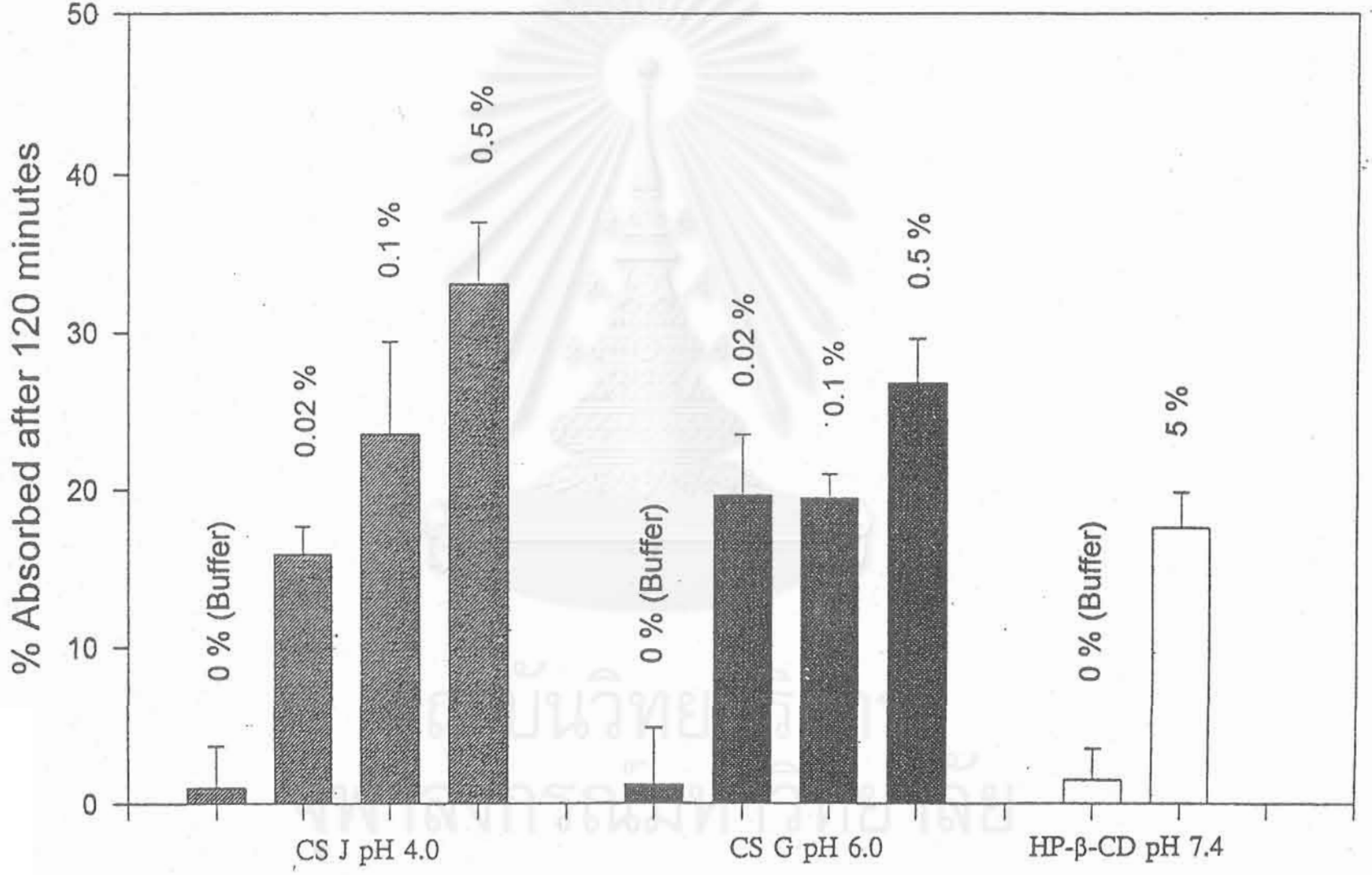


Table 6 % [D-Arg^2] Kyotorphin remaining in the perfusate when using 5%w/v HP-β-CD as an enhancer at pH 7.4

Enhancer	% [D-Arg^2] Kyotorphin remaining in the perfusate					Apparent first order rate constant, k (min^{-1})	n
	0 min	30 min	60 min	90 min	120 min		
Control (no CD)	100.0	100.6 ± 1.1	101.2 ± 1.7	99.65 ± 0.8	98.5 ± 2.0	-	4
5%w/v HP-β-CD	100.0	94.6 ± 2.2	89.2 ± 2.5	85.4 ± 2.7	82.4 ± 2.3	0.00157 ± 0.00017	4

The data show mean ± SD

Figure 14 Histogram of percent [D-Arg^2]-Kyotorphin absorbed from the nasal perfusate at 120 min in the presence of HP-β-CD, CS J and CS G at various concentrations. Value = mean ± SD (n = 4-6 rat/group)



Part II : Effects of Chitosans on the Protein and Phosphorus Release from the Rat Nasal Mucosa

This part was set in order to investigate the effects of the nasal absorption enhancers on the nasal membrane integrity as characterized by the release of protein and phospholipid which are the major components of the cell membrane. The magnitude of membrane damaging effects as determined by the membrane protein or phospholipid release may provide an accurate, simple and useful marker for predicting safety of nasal absorption enhancers (Shao et al.,1992a and b).

The total protein and phosphorus contents were quantitatively analyzed from the perfusates which were perfused through the rat nasal mucosa by *in situ* perfusion technique as previously described. The perfusion was carried out in each rat for at least 120 min with aliquots of the perfusate (200 µl each) taken at 0, 30, 60, 90 and 120 min. Each aliquot was divided into 2 parts of 100 µl for separate total protein and total phosphorus analyses. The perfusate after 120 min perfusion was also quantitated for phospholipid phosphorus content which is a direct indicator of membrane phospholipid. Quantitation of phosphorus content, both total and phospholipid phosphorus, was employed as a means to assess the extent of mucosal lipid release.

Two chitosans, CS J and CS G, were selected from the previous part for studies of their effects on protein and phosphorus release in comparison with HP-β-CD. The concentration of CS G and CS J was selected to be 0.1% w/v whereas that of HP-β-CD was fixed at 5% w/v. The phosphate buffer IPB was not allowed to use in this part because of the interference of phosphate phosphorus in the analysis. So

the other buffers were chosen. Isotonic acetate-borate buffer pH 7.4 and pH 6.0 were selected as a medium for 5%w/v HP-β-CD and 0.1%w/v CS G, respectively. Isotonic lactate buffer pH 4.0 was chosen for 0.1%w/v CS J. There were six groups for this experiment, i.e. three groups of the enhancers versus three groups of the buffers at their respective pH which served as the control for each enhancer group.

2.1 Analysis of Total Protein Contents Released from the Rat Nasal Mucosa

The calibration curve of total protein content is shown in Figure 15. This curve was plotted between the concentration of BSA solution, used as a standard protein, and the absorbance at 650 nm. It was linear and the regression coefficient (r^2) was 0.9976. The results of total protein content analysis are presented in Table 7. These are means ± SD of three rats per group. The total protein release data of the individual rats are provided in Appendix III. At 120 min, the average total protein concentration for 5%w/v HP-β-CD, 0.1%w/v CS G and 0.1%w/v CS J were 1132.38 ± 305.43, 2161.77 ± 757.74 and 1916.18 ±517.52 μg/ml, respectively. Figure 16 graphically depicts the time course of nasal protein release. The released protein concentration increased with time during the course of experiment. From each plot, the linear regression was performed from 0 to 120 min to calculate the rate of total protein release, k. Means ± SD of the rate are also shown in Table 7.

Results from Table 7 and Figure 16 also imply that perfusion with the buffers alone could cause some release of the mucosal protein, particularly with the lactate buffer pH 4.0. The acetate-borate buffer pH 7.4 caused the least amount of protein release. Perfusion of the rat nasal cavity at the flow rate of 2 ml/min was considered

Figure 15 Representative calibration curve of protein content

$y = 0.0182 + 0.0032x$ $r^2 = 0.9976$

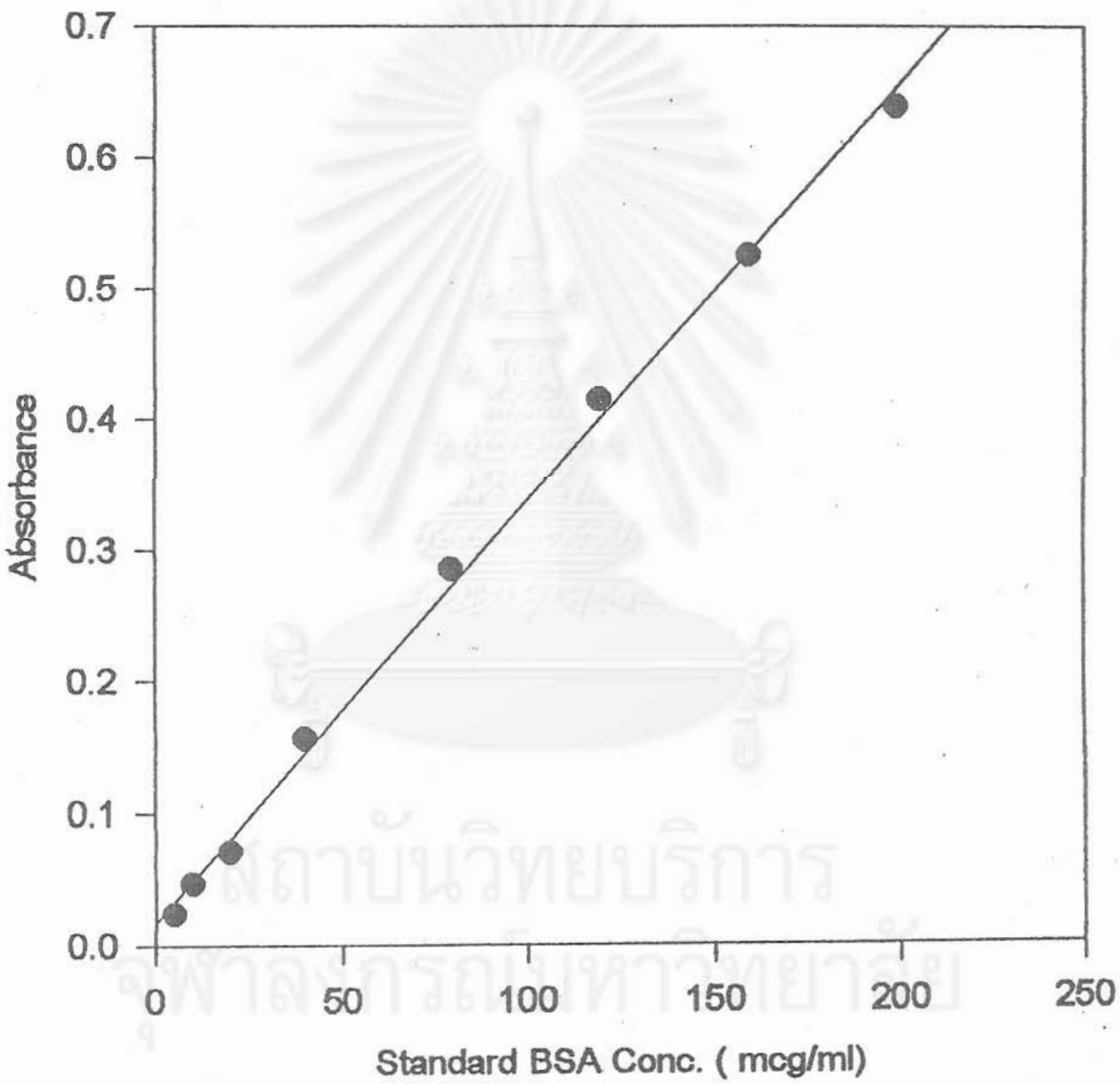


Figure 16 Release profiles of protein in the rat nasal perfusates containing 5% HP-β-CD, 0.1% CS G and 0.1% CS J. The perfusion medium were buffer pH 7.4, 6.0 and 4.0, respectively.

Value = mean ± SD (n = 3 rats/group)

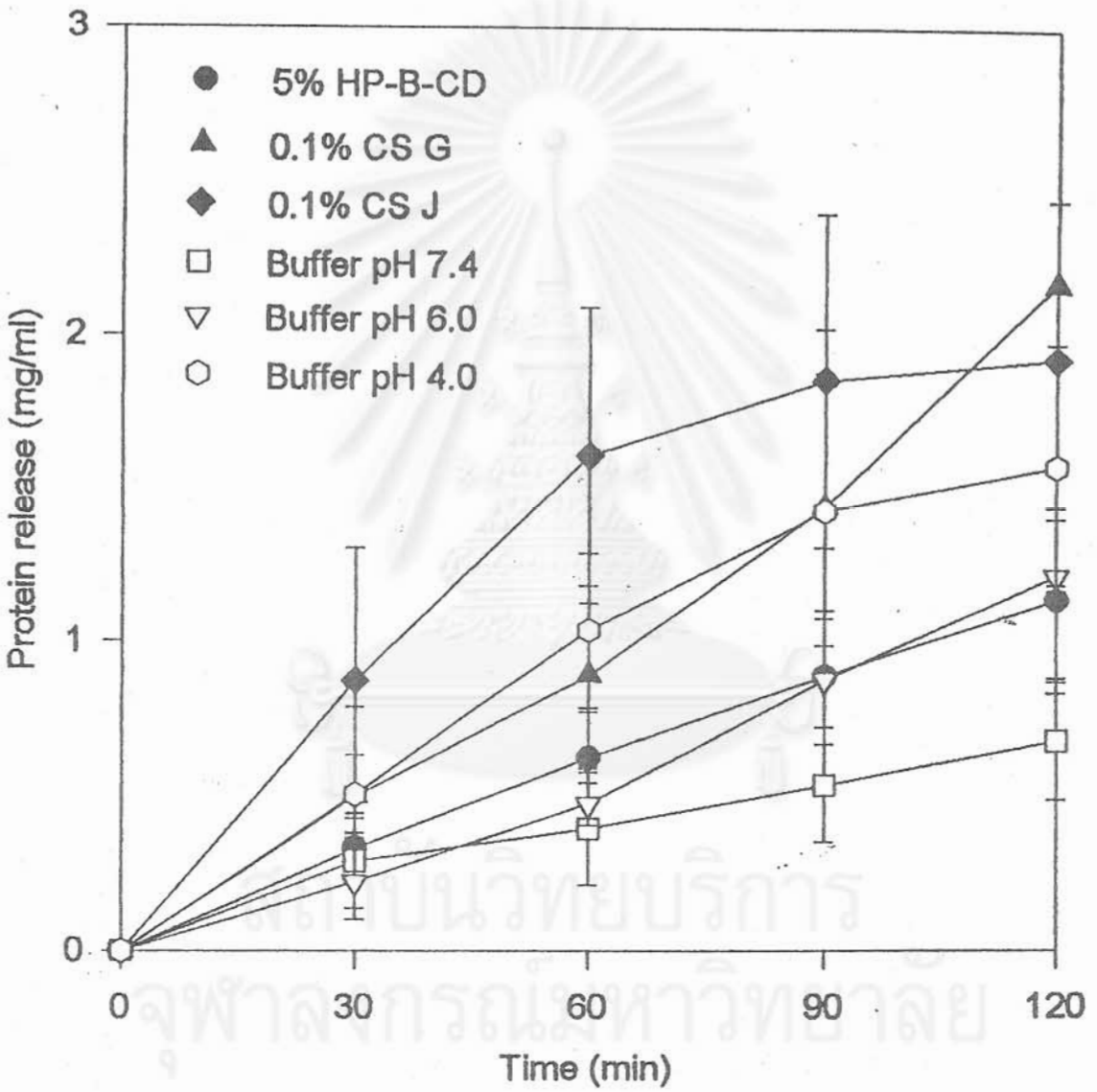


Table 7 Nasal protein release content from the perfusate of the rat containing 5% HP-β-CD, 0.1% CS G, 0.1% CS J and their buffers, pH 7.4, 6.0 and 4.0. (n = 3 rats/group)

Enhancer	Value	Protein release content (μg/ml)					Release rate, k
		0 min	30 min	60 min	90 min	120 min	(μg/ml/min)
5%	mean	0	337.58	622.57	883.22	1132.38	9.3680
HP-β-CD	SD	0	26.71	144.73	216.55	305.43	2.7596
Buffer	mean	0	294.78	395.82	537.08	676.30	5.3163
pH 7.4	SD	0	153.27	180.97	182.16	186.01	1.3111
0.1%	mean	0	568.45	918.93	1541.12	2161.77	17.6541
CS G	SD	0	192.83	252.42	449.53	757.74	5.9000
Buffer	mean	0	226.07	475.94	870.33	1212.35	10.2299
pH 6.0	SD	0	121.44	67.81	204.75	337.82	3.3099
0.1%	mean	0	976.73	1605.68	1851.24	1916.18	15.7196
CS J	SD	0	329.91	479.80	544.49	517.52	4.1891
Buffer	mean	0	574.76	1034.37	1214.28	1274.32	10.6272
pH 4.0	SD	0	35.24	253.50	206.06	175.32	1.7529

to be an extreme condition even with the buffer alone. During perfusion, a substantial amount of mucus may be washed into the perfusate resulting in the detection of mucus glycoprotein. The more acidic lactate buffer pH 4.0 may have caused an increase in the nasal mucus discharge, which in turn could lead to a greater amount of protein released. It thus appears that the type of buffers used in the experiment may contribute significantly to the total protein release and that the buffer effect should be subtracted from the individual values of the perfusion data of each enhancer.

Table 8 presents the means ± SD of the content and the rate of total protein release from the nasal mucosa of the three enhancers after the individual values have been corrected with the mean value of their buffers. The plots of these results are shown in Figure 17. When ANOVA was applied to these data at 5% significance level, it was found that there were no significant differences in the protein release content at T_{120} and the release rate among these three enhancers ($p > 0.05$).

Therefore, the data revealed that there were no significant differences among 5% HP-β-CD, 0.1% CS G and 0.1% CS J with respect to their effects on the mucosal protein release.

2.2 Analysis of Total Phosphorus Contents Released from the Rat Nasal Mucosa

Phosphorus is also an important component of the cell membrane, particularly the membrane phospholipid. The phosphorus release content therefore can be considered as an indicator of membrane damaging effect. The calibration curve was plotted between the concentrations of phosphorus, calculated from a series of standard potassium phosphate monobasic solutions, and the absorbance at 830 nm as shown in

Table 8 The corrected values of the nasal protein release content from the perfusate of the rat containing 5% HP-β-CD, 0.1% CS G and 0.1% CS J at pH 7.4, 6.0 and 4.0, respectively. (n = 3 rats/group)

Enhancer	Value	Corrected values of the protein release content (μg/ml)					Release rate, k
		0 min	30 min	60 min	90 min	120 min	(μg/ml/min)
5%	mean	0	42.80	226.75	346.14	456.08	4.0517
HP-β-CD	SD	0	26.71	144.73	216.55	305.43	2.7600
0.1%	mean	0	327.37	443.00	682.79	949.42	7.5141
CS G	SD	0	176.54	252.42	449.46	757.74	5.9510
0.1%	mean	0	392.97	571.31	636.97	641.86	3.9569
CS J	SD	0	329.91	479.80	544.49	517.52	2.7454
ANOVA results on the three enhancers						NS ($p > 0.05$)	NS ($p > 0.05$)

NS = Not significant

Figure 17 Release profiles of the corrected values of protein in the rat nasal perfusates containing 5% HP-β-CD, 0.1% CS G and 0.1% CS J. The individual values were corrected by subtraction with the mean value of each medium at the same time point.

Value = mean ± SD (n = 3 rats/group)

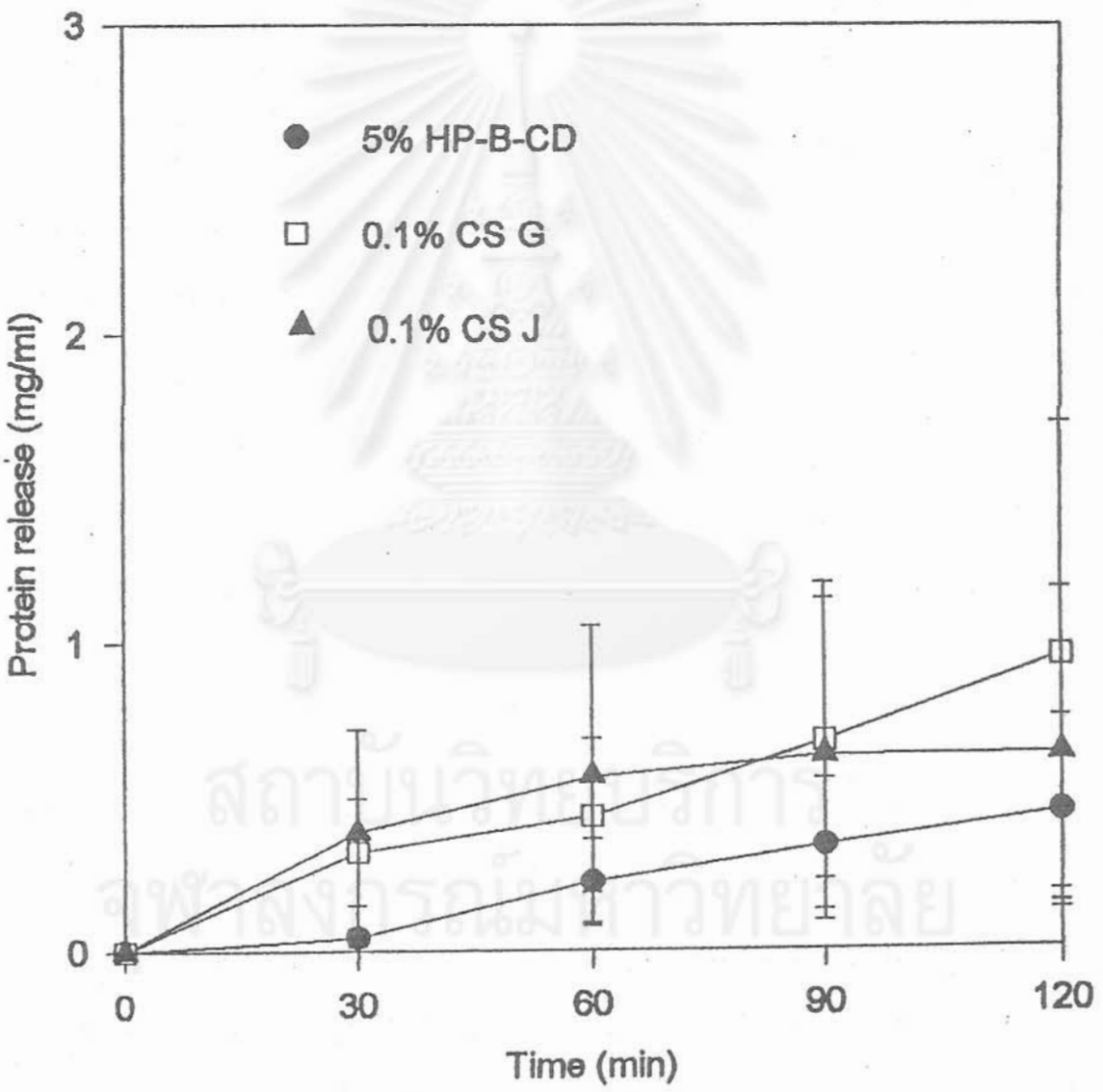


Figure 18. The plot was linear and the regression coefficient (r^2) was 0.9979. The results of the total phosphorus release at various times are presented in Table 9. They are means ± SD of the total phosphorus content released during the course of perfusion. The values for each rat are given in Appendix III. From this table, the total phosphorus content at T_{120} of 5%w/v HP-β-CD, 0.1% CS G and 0.1% CS J were 28.94 ±3.55, 25.86 ± 1.54 and 39.01 ± 1.47 μg/ml, respectively, and those of their respective buffers (pH 7.4, pH 6.0 and pH 4.0) were 8.79 ±0.84, 9.46 ± 3.47 and 24.67±3.72 μg/ml. Figure 19 shows the plots of total phosphorus release in the rat nasal perfusates versus time (min) for these enhancers and their buffers. When the linear regression of each plot was performed from 0 to 120 min, the slope of each plot was calculated as the rate of total phosphorus release, k (μg/ml/min). The rate of the release was also represented in Table 9. The values are means ± SD. From this figure, CS J appears to show the overall highest total phosphorus release content whereas CS G shows the intermediate effect and HP-β-CD shows the overall lowest release.

However, when the perfusion data of buffers alone were examined (Figure 19 and Table 9), all the three buffers also caused some release of the total phosphorus. In addition, the effect of lactate buffer pH 4.0 appears to be more intense than the borate - acetate buffers at pH 7.4 and 6.0. At 120 min perfusion, the concentration of the total phosphorus (24.67 ± 3.72 μg/ml) was nearly equal to that of 5% HP-β-CD in buffer pH 7.4 (28.94 ± 3.55 μg/ml) and 0.1% CS G in buffer pH 6.0 (25.86 ± 1.54 μg/ml). Therefore, the greater irritating effect of 0.1% CS J may have been a result of the contribution from the lactate buffer. Hence, to assess the actual membrane-

Figure 18 Representative calibration curve of phosphorus content prepared by serial dilution of KH_2PO_4 standard stock solution

$y = -0.0934 + 0.1637x$ $\quad r^2 = 0.9979$

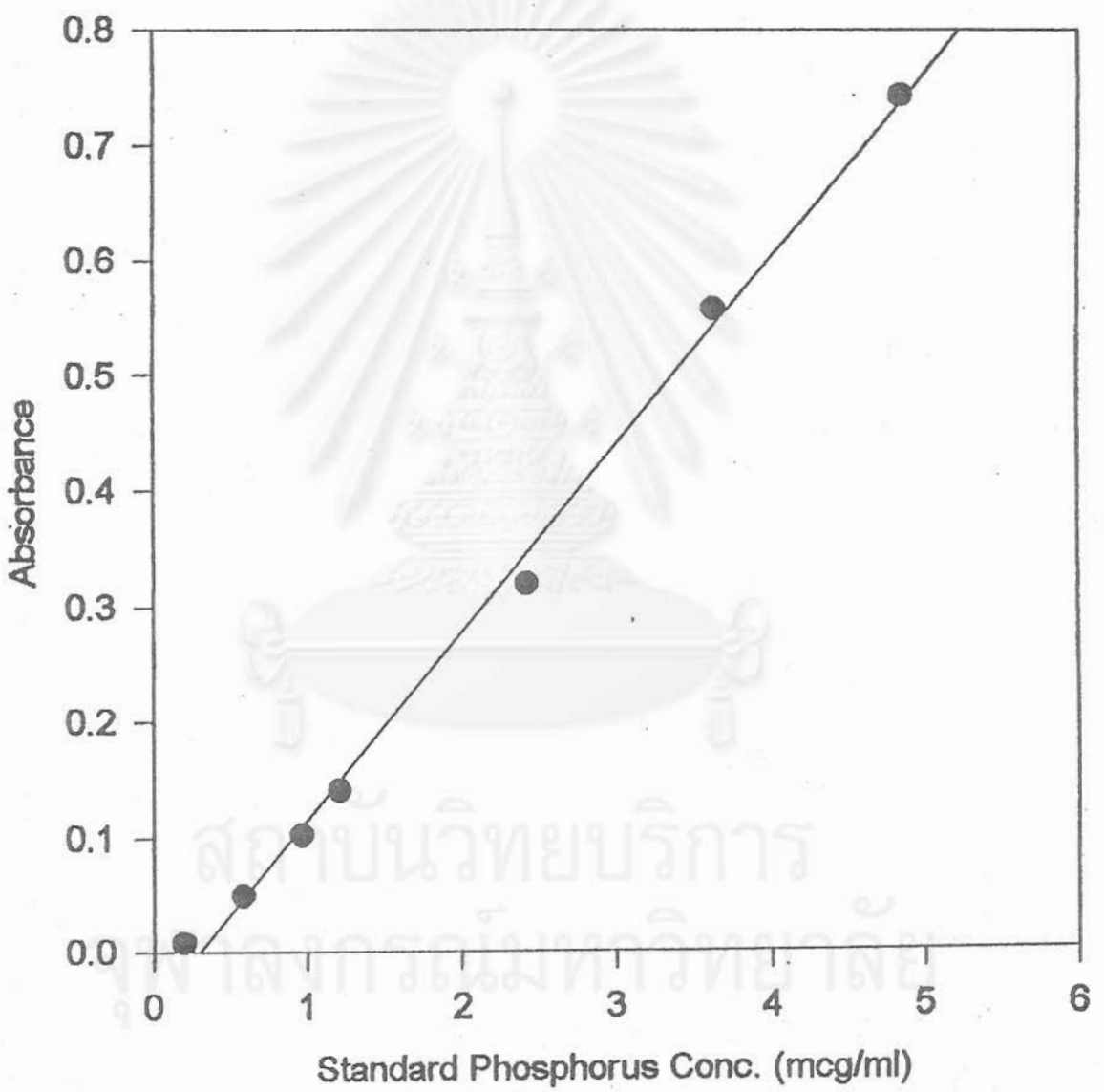
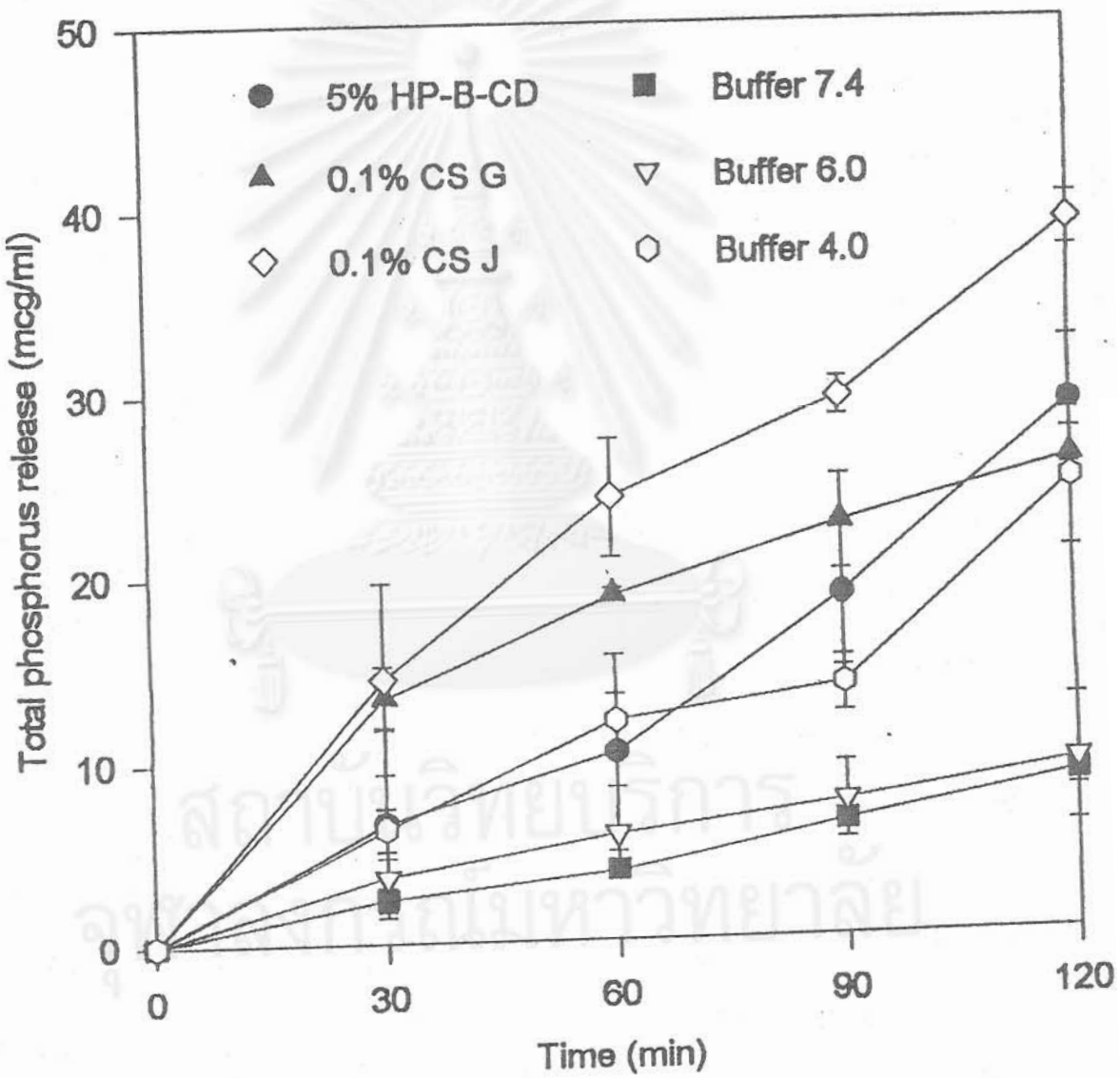


Table 9 Total phosphorus and phospholipid phosphorus content from the rat nasal perfusate containing 5% HP-β-CD, 0.1% CS G, 0.1% CS J and their respective buffers, pH 7.4, 6.0 and 4.0. (n = 3 rats/group)

Enhancer	value	Total phosphorus content (μg/ml)					Release rate, k	Phospholipid phosphorus at
		0 min	30 min	60 min	90 min	120 min	(μg/ml/min)	T_{120} (μg/ml)
5%	mean	0	6.65	10.31	18.71	28.94	0.2331	0.486
HP-β-CD	SD	0	5.19	5.37	3.91	3.55	0.0191	0.168
Buffer	mean	0	2.44	3.86	6.40	8.79	0.0718	0.026
pH 7.4	SD	0	0.67	0.49	0.54	0.84	0.0051	0.003
0.1%	mean	0	13.46	18.84	22.55	25.86	0.2027	0.439
CS G	SD	0	1.75	0.36	2.57	1.54	0.0190	0.146
Buffer	mean	0	3.71	5.88	7.52	9.46	0.0757	0.040
pH 6.0	SD	0	1.04	2.53	2.12	3.47	0.0268	0.007
0.1%	mean	0	14.48	24.12	29.45	39.01	0.3100	0.601
CS J	SD	0	5.16	3.24	1.04	1.47	0.0053	0.163
Buffer	mean	0	6.32	12.02	13.88	24.67	0.1897	0.072
pH 4.0	SD	0	1.16	1.46	1.52	3.72	0.0318	0.013

Figure 19 Release profiles of total phosphorus in the rat nasal perfusates containing 5% HP-β-CD, 0.1% CS G, 0.1% CS J and their buffers. The perfusion medium were buffer pH 7.4, 6.0 and 4.0, respectively.

Value = mean ± SD (n = 3 rats/group)



irritating potential of the enhancers, the buffer effect should be subtracted from the individual data of each enhancer. This kind of correction is pretty much similar to that previously performed on the protein release data.

Table 10 illustrates the corrected values of means ± SD of the total phosphorus release content and the release rate. These were calculated by subtracting the values of the individual rats receiving the enhancer with the mean of its respective buffer at the same time point. The release rate was then calculated after these corrections. Figure 20 shows the plots of these corrected values versus time. These values were then analyzed by ANOVA at 5% significance level. After correction for the buffer effect, no significant differences were observed among the three enhancers with respect to both the content at T_{120} and the release rate ($p > 0.05$).

2.3 Analysis of Phospholipid Phosphorus Released from the Rat Nasal Mucosa

Total phosphorus content provides only a rough indication of mucosal damages since the amount of phosphorus analyzed could come from several sources, including the intracellular and extracellular fluids as well as from the membrane. To obtain a more specific evaluation of the membrane damaging effect caused by these enhancers, assay of phospholipid phosphorus was also carried out since this substance will better reflect the amount of membrane phospholipid that has been released.

The results of the quantitative analysis of the phospholipid phosphorus content that has been released at T_{120} are shown in Table 9. These values are means ± SD of HP-β-CD, CS G, CS J, and their respective buffers at pH 7.4, 6.0 and 4.0. The data

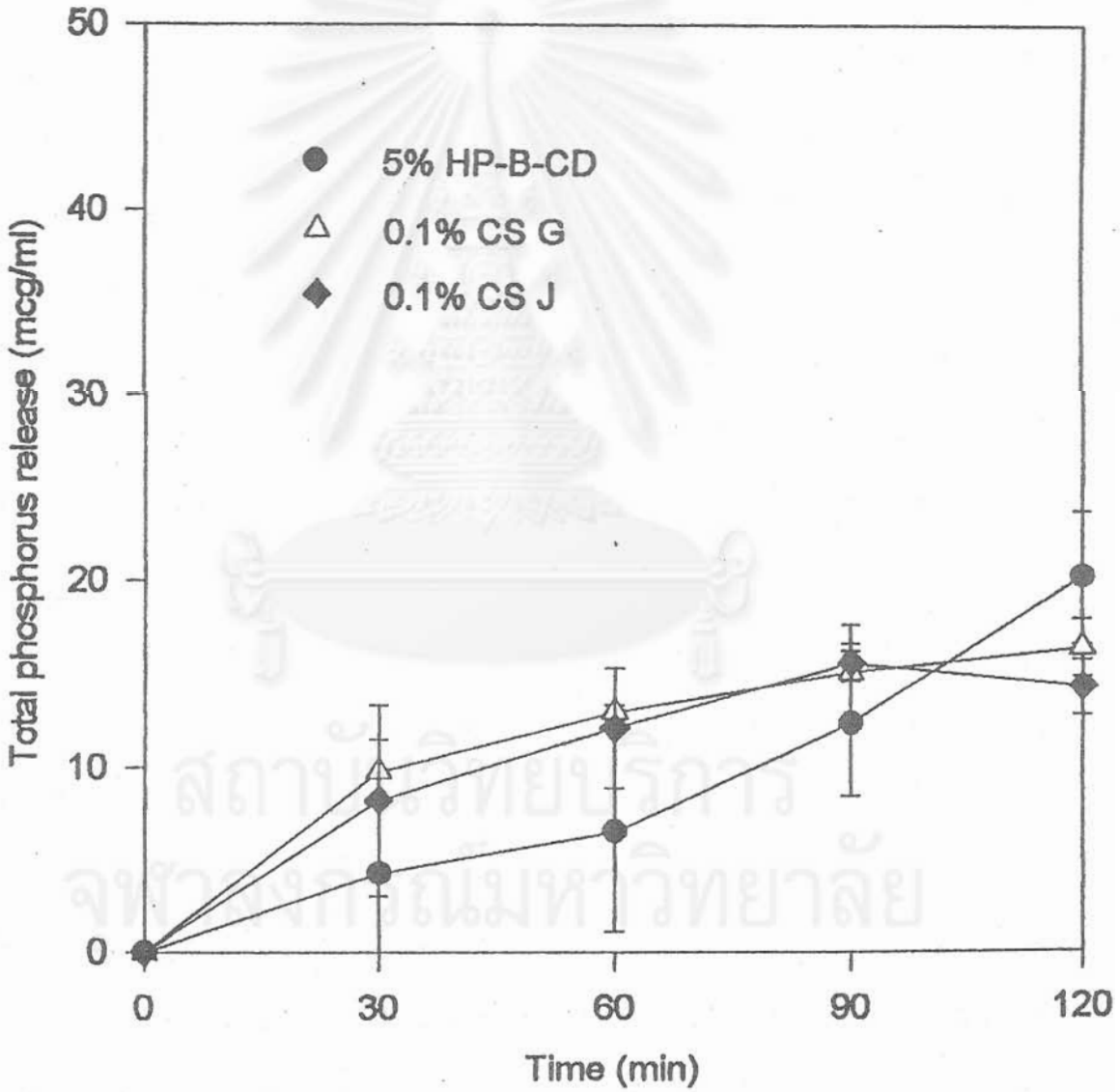
Table 10 The corrected values of the total phosphorus and phospholipid phosphorus content from the nasal perfusate of the rat containing 5% HP-β-CD, 0.1% CS G and 0.1% CS J at respective pH of 7.4, 6.0 and 4.0. (n = 3 rats/group)

Enhancer	value	Corrected values of the total phosphorus content (μg/ml)					Release rate, k	Phospholipid phosphorus at
		0 min	30 min	60 min	90 min	120 min	(μg/ml/min)	T_{120} (μg/ml)
5%	mean	0	4.22	6.46	12.31	20.15	0.1502	0.460
HP-β-CD	SD	0	5.19	5.37	3.91	3.55	0.0347	0.168
0.1%	mean	0	9.75	12.96	15.04	16.40	0.1270	0.430
CS G	SD	0	1.75	0.36	2.57	1.54	0.0190	0.139
0.1%	mean	0	8.16	12.10	15.57	14.31	0.1201	0.529
CS J	SD	0	5.16	3.24	1.04	1.47	0.0053	0.164
ANOVA results on the three enhancers						NS ($p > 0.05$)	NS ($p > 0.05$)	NS ($p > 0.05$)

NS = Not significant

Figure 20 Release profiles of corrected values of total phosphorus in the rat nasal perfusates containing 5% HP-β-CD, 0.1% CS G and 0.1% CS J. The values were corrected by subtracting the individual values with the mean value of each medium at the same time point.

Value = mean ± SD (n = 3 rats/group)



for the individual rats are provided in Appendix III. The results were 0.486 ± 0.168, 0.439 ± 0.146, 0.601 ± 0.163, 0.026 ± 0.003, 0.040 ± 0.007 and 0.072 ± 0.013 μg/ml, respectively. Figure 21 represents the histogram of the phospholipid phosphorus content at T_{120} for the three groups of the enhancers and the three groups of their respective buffers.

These values were also corrected by the mean values of their buffers at the same time point for each rat. These corrected values at T_{120} are also shown in Table 10 and in the histogram in Figure 22. When ANOVA was applied to the corrected data, there were no significant differences among HP-β-CD, CS G and CS J with respect to the phospholipid phosphorus content at T_{120} ($p > 0.05$).

For comparative purposes, the release rates of total protein and total phosphorus, the contents of total protein, total phosphorus and phospholipid phosphorus released from the nasal mucosa as extracted by the three enhancers at 120 min, are summarized in Table 11. This table also shows the results of their corresponding buffers and the corrected results of the enhancers after subtracting the buffer effect. Since there were no significant differences in all parameters among these three enhancers ($p > 0.05$), it can be concluded that 0.1% CS G in buffer pH 6.0 and 0.1% CS J in buffer pH 4.0 gave similar membrane damaging effects to the reference enhancer, 5% HP-β-CD in buffer pH 7.4.

The 5% HP-β-CD was selected as a reference enhancer due to its very small membrane damaging effects as reported by Shao et al. (1992a). The objective of their study was to delineate some of the possible enhancing mechanisms in order to understand better the effectiveness and safety of cyclodextrins as nasal absorption

Figure 21 Histogram of phospholipid phosphorus in the rat nasal perfusate at 120 min containing 5% HP-β-CD, 0.1% CS G and 0.1% CS J and their respective buffers.

Value = mean ± SD (n = 3 rats/group)

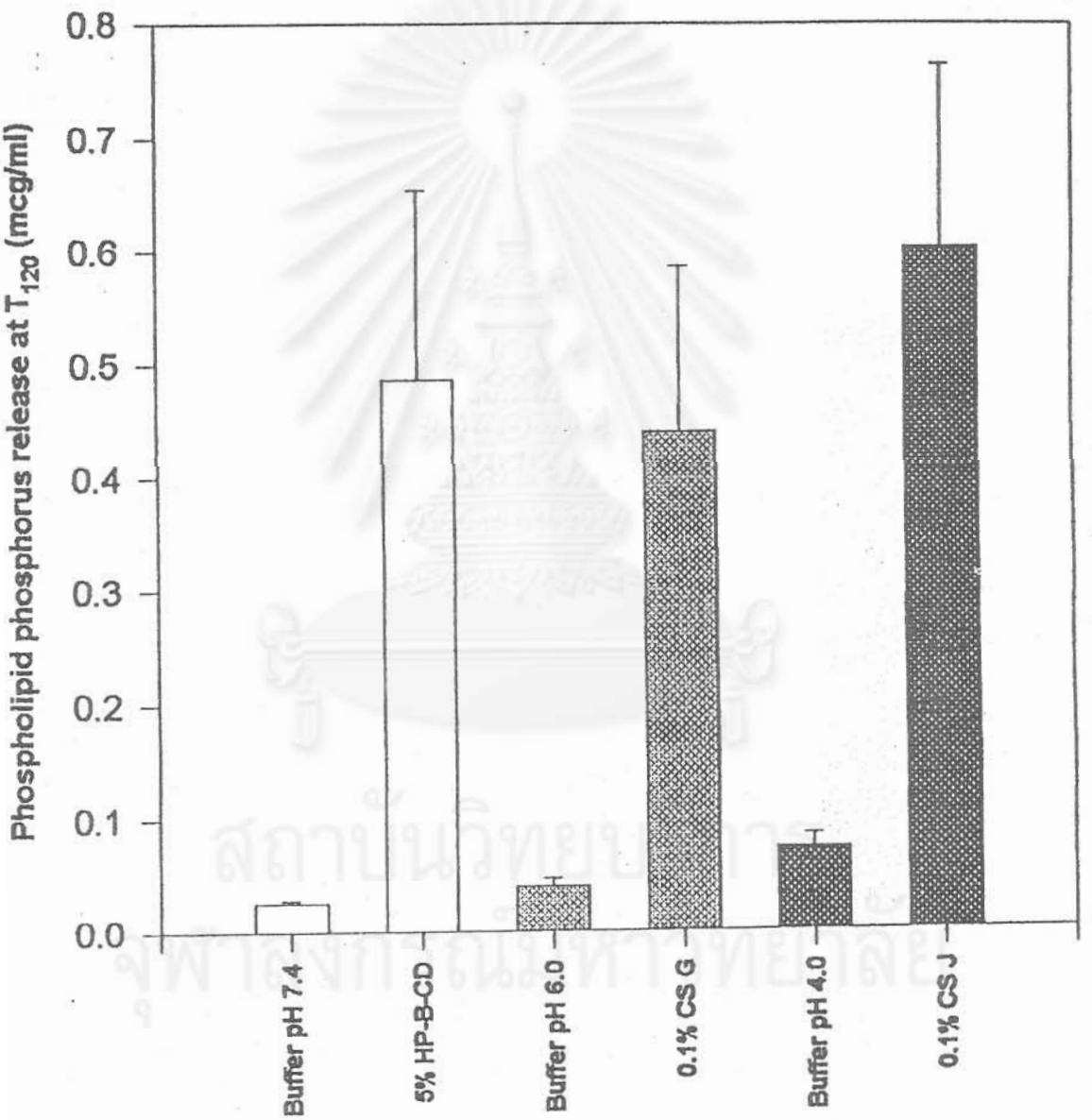


Figure 22 Histogram of corrected values of phospholipid phosphorus in the perfusate containing 5% HP-β-CD, 0.1% CS G and 0.1% CS J.

Value = mean ± SD (n = 3 rats/group)

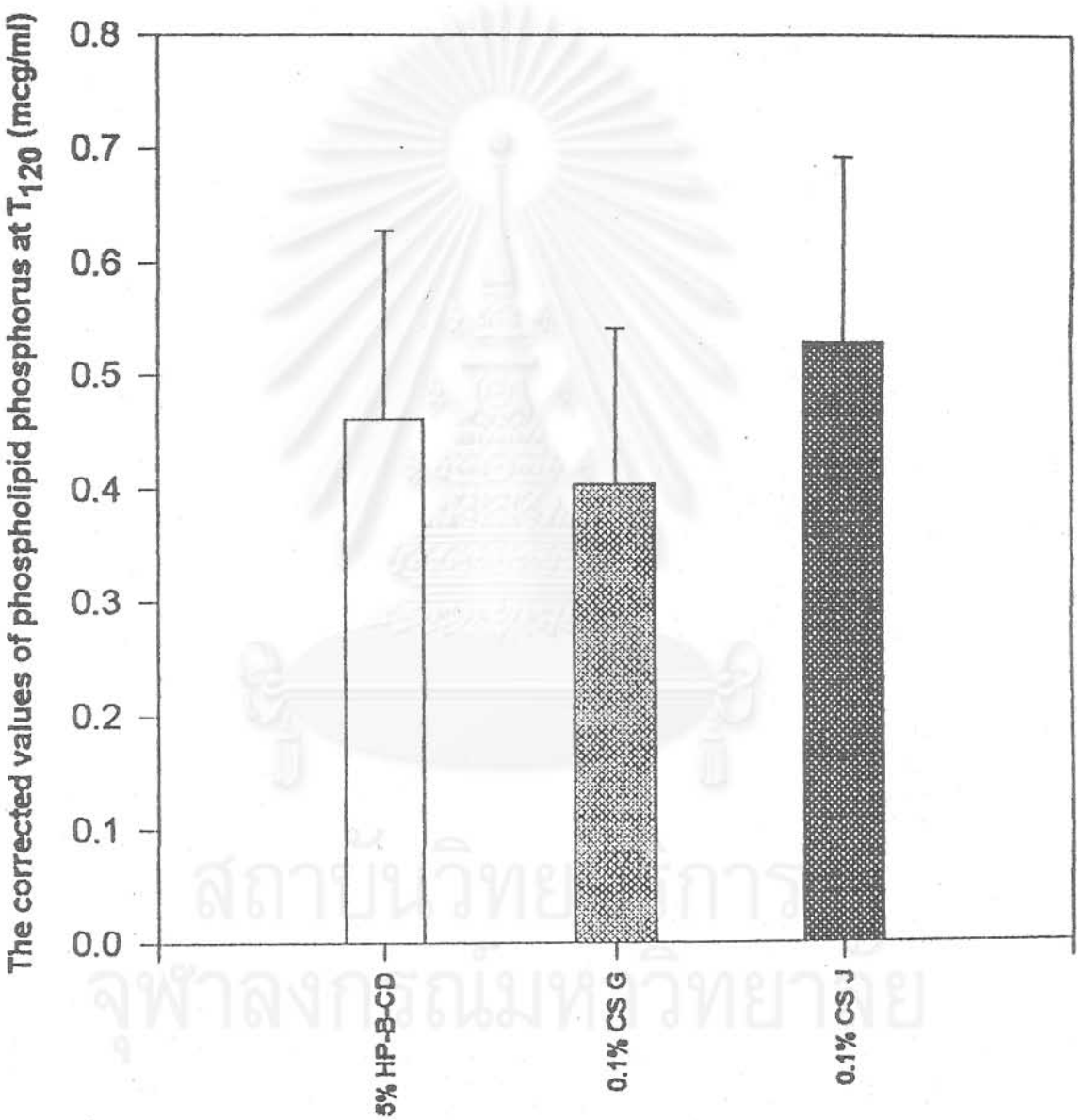


Table 11 Protein release, protein release rate, total phosphorus, total phosphorus release rate and phospholipid phosphorus content in rat nasal perfusate in the presence of various enhancers

Enhancer	Concentration/ Condition	Rates of protein release (μg/ml/min)	Rates of total phosphorus release (μg/ml/min)	Protein at T_{120} (μg/ml)	Total phosphorus at T_{120} (μg/ml)	Phospholipid phosphorus at T_{120} (μg/ml)
HP-β-CD	5%w/v, pH 7.4	9.3680 ± 2.7596	0.2331 ± 0.0191	1132.38 ± 305.43	28.94 ± 3.55	0.486 ± 0.168
CS G	0.1%w/v, pH 6.0	17.6541 ± 5.9000	0.2027 ± 0.0190	2161.77 ± 757.74	25.86 ± 1.54	0.439 ± 0.146
CS J	0.1%w/v, pH 4.0	15.7196 ± 4.1891	0.3100 ± 0.0053	1916.18 ± 517.52	39.01 ± 1.47	0.601 ± 0.163
-	A-B buffer pH 7.4	5.3163 ± 1.311	0.0718 ± 0.0051	676.30 ± 186.01	8.79 ± 0.84	0.026 ± 0.003
-	A-B bufer pH 6.0	10.2299 ± 3.3099	0.0757 ± 0.0268	1212.35 ± 337.82	9.46 ± 3.47	0.040 ± 0.007
-	Lactate buffer pH4.0	10.6272 ± 1.7529	0.1897 ± 0.0318	1274.32 ± 175.32	24.67± 3.72	0.072 ± 0.013
HP-β-CD	(corrected values)	4.0517 ± 2.7600	0.1502 ± 0.0347	456.08 ± 305.42	20.154 ± 3.551	0.460 ± 0.168
CS G	(corrected values)	7.5141 ± 5.9510	0.1270 ± 0.0190	949.42 ± 757.74	16.401 ± 1.540	0.403 ± 0.139
CS J	(corrected values)	3.9569 ± 2.7454	0.1201 ± 0.0053	641.86 ± 517.52	14.311 ± 1.466	0.529 ± 0.164

Value = mean ± SD, n = 3 rats/group

Linear regression performed from 0 to 120 min

enhancers. The analysis of protein and phosphorus release from the rat nasal mucosa caused by various cyclodextrins was one of the approaches used in their study. Five cyclodextrins, namely, dimethyl-β-cyclodextrin (DM-β-CD), α-CD , β-CD, HP-β-CD and ϒ-CD, were selected. The perfusion medium was normal saline in all the experiments and dextrose solution was used as a control. Table 12 compares the experimental results of Shao et al. (1992a) with the results of this part. Three parameters can be compared, i.e. the release rate of total protein, the release rate of total phosphorus and the total phosphorus released at 90 min. The common enhancer of these two studies was 5% HP-β-CD. However, the medium used in dissolving HP-β-CD in the paper of Shao et al.(1992a) was normal saline whereas the medium of this study was isotonic acetate-borate buffer pH 7.4. Their results of HP-β-CD with respect to the three parameters agreed quite well with our data. The slightly higher values for HP-β-CD obtained in our laboratory could be due to the different buffer effect. As can be seen from Table 12, the borate-acetate buffer pH 7.4 used in this study induced somewhat faster release of protein and phosphorus than that of the dextrose solution reported in Shao's paper, assuming that the dextrose solution caused the protein and phosphorus release to the same rate and extent as the normal saline.

By comparing the results with that of Shao et al. (1992a), it can be deduced that the two chitosans used in this study, CS G and CS J, at their respective optimum pH (pH 6.0 and 4.0), are as safe as HP-β-CD, as judged from the protein and phosphorus release data. Furthermore, their effect in enhancing the nasal absorption of [D-Arg^2]-Kyotorphin was greater than HP-β-CD. At the concentration as low as 0.02%, the enhancing activity of both CS J and Cs G was already equivalent to 5%

Table 12 Comparison of the experimental results of Part II with Shao et al (1992)

Enhancer	Rate of protein release (μg/ml/min)		Rate of total phophorus release (μg/ml/min)	
	Result of Shao*	Result of Part II	Result of Shao *	Result of Part II
DM-β-CD	132.40 ± 23.38	-	0.734 ± 0.217	-
α-CD	25.70 ± 6.87	-	0.354 ± 0.038	-
β-CD	10.67 ± 3.80	-	-	-
γ-CD	2.55 ± 0.05	-	-	-
Dextrose	1.09 ± 0.82	-	0.038 ± 0.019	-
HP-β-CD	7.46 ± 1.87	9.37 ± 2.76	0.171 ± 0.001	0.233 ± 0.019
CS G	-	17.65 ± 5.90	-	0.203 ± 0.019
CS J	-	15.72 ± 4.19	-	0.310 ± 0.005
Buffer pH 7.4	-	5.32 ± 1.31	-	0.072 ± 0.005
Buffer pH 6.0	-	10.23 ± 3.31	-	0.076 ± 0.027
Buffer pH 4.0	-	10.63 ± 1.75	-	0.190 ± 0.032

* 1. Perfusion medium was normal saline in all experiments.

2. Linear regression was performed from 15 to 90 min

Table 12 Comparison of the experimental results of Part II with Shao et al (1992) (continued)

Enhancer	Total phosphorus at T_{90} (μg/ml)	
	Result of Shao *	Result of Part II
DM-β-CD	87.22 ± 11.36	-
α-CD	42.91 ± 7.02	-
β-CD	23.71 ± 5.21	-
γ-CD	11.37 ± 0.63	-
Dextrose	7.44 ± 1.04	-
HP-β-CD	12.47 ± 0.58	18.71 ± 3.91
CS G	-	22.55 ± 2.57
CS J	-	29.45 ± 1.04
Buffer pH 7.4	-	6.40 ± 0.54
Buffer pH 6.0	-	7.52 ± 2.17
Buffer pH 4.0	-	13.88 ± 1.52

* Perfusion medium was normal saline in all experiments.

HP-β-CD. At 0.5% concentration, CS J and CS G were significantly more effective than 5% HP-β-CD ($p < 0.05$).

Shao et al.(1992a) also reported that DM-β-CD caused much greater release of protein and phosphorus than HP-β-CD. Data in Table 12 reveal that the rate of protein release of DM-β-CD was 17 folds over HP-β-CD whereas the rate of total phosphorus release was 4 folds and the concentration of total phosphorus at T_{90} was 7 folds. The enhancing effect of DM-β-CD was also much greater than HP-β-CD (Irie et al.,1992). However, more studies are needed to find out whether the two chitosans (CS G and CS J) possess the strong adjuvant property similar to DM-β-CD. At 0.1% concentration, they appear to be less membrane-irritating than 5% DM-β-CD and possess an absorption enhancing activity equal to or greater than 5% HP-β-CD with equivalent safety profile.

Part III : Effects of Chitosans on the Histological and Morphological Integrity of the Rat Nasal Mucosa

To enable comparison of the effects of different enhancers on the nasal membrane, the evaluation of histological changes was employed. This part was performed to observe morphological changes of the nasal mucosa in the presence of the enhancers selected from the previous part, i.e. CS G and CS J under the extreme experimental conditions. To examine this effect, HP-β-CD was also used as a reference enhancer in this part since it was considered to be one of the least membrane damaging (Shao et al.,1992a). There were seven investigational groups. Three groups consisted of the enhancers, i.e. 5% w/v HP-β-CD in isotonic acetate-borate buffer pH 7.4; 1% w/v CS G in isotonic acetate-borate buffer pH 6.0; and 1% w/v Cs J in isotonic lactate buffer pH 4.0. The other three groups were their respective buffers. The last one was the undosed control group. CS G and CS J solutions were prepared at the higher concentration (1% instead of 0.5 or 0.1%) in order to examine their safety under the more rigorous conditions. Three rats were used in each group. The individual nasal specimen from each rat was cross-sectioned into 5 regions as described in Chapter III. Each cross-sectioned region was separated into the left and right sides by the nasal septum. The left side was undosed and thus served as self-control for comparison with the right side which was treated with either the enhancer or its buffer.

After the rats were dosed intranasally for 2 weeks, their nasal mucosa were investigated for any morphological changes. The criteria for grading the membrane damaging effects caused by the absorption enhancers have been set up and included

the following morphological signs as shown in Table 13. These morphological manifestations were classified into 3 groups according to their level of irritation (mild, moderate or severe). For example, hypersecretion and goblet cell distention were considered to be the symptoms of only mild level of irritation whereas vascular congestion and subepithelial edema were considered to be moderate. The morphological signs for severe irritation included the observation of ectopic nuclei, sloughing of epithelial cells and/or hemorrhage (Table 13).

Table 13 Morphological signs of the rat nasal mucosa utilized as criteria for grading the membrane-damaging effects of the absorption enhancers.

Degrees	Histological lesions
i. Mild	- Hypersecretion (nasal discharge presentation) - Goblet cell distention
ii. Moderate	- Vascular congestion - Subepithelial edema - Discontinuation of epithelial cells - Cellular infiltration i.e. lymphocytes
iii. Severe	- Ectopic nuclei of epithelial cells - Sloughing of epithelial cells - Hemorrhage

The major structural features in each of the five regions of the rat nasal cavity were identified in the cross-sections produced from each rat, including the nasal septum separating the two sides of the cavity. In sections prepared from the undosed

animals, the results agree with Chandler et al.(1992a) in that a continuous epithelial layer was covering all the surfaces of the rat nasal cavity. Keratinised stratified squamous epithelium covered the surfaces in the region (i) and extended into the ventral meatus of each side of the nasal cavity in region (ii). In region (ii) the septum was covered with typical respiratory epithelium, i.e., the ciliated pseudostratified columnar epithelium with mucus filled goblet cells. The epithelium on the anterior turbinate surfaces was pseudostratified and cuboidal in form. It was only sparsely ciliated and contained few goblet cells.

Transitions to the olfactory epithelium occurred in the more posterior regions of the cavity, particularly in region (iii). In these transitional areas epithelium height was more variable. Olfactory epithelium was presented throughout the ethmoid recess in regions (iv) and (v). Thus, several types of epithelium were identified throughout the nasal cavity. Epithelium of a particular type was not always consistent between animals, particularly with respect to the epithelial height and sites of transitional zones. However, the epithelium on the left and right sides of the nasal cavity in each cross-section matched each other with respect to the distribution of the different epithelium types and height (Chandler et al.,1992a). The respiratory epithelium of the nasal septum appeared to be the most consistent in composition over a relatively large area.

Table 14 summarizes the extent of the morphological changes observed after exposure of the nasal mucosa to the enhancers. Figure 23 to 33 are the microscopic appearances obtaining from the seven groups of rats. The control group showed no signs or lesions. The epithelial cells remained unaffected in all sections studied

(Figure 23). However, the undosed group also demonstrated slight mucus secretion which was considered to be normal incidence since the rats were not kept in a tightly controlled, dust-free area. The buffer treated groups, demonstrated only a slight increase in goblet cell distention when compared to the undosed group. This sign observed in all three groups of buffers, i.e. buffer pH 7.4, 6.0 and 4.0 (Figure 24.) The results from the exposure to HP-β-CD were the moderate observation of goblet cell distention and hypersecretion (Figures 25 and 26) Some mucus secretions were found to coat on the epithelial layer in region ii . Slight subepithelial edema was also observed in this treatment group (Figure 25). Subepithelial edema is characterized as an enlargement of the subepithelial layer that could be identified by comparison with the other side of the nostril (the left). The treatment with CS G demonstrated the moderate extent of hypersecretion and extensive distention of the goblet cells (Figures 27-29). There were also some secretions coated on the epithelial layer (Figure 27). In the last group, CS J, the observed changes were characterized by moderate hypersecretion, goblet cell distention (Figure 30) and moderate vascular congestion in the subepithelial layer (Figure 31). There were also observations of slight subepithelial edema and slight cellular infiltration (Figure 32). Partial discontinuation of the epithelial cells was also slightly observed in this treatment, particularly in the deeper section (region v) of the nasal cavity (Figure 33). These symptoms are considered to reflect the moderate degree of irritation. However, they are all reversible symptoms and the mucosa can recover itself within a few days. In all cases, the epithelium remained attached to the lamina propria. All the cilia were present although the ciliary function was not evaluate in this study.

Table 14 Summary of the effects of chitosans on morphological changes of the rat nasal mucosa after two-week daily intranasal administration (n = 3 rats/group)

Treatments	Mild		Moderate				Severe		
	MS	GD	VC	SE	DE	CI	EN	H	SL
Untreated control	+	-	-	-	-	-	-	-	-
Buffer pH 4.0	+	+	-	-	-	-	-	-	-
1% CS J pH 4.0	++	++	++	+	+	+	-	-	-
Buffer pH 6.0	+	+	-	-	-	-	-	-	-
1%CS G pH 6.0	++	+++	-	-	-	-	-	-	-
Buffer pH 7.4	+	+	-	-	-	-	-	-	-
5%HP-β-CD pH 7.4	++	++	-	+	-	-	-	-	-

Extent of occurrence

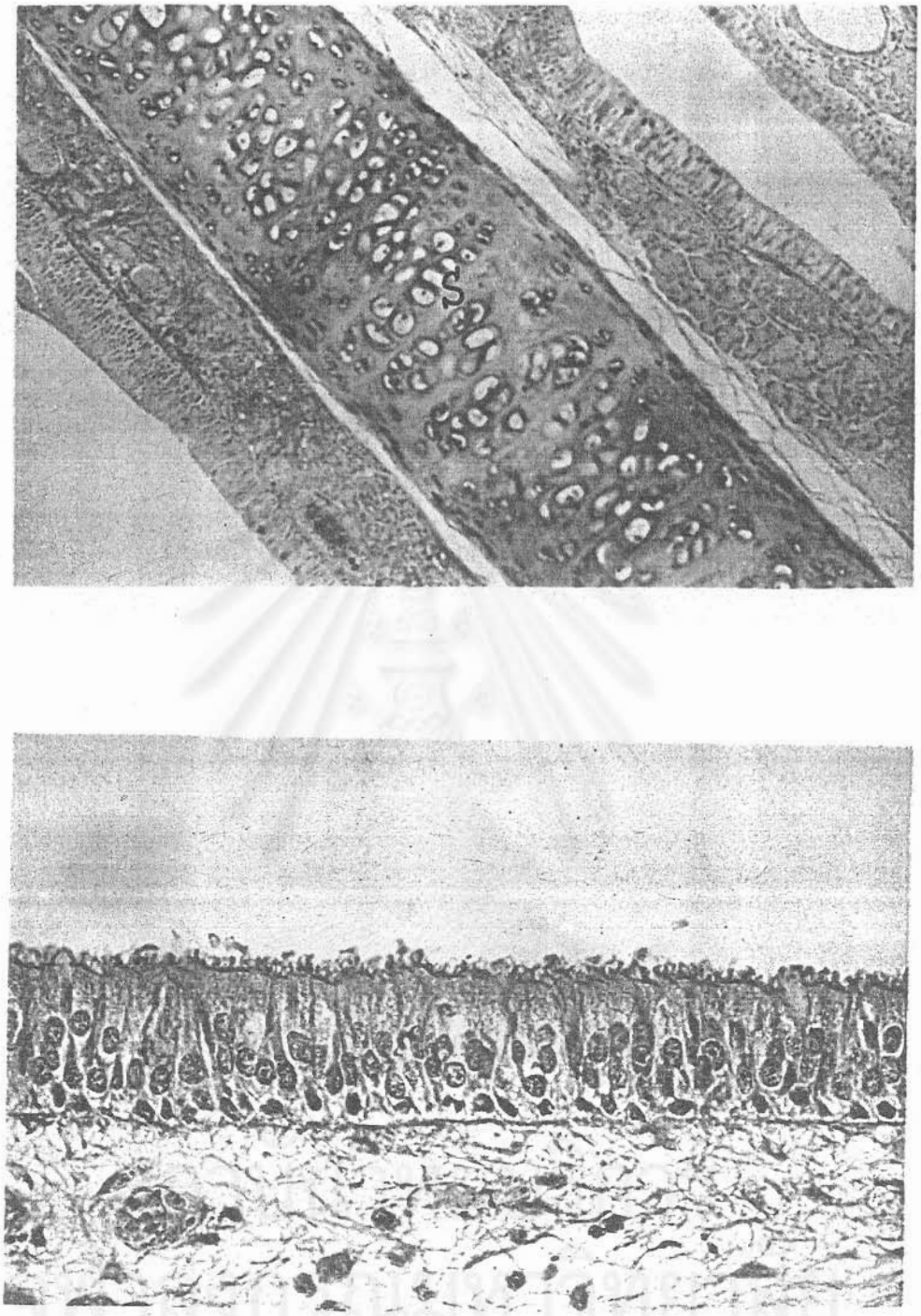
- \- Not observed
- \+ Slightly observed
- ++ Moderately observed
- +++ Extensively observed

Abbreviations

MS = Mucus secretion
GD = Goblet cell distention
VC = Vascular congestion
SE = Subepithelial edema
DE = Discontinuation of epithelium
CI = Cellular infiltration
EN = Ectopic nuclei
H = Hemorrhage
SL = Sloughing of epithelium

Results from this evaluation indicated that HP-β-CD, CS G and CS J produced some morphological changes in the rat nasal mucosa. The effects were mild to moderate irritation. The signs of severe irritation were not observed in all cases. Comparison of the results among these three enhancers indicated that CS J was somewhat more irritating than HP-β-CD. The lowest irritating enhancer was CS G, showing only the signs of mild irritation.

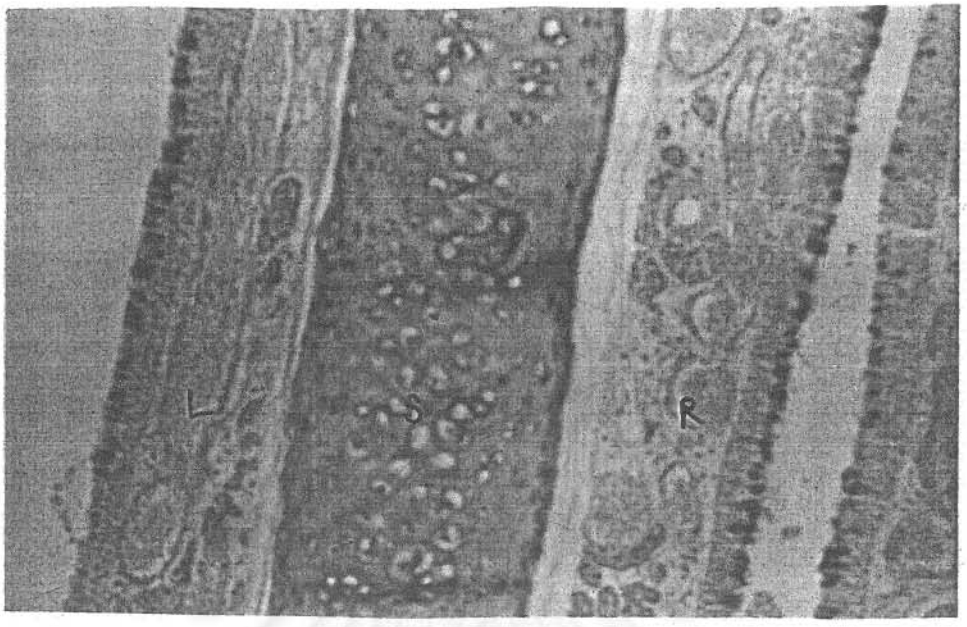
Apart from the slight subepithelial edema observed with HP-β-CD, its effects on the rat nasal mucosal integrity were nearly the same as CS G. The three buffer-treated groups also showed very little changes in the nasal mucosa morphology. Only slight mucus secretion and goblet cell distention were observed. This was not unexpected since these two symptom reflect the self-defense mechanism of the nasal mucosa in response to any foreign particles including the liquid droplets.



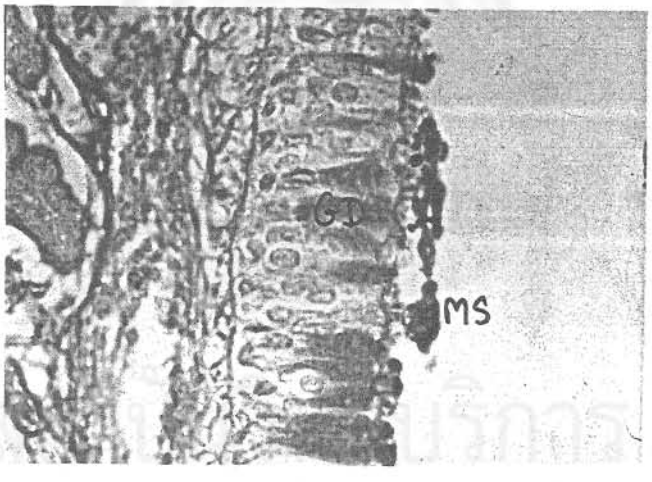
A

B

Figure 23 Light micrograph of the rat nasal mucosa of the untreated control rat from region ii. Normal appearing respiratory epithelium is present in this transverse section. The epithelium shows good intact with well pronounced cilia. Nasal septum (S). A = 100x (Alcian blue stain); B = 400x (HE stain)

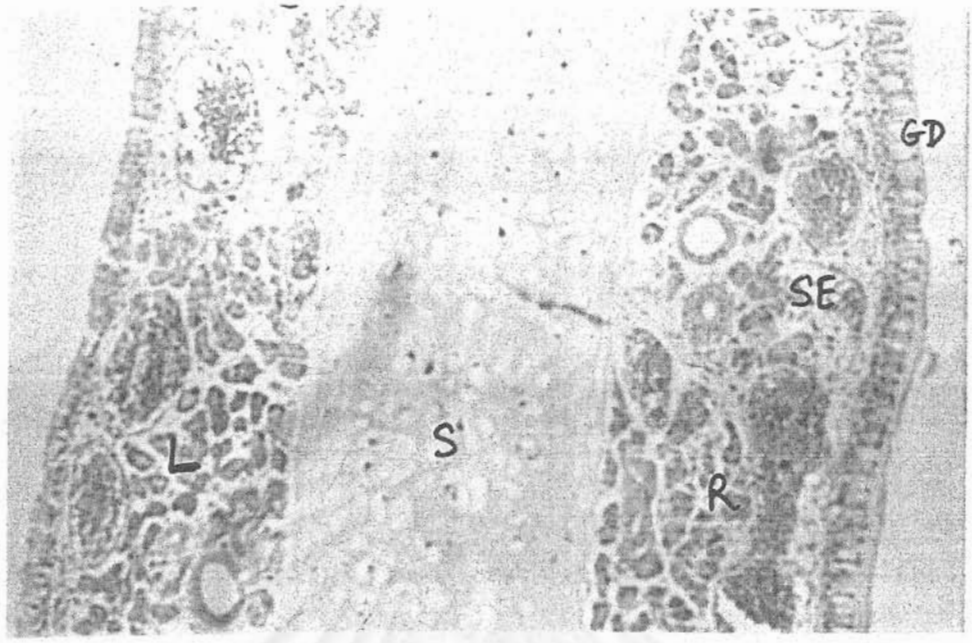


A

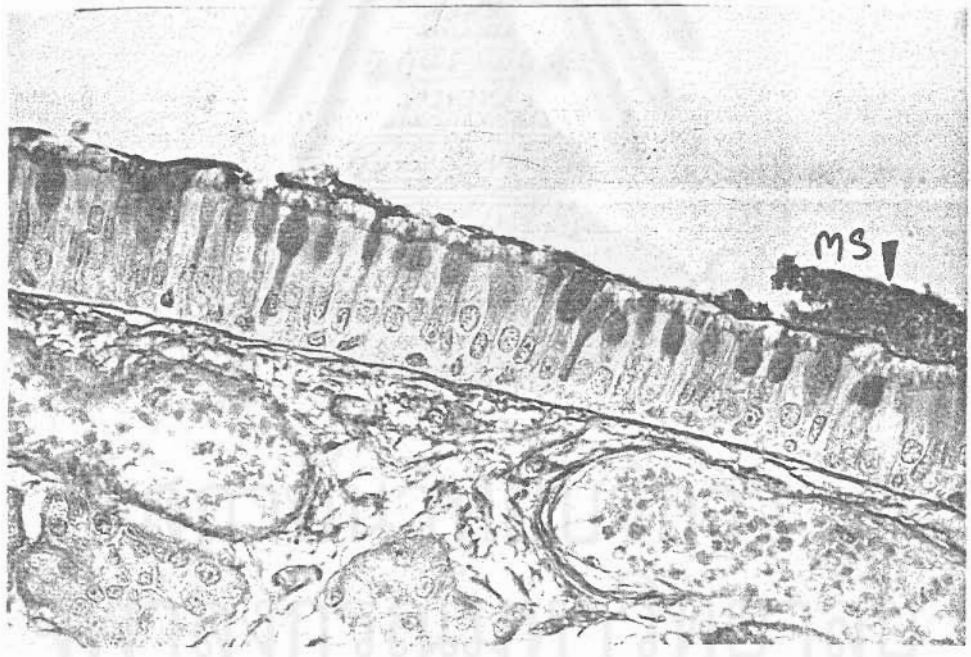


B

Figure 24 Light micrograph taken from region ii transverse section of the rat nasal mucosa following exposure to lactate buffer pH 4.0. The dosed side (R) of the cavity demonstrates more goblet cell distention (GD) and mucus hypersecretion (MS) than the undosed side (L). Nasal septum (S). Alcian blue stain. A = 100x; B = 400x. The other buffers, i.e., acetate-borate buffers pH 6.0 and 7.4, show similar appearances.



A



B

Figure 25A (100x) Light micrograph taken from region ii transverse section of the rat nasal mucosa following exposure to 5 % HP-β-CD. The dosed side (R) shows moderate goblet cell distention (GD) and slight subepithelium edema (SE). HE stain, the undosed side (L), nasal septum (S). Figure 25B (400x) illustrates mucus secretion (MS) coated on the ciliated epithelial layer only on the dosed (R) side (arrowhead).

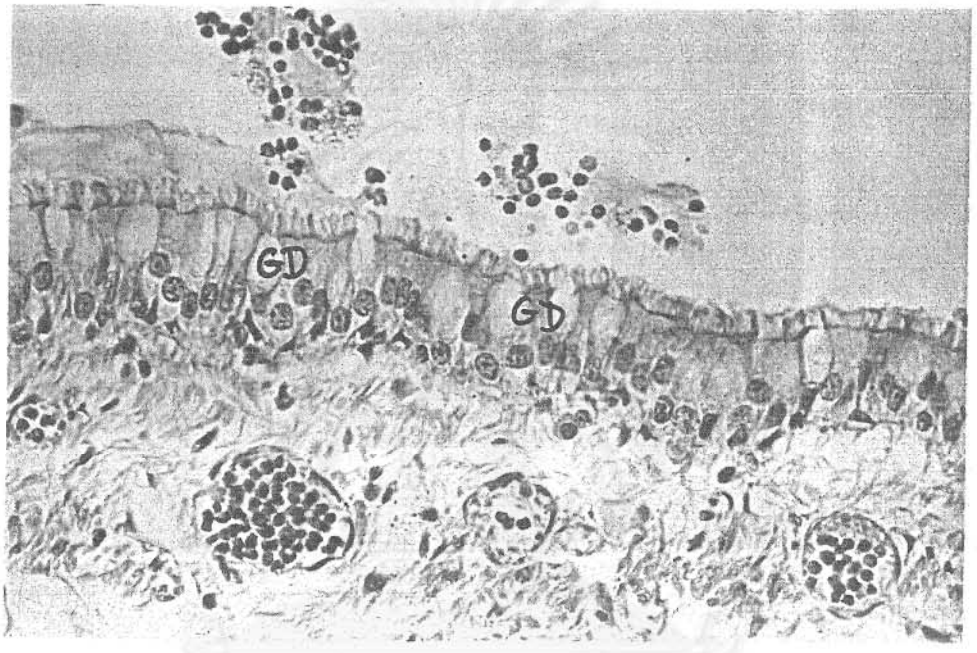
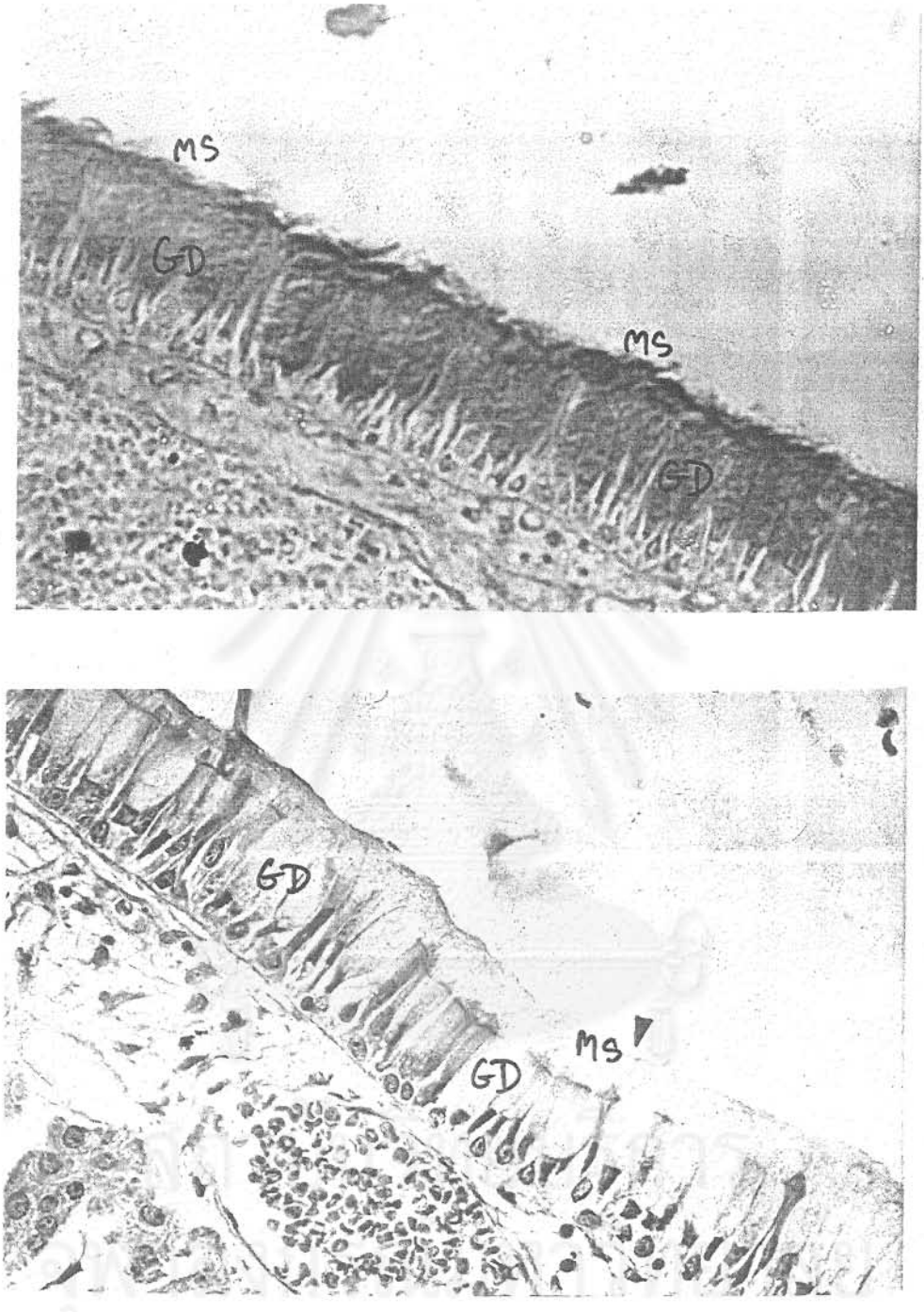


Figure 26 Light micrograph taken from region v transverse section of the rat nasal mucosa following exposure to 5 % HP-β-CD. The moderate distention of goblet cells (arrowhead) is present in the epithelium. HE stain (400x).



A

B

Figure 27 Light micrograph from region ii transverse section of the rat nasal mucosa following exposure to 1 % CS G. The dosed side (R) shows extensive distention of goblet cells (GD) and moderate hypersecretion. There is some secretion coated on the ciliated epithelium (arrowhead). Alcian blue stain (A) and HE stain (B). 400x.

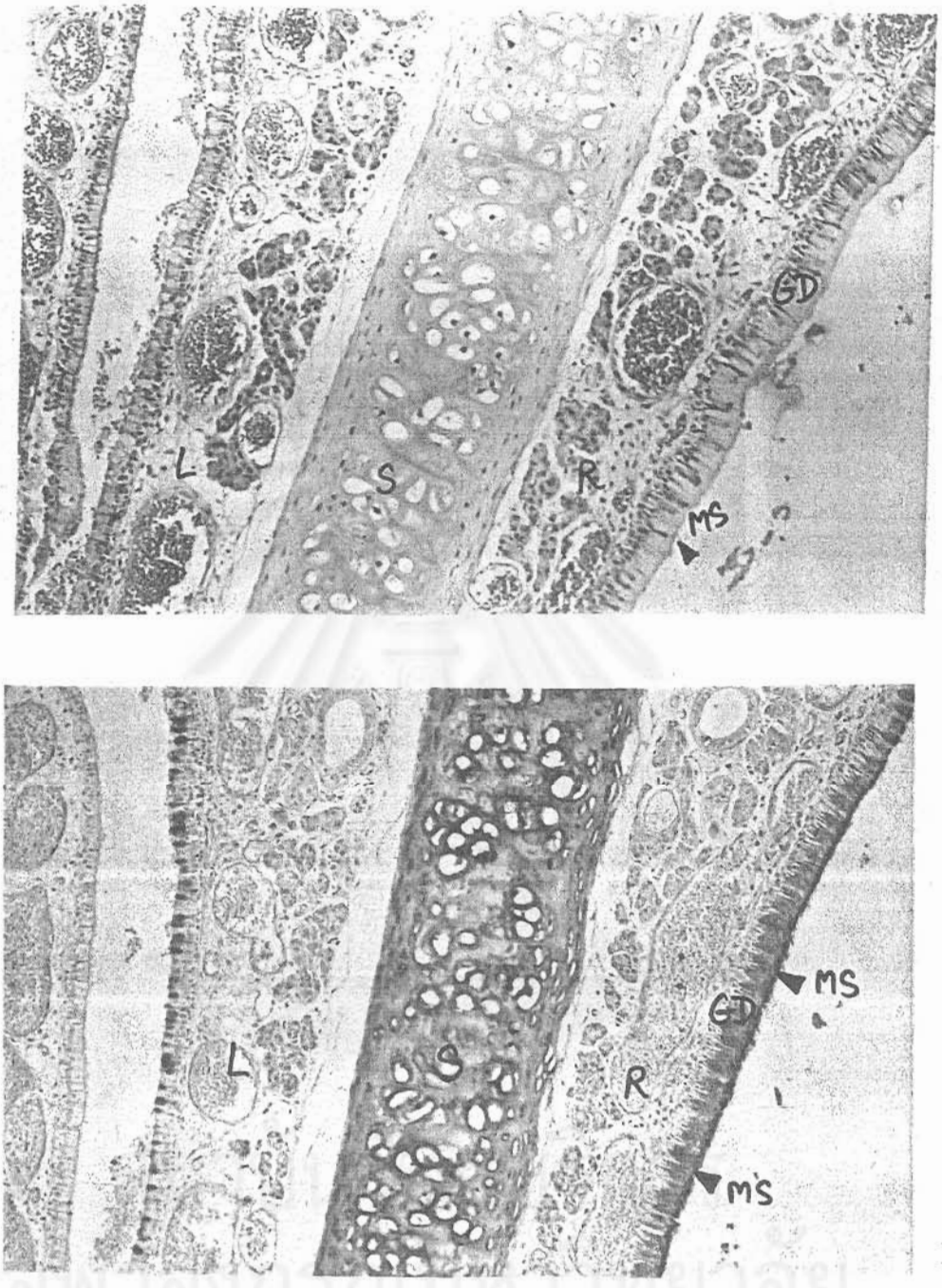


Figure 28 Light micrograph taken from region ii transverse section of the rat nasal mucosa following exposure to 1 % CS G. HE stain (A) and alcian blue stain (B). The dosed side (R) exhibits extensive distention of goblets cells and moderate hypersecretion (arrowheads). Nasal septum (S), the left side (L). A, B = 100x.

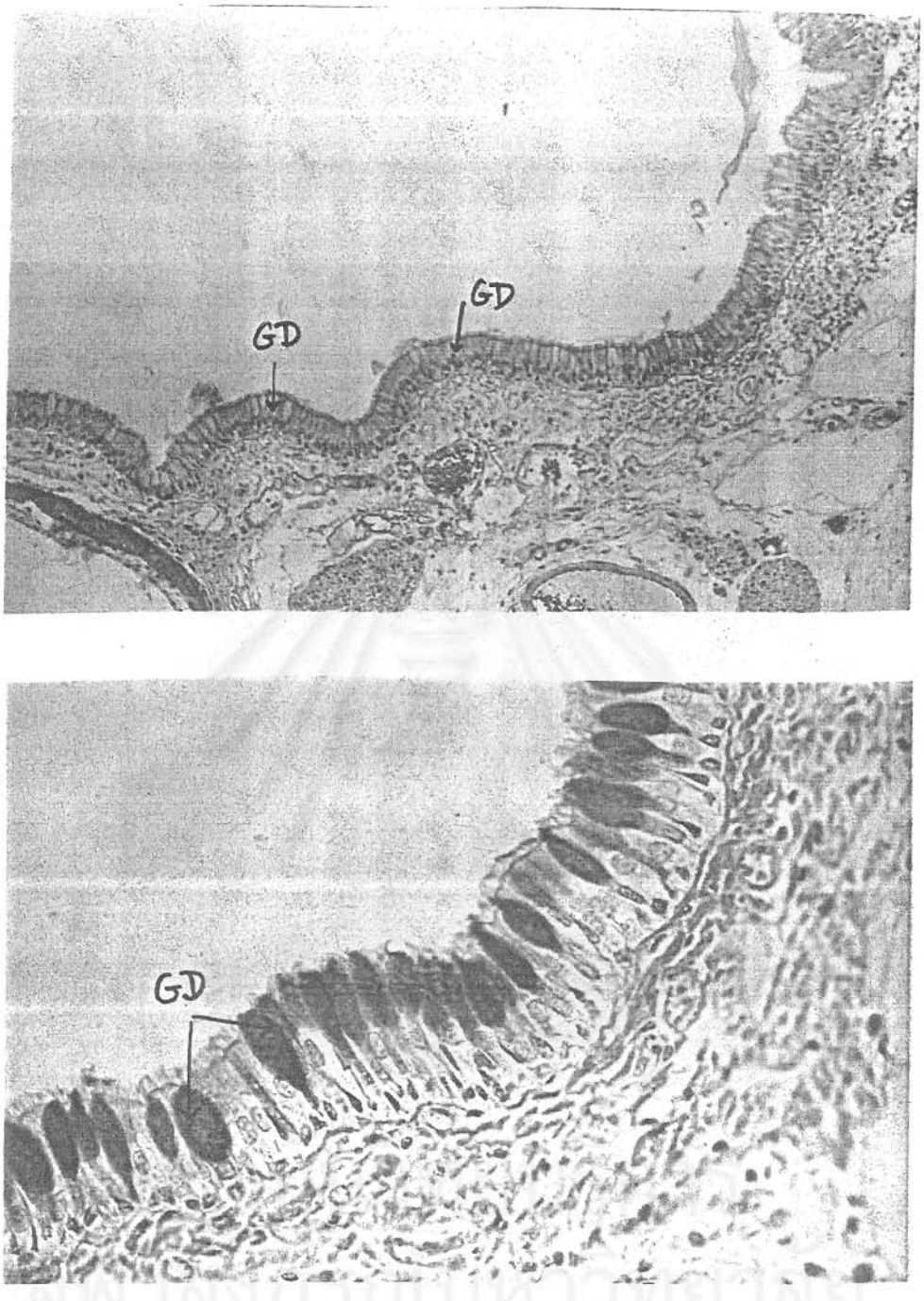


Figure 29 Light micrograph taken from region v transverse section of the rat nasal mucosa following exposure to 1 % CS G. HE stain (A) and alcian blue stain (B). The arrowhead pointing at the pale intracytoplasmic vacuole cells (A) and the blue staining (B) are indicative of increased goblet cell activities (goblet cell distention). A = 100x; B = 400x.

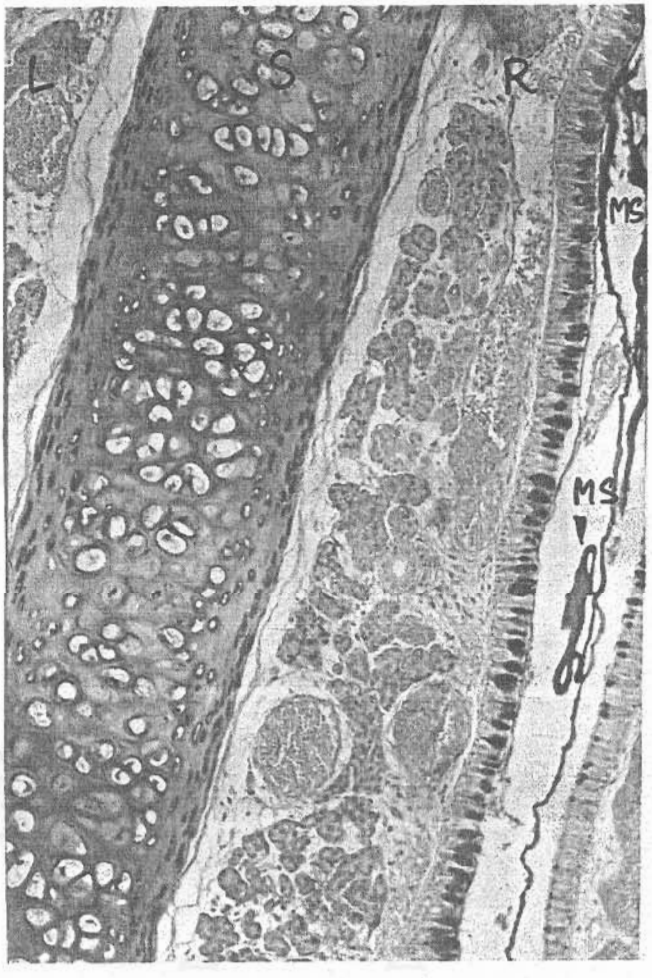


Figure 30 Light micrograph taken from region ii transverse section of the rat nasal mucosa following exposure to 1 % CS J. The dosed side (R) shows moderate goblet cell distention and hypersecretion. Mucus also shows in the lumen (arrowhead). Alcian blue stain. Nasal septum (S), the undosed side (L). 80x.

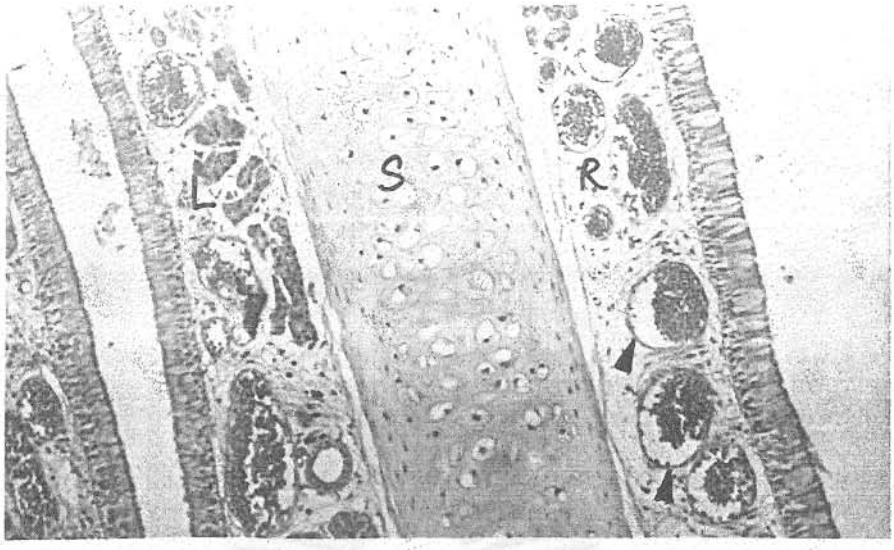


Figure 31 Light micrograph taken from region ii transverse section of the rat nasal mucosa following exposure to 1 % CS J. Vascular congestion (arrowhead) is slightly observed on the dosed side (R) of the nasal cavity (100x). HE stain. Nasal septum (S), the undosed side (L).

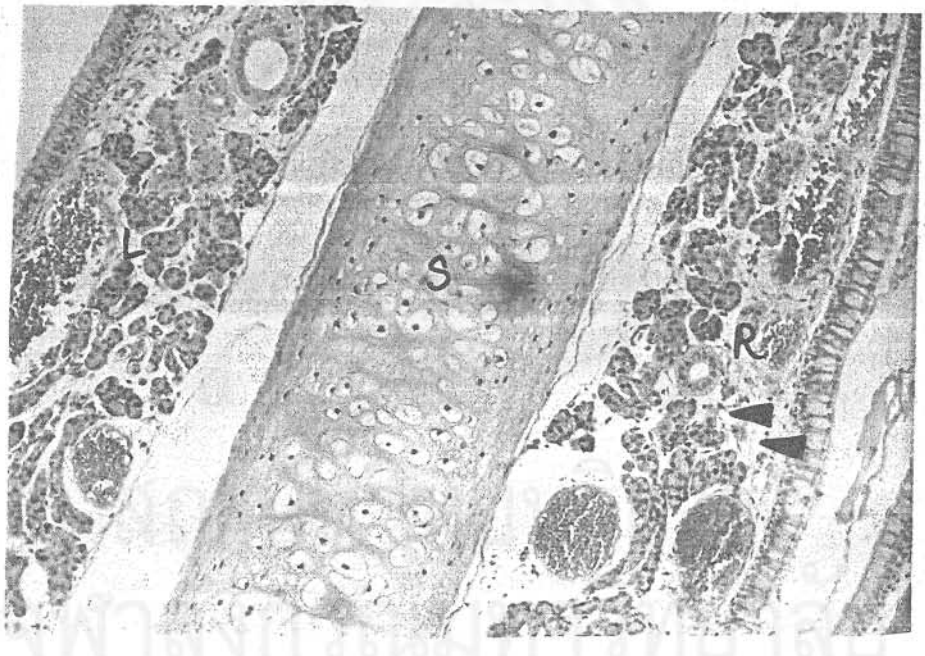
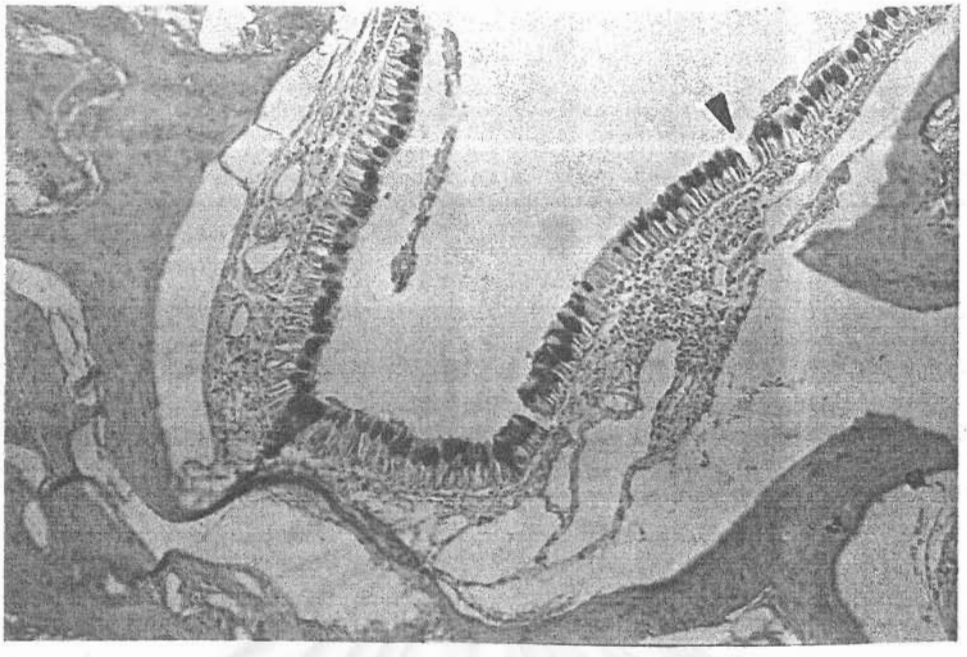
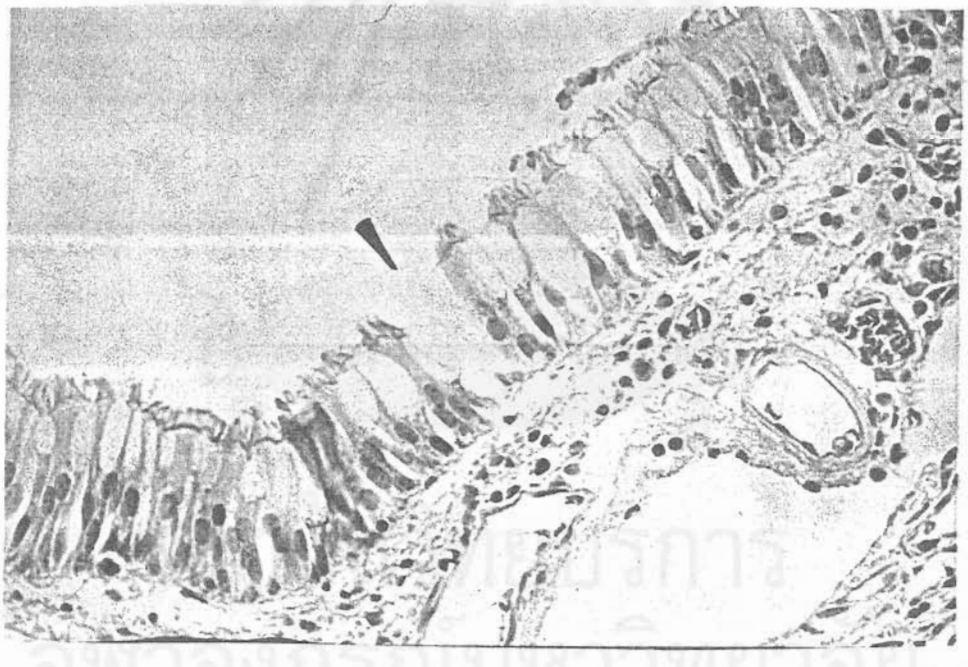


Figure 32 Light micrograph taken from region ii transverse section of the rat nasal mucosa following exposure to 1 % CS J. Subepithelial and cellular infiltration (arrowhead) are slightly observed on the dosed side (R) of the nasal cavity (100x). HE stain. Nasal septum (S), the undosed side (L).



A



B

Figure 33 Light micrograph taken from region v transverse section of the rat nasal mucosa following exposure to 1 % CS J. Alcian blue stain (A) and HE stain (B). The epithelium shows slight discontinuation of the cells (arrowhead). A = 100x; B = 400x.

Part IV. Study to Determine the Reversibility of the Nasal Membrane Integrity following Removal of Chitosans from the Rat Nasal Mucosa (Experiment 5.4 in the Proposal)

Table 15 shows the contents (U/ml) of lactate dehydrogenase (LDH) that were released from the rat nasal mucosa during the *in situ* perfusion in the presence of various enhancers (the first period) and *after* removal of the enhancer from the nasal cavity (the second period).

The control experiment was also carried out by perfusing the rat nasal cavity with only the isotonic saline pH 6.0 in both periods. This pH condition was fixed in all perfusion experiments regardless of the enhancers because preliminary results have shown that high activity of LDH was observed at this pH. Martinek (1972) reported that LDH has an optimum activity at pH 6.8 while Pujara et al. (1995) observed the loss of LDH activity at pH below 4.0. Since the perfusion could not be run at pH 6.8 due to precipitation of CS G at pH higher than 6.0, pH 6.0 was thus selected as the most favorable common pH condition for all the enhancers under study.

The concentration of CS J and CS G was set at 0.5 % w/v which was increased from 0.1 % w/v used in the previous experiments (Part II: protein and phosphorus release study). Although 0.1 % was already effective in enhancing the nasal absorption of [D-Arg^2]-Kyotorphin, the concentration was raised to 0.5 % in this study in order to see more clearly any specific effect of chitosans on the integrity of the rat nasal epithelium as indicated by the leakage of LDH. Comparison was then made with 5 % HP-β-CD as usual since it is one of the least irritating enhancers reported so far with respect to nasal morphology (Chandler et al., 1991b), mucosal component release (Shao et al., 1992a; Marttin et al., 1995), and ciliotoxicity (Merkus et al., 1991). Another cyclodextrin-type enhancer (DM-

β-CD) was also studied for comparison since it has been shown to be a very effective nasal absorption enhancer of insulin and other steroid hormones (Merkus et al.,1991; Schipper et al., 1990). The concentration of DM-β-CD used in this study was set at 1.25 % which was lower than that usually employed in the *in vivo* nasal absorption studies (2-5 %). However, preliminary results have shown that *in situ* nasal perfusion using lower concentrations of DM-β-CD can better maintain the viability of the rat throughout both perfusion periods.

Data in Table 15 reveal that perfusion with only the isotonic saline (control group) resulted in very low levels of LDH release regardless of the perfusion period. At the end of the first hour, the average LDH content was 38.33 $\pm$ 9.93 U/ml. The value of the control group agreed well with the results of Shao and Mitra (1992b) who reported that nasal perfusion with only isotonic phosphate buffer (pH 7.4) for 90 min gave LDH release of 33.0 $\pm$ 18.0 U/ml.

After the perfusate had been replaced with fresh isotonic saline and the rat nasal cavity was reperfused, the LDH content at the end of second hour increased only slightly to 70.20 $\pm$ 37.04 U/ml. When paired Student's t-test was applied at 5 % significance level, there was no difference between the LDH contents at the end of the first and second perfusion periods ($p > 0.05$).

Perfusion with 5 % HP-β-CD yielded the same results as that of control, i.e, there was very small amount of LDH release at the end of the first perfusion period, with the average content at 60 min of 50.15 $\pm$ 8.93 U/ml. Furthermore, when HP-β-CD was removed by flushing the nasal cavity and the perfusion was restarted using only the isotonic saline, there was practically no change in the release profile of LDH, with the average content measured at the end of second period of 49.73 $\pm$ 19.93 U/ml (Table 15). The value did not differ significantly from that of the first hour ($p > 0.05$).

TABLE 15 Concentration (U/mL) of lactate dehydrogenase (LDH) in the nasal perfusates. Comparison of the release profiles between the first hour of perfusion (with enhancer) and the second hour of perfusion (without enhancer).

Data : mean ± SD (n = 4 rats/group)

Control group	n	1st hour (Saline only)				2nd hour (Saline only)				ΔC_{60}
		(min) 15	30	45	60	(min) 15	30	45	60	(U/ml)
	1	20	80.72	50.27	51.95		97.89	101.25	117.95	66
Isotonic	2	53.33	35.25	36.45	37.65	13.33	20.48	21.2	61.92	24.27
saline	3	20	14.05	27.87	35.49	46.67	55.01	70.27	72.67	37.18
pH 6.0	4	40	20.65	34.33	28.24	40	13.98	40.88	28.24	0
	mean ± SD	33.33	37.67	37.23	38.33	33.33	46.84	58.40	70.20	31.86
		16.33	30.04	9.43	9.93	17.64	38.50	34.97	37.04	27.49

	n	1st hour (with CSJ)				2nd hour (Saline only)				ΔC_{60}
		(min) 15	30	45	60	(min) 15	30	45	60	(U/ml)
0.5% w/v	1	66.67	194.42	310.96	422.86	86.67	121.41	143.42	179.16	-243.7
CSJ	2	86.67	248.08	325.51	470.98	46.67	127.43	129.53	131.71	-339.27
pH 6.0	3	153.33	335.83	494.71	549.69	100	128.29	150.41	186.27	-363.42
	4	46.67	174.09	256.97	267.96	66.67	81.08	95.76	117.37	-150.59
	mean ± SD	88.34	238.11	347.04	427.87	75.00	114.55	129.78	153.63	- 274.25
		46.31	72.24	102.77	118.74	23.33	22.52	24.28	34.22	97.30

	n	1st hour (with CSG)				2nd hour (Saline only)				ΔC_{60}
		(min) 15	30	45	60	(min) 15	30	45	60	(U/ml)
0.5% w/v	1	273.33	424.44	584.79	674.62	126.67	195.39	191.96	255.18	-419.44
CSG	2	80	201.3	351.29	390.53	33.33	40.54	54.55	68.8	-321.73
pH 6.0	3	46.67	194.09	350.63	456.53	73.33	101.19	129.53	158.38	-298.15
	4	80	107.97	249.75	393.95	80	147.97	157.08	199.72	-194.23
	mean ± SD	120.00	231.95	384.12	478.91	78.33	121.27	133.28	170.52	- 308.39
		103.42	135.15	142.04	133.96	38.25	66.15	58.37	78.56	92.46

Table 15 (continued)

	n	1st hour (with HPβCD)				2nd hour (Saline only)				ΔC_{60}
		(min) 15	30	45	60	(min) 15	30	45	60	(U/ml)
5% w/v	1	40	48.11	49.79	58.13	13.33	13.81	54.29	32.88	-25.25
HPβCD	2	33.33	41.2	55.97	54.56	6.67	13.57	14.05	44.53	-10.03
pH 6.0	3	33.33	34.53	35.73	50.27	20	34.05	28.59	42.88	-7.39
	4	46.67	21.68	55.73	37.65	26.67	27.63	95.25	78.61	40.96
	mean ± SD	38.33	36.38	49.31	50.15	16.67	22.27	48.05	49.73	- 0.43
		6.39	11.26	9.49	8.93	8.61	10.24	35.60	19.93	28.69

	n	1st hour (with DMβCD)				2nd hour (Saline only)				ΔC_{60}
		(min) 15	30	45	60	(min) 15	30	45	60	(U/ml)
1.25% w/v	1	440	533.82	629.31	653.22	20	53.66	121.21	69.92	-583.3
DMβCD	2	446.67	367.26	380	386.39	33.33	47.21	47.99	55.46	-330.93
pH 6.0	3	333.33	312.09	503.91	505.71	40	47.32	61.43	49.13	-456.58
	4	393.33	526.4	475.1	496.41	60	80.98	115.65	130.92	-365.49
	mean ± SD	403.33	434.89	497.08	510.43	38.33	57.29	86.57	76.36	- 434.08
		52.35	112.27	102.83	109.53	16.67	16.08	37.26	37.40	112.72

ΔC_{60} = conc. LDH at the end of 2nd hour - conc. LDH at the end of 1st hour

On the other hand, perfusion with other enhancers (0.5 % CS J, 0.5 % CS G and 1.25 % DM-β-CD) resulted in substantial release of LDH in the nasal perfusates. The LDH contents at the end of the first hour were 427.87 ± 118.74, 478.91 ± 133.96, and 510.43 ± 109.53 U/ml for CS J, CS G and DM-β-CD, respectively (Table 15). The release profiles of LDH are shown in Figures 34 and 35 for each enhancer and the control group. When ANOVA was applied at 5 % significance level to the values of LDH at the end of the first hour, significance difference was detected among the five groups under study ($p << 0.05$). Multiple comparison using Tukey test was further employed in order to rank the effect. The results, in an increasing order, are as follows:

	Control	HP-β-CD	CS J	CS G	DM-β-CD
Enhancer conc.	0%	5%	0.5%	0.5%	1.25%
LDH (U/ml)	38.33	50.15	427.87	478.91	510.43

The lines underneath indicate that there was no significant difference in the LDH content at the end of the first hour among the groups on the same line ($p > 0.05$). Therefore, Tukey test results reveal that HP-β-CD caused minimal leakage of the enzyme and its effect was similar to saline control. This was also in agreement with Shao et al.(1992a) and Marttin et al.(1995) who reported that 5 % HP-β-CD was one of the least membrane damaging enhancers, giving minimal release of mucosal components. On the other hand, the other three enhancers caused significantly greater extent of LDH release than the control and 5 % HP-β-CD ($p < 0.05$). However, their effects were similar among each other ($p > 0.05$) as seen from the line joining the three groups.

In order to see if the effects of these enhancers on the nasal mucosal integrity were reversible, the residual enhancers were flushed from the rat

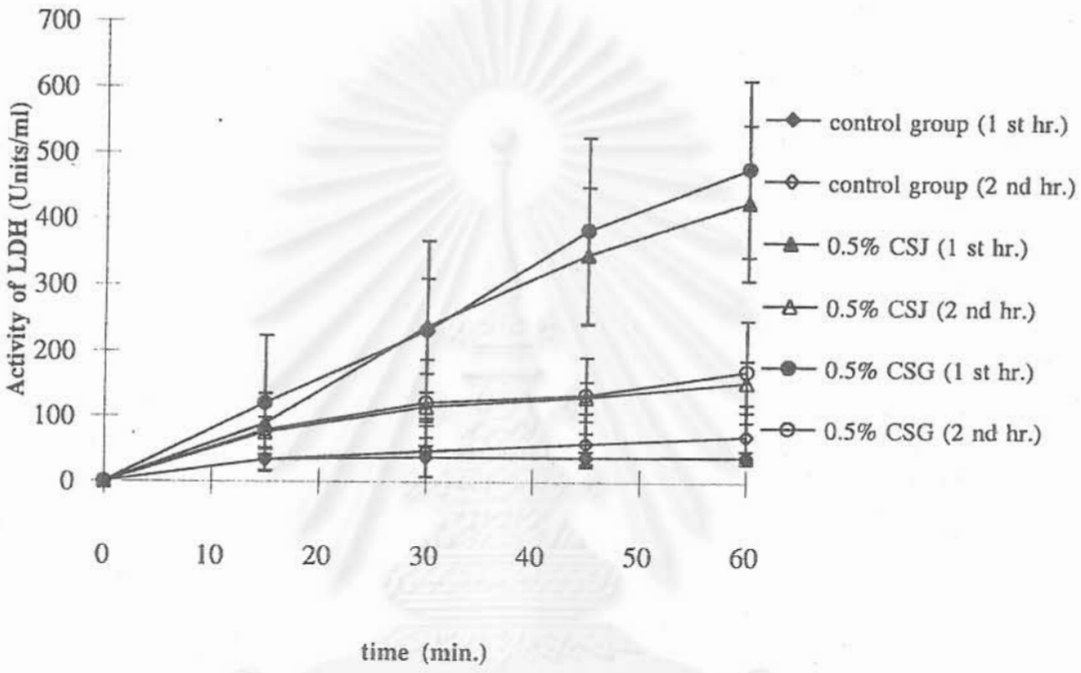


Figure 34 Plots of lactate dehydrogenase activity (LDH) released from the rat nasal mucosa during perfusion of isotonic saline in the presence of chitosans (1st hour) and *after* removal of chitosans (2nd hour). The control group was perfused with only isotonic saline in both periods. Data = mean $\pm$ SD (n = 4).

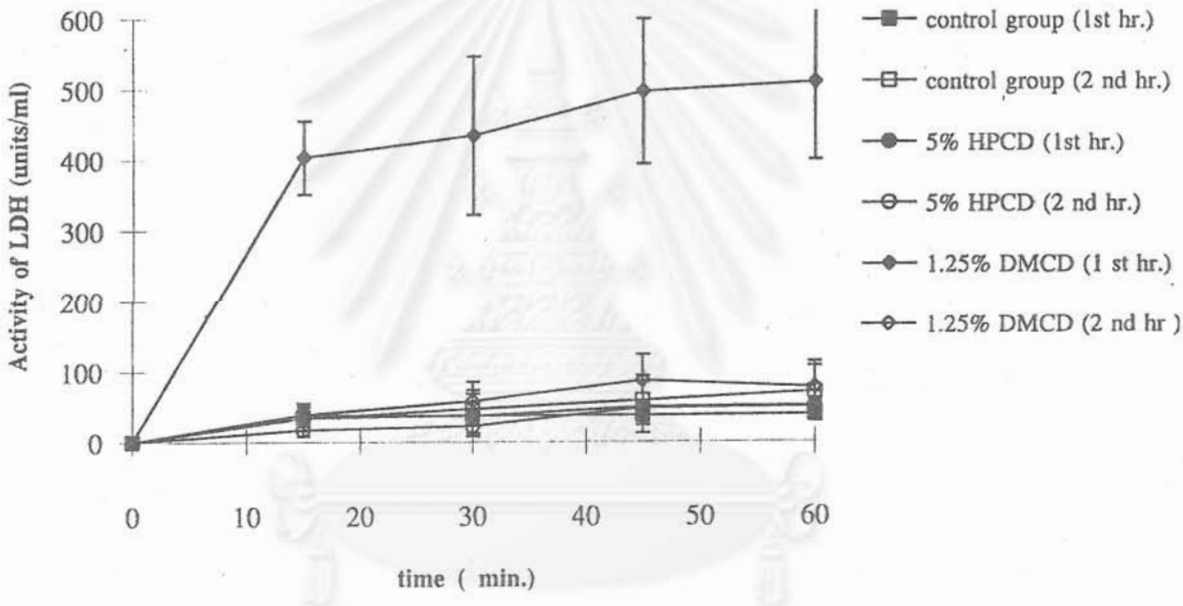


Figure 35 Plots of lactate dehydrogenase (LDH) activity released from the rat nasal mucosa during perfusion of isotonic saline in the presence of cyclodextrins (1st hour) and *after* removal of cyclodextrins (2nd hour). The control group was perfused with only isotonic saline in both periods. Data = mean $\pm$ SD (n = 4).

nasal cavity at the end of the first period and the perfusion was restarted using only the isotonic saline as the perfusing solution. Comparisons were then made between the LDH contents at the end of the first and second perfusion periods using paired Student's t-test at 5 % level for each enhancer. As previously noticed, the control and the HP-β-CD-treated groups gave the same minimum extent of LDH release regardless of the perfusion period. However, there were significant differences in the concentration of LDH at the end of perfusion between the first and second periods for all the other three enhancers (paired Student's t-test, $p < 0.05$). After removing CS J from the rat nasal cavity, the extent of LDH release at the end of perfusion dropped nearly three folds from 427.87 ± 118.74 to 153.63 ± 34.22 U/ml. In the case of CS G, the LDH content decreased from 478.91 ± 133.96 to 170.52 ± 78.56 U/ml. The value for DM-β-CD also sharply reduced from 510.43 ± 109.53 to 76.36 ± 37.40 U/ml, a value nearly similar to that of the control group at the same perfusion period (70.20 ± 37.04 U/ml). Summary of the extent of LDH release after 60 min perfusion in both periods is graphically represented in Figure 36.

The significant decrease in LDH content at the end of second perfusion indicates that, after removal of CS J, CS G and DM-β-CD, the membrane integrity and the permeability of the rat nasal mucosa appear to be reversible and that the mucosa is able to return to its normal original state. Since LDH is a cytosolic enzyme, leakage into the nasal perfusates could be a result of an increase in the membrane permeability and/or disruption of the epithelial cells caused by the absorption enhancers. However, increased membrane permeability appears to be the major mode of increasing LDH release. If cellular disruption were a major mechanism, the reversibility of the nasal mucosa should not have occurred within such a short time as observed here and substantial leakage of LDH would persist long after removal of the enhancer. Therefore, it can be concluded at this point

that the three enhancers may have a transient effect on the nasal mucosa, at least under the experimental conditions and concentrations employed in this study.

In order to compare the reversible effect of each enhancer, the difference in LDH concentration at the end of perfusion between the first and second period was calculated for each rat ($\Delta C = C_2 - C_1$; where C_1 and C_2 are LDH concentrations at the end of first and second perfusion, respectively). The data are also shown in Table 15. ANOVA was then applied to the values of ΔC at 5 % level. The results show significant differences among the five groups ($p < 0.05$) with the following rank order according to post ANOVA Tukey test:

	Control	HP-β-CD	CS J	CS G	DM-β-CD
Enhancer conc.	0%	5%	0.5%	0.5%	1.25%
ΔC (U/ml)	31.86	-0.43	-274.25	-308.39	-434.08

Tukey test result showed that 0.5 % CS J, 0.5 % CS G and 1.25 % DM-β-CD demonstrated similar transient effect on the rat nasal epithelium with respect to the LDH release ($p > 0.05$). The minus sign indicates the decrease in LDH after removal of the enhancer. On the other hand, the control and HP-β-CD-treated groups exhibited much lower ΔC values than the other three enhancers. (ΔC was even positive in the case of the control group). In fact, their Student's t-test results have shown that the LDH content did not differ significantly from the first period, indicating lack of membrane reversibility. Apparently, the lack of such reversible effect observed with HP-β-CD was due to its very mild effect on the LDH release.

Results from the first period of perfusion have shown that the effects of 0.5 % CS J and CS G on LDH release were equivalent to that of 1.25 % DM-β-CD. As previously stated, the concentration of DM-β-CD used in this study was lower than the effective concentrations used in enhancing the nasal absorption of peptide

drugs like insulin (2-5%) (Merkus et al.,1991: Schipper et al.,1992). Interestingly, 5% DM-β-CD was reported to cause a great deal of membrane damages as determined from the release of several nasal epithelial components (Shao et al., 1992a; Marttin et al., 1995), ciliostatic effect (Schipper et al., 1992), and tissue irritation studies (Yoshida et al.,1988). Shao et al. (1992a) reported that nasal perfusion of 5% DM-β-CD solution in 0.9% saline caused a drastic release of LDH, reaching about 6,120 U/ml at 90 min. In our study, 1.25% DM-β-CD caused LDH release of only 510.43 U/ml after 60 min perfusion. Marttin et al. (1995) also reported that 5% DM-β-CD induced substantial leakage of protein, albumin and cholesterol from the rat nasal mucosa to a greater extent than 2% DM-β-CD and 5% HP-β-CD. Thus, the membrane damaging effect of cyclodextrins was highly dependent on concentration and their chemical structure.

DM-β-CD was also found to be extremely hemolytic even at low concentration (Yoshida et al.,1988). This was in agreement with our results since bleeding of the nasal epithelium was observed within 30 min after perfusion with 5% DM-β-CD. The perfusate color turned light red and the animals often died prematurely, thereby discouraging further perfusion studies at this concentration. Even at 1.25 %, the nasal perfusate color appeared slightly orange in some rats, indicating some degree of hemolysis. However, no change in the perfusate color was detected in the control group as well as in the HP-β-CD- (5%) and the chitosans (0.5%)-treated groups.

Although 0.5% CS J and CS G caused greater release of LDH at the end of the first perfusion period than 5% HP-β-CD, the values of their LDH content were considered to be small compared to other enhancers like bile salts (Shao and Mitra, 1992b). Nasal perfusion of sodium deoxycholate and sodium glycocholate

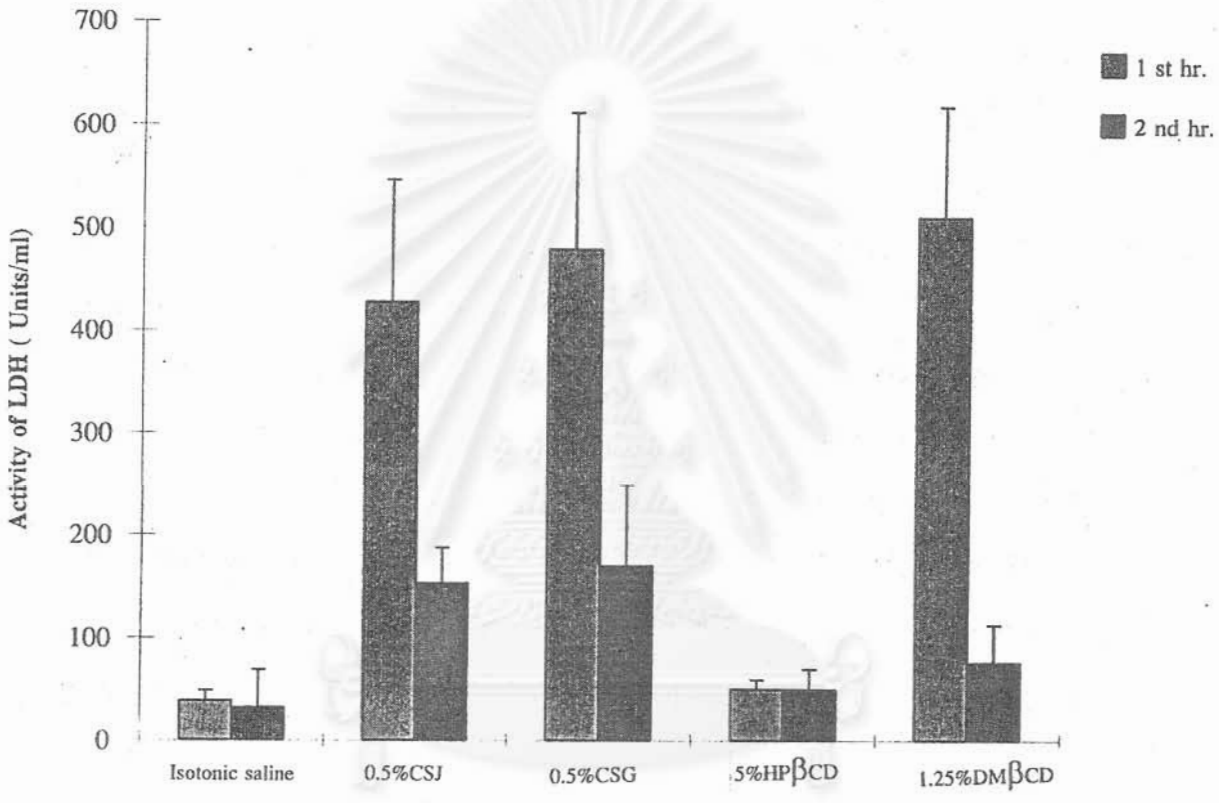


Figure 36 Comparison of lactate dehydrogenase (LDH) activities released in the nasal perfusates at 60 min between the 1st hour of perfusion (with enhancer) and the 2nd hour of perfusion (without enhancer). Data = mean $\pm$ SD (n = 4).

(15 mM each) resulted in LDH release at 90 min of 12,600 and 1,040 U/ml, respectively. These values obviously dwarfed the membrane-damaging effects of cyclodextrins and chitosans used in this study. Furthermore, it is likely that lower concentrations of chitosans will cause even less LDH release. Previous experiments have shown that nasal perfusion of 0.1% CS J and CS G resulted in the rate and extent of protein and phosphorus release which were similar to 5% HP-β-CD but much smaller than 5% DM-β-CD (See results and discussion in part II). Thus, depending on concentration, chitosans could be as safe as 5% HP-β-CD. However, HP-β-CD is not a potent absorption enhancer despite its very good safety profile. In many cases, it failed to enhance nasal absorption of several peptides even at high concentration of 5% (Verhoef et al., 1994). Therefore, the combined results from parts I - IV indicate the advantages of chitosans over the two cyclodextrins in terms of absorption enhancing efficacy and safety, i.e. at 0.1-0.5% concentrations, both CS J and CS G are more effective than 5% HP-β-CD and less membrane irritating than 5% DM-β-CD. Their enhancing effects also appear to be reversible. The extent of epithelial recovery is rapid and comparable to that of 1.25 % DM-β-CD.

Although the effects of 1.25% DM-β-CD, 0.5% CS J, and 0.5% CS G on the LDH release and membrane reversibility (ΔC) were similar ($p > 0.05$, Tukey test), DM-β-CD appeared to give the highest LDH release (510.43 U/ml). This is not unexpected since DM-β-CD is known to be highly membrane-irritating. However, its average ΔC value is also the highest (-434.08 U/ml), indicating its fairly good membrane reversibility at this relatively low concentration. It is possible that if higher concentrations of DM-β-CD were used (e.g. 2 or 5%), and if all the perfusions were carried out using more rats ($n > 4$), the statistical test results may have shown significantly greater values of LDH release and smaller values of ΔC for DM-β-CD than for 0.5% CS J and CS G.

CHAPTER V

CONCLUSION

The significance of peptides and proteins as a new class of bioactive agents has prompted a large number of investigations with a special focus on the nasal route as a potential alternative to parenteral administration. However, little is still known about the nature of the transport barriers and the mechanisms of peptide absorption via this route. Using an *in situ* nasal perfusion technique, transport of L-Tyrosyl-D-Arginine ([D-Arg^2]-Kyotorphin) across the rat nasal mucosa has been characterized. This dipeptide was chosen as a model compound in this research mainly because it is highly stable in the nasal perfusate without any detectable hydrolysis. However, even in the absence of enzymatic degradation, the *in situ* perfusion failed to show any absorption of this dipeptide from the nasal perfusate, indicating its poor membrane permeability and the need for the use of an absorption enhancer.

In general, peptides are poorly absorbed across the nasal mucosa and often require some types of absorption enhancers to effect significant nasal absorption. Therefore, various chitosans have been investigated as novel nasal absorption enhancers in this research.

The results from this study can be summarized as follows:

1. Five chitosans, namely CS J, CS L, CS H, CS G and CS HCl were evaluated for their possible absorption enhancing activities by comparing two parameters, i.e. the percent remaining of [D-Arg^2]-Kyotorphin in the rat nasal

perfusate after 120 min perfusion (T_{120}) and the apparent first order rate constant of drug absorption (k).

2. The pH for the optimum enhancing activities of CS J, CS L, CS H and CS HCl was pH 4.0 and that of CS G was pH 6.0. At their respective optimum pH, these chitosans (all at 0.5% w/v) gave varying enhancing efficacy. Comparison of their enhancing activities based on the percent [D-Arg^2]-Kyotorphin remaining in the perfusate at T_{120} using ANOVA revealed that there was a significant difference among these chitosans ($p < 0.05$) and that they could be classified into 3 groups of different enhancing effects, i.e. the first with the highest activity (CS J at pH 4.0), the second with the intermediate effect (CS G at pH 6.0) and the third group with the lowest effect (CS L, CS HCl and CS H at pH 4.0).

3. ANOVA also showed a significant difference in the apparent rate constants among the five chitosans ($p < 0.05$) with similar classification. Due to their greater absorption enhancing activities, CS J and CS G were subsequently selected as representatives for further studies.

4. The concentrations of the two selected chitosans, CS J and CS G, were varied from 0.02 to 0.5 % and the results were compared to 5% HP-β-CD, a reportedly safe absorption enhancer. Duncan's test results showed that there were no significant differences in both the percent [D-Arg^2]-Kyotorphin remaining in the perfusate at T_{120} and the apparent rate constant among 5% HP-β-CD and the two chitosans at concentrations of 0.02 and 0.1% ($p > 0.05$). However, at 0.5%, both chitosans were significantly more effective than 5 % % HP-β-CD ($p < 0.05$).

5. The membrane damaging effects of these two chitosans were evaluated by measuring the rate and extent of membrane protein and phospholipid release. ANOVA results showed that there were no significant differences in the five parameters, i.e. the contents of total protein, total phosphorus and phospholipid phosphorus released at T_{120}, as well as the rates of total protein and total phosphorus release among 0.1% CS J, 0.1% CS G and 5% HP-β-CD ($p > 0.05$). In general, the effects of CS J and CS G on the nasal membrane protein and phospholipid release were comparable to that of HP-β-CD but were much smaller than DM-β-CD when compared to the data available in the literature.

6. From histological study, CS J showed only mild to moderate alterations of the rat nasal mucosa even at a relatively high concentration of 1% w/v. CS G at 1% concentration demonstrated even less membrane irritation than CS J. Its effects were mostly in the mild level and similar to that produced by 5% HP-β-CD. The morphological signs most commonly observed for all the three enhancers were mucus hypersecretion and the distention of goblet cells. These symptoms are considered to be minor and reversible.

7. Results from the reversibility study based on comparison of the lactate dehydrogenase (LDH) activities found in the nasal perfusates before and after removal of the enhancer also showed that the two chitosans, at 0.5 % concentration, had only a transient effect on the nasal epithelial integrity. Their effects on LDH release were equivalent to that observed with 1.25% DM-β-CD ($p > 0.05$).

8. In conclusion, the cationic polysaccharide chitosans like CS J and CS G demonstrated significant nasal absorption enhancing activities even at very low

concentrations. At only 0.02%, their enhancing effects were already equivalent to 5% HP-β-CD. Evaluation of their safety profiles based on the release of nasal mucosal protein, phospholipid and LDH enzyme, together with the histological observations after two-week daily nasal administration to intact rats, have demonstrated that the two chitosans used in this study (CS J and CS G) may have a promising potential as an effective nasal absorption enhancer of poorly absorbed drugs like peptides. However, more studies are needed to validate their applicability in clinical practice.

CHAPTER VI

PLAN FOR FUTURE EXPERIMENTS

It is evident from the results of this research that chitosans, especially the free amine CS J and the glutamate salt CS G, exhibit a promising potential as a safe and effective nasal absorption enhancers of poorly absorbable drugs like peptides. However, the results obtained so far were based on the *in situ* perfusion technique. Although this technique is simple and can give a great deal of information on the mechanisms of nasal absorption enhancement exerted by the individual enhancers, the experimental conditions are far from the actual situation of the *in vivo* nasal administration. In the *in situ* method, the rat nasal cavity was perfused with a large volume of solution (5.0 ml) at a relatively high flow rate (2.0 ml/min). In actual administration, however, only a small volume (30 - 100 μl) of the preparation will be applied to the nostril of the rat or human subject. Furthermore, the observed absorption enhancing activity of chitosans was obtained from studies on a single peptide, i.e. [D-Arg^2]-Kyotorphin. More studies on other peptides are thus needed to ensure the efficacy of chitosans as nasal absorption enhancers.

Therefore, the next parts of research will concentrate on the enhancing effect of chitosans *in vivo* by actual nasal administration of a small volume of salmon calcitonin to rats. Salmon calcitonin is selected as the model peptide drug because it is known to have a very poor nasal absorption. A commercial nasal spray of salmon calcitonin is recommended as a supplemental therapy for osteoporosis and other bone diseases. However, it has nasal bioavailability of only 1 percent which makes it a very attractive

choice for use as a model peptide. The details of the experiments have been described in the proposal previously submitted (Experiments 5.6). The main purposes of these experiments are to confirm the *in situ* results that the peptide drug will be absorbed into systemic circulation and exert pharmacological action. Briefly, salmon calcitonin solution will be administered to the rat nostril at a dose of 10 IU/kg. Blood samples will be withdrawn periodically and analyzed for plasma salmon calcitonin and calcium concentrations (measurement of hypocalcemic effect). The effects of varying pH and chitosan concentrations will be studied and the results will be compared with that of the *in situ* experiments. Also, nasal bioavailabilities of salmon calcitonin will be determined and compared to that of hydroxypropyl-β-cyclodextrin (HP-β-CD), an enhancer commonly used as a reference due to its good safety profile. Comparison with another cyclodextrin, dimethyl-β-cyclodextrin (DM-β-CD), will also be made since it has been shown to possess a potent absorption enhancing activity.

Furthermore, it has been postulated that one possible mechanism that an enhancer can increase nasal absorption of peptide is to inhibit the proteolytic enzyme activity in the nasal mucosa. An experiment is thus planned to investigate this possibility by measuring the inhibitory effects of chitosans on the *in vitro* activity of leucine aminopeptidase and trypsin, two major proteolytic enzymes present in the nasal mucosa (Experiments 5.5 in the proposal). The results from these experiments should provide further insight into the mechanisms by which chitosans can enhance the nasal absorption of peptide drugs.

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APPENDIX I

The percent remaining of [D-Arg2]-Kyotorphin in the nasal perfusate whereas various chitosans were uesd as enhancers.

Control pH 3.0

Subject No.	Time (min)					k	r^2
	0	30	60	90	120		
1	100.0	94.8	91.9	90.7	86.9	0.00108	0.9630
2	100.0	92.9	87.1	81.3	75.7	0.00230	0.9996
3	100.0	95.3	90.4	86.1	78.1	0.00199	0.9756
4	100.0	95.0	89.8	85.2	79.8	0.00187	0.9980
mean ± SD	100.0	94.5 ±0.9	89.8 ±1.7	85.8 ±3.3	80.1 ±4.2	0.00181 ±0.00045	

Control pH 4.0

Subject No.	Time (min)				
	0	30	60	90	120
1	100.0	97.4	105.3	102.1	100.5
2	100.0	105.2	106.9	98.0	99.1
3	100.0	94.2	93.8	96.0	94.7
4	100.0	98.9	100.9	103.9	101.8
mean ± SD	100.0	98.9±4.0	101.7±5.1	100.0±3.2	99.0±2.7

Control pH 5.0

Subject No.	Time (min)				
	0	30	60	90	120
1	100.0	97.7	103.4	101.1	92.4
2	100.0	101.8	98.8	92.4	90.2
3	100.0	101.8	100.9	93.3	92.2
4	100.0	100.4	99.6	98.5	97.1
mean ± SD	100.0	100.4±1.7	100.7±1.7	96.3±3.6	93.0±2.5

APPENDIX I

Control pH 6.0

Subject No.	Time (min)				
	0	30	60	90	120
1	100.0	101.3	101.9	98.0	94.9
2	100.0	94.5	99.0	101.1	97.4
3	100.0	104.2	101.7	102.2	104.7
4	100.0	99.3	100.2	99.1	98.3
mean ± SD	100.0	99.8±3.5	100.7±1.2	100.1±1.6	98.8±3.6

Control pH 7.4

Subject No.	Time (min)				
	0	30	60	90	120
1	100.0	102.4	103.9	101.0	101.2
2	100.0	100.0	101.2	99.2	95.6
3	100.0	100.2	99.4	99.0	98.2
4	100.0	99.6	100.3	99.4	99.0
mean ± SD	100.0	100.6±1.1	101.2±1.7	99.65±0.8	98.5±2.0

APPENDIX I

5% HP-B-CD pH 7.4

Subject No.	Time (min)					k	r^2
	0	30	60	90	120		
1	100.0	94.8	89.6	85.9	79.8	0.00183	0.9938
2	100.0	98.1	92.4	88.5	86.0	0.00135	0.9788
3	100.0	92.6	85.4	81.0	81.6	0.00154	0.8493
4	100.0	93.0	89.2	86.0	82.4	0.00155	0.9801
mean ± SD	100.0	94.6±2.2	89.2±2.5	85.4±2.7	82.4±2.3	0.00157 ±0.00017	

0.5% CS J pH 3

Subject No.	Time (min)					k	r^2
	0	30	60	90	120		
1	100.0	95.2	83.4	78.8	69.9	0.00302	0.9807
2	100.0	97.3	83.2	80.3	78.4	0.00226	0.9021
3	100.0	97.7	88.6	81.4	79.4	0.00215	0.9576
4	100.0	84.3	72.6	60.6	54.2	0.00518	0.9955
5	100.0	91.1	83.5	74.8	68.8	0.00316	0.9985
mean ± SD	100.0	93.1 ± 5.0	82.3 ±5.2	75.2 ±7.6	70.1 ±9.0	0.00315 ± 0.00109	

0.5% CS J pH 4.0

Subject No.	Time (min)					k	r^2
	0	30	60	90	120		
1	100.0	97.8	88.3	82.0	60.8	0.00390	0.8527
2	100.0	92.7	86.5	84.8	69.3	0.00274	0.8994
3	100.0	78.2	74.0	67.4	66.4	0.00222	0.8557
4	100.0	87.6	81.0	72.7	71.2	0.00289	0.9572
mean ± SD	100.0	89.1±7.2	82.4±5.6	76.7±7.0	66.9±3.9	0.00294 ±0.00061	

0.5% CS J pH 5.0

Subject No.	Time (min)					k	r^2
	0	30	60	90	120		
1	100.0	92.7	85.3	79.4	74.2	0.00250	0.9986
2	100.0	100.4	93.7	86.1	78.6	0.00212	0.9202
3	100.0	91.5	87.0	81.5	74.3	0.00237	0.9904
4	100.0	94.3	92.3	80.1	78.5	0.00216	0.9306
mean ± SD	100.0	94.7±3.4	89.6±3.5	81.8±2.6	76.4±2.2	0.00229 ±0.00016	

0.5% CS J pH 6.0

Subject No.	Time (min)					k	r^2
	0	30	60	90	120		
1	100.0	89.2	84.8	79.8	75.8	0.00222	0.9699
2	100.0	87.2	84.5	77.8	72.5	0.00252	0.9656
3	100.0	95.4	91.6	87.7	81.2	0.00167	0.9825
4	100.0	94.2	91.3	84.2	79.8	0.00188	0.9862
mean ± SD	100.0	~~95.1~~ 91.5 ±3.4	88.0±3.4	82.4±3.8	77.3±3.4	0.00207 ±0.00032	

APPENDIX I

0.5% CS G pH 4.0

Subject No.	Time (min)					k	r^2
	0	30	60	90	120		
1	100.0	95.0	81.0	78.5	75.2	0.00254	0.9286
2	100.0	93.5	90.1	87.4	80.5	0.00167	0.9718
3	100.0	96.8	91.3	86.1	81.8	0.00173	0.9920
4	100.0	93.6	89.2	86.4	83.9	0.00144	0.9689
5	100.0	93.5	89.2	88.0	83.4	0.00142	0.9643
6	100.0	94.4	86.2	79.7	76.0	0.00239	0.9904
mean ± SD	100.0	94.5±1.2	87.8±3.4	84.4±3.8	80.1±3.4	0.00186±0.00044	

0.5% CS G pH 5.0

Subject No.	Time (min)					k	r^2
	0	30	60	90	120		
1	100.0	97.2	94.9	90.3	86.1	0.00124	0.9759
2	100.0	93.8	92.8	87.2	83.6	0.00144	0.9708
3	100.0	97.6	96.2	90.9	87.0	0.00116	0.9504
4	100.0	92.4	90.2	86.2	82.6	0.00156	0.9758
5	100.0	93.7	84.3	74.8	68.3	0.00329	0.9920
6	100.0	94.1	81.3	76.8	72.1	0.00286	0.9712
mean ± SD	100.0	94.8±1.9	90.0±5.4	84.4±6.3	80.0±7.1	0.00192±0.00083	

0.5% CS G pH 6.0

Subject No.	Time (min)					k	r^2
	0	30	60	90	120		
1	100.0	92.4	88.5	85.2	77.8	0.00194	0.9764
2	100.0	90.2	85.8	83.1	69.9	0.00266	0.9266
3	100.0	95.6	89.5	84.7	72.8	0.00252	0.9383
4	100.0	89.4	81.6	76.4	71.9	0.00272	0.9822
5	100.0	99.8	82.2	71.8	70.9	0.00339	0.9078
6	100.0	103.0	93.0	79.6	76.1	0.00268	0.8798
mean ± SD	100.0	95.1±5.0	86.8±4.0	80.1±4.8	73.2±2.8	0.00265±0.00042	

APPENDIX I

0.1% CS J pH 4.0

Subject No.	Time (min)					k	r^2
	0	30	60	90	120		
1	100.0	94.4	88.3	79.5	71.8	0.00278	0.9826
2	100.0	97.6	87.9	80.8	73.6	0.00267	0.9737
3	100.0	97.7	90.6	81.7	73.9	0.00261	0.9583
4	100.0	99.7	92.8	90.2	86.6	0.00129	0.9499
mean ± SD	100.0	97.4±1.9	89.9±2.0	83.0±4.2	76.5±5.9	0.00234±0.00061	

0.02% CS J pH 4.0

Subject No.	Time (min)					k	r^2
	0	30	60	90	120		
1	100.0	92.1	92.0	89.9	86.9	0.00102	0.8665
2	100.0	101.0	92.5	88.7	82.4	0.00179	0.8806
3	100.0	98.2	93.7	89.5	84.3	0.00145	0.9724
4	100.0	97.6	92.4	88.0	82.7	0.00161	0.9823
mean ± SD	100.0	97.2±3.2	92.6±0.6	89.0±0.7	84.1±1.8	0.00147±0.00028	

APPENDIX I

0.1% CS G pH 6.0

Subject No.	Time (min)					k	r^2
	0	30	60	90	120		
1	100.0	95.3	86.8	83.8	81.1	0.00182	0.9628
2	100.0	96.8	91.0	88.0	78.0	0.00197	0.9378
3	100.0	96.2	83.7	83.2	81.2	0.00187	0.8711
4	100.0	94.9	90.8	85.6	82.0	0.00167	0.9981
mean ± SD	100.0	95.8±0.7	88.1±3.0	85.2±1.9	80.6±1.5	0.00183±0.00011	

0.02% CS G pH 6.0

Subject No.	Time (min)					k	r^2
	0	30	60	90	120		
1	100.0	92.1	77.4	73.5	74.1	0.00275	0.8726
2	100.0	100.5	84.4	80.4	80.7	0.00202	0.9229
3	100.0	92.6	93.1	87.3	84.6	0.00131	0.9279
4	100.0	92.4	87.8	87.0	82.1	0.00152	0.9429
mean ± SD	100.0	94.4±3.5	85.7±5.7	82.0±5.6	80.4±3.9	0.00190±0.00055	

APPENDIX II

Preparation of buffer solutions and reagents used in Parts I - III

Preparation of isotonic phosphate buffers (IPB)

IPB pH 3.0 (0.15 M):

85% H_3PO_4	0.56	ml
NaH_2PO_4	9.19	gm
NaCl	0.78	gm
H_2O qs. to	500.0	ml

IPB pH 4.0 (0.15 M):

85% H_3PO_4	40.0	µl
NaH_2PO_4	10.22	gm
NaCl	0.42	gm
H_2O qs. to	500.0	ml

IPB pH 5.0 (0.15 M):

NaH_2PO_4	0.125	gm
Na_2HPO_4	10.28	gm
NaCl	0.59	gm
H_2O qs. to	500.0	ml

IPB pH 6.0 (0.15 M):

NaH_2PO_4	7.20	gm
Na_2HPO_4	0.947	gm
NaCl	5.20	gm
H_2O qs. to	1000.0	ml

IPB pH 7.4 (0.15 M):

NaH_2PO_4	1.60	gm
Na_2HPO_4	7.58	gm
NaCl	4.40	gm
H_2O qs. to	1000.0	ml

Isotonic lactate buffer pH 4.0 (0.15 M):

Lactic acid (90%)	12.41	gm
NaOH	3.48	gm
NaCl	1.70	gm
H_2O qs. to	1000.0	ml

Isotonic acetate-borate buffer pH 6.05 (0.15 M):

Sodium acetate	2.00	gm
Boric acid	17.10	gm
H_2O qs. to	1000.0	ml

Isotonic acetate-borate buffer pH 7.4 (0.15 M):

Sodium acetate	19.05	gm
Boric acid	0.95	gm
H_2O qs. to	1000.0	ml

Preparation of reagents used in protein determination

Solution A: 2 gm potassium sodium tartrate and 100 gm Na_2CO_3 are dissolved in 50 ml of 1 N sodium hydroxide and diluted with water to 1 L.

Solution B: 2 gm potassium sodium tartrate and 1 gm $CuSO_4.5H_2O$ are dissolved in 90 ml water and 10 ml 1 N sodium hydroxide is added.

Solution C: 1 volume of Folin-Ciocalteus phenol reagent is diluted with 15 volume of water. This solution (prepared daily) should be between 0.15 N and 0.18 N when titrated to pH 10 with 1 N NaOH. If the acidity exceeds 0.18 N, it should be adjusted with NaOH.

Preparation of Fiske and Subbarow Reducer reagent used in phosphorus determination

The solution contains 1-amino-2-naphthol-4-sulfonic acid (0.8 %) and 5 gm of sodium sulfite and sodium bisulfite dissolved in 31.5 ml distilled water.

Preparation of Bouin's fixative solution

Picric acid, saturated aqueous solution	750.0 ml

37.4 % Formalin	250.0 ml
Glacial acetic acid	50.0 ml

Preparation of formic acid-sodium citrate decalcifying solution

Solution A:

Sodium citrate	50.0 gm
Distilled water	250.0 ml

Solution B:

Formic acid, 90 %	125.0 ml
Distilled water	125.0 ml

- Mix solutions A and B in equal portion for use
- Change solution daily for best results.

APPENDIX III

The rate (μg/ml/min) and concentration (μg/ml) of total protein released from the rat nasal mucosa during 120 min *in situ* perfusion in the presence of various absorption enhancers.

Enhancer	Subj.	Protein release (μg/ml) at time					k	r^2
	No.	0	30	60	90	120		
5%HP-β-CD	1	0	339.83	826.85	1189.01	1546.18	13.1385	0.9967
in	2	0	369.10	509.38	715.81	818.21	6.6104	0.9510
A-B buffer	3	0	303.80	531.47	744.85	1032.75	8.3552	0.9961
pH 7.4	mean	0	337.58	622.57	883.22	1132.38	9.3680	
	±SD	0	26.71	144.73	216.55	305.43	2.7596	
A-B buffer	1	0	305.26	417.11	626.35	674.14	5.5646	0.9429
pH 7.4	2	0	102.04	164.30	283.17	449.57	3.6009	0.9719
	3	0	477.04	606.04	701.72	905.19	6.7835	0.9014
	mean	0	294.78	395.82	537.08	676.30	5.3163	
	±SD	0	153.27	180.97	182.16	186.01	1.3111	
5%HP-β-CD	1	0	45.055	431.03	651.93	869.88	7.8221	0.9640
(corrected	2	0	74.32	113.56	178.73	141.91	1.2941	0.8024
with buffer)	3	0	9.02	135.65	207.77	356.45	3.0389	0.9423
	mean	0	42.80	226.75	346.14	456.08	4.0517	
	±SD	0	26.71	144.73	216.55	305.43	2.7600	

Enhancer	Subj.	Protein release (μg/ml) at time					k	r^2
	No.	0	30	60	90	120		
0.1%CS G	1	0	315.98	590.98	997.98	1498.66	12.2644	0.9851
in	2	0	605.40	1205.00	1526.58	1764.30	14.8326	0.9625
A-B buffer	3	0	783.96	960.82	2098.81	3222.35	25.8652	0.9496
pH 6.0	mean	0	568.45	885.54	1541.12	2161.77	17.6541	
	±SD	0	192.83	252.416	449.53	757.74	5.9000	
A-B buffer	1	0	287.52	393.16	842.97	1231.24	10.0598	0.9629
pH 6.0	2	0	56.46	475.38	1133.66	1616.32	14.3661	0.9416
	3	0	334.24	559.27	634.37	789.50	6.2638	0.9387
	mean	0	226.07	475.94	870.33	1212.35	10.2299	
	±SD	0	121.44	67.82	204.75	337.81	3.3099	
0.1%CS G	1	0	89.91	115.04	127.65	286.31	2.0345	0.8669
(corrected	2	0	379.33	729.06	692.25	551.95	4.7227	0.5697
with buffer)	3	0	512.89	484.88	1228.48	2010.00	15.7850	0.9123
	mean	0	327.37	443.00	682.79	949.42	7.5141	
	±SD	0	176.54	252.41	449.46	757.74	5.951	

Enhancer	Subj.	Protein release (μg/ml) at time					k	r^2
	No.	0	30	60	90	120		
0.1%CS J	1	0	817.02	1306.04	1471.10	1557.06	12.5607	0.8581
in	2	0	660.68	1228.27	1461.37	1543.45	12.9586	0.9033
Lactate buffer	3	0	1425.48	2282.74	2621.25	2648.03	21.6394	0.8417
pH 4.0	mean	0	976.73	1605.68	1851.24	1916.18	15.7196	
	±SD	0	329.91	479.80	544.49	517.52	4.1891	
Lactate buffer	1	0	569.92	1094.80	1327.65	1368.57	11.6496	0.9011
pH 4.0	2	0	620.14	1310.19	1390.07	1425.80	12.0718	0.8402
	3	0	534.24	698.13	925.10	1028.60	8.1602	0.9130
	mean	0	574.76	1034.37	1214.282	1274.32	10.6272	
	±SD	0	35.24	253.50	06.06	175.32	1.7529	
0.1%CS J	1	0	242.26	271.67	256.83	282.74	1.9668	0.4971
(corrected	2	0	85.92	193.90	247.10	269.13	2.0648	0.8787
with buffer)	3	0	850.72	1248.37	1406.97	1373.71	7.839	0.5345
	mean	0	392.97	571.31	636.97	641.86	3.9569	
	±SD	0	329.91	479.80	544.49	517.52	2.7454	

The rate (μg/ml/min) and concentration (μg/ml) of total phosphorus released from the rat nasal mucosa during 120 min *in situ* perfusion in the presence of various absorption enhancers.

Enhancer	Subj.	Total phosphorus release (μg/ml) at time					k	r^2
	No.	0	30	60	90	120		
5%HP-β-CD	1	0	3.222	5.569	15.303	30.712	0.2450	0.8742
in	2	0	13.986	17.815	24.179	32.123	0.2481	0.9602
A-B pH 7.4	3	0	2.749	7.559	16.642	23.985	0.2062	0.9614
	mean	0	6.652	10.134	18.708	28.940	0.2331	
	±SD	0	5.189	5.336	3.907	3.551	0.0191	
A-B pH7.4	1	0	2.813	4.419	6.481	9.442	0.0752	0.9902
	2	0	1.496	3.225	5.705	7.594	0.0646	0.9924
	3	0	3.000	3.930	7.017	9.321	0.0755	0.9820
	mean	0	2.436	3.858	6.401	8.786	0.0718	
	±SD	0	0.669	0.490	0.539	0.844	0.0051	
5%HP-β-CD	1	0	0.786	1.711	8.902	21.926	0.1732	0.7910
(corrected with	2	0	11.550	13.958	17.775	23.338	0.1763	0.9292
buffer)	3	0	0.313	3.701	10.241	15.199	0.1011	0.5436
	mean	0	4.216	6.457	12.306	20.154	0.1502	
	±SD	0	5.189	5.366	3.906	3.551	0.0347	

Enhancer	Subj.	Total phosphorus release (μg/ml) at time					k	r^2
	No.	0	30	60	90	120		
0.1%CS G	1	0	11.363	19.208	24.522	26.624	0.2214	0.9329
in	2	0	15.642	18.958	24.220	27.241	0.2102	0.8808
A-B pH 6.0	3	0	13.362	18.357	18.917	23.709	0.1766	0.8502
	mean	0	13.456	18.841	22.553	25.858	0.2027	
	±SD	0	1.748	0.357	2.574	1.540	0.0190	
A-B pH 6.0	1	0	2.868	4.003	5.268	5.899	0.0473	0.9279
	2	0	3.081	4.183	6.927	8.307	0.0682	0.9793
	3	0	5.180	9.467	10.349	14.164	0.1117	0.9551
	mean	0	3.710	5.884	7.515	9.457	0.0757	
	±SD	0	1.043	2.534	2.116	3.471	0.0268	
0.1%CS G	1	0	7.654	13.326	17.007	17.168	0.1456	0.9015
(corrected	2	0	11.932	13.076	16.706	17.784	0.1345	0.8108
with buffer)	3	0	9.653	12.476	11.402	14.253	0.1008	0.7304
	mean	0	9.746	12.959	15.038	16.401	0.1270	
	±SD	0	1.748	0.357	2.574	1.540	0.0190	

Enhancer	Subj.	Total phosphorus release (μg/ml) at time					k	r^2
	No.	0	30	60	90	120		
0.1%CS J	1	0	12.954	22.308	28.892	39.276	0.3150	0.9885
in	2	0	9.066	21.368	28.540	37.107	0.3123	0.9930
Lactate buffer	3	0	21.423	28.670	30.907	40.669	0.3027	0.8875
pH 4.0	mean	0	14.481	24.115	29.446	39.011	0.3100	
	±SD	0	5.159	3.243	1.043	1.466	0.0053	
Lactate buffer	1	0	6.640	11.266	11.961	24.011	0.1778	0.9178
pH 4.0	2	0	7.550	10.732	14.000	20.480	0.1580	0.9737
	3	0	4.760	14.060	15.672	29.527	0.2332	0.9448
	mean	0	6.317	12.020	13.878	24.673	0.1897	
	±SD	0	1.162	1.459	1.517	3.723	0.0318	
0.1%CS J	1	0	6.637	10.289	15.015	14.570	0.1251	0.9082
(corrected	2	0	2.749	9.349	14.663	12.401	0.1224	0.8585
with buffer)	3	0	15.106	16.650	17.029	15.963	0.1128	0.5411
	mean	0	8.164	12.096	15.569	14.311	0.1201	
	±SD	0	5.159	3.243	1.042	1.466	0.0053	

Concentration (μg/ml) of phospholipid phosphorus released in the nasal perfusates after 120 min perfusion in the presence of various absorption enhancers.

Enhancer /Condition	Phospholipid phosphorus at T_{120} of subject No. (μg/ml)			mean ± SD
	1	2	3	
5%HP-β-CD in A-B buffer pH 7.4	0.565	0.252	0.641	0.486 ± 0.168
A-B buffer pH 7.4	0.022	0.030	0.026	0.026 ± 0.003
5%HP-β-CD (corrected with buffer)	0.539	0.226	0.615	0.460 ± 0.168
0.1%CS G in A-B buffer pH6.0	0.622	0.264	0.432	0.439 ± 0.146
A-B buffer pH 6.0	0.046	0.031	0.044	0.040 ± 0.007
0.1%CS G (corrected with buffer)	0.582	0.244	0.383	0.403 ± 0.139
0.1%CS J in Lactate buffer pH 4.0	0.743	0.688	0.372	0.601 ± 0.163
Lactate buffer pH 4.0	0.071	0.089	0.057	0.072 ± 0.013
0.1%CS J (corrected with buffer)	0.671	0.616	0.300	0.529 ± 0.164