GASTROINTESTINAL ABSORPTION OF HEPARINS

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PREFACE

This dissertation has been organized as a series of manuscripts that was or will be submitted for publication in scientific journals. Some repetition of introductory and methodological material is unavoidable.

ABSTRACT

Heparin, a highly sulfated and acidic glycosaminoglycan, has been clinically used in parenteral formulations to prevent and/or treat thromboembolic disorders for more than 90 years. Actions of heparin are not limited to anticoagulation and antithrombosis. Rather heparin has several other important actions which include fat clearing, antitumor and anti-inflammatory effects. However, use of heparin for such applications has been limited by its route of administration.

Historically, it has been believed that heparin is not absorbed following oral administration and therefore is only available for clinical use by parenteral administration. This belief has been challenged several times by our laboratory and other researchers showing heparin binding to endothelium following oral administration as well as prevention of thrombosis and lowering blood pressure, etc. However, the site of oral heparin absorption and the mechanism responsible for absorption have not been investigated. This *in vitro* study was designed to address these important questions.

We mounted pieces of rat gastrointestinal mucosa in a vertical diffusion Ussing chamber with both sides of the mucosal membrane exposed to Kreb's bicarbonate buffer solution containing mannitol on the mucosal side (lumen) and glucose on the serosal side. Electrical properties across the membrane including potential difference (PD), resistance (R), and short circuit current (Isc) were recorded following heparin addition to the mucosal buffer under different mucosal buffer pH conditions. Mucosal and serosal buffer and tissue were collected and extracted for heparin and heparin recovery was performed by gel electrophoresis and anticoagulation tests.

The first chapter (chapter 4) was designed to investigate if stomach mucosal tissue is a site for unfractionated heparin (UFH) absorption when mounted in the Ussing chamber. We found that stomach mucosa is able to transport UFH in an intact form when both mucosal and serosal buffers are at neutral pH of 7.4. When the mucosal buffer pH is made more acidic, at pH 4, transport is facilitated.

The second study (chapter 5) was designed to investigate if stomach mucosal tissue is also capable of transporting low molecular weight heparins (LMWHs). We showed that LMWHS were transported across stomach mucosa. However, the rate of transport was faster at mucosal buffer pH of 7.4 compared to pH 4.

The third study (chapter 6) investigated the effect of molecular weight on rate of heparin transport across stomach mucosal tissue since pH dependency of this transport was evident from both previous studies. This study suggested that decreasing the molecular weight increases the rate of heparin transport across stomach tissue under neutral pH but not acidic pH conditions.

The fourth study (chapter 7) investigated how UFH is transported across the ileal mucosa and if Peyer's patches contribute to this transport. It was shown that UFH is transported across ileal mucosa containing Peyer's patches at a rate faster than ileal mucosa without Peyer's patches. Making the mucosal buffer pH acidic facilitated UFH transport in the absence of Peyer's patches but not when ileal mucosa contained Peyer's patches.

The final study (chapter 8) investigated the mechanism of UFH transport across stomach mucosa mounted in the Ussing chamber using pharmacological inhibitors sodium fluoride, colchicine, and amiloride. Results showed that UFH is transported across the

stomach mucosa at physiological acidic pH by an active transport mechanism using metabolic energy, cytoplasmic tubule formation, and sodium-coupled systems.

From this, we conclude that oral heparins are absorbed across the gastrointestinal tract. The acidic environment of the stomach is a better absorption site for UFH. On the other hand, the more basic environment of the intestine is a better site for absorption of LMWHs. UFH is mainly absorbed across the stomach mucosa by an active transport mechanism using metabolic energy, cytoplasmic tubule formation, and sodium-coupled systems. Considering the very much larger surface area of the intestine than the stomach and that intestine, especially the ileum, contains many Peyer's patches where UFH transport is not pH-dependent, larger amounts of UFH may be transported across the intestinal tissue compared to the stomach.

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My special gratitude to my parents, Dr. Hadi Moazed and Malikeh Jafarnejadi, whose love and affection is always the source of inspiration and encouragement in all aspects of my life.

I dedicate this work to my loving husband, my spiritual master Hossein Azinfar, and to my precious son, Armin

My darling husband, Hossein, you direct my understanding of life. You supported and encouraged me through all of my desires and goals even when my dreams seemed totally out of reach. I am so very blessed to have you as my husband. Without you in my life I would have been a lost human being not knowing your sincere and secure love.

My dear son, Armin, you hold a place in my heart where no one's ever been.
You are the most precious son a mother could ask for. I love you more than my life. You have brought me more pleasure in a single moment of your life, than all other moments combined.

Hossein and Armin, you both are my link to life and the continuity of life, and of love.

I love you both very much

Table of Contents

List of Tables

List of Figures

Abbreviations

AA, arachidonic acid

ADP, adenosine diphosphate

ADPase, adenine nucleotidases

ANGII, angiotensin II

APC, activated protein C

APTT, activated partial thromboplastin times

AT, antithrombin

ATP, adenosine-5'-triphosphate

bFGF, basic fibroblast growth factor

BSA, bovine serum albumin

Ca^{2+}, calcium

CaM kinase II, Ca^{2+}/calmodulin dependent protein kinase II

cAMP, adenosine 3', 5'-cyclic monophosphate

cdc2, cyclin-dependent c2 protein

cdk2, cyclin-dependent kinase 2

Cl^-, chloride

CS, Chondroitin sulfate

DAG, diacylglycerol

DS, Dermatan sulfate

EDTA acid, ethylenediaminetetraacetic acid

EGFR, epidermal growth factor receptor

EPCR, endothelial protein C receptor

ET, endothelin

Factor VIIai, recombinant factor VIIa

FFAs, free fatty acids

FGFR-1, fibroblast growth factor receptor-1

FITC, Fluorescein isothiocyanate

fMLP, N-formyl-methionyl-leucyl-phenylalanine

GAGs, glycosaminoglycans

GI, gastrointestinal

Glycoprotein Iba, GPIba

HA, Hyaluronic acid

HB-EGF, heparin-binding EGF-like growth factor

HCII, Heparin cofactor II

HIV, human immunodeficiency virus

HK, high-molecular-weight kininogen

$hLpL^{HBM}$, human LpL minigene

HRP, horseradish peroxidase

ICAMs, intercellular adhesion molecules

IgG, immunoglobulin G

IP3, inositol triphosphate

KH2, reduced form of vitamin K

KO, vitamin K epoxide

LMWHs, Low molecular weight heparins

L-NAME, N^G-nitro-L-arginine-methyl ester

LPL, Lipoprotein lipase

M cells, microfold cells

MAPK, mitogen activated protein kinase

MBO, mixed backbone oligonucleotide

MHC, major histocompatibility

MMPs, matrix metalloproteinases

MWCO, Molecular weight cut off

NaF, sodium fluoride

NAPc2, recombinant nematode anticoagulant peptide

NF-κB, Nuclear factor κB

NK, natural killer cell

ONs, oligonucleotides

PAF, platelet activating factor

PAI-1, plasminogen activator inhibitor-I

PAR, protease-activated receptors

PAs, plasminogen activators

PC, phosphatidylcholine

PCI, Protein C inhibitor

PDGF, platelet-derived growth factor

PF4, platelet factor 4

PGE_2, prostaglandin E_2

PGI2, prostacyclin

PIP2, phosphatidylinositol-4,5-bisphosphate

PKA, protein kinase A

PKC, protein kinase C

PLC, phospholipase C

SK, Streptokinases

SNAC, sodium *N*-[8-(2-hydroxybenzoyl) amino] caprylate

SNAD, sodium *N*-[10-(2-hydroxybenzoyl) amino] decanoate

TAFI, thrombin activatable fibrinolysis inhibitor

TF, tissue factor

TFPI, tissue factor pathway inhibitor

TGF-β, transforming growth factor-β

t-PA, tissue-type plasminogen activator

TRAP, thrombin receptor agonist peptide

TxA_2, thromboxane A_2

UFH, unfractionated heparin

UK, urokinase

u-PA, urokinase-type plasminogen activator

VEGF, Vascular endothelial growth factor

VSMCs, vascular smooth muscle cells

vWF, von Willebrand's factor

ZPI, protein Z-dependent protease inhibitor

α_1PI, α_1-Protease inhibitor

α_2AP, α_2-antiplasmin

α_2-M, α_2-macroglobulin

Chapter 1 Introduction

1.1 Hemostasis

Hemostasis is the physiological process of cessation of blood loss from an injured blood vessel as well as maintenance of vascular integrity so that a closed, high-pressure circulatory system is reserved after vascular damage. It is essential for survival. When a vessel wall is injured such that the vascular endothelium is disrupted and blood extravasates from the circulation into the tissue, a series of events are initiated in the vessel wall and in blood to seal the cut. Circulating platelets are recruited to the site of injury along with activation of blood coagulation systems; thrombin and fibrin accumulate which seal the defect (Furie and Furie, 2008).

The components of hemostasis therefore, include blood vessels, platelets, plasma coagulation factors and their inhibitors and the fibrinolytic system. The hemostatic system is divided into primary hemostasis which mainly involves the vascular system and platelets, secondary hemostasis which is mainly related to activation of coagulation pathways and fibrin formation, and tertiary hemostasis which is related to fibrinolysis and dissolution of the blood clot when it is no longer required for hemostasis. These events happen concomitantly under normal conditions although classified as separate events and together maintain normal blood vessel function and a non-thrombotic state (Arnout et al., 2006; Stassen et al., 2004; Furie and Furie, 2008).

1.1.1 Primary Hemostasis

1.1.1.1 Vascular Spasm

Blood vessel contraction is the very first step in hemostasis that controls the rate and volume of blood loss through the injured vessel. The endothelial cell serves as the modulator of the vascular smooth muscle contractile state and as a result is a critical determinant of the blood flow.

Under normal conditions, the endothelium releases factors that relax vascular smooth muscle including prostacyclin, endothelium-derived relaxing factor (nitric oxide), and endothelium-derived hyperpolarizing factor and therefore maintain a low basal vascular tone. When a blood vessel is injured however, endothelium synthesizes and releases vasoconstrictor substances including endothelin, endothelium-derived constricting factor(s), and also reduces the synthesis of vasodilator products and therefore increases vessel tone (Loscalzo, 1995). Moreover, damage to the vessel wall sends pain stimuli to the spinal cord which leads to vasoconstriction by reflex mechanisms through sympathetic fibers (Hassler, 1969).

1.1.1.2 Platelets

Platelets, also referred to as thrombocytes, are cytoplasmic fragments of bone marrow megakaryocytes (Schulze and Shivdasani, 2005). They mature in the peripheral blood where they endocytose and store in their α-granules several plasma proteins including fibrinogen, von Willebrand's factor (vWF), factor V, albumin, platelet-specific proteins including β-thromboglobulin, thrombospondin as well as mitogenic proteins such as

platelet-derived growth factor (PDGF) and transforming growth factor-β (TGF-β) involved in tissue repair (Harrison and Cramer, 1993; Blair and Flaumenhaft, 2009). In addition to α-granules, platelets contain dense granules containing adenine nucleotides, serotonin, calcium, and lysosomes.

1.1.1.2.1 Platelet Adhesion

Intact endothelium is thrombotic resistant. Blood soluble or cellular components, including platelets, circulate in the blood as inactive and inert constituents which do not interact with endothelium (Born and Schwartz, 1997; Ruggeri, 2002). This may be due to (1) the negatively charged surface of endothelial cells which repels negatively charged, unactivated platelets (Austin, 2009; van Hinsbergh, 2001); (2) endothelial cell synthesis of platelet inhibitors [prostacyclin (PGI2), nitric oxide, adenine nucleotidases (ADPase)] and fibrin formation [tissue factor pathway inhibitor (TFPI), thrombomodulin]; and (3) endothelial cell synthesis and release of activators of fibrin degradation [tissue-type plasminogen activator (t-PA), urokinase-type plasminogen activator (u-PA)] (Marcus et al., 1997; Nosaka et al., 1996; Krötz et al., 2004; Patterson et al., 2006; Esmon et al., 1982; van Hinsbergh, 1988; Oliver et al., 2005). Upon tissue injury however, the system becomes activated. Endothelial cells express protein receptors on their plasma membrane and exocytose Weibel-Palade bodies. The structures that lie beneath endothelial cells are also exposed to the blood.

Platelets adhere promptly where endothelial cells are altered or extracellular matrix substrates are exposed (Ruggeri, 2002; Gawaz et al., 2005; Wagner and Burger, 2003).

Depending on the matrix proteins exposed to blood and the hemodynamic conditions, platelet adherence to the damaged surface is achieved via the synergistic function of different platelet glycoprotein receptors with matrix proteins (ligands) on the vessel wall (Ruggeri and Mendolicchio, 2007). The extracellular matrix components that react with platelets include different collagen types, vWF, fibronectin (Beumer et al., 1995) and other adhesive proteins such as laminin (Hindriks et al., 1992) fibulin (Godyna et al., 1996), thrombospondin (Jurk et al., 2003) fibrinogen/fibrin (Savage et al., 1996) and vitronectin (Asch and Podack, 1990).

vWF is a large multimeric glycoprotein produced constitutively in endothelial cells and presented as a component of the extracellular matrix. It is responsible for platelet adhesion under conditions of elevated shear stress (Savage et al., 1998; Savage et al., 1996; Ruggeri and Ruggeri, 2004; De Meyer, et al., 2009) primarily through the tethering of glycoprotein Iba (GPIba) in the platelet membrane GPIb-IX-V receptor complex (Andrews et al., 2003) to the A1 domain of immobilized VWF exposed to flowing blood (Reininger, 2008; Ruggeri, 2007).

Receptors present on the platelet membrane that interact with collagen include the integrin $\alpha 2\beta 1$ (Santoro, 1986; Kunicki et al., 1988; Nieuwenhuis et al., 1985), GPVI (Moroi et al., 1989; Moroi et al., 1996; Offermanns, 2006), GPIV (CD-36) (Asch et al., 1993; Tandon et al., 1989; Diaz-Ricart et al., 1993) and the 65-kDa protein (p65) specific for type I collagen (Chiang et al., 1997). Platelets also possess two main receptors for fibronectin protein, $\alpha 5\beta 1$ and $\alpha IIb\beta 3$ (Ginsberg et al., 1993). Fibrinogen mediates

platelet adhesion through selective interaction with αIIbβ3 present on platelet membranes (Savage et al., 1996). Fibrin that is a cross-linked insoluble polymer of fibrinogen, mediates platelet adhesion through a synergistic action with immobilized vWF (Hantgan et al., 1990).

Different forms of subendothelial laminin that are potential substrates for platelet adhesion at sites of vascular injury include laminins 8 (α4β1γ1) and 10 (α5β1γ1) (Nigatu et al., 2007). Inoue et al., (2006) found a synergistic role for α6β1 integrin and GPVI on the platelet membrane as laminin receptors. Thrombospondins are a family of adhesive proteins (Adams and Lawler, 1993). Adhesion to thrombospondin-1 is mediated by GPIb with a minor contribution by GPIV (CD36). Thrombospondin-2 is also a relevant constituent of extracellular matrices and when it is absent, mice are defective in the hemostatic mechanism (Kyriakides et al., 1998; Zaverio and Ruggeri, 2007).

1.1.1.2.2 Platelet activation and aggregation

Under conditions of high sheer stress, the firm anchoring of platelets to the damaged vessel wall involves the platelet vWF receptor GPIb/V/IX and the collagen receptor GPVI (Jackson et al., 2003; Ruggeri, 2003; Nieswandt and Watson, 2003) resulting in the formation of a platelet monolayer. Binding of platelets to collagen via GPVI activates phospholipase C (PLC) leading to the formation of intracellular messengers inositol triphosphate (IP3) and diacylglycerol (DAG) through the hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP2). IP3 alone mobilizes small amounts of intracellular calcium (Ca^{2+}), and DAG activates protein kinase C (PKC). Increase in

intracellular Ca^{2+} (1) leads to secretion of α granules releasing several procoagulant proteins such as FV, FVIII, and fibrinogen which begin coagulation on the surface of platelets producing thrombin (Heemskerk et al., 2002); (2) leads to degranulation of dense granules that release mediators such as adenosine diphosphate (ADP) and serotonin (Offermanns, 2006). (3) phospholipase A2 pathway becomes activated resulting in formation of platelet activating factor (PAF) and arachidonic acid (AA) from the membrane component, phosphatidylcholine (PC). AA becomes oxidized to thromboxane A_2 (TxA_2) via the cyclooxygenase pathway.

Thrombin, TxA_2, and ADP are the most important mediators of platelet recruitment and activation. They induce platelet-shape change, degranulation, and α IIb/β_3-mediated aggregation through three major G protein-mediated signalling pathways that are initiated by the activation of the G proteins G(q), G(13), and G(i). TxA_2 and thrombin activate mainly G_q and G_{12}/G_{13} via the TxA_2 receptor (Offermanns et al., 1994; Knezevic et al., 1993; Djellas et al., 1999) or the protease-activated receptors (PAR) (Coughlin, 2005; Offermanns et al., 1994; Klages et al., 1999) whereas ADP activates G_q and G_i through its receptors $P2Y_1$ and $P2Y_{12}$ (Murugappa and Kunapuli, 2006; Gachet et al., 2006; Savi et al., 1998; Hechler et al., 1998; Ohlmann et al., 1995; Jantzen et al., 2001).

It should be noted that although massive blood loss from an injured vessel is prevented by platelet aggregates, the complete cessation of blood loss is not possible without formation of a fibrin clot or thrombus.

1.1.2 Secondary Hemostasis

Platelet activation enhances activation of the coagulation cascade. Activation and secretion of platelets not only provides additional coagulation factors including factor V, factor VIII, or fibrinogen (Bouchard et al., 2002), but also translocates phosphatidylserine to the platelet surface which has high affinity for procoagulant proteins such as factor IX, factor X, and prothrombin (Bouchard and Tracy, 2001; Heemskerk et al., 2002). The coagulation system takes place through consecutive activation of several enzymes cited in Table 1.

Table 1.1 Enzymes and cofactors involved in the coagulation cascade of the hemostatic process.

No	Name
I	Fibrinogen
II	Prothrombin
III	Thromboplastin
IV	Ca
V	Proaccelerin
VII	Proconvertin
VIII	Antihemophilic A
IX	Antihemophilic B
X	Stuart Power factor
XI	PTA (plasmathromboplastin antecedant)
XII	Hageman factor
XIII	Fibrin stabilizing factor (Laki-Lorand factor)

1.1.2.1 Contact Activation Pathway or Intrinsic Pathway

Factor XII (FXII) becomes activated when it contacts damaged endothelium (Gailani and Broze, 2001; Gailani and Renne´, 2007; Renne´ and Gailani, 2007). FXIIa activates factor XI (FXI) which is in complex with high-molecular-weight kininogen (HK)-FXI

complex attached to the damaged endothelium (Schmaier and Mccrae, 2007). Activated factor XI (FXIa) then converts factor IX (FIX) to activated factor IX (FIXa). FIXa in turn, with combination of a protein cofactor (FVIIIa), phospholipid and calcium, makes the tenase complex, which activates the zymogen factor X (FX) to activated FX (FXa) (Figure 1).

1.1.2.2 Extrinsic Pathway

Endothelial cells produce tissue factor (TF or thromboplastin), the extracellular domain of which is exposed on the damaged cell surface, and binds to circulating factor VII (FVII) (Mackman, 2004; Morrissey, 2004; Mackman et al., 2007). The resulting proteolytically active TF-FVIIa complex in the presence of calcium converts the zymogens FX and FIX to their active forms, FXa and FIXa respectively. FIXa then becomes a part of the tenase complex resulting in activation of more FX. This bypasses the contact activation pathway.

1.1.2.3 Common Pathway

The intrinsic and extrinsic pathways converge at the level of factor X activation. FXa in combination with activated protein cofactor FV (FVa), phospholipid and, Ca^{2+} makes the prothrombinase complex. Prothrombinase complex rapidly cleaves prothrombin to thrombin. Finally, thrombin activates factor XIII to FXIIIa and facilitates the conversion of fibrinogen to fibrin (Mann, 1999; Furie and Furie, 1992; Dahlback, 2000; Davie and Kulman, 2006; Gailani and Renné, 2007). Fibrin mesh formed around the platelet

aggregates makes a stable thrombus that seals off the injured vessel wall and completely halts the loss of blood.

The amount of thrombin produced by FXa generated by the extrinsic pathway produces only small amounts of fibrin. However, it is enough to initiate several positive feedback loops that induce further thrombin production. Thrombin increases the rate of prothrombin activation in several ways. It induces the activation of FV to FVa, and FVIII to FVIIIa (Beguin et al., 1988; Hirsh et al., 2001). Moreover, thrombin directly and indirectly activates FX by activating FXI and FIX (Ofosu et al., 1987). FX itself has a positive feedback on activation of FVII (Dee Unglaub Silverthorn, 2003).

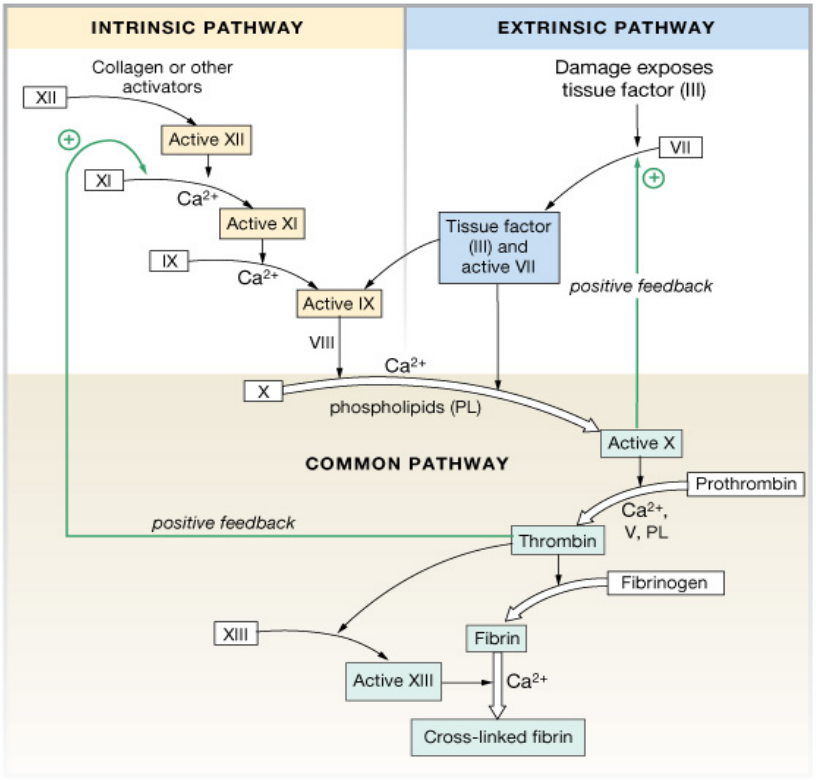


Figure 1.1 Adapted from Human Physiology: an integrated approach with interactive physiology. Third edition (2003), Chapter 16: Blood. Dee Unglaub Silverthorn, PhD.

1.1.3 Tertiary Hemostasis (Counter-Regulatory Systems)

While coagulation serves to restrict the loss of blood from a damaged vessel by formation of a stable fibrin clot, excessive coagulation or thrombosis should be prevented. Therefore, several inhibitory systems of fibrin formation as well as a fibrinolytic system exist in the circulation to control formation and degradation of fibrin clots respectively (Rau et al., 2007).

1.1.3.1 Inhibitory Systems of Fibrin Formation

Formation of fibrin is mainly regulated by antithrombin (AT) (Pike et al., 2005), the protein C pathway (Dahlback and Villoutreix, 2005), and TFPI (Sandset, 1996). Heparin cofactor II (HCII) (Tollefsen, 2007) and protein Z-dependent protease inhibitor (ZPI) (Broze, 2001) also play a role although to a lesser extent. Protein C inhibitor (PCI) may also contribute by inhibiting thrombin (Cooper et al., 1995; van Meijer et al., 1997).

The AT is the most important physiological inhibitory system of fibrin formation. It mainly inhibits thrombin, although it is capable of inhibiting FIXa, Xa and XIa. AT anticoagulant activity depends on its cofactor, heparin, which is discussed in detail later (McCoy et al., 2003; Belzar et al., 2000).

Protein C zymogen binds to its endothelial protein C receptor (EPCR). Thrombin, generated via the coagulation pathway, binds to thrombomodulin on damaged endothelial cells via its exosite I which is nessecary for its binding to fibrinogen. Therefore upon binding to thrombomodulin, thrombin losses its procoagulant activity. However,

thrombomodulin bound thrombin activates zymogen protein C to activated protein C (APC). When activated, protein C complexes with protein S that converts FVa and FVIIIa to inactive forms that can no longer participate in the prothrombinase and tenase complex (Esmon 2003; Lu et al., 1996; Stearns-Kurosawa et al., 1996; Fuentes-Prior et al., 2000; Fukudome et al., 1996).

The TFPI is a protease inhibitor responsible for inhibiting FVIIa-TF. It also inhibits FXa. When complexed to Xa, TFPI is a much more potent inhibitor of the VIIa-TF than by itself (Bajaj et al., 2001; Hisao Kato, 2008).

The HCII is a heparin binding protein that inhibits thrombin. It also maintains inhibitory function of thrombin when binding to other polyanionic compounds such as dermatan sulfate through a unique hexasaccharide sequence within the molecule (Maimone and Tollefsen, 1990; Tollefsen, 2007).

The ZPI rapidly inhibits FXa and FXIa in the presence and absence of cofactors protein Z, phospholipids and Ca^{2+} respectively. Its inhibitory action on FXIa can be accelerated 2-fold by heparin (Han et al., 2000).

The PCI is a heparin binding serine protease inhibitor that is capable of inhibiting many proteases, including APC (Aznar et al., 1996), thrombin, thrombin bound to thrombomodulin (Rezaie et al., 1995), tPA and uPA (Espana et al., 1993). The PCI can function as both anticoagulant and procoagulant depending on the target protease and

cofactors present. In the presence of heparin, PCI inhibits the conversion of fibrinogen to fibrin by thrombin and thus, acts as an anticoagulant. However, PCI inhibits the activation of PC by thrombin in the presence of thrombomodulin and thus, acts as a procoagulant (Pike et al., 2005).

1.1.3.2 Fibrinolytic Pathway

Fibrinolysis is the physiological process of dissolution of the fibrin clot to prevent the excessive fibrin formation or thrombosis (Cesarman-Maus and Hajjar, 2005). The main serine protease responsible for degrading fibrin is plasmin, which circulates as a zymogen, plasminogen. When fibrin is present, tPA cleaves plasminogen to plasmin, which then converts fibrin to fibrin degradation products. The degradation of fibrin limits further activation of plasminogen since fibrin is a necessary cofactor for the reaction (Wiman and Collen, 1978; Levi et al., 2004; Loskutoff and Quigley, 2000). Plasminogen can also be converted to plasmin by the serine protease, uPA (Dano et al., 2005) and streptokinase (Ohman et al., 2001).

Dissolution of the fibrin clot is concisely controlled by α_2-antiplasmin (α_2AP) (Coughlin, 2005), plasminogen activator inhibitor-I (PAI-1) (Wiman and Collen, 1978; Vaughan, 2001) and thrombin activatable fibrinolysis inhibitor (TAFI) (Mosnier and Bouma, 2006). The PCI is also capable of inhibiting tPA and uPA (Heeb et al., 1987; Espana et al., 1989). The α_2AP is the primary physiological inhibitor of plasmin, but can also inhibit APC (Aoki, 1984; Miles et al., 1982). When α_2AP is overwhelmed, α_2-macroglobulin (α_2-

M), another protease inhibitor, may also inhibit plasmin activity (Hertig and Rondeau, 2004).

The PAI-1 is the main physiological inhibitor of plasminogen activation through inhibition of both tPA and uPA (Heimark et al., 1980; Vaughan, 2001; Cesarman-Maus and Hajjar, 2005). Upon activation, platelets release PAI-1 to protect the breakdown of the premature thrombus. However, when the vessel injury is sealed off by a stable fibrin clot, tPA and plasminogen/plasmin are bound to fibrin within the thrombus, and thus will be protected from inhibition by PAI-1, resulting in fibrin dissolution (Heimark et al., 1980; Vaughan, 2001; Cesarman-Maus and Hajjar, 2005).

The TAFI is a glycoprotein shown to inhibit the fibrinolytic system when activated by high concentrations of thrombin or plasmin (Bajzar et al., 1995; Bajzar et al., 1996). The activation of TAFI by thrombin is greatly enhanced in the presence of thrombomodulin (Bajzar et al., 1996; Hosaka et al., 1998). Activated TAFI inhibits t-PA and therefore, inhibits fibrin degradation (Bouma and Mosnier, 2003/2004).

α_1-Protease inhibitor (α_1PI), historically known as α_1-antitrypsin, has been shown to inhibit APC in a heparin-independent manner (Heeb and Griffin, 1988).

1.2 Pathological Thrombus Formation

Thrombosis is the major cause of morbidity and mortality (Weitz et al., 2008). It is a condition where the fine balance between the process of coagulation and anticoagulation

is disrupted leading to increased coagulation and a variety of pathophysiological conditions such as stroke, pulmonary embolisam, heart attack and other serious conditions (Chakrabarti and Das, 2007).

1.3 Antithrombotic Drugs

Antithrombotic drugs are classified into 3 major categories; antiplatelets, thrombolytics, and anticoagulants. Arterial thrombosis is mainly made up of platelet aggregates with small amounts of fibrin (Baumgartner, 1973; Freiman, 1987; Badimon and Badimon, 1989; Lassila et al., 1990; Hirsh et al., 2001; Weitz et al., 2008). Venous thrombosis, in contrast, is mainly made up of fibrin mesh and red blood cells with only a few platelets (Freiman, 1987; Badimon and Badimon, 1989; Lassila et al., 1990; Hirsh et al., 2001; Weitz et al., 2008). Therefore, antithrombotics of choice used in the treatment of arterial thrombosis primarily consists of antiplatelet agents whereas anticoagulants are used in the treatment of venous thrombosis. Fibrinolytics are used in conditions where there is an urgent need to lyse the thrombus in order to maintain the blood flow such as in early stroke, acute myocardial infarction as well as in the treatment of massive pulmonary embolism (Weitz et al., 2008).

1.3.1 Antiplatelets

Antiplatelets currently available are aspirin, thienopyridine derivatives (clopidogrel and ticlopidine), dipyridamole and the glycoprotein IIb/IIIa receptor antagonists (abciximab and tirofiban). Some drawbacks associated with these drugs have led to the development of new antiplatelet agents targeting the TxA_2, ADP or thrombin receptors on platelets.

Aspirin (acetylsalicylic acid) irreversibly acetylates and inhibits prostaglandin H synthase (cyclooxygenase-1) in platelets, and thereby blocks the formation of TxA_2 (Patrono, 1994; Vane and Botting, 2003). When TxA_2 is absent to bind to its receptor, platelet aggregation is inhibited. TxA_2 receptors on platelets also bind to prostanoids such as prostaglandin F2α and cause platelet aggregation. Aspirin however, has no effect on the formation of prostanoids (Hankey and Eikelboom, 2006; Macchi et al., 2006; Hovens et al., 2007). Some patients are resistant to aspirin or aspirin inhibition of TxA_2 is not complete (Hankey and Eikelboom, 2006; Macchi et al., 2006; Hovens et al., 2007). TxA_2 receptor antagonists were therefore developed. S18886 is an orally active selective inhibitor of the TxA_2 receptor that prevents platelet aggregation (Gaussem et al., 2005).

The **thienopyridine derivatives (clopidogrel and ticlopidine)** are metabolised in the liver to active compounds which covalently bind to the ADP receptor (P2Y12) on platelets and dramatically reduce platelet activation (Richter et al., 2004; Gardell, 1994; Savi and Herbert, 2005). It has been shown however, that the ADP-induced platelet aggregation is not completely inhibited by these drugs at usual clinical doses (Savi and Herbert, 2005; Gurbel et al., 2003; Gurbel et al., 2006; Geisler and Gawaz, 2007). Moreover, thienopyridines are not fast acting because of the time needed for their activation in liver (Savi and Herbert, 2005). The action of thienopyridines is also not turned off rapidly since they irreversibly inhibit their target (Peters et al., 2003; Fox et al., 2004; Kapetanakis et al., 2005). New ADP receptor antagonists were therefore developed.

Prasugrel is a new ADP receptor antagonist that like thienopyridines needs activation by liver cytochrome P450 enzyme, R-138727 (Jakubowski et al., 2007). Activation of prasugrel however, is more efficient than that of clopidogrel (Jakubowski et al., 2007; Tantry et al., 2006).

Cangrelor and AZD6140 are direct competitive inhibitors of $P2Y_{12}$ that unlike clopidogrel or prasugrel do not need activation by hepatic enzymes (van Giezen and Humphries, 2005; Tantry et al., 2007). Consequently, the onset and offset of their action is quite fast after intravenous or oral administration respectively (van Giezen and Humphries, 2005; Fugate and Cudd, 2006; Tantry et al., 2007).

Dipyridamole inhibits phosphodiesterase (De La Cruz et al., 1994), which inactivates adenosine 3', 5'-cyclic monophosphate (cAMP). Increased intraplatelet concentrations of cAMP reduce the activation of cytoplasmic second messengers. Dipyridamole also stimulates prostacyclin release and inhibits TxA_2 formation (Harker and Kadatz, 1983; Costantini et al., 1990).

Glycoprotein IIb/IIIa receptor antagonists block the final common pathway for platelet aggregation (Frelinger et al., 2001). *Abciximab* is a humanised mouse antibody fragment with a high binding affinity for the glycoprotein IIb/IIIa receptor (Schrör and Weber, 2003; Hashemzadeh et al., 2008). *Tirofiban* (a non-peptide derivative of tyrosine) and eptifibatide (a synthetic heptapeptide) mimic part of the structure of fibrinogen that interacts with the glycoprotein IIb/IIIa receptor and thus compete with ligand binding of

fibrinogen to the glycoprotein IIb/IIIa receptor (Schrör and Weber, 2003; Hashemzadeh et al., 2008).

Thrombin receptor (PAR-1) antagonists, SCH-530348 (Chackalamannil et al., 2003) and E5555 (Weitz et al., 2008) are a new class of antiplatelet agents in the process of development. These drugs reversibly bind to PAR-1 receptors and block thrombin- and thrombin receptor agonist peptide (TRAP)-induced platelet aggregation (Chackalamannil et al., 2003; Weitz et al., 2008).

1.3.2 Fibrinolytics

Fibrinolytic therapy can be achieved via two strategies; drugs that enhance the endogenous fibrinolytic activity or drugs that enhance plasminogen activation.

1.3.2.1 Enhancing the Endogenous Fibrinolytic Activity

Inhibitors of PAI-1: PAI-1 inhibits both t-PA and u-PA. Inhibitors of PAI-1 decrease the PAI-1 activity either by reducing its gene expression (Fujii et al., 1993; Brown et al., 1995), or by reducing its activity (Kvassman et al., 1995; Eitzman et al., 1995) and therefore increase plasmin production from plasminogen.

Inhibitors of TAFI: TAFI inhibits binding of plasminogen or plasmin to fibrin by cleaving carboxy-terminal lysine residues from fibrin (Sakharov et al., 1997). Inhibitors of TAFI therefore increase the fibrinolytic activity (Redlitz et al., 1996; Klement et al., 1999; Nagashima et al., 2000; Guimaraes et al., 2006).

Inhibitors of activated factor XIII (XIIIa): Factor XIIIa cross-links the α- and γ-chains of fibrinogen and thereby makes the fibrin polymer resistant to degradation by plasmin (Mosesson, 1992). Consequently, inhibitors of factor XIIIa increase fibrinolysis (Muszbek et al., 1996; Finney et al., 1997).

1.3.2.2 Enhancing Plasminogen Activation

The tPA, is a family of drugs that include *Alteplase (Activase®; rtPA)* a recombinant form of human tPA (Dunn and Goa, 2001; Wagstaff et al., 1995), *Retaplase (Retavase®)* a derivative of *rtPA* with a smaller size and a faster onset of action, (Noble and McTavish, 1996) and Tenecteplase (TNK-tPA) another derivative of *rtPA* with longer half-life and greater affinity for binding to fibrin (Dunn and Goa, 2001).

Streptokinases (SK) are antigenic and include natural SK that is purified from streptococci bacteria and Anistreplase (Eminase®) that is a mixture of SK and plasminogen (Ohman et al., 2001; Bizjak and Mauro, 1998) with a longer activity than natural SK. These drugs are not specific for fibrin and can cause massive fibrinogenolysis (Ohman et al., 2001). Therefore they are not as desirable as tPA compounds.

The u-PA (Abbokinase®; UK), Like SK drugs, urokinase (UK) drugs cause massive fibrinogenolysis and therefore are not desirable (Maizel and Bookstein, 1986). However, unlike SKs, UKs are not antigenic (Ohman et al., 2001).

The tPAs are the most commonly used thrombolytic drugs and based on advances with tPA derivatives, new thrombolytic drugs are under development which include alfimeprase, a direct acting fibrinolytic that accelerates clot lysis (Deitcher et al., 2006), as well as BB10153 (Comer et al., 2005) and desmoteplase (Stewart et al., 1998; Mellott et al., 1995) that have high specificity for fibrin.

1.3.3 Anticoagulants

Currently available clinical anticoagulants include rapidly acting parenteral anticoagulants such as heparin and its derivatives, and oral vitamin K antagonists such as warfarin, preferred for long term anticoagulation (Hirsh et al., 1998; Weitz et al., 2008). Sodium citrate, sodium oxalate, sodium fluoride and ethylenediaminetetraacetic acid (EDTA) are anticoagulants that are used only for blood collection or preservation.

1.3.3.1 Vitamin K Antagonists

The current oral coumarin anticoagulants, such as warfarin, acenocoumarol and phenprocoumon are among the most widely prescribed drugs in modern medicine (Daly, 2009). Coumarin derivatives and indanediones are oral anticoagulants that are absorbed from the gut (Breckenridge, 1978; Kelly and O'Malley, 1979; Daly, 2009), and are vitamin K antagonists that interfere with the cyclic interconversion of vitamin K (Hirsh et al., 2001).

Coagulation factors II, VII, IX, and X, are dependent on vitamin K for their biological activity. Glutamate residues on the N-terminal region of these proteins require

posttranslational γ-carboxylation by a reduced form of vitamin K, (vitamin KH2) (Whitlon et al., 1978; Fasco et al., 1982; Choonara et al., 1988; Trivedi et al., 1988; Stenflo et al., 1974; Nelsestuen et al., 1974; Hirsh et al., 2001). The carboxylation reaction takes place when vitamin KH_2 becomes oxidized to vitamin K epoxide (KO) (Oldenburg et al., 2008). The KO is then converted to vitamin KH2 by two reduction reactions which have different degrees of sensitivity to vitamin K antagonists (Oldenburg et al., 2008).

In the first reaction that is strongly inhibited by vitamin K antagonists, the KO is reduced to vitamin K1 (Whitlon et al., 1978; Fasco et al., 1982; Choonara et al., 1988; Daly, 2009; Oldenburg et al., 2008). In the second reaction that is not very sensitive to inhibition by vitamin K antagonists, vitamin K1 becomes reduced to vitamin KH2. Therefore vitamin K antagonists produce their anticoagulant effect by preventing the formation of vitamin KH_2 that is necessary for the γ-carboxylation of the vitamin K-dependent coagulant proteins (Friedman et al., 1977; Malhotra et al., 1985; Daly, 2009). Decarboxylated coagulant proteins do not easily bind to cofactors on phospholipid surfaces and have therefore reduced procoagulant activity (Friedman et al., 1977; Malhotra et al., 1985; Nelsestuen, 1976; Prendergast and Mann, 1977; Borowski et al., 1986; Hirsh et al., 2001; Simmelink et al., 2002).

Oral anticoagulants also work by inhibiting the synthesis of endogenous anticoagulant proteins C and S. It is possible that the inhibition of proteins C and S contributes to a

transient pro-coagulant effect of the oral anticoagulants when they are first administered (Hirsh et al., 2001; Stearns-Kurosawa et al., 2002; Simmelink et al., 2002).

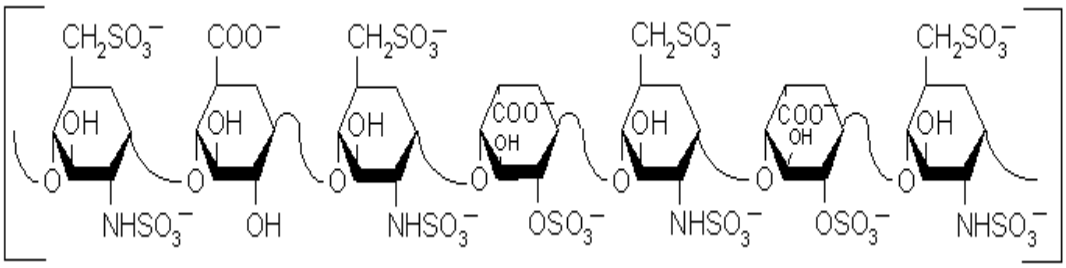
It has been shown that the patient's genetic factors as well as non-genetic factors determine the dose of the drug required for each patient (O'Reilly and Aggeler, 1970; Aithal et al., 1999; Mannucci, 1999; Hirsh et al., 2001). Thus, dosing should be determined individually on the basis of the patient's response (Daly, 2009; Rost et al., 2004).

1.3.3.2 Heparin and Derivatives

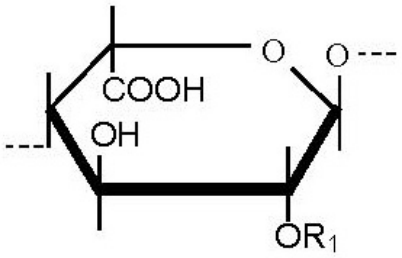
Heparin was discovered during World War I in 1916 by Jay McLean, a second-year medical student working under the direction of Wiliam Howell at Johns Hopkins University in Baltimore (McLean, 1916; Linhardt, 1991). McLean originally isolated heparin from canine liver cells, hence its name (*hepar* or "ηπαρ" is Greek for "liver"). Heparin belongs to the family of glycosaminoglycans (GAGs), a class of natural products extracted from animal organs that also includes heparan sulphate, chondroitin sulphate, dermatan sulphate, keratan sulphate, and hyaluronic acid (Hook et al., 1984; Garg and Lyon, 1991). Heparin is stored within the secretory granules of basophils and mast cells (Page, 1991; Amihai et al., 2001; Guyton and Hall, 2006). Native heparin or unfractionated heparin (UFH) is a polymer with a molecular weight ranging from 3000 to 30,000 Da, with a mean molecular weight of 15000 Da (Andersson et al., 1979; Harenberg, 1990; Johnson and Mulloy, 1976; Linhardt and Gunay, 1999). The polymeric chain of heparin consists of repeating disaccharide units of a D-glucosamine derivative

and uronic acid linked by 1—>4 interglycosidic bonds (Taylor and Gallo, 2006; Rhodes and Simons, 2007) (Figure 2). The uronic acid residue could be either D-glucuronic acid or L-iduronic acid (Garg et al., 2000; Taylor and Gallo, 2006). A few hydroxyl groups on each of these monosaccharide residues has various *O*-sulfonate, *N*-sulfonate, and *N*-acetyl substituents giving rise to a polymer that is highly negatively charged (Cox and Nelson, 2004) (Figure 2).

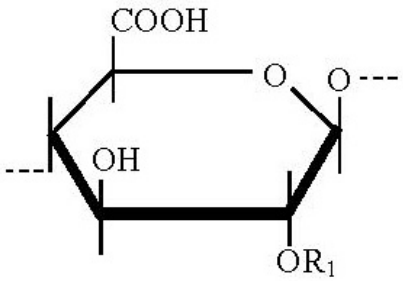
Low molecular weight heparins (LMWHs) are derived from heparin by chemical or enzymatic depolymerization (Linhardt and Gunay,1999) and have a molecular weight ranging from 1000 to 10 000 Da with a mean of 4500 to 5000 Da (Hirsh et al., 2001). Depending on the type of fractionation method used, different LMWHs have different chemical structure, physical and biological properties (Barrowcliffe, 1995; Donayre, 1996; Hunt, 1998; Ramos-Sánchez, 1995; Fareed et al., 1998). While UFH has both anti-factor Xa and anti-factor IIa anticoagulant activity, LMWHs have primarily anti-factor Xa activity (Johnson et al., 1976; Andersson et al., 1976; Hirsh et al., 2001). LMWHs also differ from UFH in their half-life, tissue binding, clinically effective doses, side effects observed, and choice of effective neutralizing agents (Hirsh et al, 1998; Hirsh et al., 2001). Heparin and its derivatives, LMWHs, are used parenterally as drugs of choice when there is a need for an immediate anticoagulant effect (Hirsh et al., 2001; Büller et al., 2004; Turpie et al., 2004; Otero-Fernández et al., 2008).



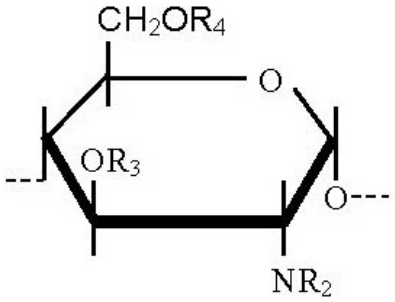
(a) α-L-iduronic acid



(b) β-D-glucuronic acid



(c) α-D-glucosamine derivatives



R_1 = H or SO_3^-
R_2 = H, $COCH_3$ or SO_3^-
R_3 = H or SO_3^-
R_4 = H or SO_3^-

Figure 1.2 Heparin structure. Heparin consists of alternating disaccaride units of D-glucosamine and either D-glucuronic acid or L-iduronic acid. These basic sugars are then *O*-sulfated, *N*-sulfated, and *N*-acetylated at various positions making a long, linear polymeric chain that's highly negatively charged. Adapted from Silva and Dietrich, 1975.

1.3.3.2.1 Anticoagulant and Antithrombotic Effects of Heparin

Heparin performs the majority of its anticoagulant effect by activating AT (Abildgaard, 1968). Upon binding to lysine site on AT through a unique glucosamine unit (Rosenberg and Bauer, 1994; Lindahl et al., 1979; Casu et al., 1981; Rosenberg and Lam, 1979) contained within a pentasaccharide sequence in heparin (Choay et al., 1981), heparin makes a conformational change at the arginine-reactive site on AT converting it from a slow, progressive inhibitor to a very rapid inhibitor of the active center serine of thrombin and factor Xa (Lindahl et al., 1979; Rosenberg and Lam, 1979; Hirsh et al., 2001). Thrombin is more sensitive by ten-fold to inhibition by the heparin-AT complex than factor Xa (Hirsh et al., 2001). Moreover, heparin molecules should contain at least 18 saccharides to be able to inactivate thrombin but not factor Xa. This is due to the necessity for heparin to bind to both AT and the coagulation enzyme simultaneously for inactivation of thrombin but not factor Xa (Casu et al., 1981). Consequently, large heparin molecules containing the pentasaccharide sequence have the ability to inactivate both thrombin and factor Xa versus very small heparin fragments containing the pentasaccharide sequence that mostly catalyze inhibition of factor Xa (Lindahl et al., 1984; Lane et al., 1984; Oosta et al., 1981; Nesheim, 1983, Hirsh er al., 2001). Upon inactivation by heparin, thrombin is not only unable to produce fibrin but also can not induce activation of factor V and factor VIII (Ofosu et al., 1987; Ofosu et al., 1989; Beguin et al., 1988; Hirsh et al., 2001). The heparin-AT complex also inactivates factors IXa, XIa, and XIIa although to a lesser degree (Rosenberg and Bauer, 1994).

Heparin's anticoagulant effect at therapeutic concentrations through activation of AT is due to only about one third of an administered dose of heparin (Lam et al., 1976; Andersson et al., 1976; Hirsh et al., 2001). The remaining two thirds of an administered dose of heparin have minimal anticoagulant activity at therapeutic concentrations. However, at concentrations greater than those obtained clinically, heparin catalyzes the antithrombotic activity of another plasma protein, HCII (Tollefsen et al., 1982; Hirsh et al., 2001).

The UFH and LMWHs also have antithrombotic action by inducing vascular endothelial cells to secret TFPI (Lupu et al., 1999; Altman et al., 1998; Gori et al., 1999; Young et al., 1992; Hirsh et al., 2001). Release of TFPI is dependent on the chain length and the degree of sulfation of heparin molecules (Barzu et al., 1985; de Swart et al., 1982; Olsson et al., 1963; Hirsh et al., 2001). Heparin also binds to vWF and inhibits platelet function (Sobel et al., 1991; Hirsh et al., 2001).

1.3.3.2.2 Limitations to Heparin

The anticoagulant response to heparin is variable (Hirsh et al., 1976; Hirsh et al., 2001) with some patients being resistant to heparin therapy (Levine et al., 1994; Young et al., 1992) requiring higher than average amounts of heparin daily (Levine et al., 1994; Anand et al., 1997; Hirsh et al., 1994). Heparin has a high binding affinity for a number of proteins and also surfaces. Heparin binds to plasma proteins (Young et al., 1994), and to proteins released from platelets (Lane, 1989). Heparin also binds to macrophages (Friedman and Arsenis, 1974) and endothelial cells (Glimelius et al., 1978; Mahadoo et

al., 1977) leading to its clearance in a dose-dependent manner. Therefore, heparin resistance occurs in situations where there is AT insufficiency (Olson et al., 1998), increased heparin clearance (Whitfield et al., 1983), increased heparin binding proteins (Lijnen et al., 1983; Marci and Prager, 1993; Young et al., 1992), increased factor VIII (Levine et al., 1994; Edson et al., 1967), fibrinogen (Edson et al., 1967), and platelet factor 4 (PF4) (Levine et al., 1984). Heparin-induced thrombocytopenia and therefore bleeding occurs when heparin-PF4 complex acts as an antigen causing antibody production to platelets (Visentin et al., 1994; Greinacher et al., 1994). Moreover, heparin-induced osteopenia occurs when heparin binds to osteoblasts and reduces bone formation (Shaughnessy et al., 1995), and is also responsible for activation of osteoclasts.

1.3.3.3 New Anticoagulants

New anticoagulants have been made and classified as inhibitors of initiation of coagulation, inhibitors of propagation of coagulation, and inhibitors of fibrin formation (Weitz et al., 2008). Clinical trials addressing the efficacy and safety of these drugs as therapeutic agents are currently underway.

1.3.3.3.1 Inhibitors of Initiation of Coagulation

Drugs in this category include **tifacogin** that is a recombinant form of TFPI expressed in *Saccharomyces cerevisae* (Abraham et al., 2001; Abraham et al., 2003), **recombinant nematode anticoagulant peptide (NAPc2)** which is an 85-amino acid polypeptide isolated from the canine hookworm, *Ancylostoma caninum*, and expressed in yeast (Cappello et al., 1995; Bergum et al., 2001; Vlasuk et al., 2003), and **recombinant factor**

VIIa (factor VIIai) that has an irreversibly blocked active site (Taylor, 1996; Jang et al., 1995). These parenteral drugs inhibit the initiation of coagulation by inhibiting factor VIIa/TF complex (Abraham et al., 2001; Abraham et al., 2003; Weitz et al., 2008).

1.3.3.3.2 Inhibitors of Propagation of Coagulation

Direct Factor IXa Inhibitors consisting of the parenteral RNA aptamer, RB006 (Rusconi et al., 2002; Dyke et al., 2006), and the oral TTP889 (Eriksson and Quinlan, 2006).

Direct and Indirect Factor Xa Inhibitors. Indirect Factor Xa Inhibitors are synthetic analogs of the antithrombin-binding pentasaccharide found in UFH or LMWH (Boneu et al., 1995) catalyzing factor Xa inhibition by AT (Weitz et al., 2008). These include fondaparinux (Boneu et al., 1995; Cohen et al., 2006; Buller et al., 2004; Buller et al., 2003), idraparinux (Herbert et al., 1998), SSR126517E (Weitz et al., 2008), and SR123781A (Herbert et al., 2001; Becker et al., 1999). Direct Factor Xa Inhibitors are small molecules that block the active site of factor Xa in a reversible manner (Hirsh et al., 2001). These include parenteral DX9065a (Herbert et al., 1996; Becker et al., 2006) and otamixaban, (Paccaly et al., 2005; Hinder et al., 2006; Cohen et al., 2007) as well as oral Razaxaban (Lassen et al., 2003), Apixaban (Kan et al., 2006), Rivaroxaban (Kubitza et al., 2005; Eriksson et al., 2007; Eriksson et al., 2006), LY-517717 (Agnelli et al., 2007), YM 150 (Eriksson et al., 2007), DU-176b (Turpie, 2007), and PRT 054021(Turpie et al., 2007; Buller, 2006).

Factor Va Inhibitors (Weitz et al., 2008) including Drotrecogin Alfa (Activated), a direct inhibitor of FVa (Bernard et al., 2001; Abraham et al., 2005), and ART-123, an indirect inhibitor of FVa (Parkinson et al., 1990; Kearon et al., 2005).

1.3.3.3.3 Inhibitors of Fibrin Formation, this group of drugs consist of direct and indirect thrombin inhibitors, an enzyme that converts fibrinogen to fibrin (Weitz et al., 2008).

Direct thrombin inhibitors that directly bind to thrombin and prevent its interaction with the substrate include parenteral agents such as hirudin, argatroban, and bivalirudin that have already been licensed in North America for some limited applications (Weitz et al., 2008) as well as flovagatran (Weitz et al., 2008) and pegmusirudin (Avgerinos et al., 2001) currently undergoing evaluation. There are also two new oral direct thrombin inhibitors, ximelagatran (Gustafsson et al., 2001; Testa et al., 2007; Lee et al., 2005; Gustafsson, 2003) and dabigatran etexilate (Gustafsson, 2003; Weitz, 2006; Stangier et al., 2007), as well as one new oral **indirect thrombin inhibitor**, odiparcil, acting via inhibition of HCII (Tommey et al., 2006; Bates et al., 2007).

1.4 Other Actions of Heparin

It is now well known that besides anticoagulation, heparin has many other functions including lipid clearing effects, anti-proliferative, anti-inflammatory, anti-tumor effects, etc.

1.4.1 Heparin and Lipoprotein Lipase Activity

Lipoprotein lipase (LPL) is the major enzymatic activity involved in the hydrolysis of triglycerides into free fatty acids (FFAs) (Goldberg, 1996). Heparin binding is required for maintaining LPL stability and activity (Lutz et al., 2001; Morris et al., 2005). Heparin totally arrests LPL intracellular degradation and increases the quantitative secretion of the mature form of the enzyme (Engelberg, 1996). When mutated at the heparin binding site, a human LpL minigene ($hLpL^{HBM}$) expressed in mice accumulated in peripheral blood with no activity as assessed by increased postprandial FFAs, decreased lipid uptake in muscle tissue, and increased lipid uptake in kidneys compared with mice expressing native hLpL in the muscle (Lutz et al., 2001).

1.4.2 Heparin and Proliferation and Migration of Smooth Muscle Cells Associated With Atherogenesis

The proliferation and migration of vascular smooth muscle cells (VSMCs) are fundamental processes in atherogenesis (Ross, 1993; Schwartz et al., 1995; Rauch et al., 2004). Heparin has repeatedly been reported to have antiproliferative effect on VSMCs (Rudijanto, 2007; Fasciano et al., 2005) both in culture systems (Hoover et al., 1980; Castellot et al., 1981), and in animal models (Schoen et al., 1991; Clowes and Clowes, 1987; Clowes and Karnovsky, 1977; Guyton et al., 1980).

SMCs proliferate in response to growth factors, cytokines, and extracellular matrix proteins (Ross, 1993). Attachment of cells to mitogenic substrates such as angiotensin II (ANGII), PDGF, and thrombin leads to the generation of PIP2 which is a substrate for

PLC to generate IP3 and DAG. DAG activates PKC, and IP3 increases SMC intracellular Ca^{2+} (Marks, 1992). Ca^{2+} is an important regulator of cell proliferation and migration (Pfeilschifter et al., 1989). An increase in intracellular Ca^{2+} stimulates transcription factors like c-*fos* involved in the induction of mitogenic genes (Roche and Prentki, 1994).

Heparin binds to specific receptors on VSMCs and is rapidly internalized by receptor-mediated endocytosis (Letourneur et al., 1995). Studies have shown that heparin blocks IP3-induced Ca^{2+} release (Ghosh et al., 1988; Kobayashi et al., 1988; Kobayashi et al., 1989). Furthermore, it has been reported that the antiproliferative action of heparin in VSMCs is mediated via regulation of a Ca^{2+}-mediated mitogenic signaling pathways (Mishra-Gorur et al., 2002). IP3-increased intracellular Ca^{2+} causes binding of calmodulin to Ca^{2+}/calmodulin dependent protein kinase II (CaM kinase II) which in turn, leads to the autophosphorylation on Thr286/287 and CaM kinase II activation (Schulman, 1993). Activated CaM kinase II has been implicated in the VSMC migration (Pauly et al., 1995) and proliferation (Tombes et al., 1995). Heparin inhibits CaM kinase II phosphorylation by activating protein phosphatases 1 and 2A (Mishra-Gorur et al., 2002). Activated CaM kinase II has also been shown to activate mitogen activated protein kinase (MAPK), a key molecule in mitogenic signalling (Abraham et al., 1997). Heparin inhibits MAPK activation in rat VSMCs (Ottlinger et al., 1993; Seger and Krebs, 1995; Pukac et al., 1992; Castellot et al., 1989), and therefore down-regulates the expression levels of c-*fos,* c-*jun, myb,* and *myc* genes involved in cell growth (Pukac et al., 1990). Heparin strongly down-modulated the levels of cell cycle regulatory proteins such as

cyclin D1 mRNA and protein, cyclin-dependent kinase 2 (cdk2) mRNA and cyclin-dependent c2 (cdc2) protein (Vadiveloo et al., 1997; Fasciano et al., 2005).

The VSMCs proliferate and migrate in response to matrix metalloproteinases (MMPs). PDGF up-regulates the transcription of MMPs (Stetler-Stevenson et al., 1993). Heparin binds to an amino acid sequence in PDGF, causing inactivation of the growth factor (Fager et al., 1992a). Moreover, heparin prevents PDGF binding to its cell surface receptor (Fager et al., 1992b), and inhibits the expression of MMPs in primate arterial SMCs (Kenagy et al., 1994).

It has also been shown that thrombin and FXa induce proliferation and migration of SMCs by releasing heparin-binding EGF-like growth factor (HB-EGF) and basic fibroblast growth factor (bFGF), which then act as ligands for the epidermal growth factor receptor (EGFR) and fibroblast growth factor receptor-1 (FGFR-1) in rats (Prenzel et al., 1999; Kalmes et al., 2000) and humans respectively (Rauch et al., 2004). Heparin inhibits mitogenesis and migration of SMCs *in vivo* and *in vitro* (Clowes and Karnowsky, 1977; Majack and Clowes, 1984; Clowes and Clowes, 1987) by binding to HB-EGF and therefore interfering with its pathway (Kalmes et al., 2000; Rauch et al., 2004). Heparin also inhibits the activation of FGFR-1 by competing with heparan sulfate proteoglycans for released bFGF (Rauch et al., 2004; Ross, 1993).

Na+-H+ exchange in vascular SMC is important in SMC-growth activated by ANG II, thrombin and endothelin (ET). Heparin inhibited Na+-H+ exchange in rat aortic SMC (Zaragoza et al., 1990).

1.4.3 Anti-Inflammatory Actions of Heparin and Atherosclerosis

Heparins anti-inflammatory activity was repeatedly shown in clinical trials where heparin and LMWHs were shown to be beneficial in inflammatory diseases such as bronchial asthma (Boyle et al., 1964; Fine et al., 1968; Ahmed et al., 1999), ulcerative colitis (Gaffney et al., 1995; Evans et al., 1997; Folwaczny et al., 1999; Torkvist et al., 1999; Vrij et al., 2001), and burns (Salba, 2001).

The mechanism (s) by which heparin performs its anti-inflammatory action is not completely understood. However, there are studies suggesting some potential mechanisms. Heparin is a highly negatively charged and acidic molecule with both specific and nonspecific high affinity for binding to an ever growing list of proteins. Heparin in pharmacological doses was able to bind and neutralize many chemokines, cytokines and complement factors, therefore preventing interaction with their respective receptors (Weiler et al., 1992; Nissen et al., 1999; Elsayed and Becker, 2003).

Leukocyte adhesion to endothelial cells and activation are important phenomena in an inflammatory response (Tyrell et al., 1999). Heparin and LMWH are able to inhibit neutrophil adherence to resting and stimulated endothelial cells (Bazzoni et al., 1993; Silvestro et al., 1994; Lever et al., 2000). Glycoprotein adhesion molecules L-selectin

(constitutively expressed by neutrophils and monocytes), and P- and E-selectin (expressed on activated endothelial cells) mediate a *loose* interaction of endothelium and neutrophils during the first step of an inflammatory response (Bevilacqua and Nelson, 1993; Lorant et al., 1993; Moore et al., 1995). Heparin and LMWHs bind to stimulated endothelial cells expressing P-selectin (Nelson et al., 1993; Young et al., 1999; Koenig et al., 1998; Xie et al., 2000; Wang et al., 2002; Gao et al., 2005) and therefore inhibit neutrophils adherence (Silvestro et al., 1994).

β2-integrins (CD11b/CD18, CD11c/CD18 complexes) expressed constitutively on the surface of neutrophils mediate the *firm* attachment of neutrophils to the intercellular adhesion molecules (ICAMs) on endothelial surfaces. This *firm* attachment of neutrophils to the endothelial cells is necessary for transendothelial migration of neutrophils into the interstitial space (Diamond et al., 1993; Lefer, 1999). Heparin binds to CD11b/CD18 and inhibits neutrophil adhesion to endothelial cells (Diamond et al., 1995; Peter et al., 1999). Nuclear factor κB (NF-κB) is an inducible transcription factor residing in the cell cytosol (Wulczyn et al., 1996). NF-κB mediates the expression of genes involved in inflammatory responses, including ICAMs, L- and P-selectins, when translocated into the cell nucleus via a positively charged nuclear localization sequence present within its protein sequence (Lin et al., 1995). Heparin has been shown to be internalized and bound in the cytosol of the endothelial cells (Young et al., 1999), VSMCs (Letourneur et al., 1995), hepatic cells (Dudas et al., 2000) and cardiac myocytes (Akimoto et al., 1996). Heparin binds to the cationic nuclear localization sequence inhibiting NF-κB translocation from cytoplasm into the nucleus of human endothelial cells (Manduteanu et

al., 2003) and T cells (Hecht et al., 2004). This has been shown to down-regulate the expression of proinflammatory cytokines (Hochart et al., 2006).

1.4.4 Heparin and cancer

The growth of a primary tumor depends on its proliferation potential, activity of the immune system, and on tumor angiogenesis. Heparin has an anti-proliferative effect on VSMCs, mesangial cells, fibroblast, and epithelial cells (Tiozzo et al., 1989; Au et al., 1993; Bennett et al., 1994; Miralem et al., 1996). Heparin inhibits phosphorylation of MAPK and therefore inhibits the expression of proto-oncogenes c-fos and c-myc in the PKC pathway (Castellot et al., 1989; Pukac et al., 1990; Pukac et al., 1992; Imai et al., 1993; Miralem et al., 1996; Ottlinger et al., 1993; Daum et al., 1997; Mishra-Gorur and Castellot, 1999; Smorenburg and Van Noorden, 2001).

Angiogenesis is a complicated process including activation, proliferation, and migration of endothelial cells, degradation of extracelluar matrix as well as formation of the capillary vessel lumen (Diaz-Flores et al., 1994). Heparin can interfere with these processes. Tumors produce a number of high affinity growth factors for receptors on endothelial cells stimulating angiogenesis (Senger et al., 1986; Rosen and Goldberg, 1997; Kumar et al., 1998; Schmidt et al., 1999). Vascular endothelial growth factor (VEGF) is secreted by various human and animal tumor cell lines and facilitates tumor growth via stimulating angiogenesis (Claffey et al., 1996). There are two high affinity VEGF receptors on endothelial cells, KDR/flk-1 and flt-1 (Keyt et al., 1996). The KDR receptor is implicated in the VEGF induction of endothelial cell proliferation. A highly

positively charged amino acid surface (arginine, lysine, and histidine) is critical for VEGF binding to KDR/flk-1. Heparin is strongly anionic and it binds to and inhibits the activity of these cationic amino acids. b-FGF is another growth factor whose activity is inhibited by heparin (Jayson and Gallagher, 1997). Many human and animal tumors activate coagulation. Fibrin provides a framework for invasive cancer and endothelial cells and regulates the formation of a capillary-like tubular structure (Nehls and Herrmann, 1996; Shats et al., 1997). Heparin affects angiogenesis by altering the structure of this matrix (Collen et al., 2000). Another anti-angiogenic aspect of heparin via anticoagulant function is inhibition of the expression of TF (Pepe et al., 1997). The TF/factor VIIa complex enhances tumor cell migration and invasion and up-regulation of VEGF (Ruf and Mueller, 1996). Heparin enhances activity of the TFPI (Engelberg, 1999; Novotny et al., 1991; Pepe et al., 1997). Invasion into the extracellular matrix by endothelial cells is also important for angiogenesis and involves plasminogen activators (PAs), MMPs, and cathepsins (Keppler et al., 1996; Mignatti and Rifkin, 1996; Rabbani, 1998). Heparins have both stimulatory and inhibitory action on the expression of PAs and MMPs (Clowes et al., 1992; Kenagy et al., 1994; Putnins et al., 1996; Brunner et al., 1998; Gogly et al., 1998).

Experiments demonstrated that the level of natural killer (NK) cell reactivity is a significant factor in the survival or destruction of metastatic cells in the blood stream (Gorelik et al., 1984). Thrombin is generated by tumors, and the resultant fibrin formation around tumor cells impedes NK cell activity (Engelberg, 1999). It has recently

been found that heparin activates NK cells in both *in vitro* and *in vivo* conditions (Bobek and Kovarik, 2004).

1.5 Oral Heparin

Heparin is traditionally given by intravenous and subcutaneous routes (Emmanuele and Fareed 1987) due to the belief that it is not absorbed from the gastrointestinal (GI) tract and therefore considered ineffective given by the oral route (Fisher and Astrup 1939; Dal pozzo et al., 1989; Money and York, 2001; Hirsh et al., 2001). This is based on the assumption that the high negative charge and molecular weight of heparin prevent its absorption (Money and York, 2001). Furthermore, it is believed that heparin is destroyed by stomach acids (Emmanuele and Fareed 1987; Dal Pozzo et al., 1989). Little observed change in plasma activated partial thromboplastin times (APTT) following oral administration, an assay commonly used to measure heparin in plasma, has also supported this presumption (Leone-Bay et al., 1998). Since continuous intravenous UFH for long periods of time is not practical, long term subcutaneous administration of LMWHs or use of oral agents such as warfarin have been used to prevent thrombosis. Unfortunately, individual patient response to warfarin therapy varies depending on genetic and dietary factors as well as various disease states, and the dosage must be monitored closely for the duration of treatment (Daly, 2009; Rost et al., 2004; Hirsh et al., 2001; Aithal et al., 1999; Mannucci, 1999; O'Reilly and Aggeler, 1970). Moreover, subcutaneous injection of LMWHs produces discomfort in patients. The pain associated with intravenous or subcutaneous routes of heparin administration would be avoided with an oral heparin formulation (Linhardt et al., 1990; Gonze et al., 2000). Based on the

assumption of the ineffectiveness of orally administered heparin, there has been considerable effort directed towards increasing heparin absorption following oral administration with the goal being to detect evidence of heparin in plasma.

1.6 Increasing Oral Heparin Absorption

The numerous therapeutic applications of heparin are more feasible with an oral heparin formulation, therefore, a number of formulation strategies have been investigated to enhance heparin's oral bioavailability in *in vivo* animal models (Motlekar and Youan, 2006) such as mice (Folkman et al., 1983; Chander et al., 1989), rats (Schmitz et al., 2005; Larsen et al., 1986a; Windsor and Cronheim, 1961; Engel and Riggi, 1969; Vasdev et al., 1992; Brayden et al., 1997; Leone-Bay et al., 1998; Li et al., 2000; Costantini et al., 2000; Kast et al., 2003; Thanou et al., 2001; Yang et al., 2005; Motlekar et al., 2005), rabbits (Andriuoli et al., 1992; Jiao et al., 2002; Welt et al., 2001), dogs (Ueno et al., 1982), pigs (Thanou et al., 2001), primates (Leone-Bay et al., 1998) and humans (Dryjski et al., 1989; Horwitz et al., 1992; Imiela et al., 1995; Cui and Jiang, 1999; Berkowitz et al., 2003).

Investigators have administered bile salts and its derivatives (Kim et al., 2006; Lee et al., 2001; Park et al., 2004) and polycationic lipophilic-core dendrons (Hayes et al., 2006) to increase heparin penetration through cell membranes. Since heparin is a very large hydrophilic macromolecule, it was speculated that its absorption may be limited to the paracellular pathway consisting of the tight junctions holding aqueous pores (Goldberg and Gomez-Orellana, 2003). Therefore researchers have tried to modify tight junctions

using several absorption enhancers such as labrasol (Rama Prasad et al., 2004), sulfonated surfactants (Engel and Riggi, 1969), EDTA acid (Windsor and Cronheim, 1961), saponins (Cho et al., 2003), chitosan derivatives (Thanou et al., 2001), thiolated polycarbophil system (Schmitz et al., 2005; Kast et al., 2003; Bernkop-Schnurch et al., 2003), carbopol 934P (Thanou et al., 2001), sodium caprate (Motlekar et al., 2005), and Zonula occludens toxin synthetic peptide derivative AT1002 obtained from the bacterium *vibrio cholerae* which was shown to open the tight junctions in a reversible manner (Motlekar et al., 2006).

Researchers have also tried to increase the lipophilicity of heparin by covalently attaching it to lipophilic molecules such as dimethyl sulfoxide and deoxycholic acid conjugates (Kim et al., 2006; Lee et al., 2001; Park et al., 2004). Heparin has also been conjugated with carriers such as organic acids (Dal Pozzo et al., 1989), sodium *N*-[10-(2-hydroxybenzoyl) amino] decanoate (Gonze et al., 1998) (SNAD), sodium *N*-[8-(2-hydroxybenzoyl) amino] caprylate (SNAC) (Rivera et al., 1997; Berkowitz et al., 2003; Pineo et al., 2004), diamine salts (Andriuoli et al., 1992). Furthermore, heparin was incorporated into microemulsion formulations (Kim et al., 2006; Andersson and Lofroth, 2003), polyion complex micelles (Dufresne and Leroux, 2004; Duncan, 2003), liposomes (Ueno et al., 1982; Gregoriadis, 1980) and dendrons (Hayes et al., 2006).

Heparin was also protected against the acidic pH of the stomach following oral administration by enteric coating (Doutremepuich et al., 1985; Andriuoli et al., 1990), use

of alginate/chitosan/PEG microparticles (Chandy et al., 2002) and polymeric nanoparticles (Jiao et al., 2002).

1.7 Evidence of Oral Heparin Absorption

Despite the efforts cited above to increase the oral absorption of heparin, results from our lab and research by several other investigators have challenged the belief that heparin is ineffective following oral administration. The studies outlined below demonstrate that heparin may be effective when administered by the oral route.

1.7.1 Target Organ of Orally Administered Heparin-Endothelium

Early studies of thrombus formation in glass vascular shunts showed that the presence of anticoagulant activity in blood did not immediately prevent thrombus growth. Furthermore, disappearance of the anticoagulant activity did not result in thrombus formation (Best et. al., 1938). An additional factor was obviously involved in the anti-thrombotic action of heparin- the vessel wall. Srinivisan et al., (1968) observed that blood vessel injury, resulting in thrombosis, was accompanied by a marked decrease in the normal negative charge of the vessel wall. Imposition of a positive charge on the vessel lumen or prostheses produced a thrombus. This decrease in negative charge was restored to normal by heparin. Because of its high negative charge, heparin interacts with the vascular wall, formed elements and the plasma proteins.

Work from our lab and that of others have shown that heparin binds avidly to endothelium following both *in vivo* and *in vitro* administration (Hiebert and Jaques

1976[a]; Hiebert and Jaques 1976[b]; Glimelus et al., 1978; Barzu et. al., 1985; Hiebert and McDuffie 1989; Barzu et. al., 1984; Van Rijn et. al., 1987; Vannucchi et. al., 1986; Jaques et. al., 1991; Hiebert et. al., 1993; Dawes and Pepper 1992; Vannucchi et. al., 1988; Barzu et. al., 1987). LMWHs are also bound and internalized by endothelium although with less affinity than UFH (Palm et. al., 1990; Barzu et. al., 1985; Hiebert and McDuffie 1989). Jaques et. al., 1991 and Hiebert et. al., 1993, measured chemical heparin levels in both plasma and endothelium following administration by various routes. A large portion of the intravenous dose and almost all of the oral dose was found in the endothelium from the rat aorta and vena cava at as little as 2.4 and 6 minutes, whereas the amounts found in plasma after an oral dose did not exceed 1% of the administered dose during the 2h period. The concentration of heparin in endothelium was on the order of 100 times greater than that in plasma following intravenous injection and 1000 times greater following subcutaneous, intraperitoneal, intrapulmonary, or intragastric administration (Hiebert et al., 1993). These findings have been supported by administration of cold and [^{14}C] porcine UFH to rats (Hiebert et. al., 2000). These results show that heparin enters the circulation following the administration by the oral route. Furthermore, endothelium is the target organ for heparin in the body (Araki et. al., 1992; Norrby 1993). The finding, in the endothelium, of a large portion of an oral dose of heparin within 2.4 min following placing the drug in the stomach means that heparins are absorbed rapidly possibly via a unique transport mechanism. Since heparin is taken up rapidly by the endothelium when administered orally, and because of the very large endothelial surface in the splanchnic area, very little remains in the plasma that reaches the general circulation.

Results of these studies are quite unique in that the method used to measure the heparin level differed from previous procedures, which indirectly estimated plasma heparin concentrations by determining inhibition of coagulation. Activation of antithrombin and activated factor X inhibition have been shown, by many investigators, to be associated with only a few of the separate individual sulfated polysaccharides that are present in the drug (Jaques 1980; Jaques 1967), less than 5% for each action in heparin. Jaques et al., (Jaques et al., 1991) and Hiebert et al., (Hiebert et al., 1993) measured the chemical amount of the drug. This type of measurement involves the total drug rather than a small fraction, and includes all the chain lengths that are individually responsible for the wide variety of biological activities.

Similar observations as shown for heparin were obtained for oral dextran sulfate, a polyanion similar in structure to heparin. Lorensten et al., (Lorensten et al., 1989) gave dextran sulfate (molecular weight 8000) to human volunteers and failed to obtain evidence of dextran sulphate in plasma at 0.25 to 12 h but did observe a positive plasma LPL release at 3 to 4 h, indicating absorption and endothelial interaction of the drug. Thus, previous studies have drawn quite different conclusions describing the ineffectiveness of oral heparin, since they were not able to conduct analyses on the body compartment (endothelium) that incorporates immediately almost all the drug that is absorbed.

Experiments using ^{125}I-labelled bovine heparin were conducted to determine the fate of heparin following uptake by cultured porcine aortic endothelial cells (Hiebert et. al.,

1993). Heparin was recovered from cells following addition of radiolabel to culture medium for 2 days. Following removal of heparin-containing medium, washing of cells with buffer and addition of fresh medium without heparin, the level of heparin present in cells decreased with time. Moreover, there was a concomitant increase in radioactivity in the medium. Radioactivity was recovered from both peri-cellular and cellular fractions indicating that heparin not only attaches to the cellular surface but also is internalized. Therefore from *in vitro* experiments, it is obvious that the interaction of heparin with components of the vessel wall is not simply the fixation of heparin to endothelium but a dynamic interchange with extracellular fluid and external and internal cell components. These results indicated that heparin, taken up by endothelial cells, is returned to plasma or medium. Some of the heparin that is internalized, remains associated with the cells for long periods of time (Hiebert and McDuffie 1989) as indicated by the presence of metachromatic inclusions when stained with Toluidine Blue for 5 days after heparin uptake. Similar results were obtained with ^{125}I-labelled unfractionated porcine mucosal heparin or a low molecular weight porcine heparin, CY222 (Hiebert and McDuffie 1990).

1.7.2 Heparin in Non-Gut Tissues Following Oral Administration

Hiebert et al., (Hiebert et al., 2000) took the advantage of radiolabelled heparin to better understand heparin absorption from the gut. They administered porcine mucosal heparin along with [^{14}C] heparin to rats by the oral route and measured both radiolabelled and chemical heparin.

The lung showed a relatively high level of radioactivity at 3 minutes. When heparin was given in gelatin capsules, results at 24 h showed no difference in radiolabel recovered from gut and non-gut tissue compared with [^{14}C] heparin given by stomach tube, suggesting that it was unlikely that aspiration played a significant role in the final distribution of heparin in non-gut tissue. Distribution to the lung, however, may be a result of the high degree of vascularity of the tissue. Chemical determinations also detected relatively high amounts of heparin in the lung.

Substantial amounts of radiolabel was detected in the **liver** from 15 min to 24 h which was not confirmed by chemical determination of heparin. This discrepancy was explained to be either a result of high levels of endogenous heparin and GAGs in the liver making chemical detection difficult, or that some heparin is broken down into heparin metabolites not detected chemically by agarose gel electrophoresis. Uptake of [^{14}C] heparin in the liver was in agreement with previous studies demonstrating uptake and long-term heparin retention in liver of rats, rabbits, and guinea pigs (Asplund et al., 1939), retention in the liver of [^{35}S] heparin and [^{35}S] heparin fragments 5 h after intravenous administration (Larsen et. al., 1986^{b}), and uptake by liver Kupffer cells (Loomis 1961; Hiebert 1981; Asplund et. al., 1939) and liver endothelial cells. **Kidney** also showed low levels of radiolabel and chemical heparin from 15 min onward, which was in agreement with previous studies (Asplund et. al., 1939; Larsen et al., 1986^{b}).

At 24 h after [^{14}C] administration, radiolabel was found in many organs including heart, lung, scrotum, thymus, spleen, brain, tongue, bone marrow, muscle, hair, and skin, with

trace amounts in trachea, bile ducts, aortic and vena caval walls (without endothelium), ureters, and bladder (Hiebert et al., 2000). Polyacrylamide gel electrophoresis analysis of GAGs recovered from lung, liver, and kidney suggested that the majority of heparin was absorbed as complete molecules and not broken down into smaller fragments prior to digestion. These results suggest wide tissue distribution of heparin.

1.7.3 Low Recovery of Oral Heparin in the 24 h Gut Samples

When all the components of the GI tract were considered, including the tongue, esophagus, and feces, only 7% of the radiolabel could be accounted for in the GI tract at 24 h, with 4.7% present in the gut washings and feces and 2.3% present in the gut tissue. Hiebert et al., (2000) mentioned that the latter fraction may also represent absorption, although further studies are required to determine if this fraction was simply attached to the gut wall, attached to the vasculature, or otherwise incorporated into the tissue. Recovery of 7% at 24 h is considerably less than 34.9-43.9% observed from the GI tract at 3-6 min after administration, again suggesting absorption into the body. The percent recovered from non-gut tissue increased with the number of tissues sampled at 24h; 0.5% found when urine, plasma, and five tissues were examined VS >5.1% when an additional 12 non-gut tissues were observed, again supporting the idea that the heparin is absorbed and widely distributed. When samples obtained from the GI tract were compared upon administration of ^{14}C radiolabel and cold heparin by stomach tube, amounts and concentrations of heparins found in the colon are much less than that found in the stomach again suggesting that absorption has occurred.

1.7.4 Antithrombotic Activity of Oral Heparin

Absorption of heparin following oral administration is supported by observations of antithrombotic activity in a rat jugular vein model. Application of an irritant to the jugular vein of rats resulted in a high and reproducible incidence of thrombosis. In this model, when the vein was examined 4 h after thrombus initiation, both bovine and porcine UFH significantly reduced thrombotic incidence when administered by stomach tube (Hiebert et al., 2000; Hiebert et al., 1996). A dose-dependent decrease in thrombosis was observed with oral bovine UFH with thrombotic incidence reduced by 50% at 7.5 mg/kg and thrombosis completely abolished at 60 mg/kg (Hiebert et al., 1996). A similar reduction in thrombosis incidence was seen with oral porcine UFH at 7.5 mg/kg.

The dose required to obtain antithrombotic activity was reduced when repeated doses of oral UFH were administered (Hiebert et al., 2008). Bovine lung UFH was administered by oral gavage to rats in 3 doses of 7.5 mg/kg each 12, 24, 48, and 72 h apart; and in 3 or 15 doses of 1 mg/kg every 48 h. The last dose was given immediately after thrombus initiation in the jugular vein, and the vessel was examined for thrombosis 4 h later. Thrombotic incidence was most reduced at 48 h dose-intervals and was significantly less than single dose treatment when 3 doses of 7.5 mg/kg each bovine lung heparin was given to rats.

Studies with oral LMWHs in the same thrombosis model show that thrombosis can be prevented at much lower doses when compared to oral UFH. A dose-dependent reduction in antithrombotic activity was demonstrated for the LMWHs reviparin and logiparin, with a 50% reduction in thrombotic incidence at 0.025, and 0.1 mg/kg respectively (Hiebert et.

al., 2000; Hiebert et. al., 2001). A similar reduction in thrombotic incidence was demonstrated following administration of 1 mg/kg of ardeparin. As was observed with UFH, only trace amounts were present in plasma although the drug could be recovered from endothelium in a dose-dependent manner. Heparins were also shown to reduce thrombosis incidence in venous stasis (Costantini et al., 2000), and carotid arterial thrombosis models (Pinel et al., 2004). Oral dextran sulphate, a polyanionic compound similar to heparin of molecular weight 8000, also showed reduction in thrombosis incidence (Jaques et. al., 1991).

1.7.5 Other Evidence of Oral Heparin Absorption

Other investigators have supported the idea that oral heparin is systemically effective. Administering LMWH or UFH (0.5 mg/kg) in drinking water, given to spontaneously hypertensive rats, returned the systolic blood pressure to normal (Vasdev et. al., 1992; Vasdev et. al., 1994). Oral heparin was reported to be effective in treatment of rheumatoid arthritis and also in promoting the survival of skin allografts (Gorski and Lagodzinski 1991; Gorski et, al., 1994). Oral heparin (40,000 units) decreased plasma triglycerides in five human subjects with little change in APTT (Johnson et. al., 1997).

1.8 Macromolecular Absorption

It was commonly assumed that macromolecules like carbohydrates or proteins are completely digested to their building blocks within the lumen of the GI tract before absorption occurs. The oral absorption of GAGs was consistently debated over the years. This may be due to lack of sensitive analytical methods. It is no longer tenable to regard

the GI tract as an impenetrable "physical" barrier, since many particles and large molecules previously regarded as "nonabsorbable" are in fact absorbed (Gardner 1988).

1.8.1 Evidence for Intact Protein Absorption

It is now known that intestinal peptide transport is a major process. Peptide transport systems, distinct from free amino acid carriers, have been characterized in intestinal brush-border membranes, with the terminal stages of protein digestion occurring intracellularly after transport of peptides into the mucosal absorptive cells (Matthews 1975; Matthews 1977; Matthews and Payne 1980). Also, there now is irrefutable evidence that small amounts of intact peptide and proteins can escape total digestion to amino acids and do enter the circulation under normal circumstances (Gardner, 1984; Gardner, 1987). Therefore, intact protein absorption is now regarded as a normal physiological process in humans and animals.

The most compelling single item of evidence showing that intact proteins or macromolecular protein fragments are absorbed is provided by the demonstration, repeatedly made by numerous independent workers, that antibodies to many food proteins and their immune complexes occur in the circulation of all individuals (Paganelli and Levinsky 1980; Sass et al., 1987). Moreover, analyses of plasma by radioimmunoassay showed the presence of orally administered proteins, such as ovalbumin in blood (Husby et al., 1987; Jakobsson et al., 1986). Hence, it is impossible to escape the conclusion that immunologically small amounts of intact protein (or

immunologically identifiable large fragments thereof) have been absorbed (Gardner, 1988).

The above conclusion was reinforced by numerous animal and isolated tissue experiments. For example, Mclean and Ash, 1986; and Mclean and Ash, 1987 have reported the presence of intact (or largely intact) horseradish peroxidase (HRP) in blood and in several peripheral tissues in fish *in vivo*. Several other studies have demonstrated passage of high-molecular-weight fragments of protein across isolated animal jejunum (Warshaw et al., 1974; Heyman et al., 1982; Ducroc et al., 1983). Moreover, laboratory and clinical findings have indicated that protein antigens are capable of crossing the gastric epithelium immunologically intact and interacting with immune cells in the lamina propria. It was demonstrated that the stomach is capable of mounting a type I hypersensitivity reaction to luminal antigen challenge (Catto-Smith et al., 1989; Reimann and Lewin, 1988).

1.8.1.1 Possible Routes and Mechanisms for Protein Absorption

1.8.1.1.1 Paracellular or Transcellular Routes

Both the paracellular pathway through the "tight junctions", and the transcellular pathway may be involved in intact protein absorption (Gardner, 1988). However, most evidence favors the latter route as dominant, especially in healthy individuals, although the process is a complex one involving metabolic energy expenditure, cytoplasmic tubule formation, and processing.

Bockman and Winborn, 1966 observed ferritin passing through hamster intestinal cells by pinocytosis, with none passing between the cells. Likewise, Heyman et al., 1982 suggested that only a small fraction of absorbed HRP crossed by the paracellular route in their rabbit ileal experiments *in vitro*. Hence, as indicated below, the transcellular route seems to be more important than the paracellular route, although increases in the paracellular route caused by disease may even make it a predominant route.

1.8.1.1.2 Endocytotic Mechanisms

Histochemical and electron microscopic observations on macromolecular transport indicated that protein molecules bind to receptors on the surface of the apical (brush-broder) membrane (Walker and Isselbacher 1974). The membrane invaginates to form phagosomes or vesicles encapsulating the protein. The phagosomes migrate in the cytoplasm to lysosomes via a system of cytoplasmic microtubules. Most fuse to form phagolysosomes or secondary lysosomes, in which proteolysis occurs by a series of cathepsins and other acid proteases. Some apparently fail to fuse or use a separate pathway and leave the cells by exocytosis at the basolateral membrane. All these steps are energy dependent. A similar process occurs in neonatal animals but large numbers of vacuoles are formed. At the neonatal stage, as an example, immunoglobulin G (IgG) receptors exist on the brush-border membrane and it is thought that binding to them (and their inclusion in the vesicles) specially protects the engulfed IgG from proteolysis in the phagolysosomes (Gardner, 1988). In the experiments of Heyman et al., 1982, 97% of the peroxidase entering the cells was shown to be degraded to fragments of 2000-4000 daltons. Hence it appears that lysosomal proteolysis is a major factor in minimizing entry

of intact protein into the circulation, although the mechanism of this process has not been extensively studied in the gastrointestinal cells.

1.8.1.2 Pathways in the Gastrointestinal Tract for Uptake of Intact Proteins

The lumen of the gastrointestinal tract contains a variety of macromolecules. A number of studies have described the existence of pathways for the uptake of intact macromolecules from the lumen across the mucosa of the stomach (Curtis and Gall, 1992; Curtis et al., 1993) and small intestine (Heyman et al., 1982; Ramage et al., 1988; Kimm et al., 1996; Kimm et al., 1994; Warshaw et al., 1971).

1.8.1.2.1 Protein Uptake in Stomach

Macromolecular uptake in the upper GI tract is a highly regulated phenomenon. In the stomach the uptake of intact protein involves an active transcellular, energy dependent mechanism that utilizes the microtubular network (Curtis and Gall 1992) and is regulated by cyclooxygenase metabolites (Curtis et al., 1993). The uptake of immunologically intact bovine serum albumin (BSA) was significantly reduced by sodium fluoride (NaF), an inhibitor of adenosine-5'-triphosphate (ATP) production and endocytosis; colchicine, which inhibits polymerization of cytoskeletal microtubules; and low temperature (4°C), a general metabolic inhibitor (Curtis and Gall 1992). The role of paracellular permeability in BSA transport was measured by calculating conductance in the inhibition studies. NaF or colchicine did not alter conductance, but it was significantly reduced by 4°C. Moreover, the intact BSA fluxes did not differ significantly between the three groups examined. Thus, despite the variable effect on conductance, inhibition of BSA movement

occurred to a similar extent with all three metabolic inhibitors, indicative of transport independent of the paracellular processes. This result agrees with other work by Atisook and Madara, 1991 in the intestine. They suggested that tight junctions might be permeable to small oligopeptides but selectively exclude larger macromolecules. They noted permeation of an 11-amino acid hemepeptide (MP-11, molecular weight 1900) through tight junctions but exclusion of HRP (molecular weight 40,000). Assuming a commonality of tight junction function along the GI tract, it is predictable that BSA (molecular weight 66,200) would also be excluded from paracellular transport (Curtis and Gall 1992).

The effect of two structurally distinct cyclooxygenase inhibitors, indomethacin and piroxicam, on the movement of intact BSA protein across the rat gastric mucosa *in vitro* were investigated (Curtis et al., 1993). Cyclooxygenase inhibition significantly reduced tissue prostaglandin E_2 (PGE_2) synthetic capacity. Both inhibitors significantly decreased uptake of immunologically intact BSA. However, neither PGE_2 nor PGI_2, applied to both the mucosal and serosal surfaces of the tissue altered macromolecular uptake, although PGE_2 and PGI_2 are the most prevalent prostaglandins in the GI tract. These findings suggested that cyclooxygenase metabolites other than PGE_2 and PGI_2 might be responsible for regulating intact protein transport, although rapid metabolism of these compounds may have negated their effects.

1.8.1.2.2 Protein Uptake in Jejunum

In the jejunum, mucosal to serosal macromolecular transport is a saturable transcellular process that is both microtubule and energy dependent (Heyman et al., 1982; Kimm et al., 1994) and is regulated by the enteric nervous system acting via muscarinic pathways (Kimm et al., 1994). Metabolic inhibition by 4°C and NaF, as well as inhibition of microtubule assembly by colchicine, significantly decreased uptake of intact BSA (Kimm et al., 1994) in agreement with previous results showing metabolic inhibition to decrease uptake of both intact HRP and β-lactoglobulin (Heyman et al., 1982; Marcon-Genty et al., 1989). Macromolecular uptake of intact BSA by rat jejunum was not through the bulk flow via paracellular route, since (1) metabolic inhibition decreased intact BSA uptake in the absence of a change in conductance, (2) glucose addition to the mucosal chamber which up-regulates paracellular pathways, did not affect intact BSA uptake (Kimm et al., 1994). Treatment of the rat jejunum with the nerve blocker tetrodotoxin, which binds non-competitively to electrogenic sodium channels and renders all neurons non-functional, significantly reduced antigen uptake (Kimm et al., 1994). Atropine, a muscarinic cholinoceptor antagonist, significantly reduced antigen uptake to similar extent as tetrodotoxin, whereas hexamethonium, a nicotinic cholinoceptor antagonist, had no effect.

Nitric oxide has also been shown to play a role in the regulation of jejunal macromolecular uptake (Kimm et al., 1996). Nitric oxide is known as a major non-adrenergic, non-cholinergic neurotransmitter in the intestine. N^G-nitro-L-arginine-methyl ester (L-NAME, the inhibitor of the nitric oxide synthase (which is an enzyme responsible for production of nitric oxide) was shown to significantly increase intact BSA

uptake in the rat jejunum (Kimm et al., 1996). The L-NAME effect was reversed by L-arginine (substrate for nitric oxide production by nitric oxide synthase). In contrast to the previous results by Kimm et al., 1994 showing up-regulation of macromolecular transport across rat jejunum by cholinergic nerve fibers, these findings indicated that nitric oxide down-regulates intact macromolecular flux in the small intestine (Kimm et al., 1996).

1.8.1.2.3 Protein Uptake in Ileum

In the ileum, mucosal to serosal macromolecular uptake has also been shown to be both microtubule and energy dependent (Macron-Genty et al., 1989) and under neural regulation acting through muscarinic pathways (Bijlsma et al., 1996). Transport of intact β-lactoglobulin was inhibited by the metabolic inhibitors deoxyglucose and azide added simultaneously, was reduced by the microtubule assembly inhibitor colchicine, and was enhanced by ammonia, which inhibits lysosomal proteolytic activity (Marcon-Genty et al., 1989). Furthermore, experiments using the adenylyl cyclase activator forskolin suggested that adenosine 3', 5'-cyclic monophosphate (cAMP) plays a role in the regulation of ileal macromolecular uptake (Bijlsma et al., 1996). Bijlsma et al., (1996) investigated the effects of the secretagogues forskolin and carbachol on HRP protein uptake in the isolated ileum of rats. Forskolin is a direct activator of adenylyl cyclase, which induces chloride (Cl^-) secretion by stimulating cAMP-dependent protein kinase A (PKA), and carbachol is a cholinergic agonist that causes Cl^- secretion by increasing intracellular Ca^{2+} and activation of PKC (Bajnath et al., 1992). In the absence of secretagogues, enzymatically active HRP was transported across the mucosa. Electron micrographs showed vesicles filled with active HRP in enterocytes but no HRP activity

in intercellular spaces. Forskolin decreased HRP activity in the cells. Carbachol, however, increased the amount of HRP-filled vesicles in enterocytes and induced HRP filling in some intercellular spaces and tight junctions in the upper parts of the villi. The transepithelial flux of intact HRP increased more than 2.5-fold. This effect was suppressed by atropine. These results indicated that cholinergic activation could increase the uptake of intact protein via endocytosis and the transepithelial passage by the induction of a diffusional paracellular pathway (Bijlsma et al., 1996).

1.8.1.2.3.1 The Gut as an Immune Organ-The M Cell Route

The mucosal tissues of the small intestine (jejunum and ileum) are sites of intense immunological activity and are regarded as important immune organs. Peyer's patches are gut-associated lymphoid tissues in the mucosa covered by a special epithelium containing specialized microfold cells or M cells. M cells in the epithelium are especially differentiated to take up and transport antigenic macromolecules and microorganisms from the lumen across the epithelial barrier (Kernei′s et al., 1997). The hallmark of M cells is the presence of a large invaginated subdomain of the basolateral membrane that amplifies the cell surface and forms an intraepithelial "pocket"(Neutra et al., 2001). The pocket membrane provides a docking site for specific subpopulation of intraepithelial B- and T-lymphocytes. The apical membrane of M cells are designed to promote adherence and uptake of foreign macromolecules, particles, and microorganisms. They generally lack brush borders and instead have variable microvilli or microfolds interspersed with large plasma membrane subdomains that participate in clathrin-mediated endocytosis of ligand-coated particles, adherent macromolecules, and viruses (Neutra et. al., 2001).

Vesicles are particularly abundant in the M cell, a reflection of their endocytotic capacity, and there appear to be fewer lysosomes in the cytoplasm, which is consistent with a diminished rate of intracellular protein degradation (Wolf and Bye 1984). If macromolecules enter M cells, then they may be carried through and released into the pocket and only rarely go into phagolysosomes. However, M cells do have a few lysosomes, and may be able to digest some of the material they take up (Allan et al., 1993). This means that M cells, which have major histocompatibility (MHC) class II determinants on their surfaces, may be able to present antigen directly to lymphocytes. This is a mechanism by which uncommitted lymphocytes may come in contact with antigen taken up by M cells.

1.8.1.2.4 Protein Uptake in Colon

Much less is known about macromolecular uptake in the colon compared to the small intestine. Studies have demonstrated the *in vivo* uptake of intact human growth hormone across rat colon (Moore et al., 1986) and bioactive arginine vasopressin has been detected in rabbit plasma following injection into ligated colonic loops (Lundin and Vilhardt, 1986). In addition, the bacterial chemotactic peptide N-formyl-methionyl-leucyl-phenylalanine (fMLP) has been shown to be absorbed intact across rat colon (Ferry et al., 1989). Apical to basal transepithelial absorption of intact HRP across human colon carcinoma cell line (Caco-2) monolayers was not altered by 4°C or exposure to monensin, a drug known to inhibit receptor mediated endocytosis (Heyman et al., 1990).

In contrast, basal to apical movement of intact HRP across Caco-2 monolayers was eight times greater than that observed in the apical to basal direction and is reduced at 4°C.

Hardin et al., 1999 investigated the mechanisms involved in the transport of large antigenically intact macromolecules across the proximal and distal colonic epithelium in the rabbit. Intact BSA transport in proximal and distal colonic tissue showed saturable kinetics. Immunologically intact BSA transport in the distal segment was significantly less than that in the proximal segment. Intact BSA transport in the proximal colon was significantly reduced following treatment with NaF, colchicine, and tetrodotoxin. Cholinergic blockade had no effect on the uptake of intact BSA. These findings suggested that intact BSA transport in the proximal colon is an energy dependent process that utilizes microtubules and is regulated by the enteric nervous system (Hardin et al., 1999).

1.8.1.3 Serosal to Mucosal Transport in the Small Intestine

The transport of intact proteins in the small intestine also occurs in the serosal to mucosal direction (Heyman et al., 1982; Kimm et al., 1997). As shown for mucosal to serosal uptake, jejunal serosal to mucosal macromolecular transport is both saturable, and energy and microtubule dependent (Kimm et al., 1997). NaF and colchicine inhibited the transport of intact BSA from the serosa to mucosa in the rat jejunum. However, unlike mucosal to serosal uptake, the serosal to mucosal flux of intact protein is not under neural control (Kimm et al., 1997).

1.8.2 Oligonucleotide Transport in the Gastrointestinal Tract

There is some controversy concerning oligonucleotides (ONs) absorption from the GI tract. Agrawal et al., 1995 reported significant absorption of a naked, hybrid or mixed backbone oligonucleotide (MBO) after lavage. The ON was present as an undegraded molecule in the stomach and small intestine up to 6 h after administration, but was significantly degraded thereafter. After intestinal absorption, the ON was also evident as an intact parent molecule in portal venous plasma. Intact and degraded products of the MBO were detected in systemic plasma and tissues.

Hughes et al., 1995 concluded that ON absorption in rat jejunum occurs via the paracelluar pathway rather than an active mechanism. In contrast, a transcellular transport hypothesis was presented by Beck et al., 1996 who examined the cellular uptake of phosphodiester and phosphorothioate ONs in washed Caco-2 cells. Wu-Pong et al., 1999 however, examined paracellular ON absorption through human jejunum, rat jejunum, ileum and colon. Inconsistent with observations of Agrawal et al., 1995, they concluded that mucosal to serosal transport was inefficient and did not appear to be saturable up to 500 μM in the intestinal tissue, a finding which, is also suggestive of either MBO paracellular diffusion or the involvement of a transporter of relatively high capacity. However, other data in the same study argued for a transcellular component in MBO absorption: 1) tight junction openers unexpectedly did not increase MBO transport; 2) mucosal to serosal transport appeared to exceed rather than equal, seorsal to mucosal transport; and 3) fluorescein isothiocyanate (FITC)-MBO distribution in both human and rat jejunum clearly showed intracellular distribution of fluorophore in the absence of

significant FITC-MBO degradation. Thus, the authors concluded that MBO absorption is likely to include a transcellular mechanism, although a paracelluar component was not excluded based on these results.

1.8.3 Glycosaminoglycan Absorption from Gastrointestinal Tract

The GAGs of physiological significance are hyaluronic acid, dermatan sulfate, chondroitin sulfate, heparin, heparan sulfate, and keratan sulfate. Absorption of GAGs across the GI tract have been repeatedly reported in the literature.

Hyaluronic acid (HA) is typically used as a medical device for treatment of degenerative joint conditions, and is used in eye surgery and wound healing (Bucci and Turpin, 2004; Laurent and Fraser, 1992; Kogan et al., 2007; Campbell et al., 2007; Samson et al., 2007). HA is administered by injectable or topical routes of administration for these uses. Because of the large molecular weight and size of individual HA molecules (usually 1 MDa or more), and rapid clearance from the bloodstream by the liver (Laurent and Fraser, 1992), it has been assumed that oral HA would exhibit poor systemic uptake and/or clinical utility. However, this assumption has not been tested or reported in the scientific literature using modern techniques until recently. Balogh et al., (2008) reported the first evidence for uptake into the bloodstream and distribution to tissues, especially connective tissues, of orally administered, high-molecular weight HA.

Dermatan sulfate (DS) inhibits blood clotting both by potentiating the inactivation of thrombin by HCII (Tollefsen et al., 1983) and by reducing thrombin generation in plasma (Ofosu et al., 1984). Animal studies have indicated that it is an effective antithrombotic

agent at concentrations which are not effective in *in vitro* tests of anticoagulant activity (Buchanan et al., 1985; Maggi et al., 1987) and that it is also able to inhibit growth of established thrombi (Van Ryn-McKenna et al., 1989). Despite possessing anti-platelet activity (Sie et al., 1982) it appears to be less haemorrhagic than heparin at equivalent antithrombotic doses (Fernandez et al., 1986). Thus, DS is a potential antithrombotic agent with a different spectrum of pharmacological activity from that of heparin. The pharmacokinetics of DS, MF701, were studied by the Dawes group (Dawes et al., 1989[a]; Dawes et al., 1989[b]; Dawes et al., 1991) in healthy patients after administration of single intravenous bolus, intramuscular and oral doses. Plasma drug concentrations were measured using a competitive binding assay and a range of biological activity assays, including a sensitive catalysed thrombin inhibition test. They concluded that DS is the only GAG known to generate significant plasma concentrations following oral administration.

Chondroitin sulfate (CS) is an important structural component of cartilage and provides much of its resistance to compression. *In vivo* studies have produced controversies concerning the absorption of the CS from the GI tract. Some studies show an elevation of plasma, urinary and synovial CS concentrations after oral administration (Conte et al., 1991; Conte et al., 1995). Conversely, others failed to show absorption following oral administration (Konador and Kawiak, 1977). The presence of low molecular weight fragments derived from CS in urine or plasma following oral dosing was also shown (Conte et al., 1995).

Dextran sulfate, a polyanionic compound similar to heparin of molecular weight 8000, is absorbed after oral administration to human immunodeficiency virus (HIV)-positive subjects (Hiebert et al., 1999). Dextran sulfate was recovered from plasma when it was orally administered in a single dose of 4 g per day for 5 days, or in a multiple doses of 1g for 4 times a day for 29 to 335 days. As well, oral dextran sulphate reduced thrombosis incidence in a rat model (Jaques et. al., 1991).

Pentosan polysulfate sodium, another heparin like GAG, is orally administered in pill form. It prevents cell permeability to irritant solutes and thus protect against interstitial cystitis or painful bladder syndrome. There has been some controversy against its oral bioavailability since greater than 94% of drug was shown to be excreted intact in feces following oral administration (Simon et al., 2005). However, Anderson and Perry, 2006, supported use of pentosan polysulfate as the only oral treatment for interstitial cystitis approved by US Food and Drug Administration.

1.8.3.1 Absorption of Heparin from the Gastrointestinal Tract

Many previous studies on heparin absorption from the GI tract examined the small intestine, the anatomically favored and major site of absorption for most compounds (Thomson et al., 2003[a]; Thomson et al., 2003[b]). Sue et al., reported that when heparin was introduced into the GI tract, increases in whole blood clotting time and plasma anti-Xa activity were observed. The anticoagulant effect was greater when heparin was placed in the stomach versus the small intestine (Sue et al., 1976). Loomis instilled sodium heparin into dog duodenal loops with buffers of pH 4 or pH 8 and reported that the

systemic anticoagulant effect was obtained in the presence of acidic buffer of pH 4 but not the alkaline buffer of pH 8 (Loomis, 1959). Koh and Bharucha reported that complexes of heparin with weak organic bases and amino acids were absorbable from the jejunum of rabbits (Koh and Bacharicha, 1972). Results from our lab showed that more heparin is recovered from endothelium when UFH is placed in the stomach with the pyloric sphincter tied versus the duodenum for 15 minutes (Hiebert et al., 2007). More chemical or [^{14}C] UFH was found in the stomach versus duodenum, jejunum, ileum or colon up to 24 h (Hiebert et al., 2000). Despite these few studies, the site and mechanism of heparin absorption is unknown.

Chapter 2: Hypotheses and Objectives

2.1 Hypotheses

1. Orally administered heparins have antithrombotic activity and are effective in treatment of hypertension, rheumatoid arthritis, and in survival of skin allografts. **Therefore, heparins are absorbed by the GI tract.**
2. Greater anticoagulant activity and greater amount of heparin bound to endothelium is found following heparin administration into the stomach versus small intestine. **Therefore, heparins are absorbed in the stomach.**
3. LMWHs have smaller molecular weight than UFH. They also achieve antithrombotic activity at much lower doses than UFH after oral administration. **Therefore decreasing the molecular size facilitates the absorption of heparin through the gastric mucosal membrane.**
4. Intestine has a larger surface area than the stomach. As well, intestine has areas containing Peyer's patches with increased permeability. **Therefore, heparins are absorbed by the intestine.**
5. LMWHs do not bind as readily to proteins (Fareed et al., 1998) as UFH. As a result, they may be less likely to attach to epithelium in the stomach thus entering the duodenum and lower levels of the gut more easily. **Therefore, the site of heparins absorbtion likely differs for UFH and LMWH.**
6. Intact protein macromolecules cross the GI tract via an active transport mechanism utilizing energy and the cellular microtubular system. **Therefore, heparins cross the GI membranes predominantly via an active transport mechanism**.

2.2 Objectives

1. To determine whether orally administered UFH crosses rat gastric mucosa when mounted in an Ussing chamber, and to better understand the site of UFH absorption by manipulating the pH of the mucosal buffer.
2. To investigate if LMWHs cross rat gastric mucosa mounted in an Ussing chamber, and to better understand the site of LMWH absorption by manipulating the pH of the mucosal buffer.
3. To determine if LMWHs move across rat gastric mucosa faster than UFH.
4. To investigate if UFH crosses rat ileal mucosa to determine if ileum could be involved in UFH absorption. In addition, to determine if UFH movement across ileal mucosa is dependent on the presence of Peyer's patches, ileal preparations with or without patches will be used.
5. To determine if mechanism(s) of active transport or passive diffusion are involved in the process of UFH absorption across the gastric mucosa.

Chapter 3: Materials and Methods

3.1 Chemicals for transport studies

Materials for gel electrophoresis; petroleum ether, glacial acetic acid, and acetone were obtained from VWR Canlab, Mississauga, ON, Canada; sodium barbital, hexadecyltrimethylammonium bromide, toluidine blue, and HCl were from Sigma-Aldrich, ON, Canada; and agarose was from Bio-Rad, Mississauga, ON, Canada. Materials for Kreb's buffer, $MgCl_2$-$6H_2O$, $CaCl_2$-$2H_2O$, NaCl, KCl, Na_2 HPO_4, NaH_2PO_4-H_2O, $NaHCO_3$, mannitol, D-glucose, were from VWR Canlab. Molecular weight cut off (MWCO) 1000 dialysis tubing was purchased from Spectrum Laboratories Inc., RanchoDominguez, CA, USA. Materials for heparin extraction from mucosal tissues; protease from Streptomyces griseus was from Sigma-Aldrich; Tris, $CaCl_2$, isopropanol, and methanol were from VWR Canlab. The chromogenic assay kit (Accucolor™ Heparin®) for measurement of anti-Xa activity was obtained from Sigma-Aldrich. Reagents for measurement of anti-IIa activity, as outlined below, were from DiaPharma Group, Inc. West Chester, OH. QIAprep® Spin M13 Kit (50) columns for desalting the buffers were from Qiagen, ON, Canada. Chemicals for anaesthesia, chloral hydrate, sodium pentobarbital, magnesium sulfate, ethanol and propylene glycol were obtained from Sigma-Aldrich.

3.2 Buffer preparation

3.2.1 Kreb's Bicarbonate Buffer Stock Solutions

Test substances were added to Kreb's bicarbonate buffer. Kreb's bicarbonate buffer stock solutions were prepared as described in Table 3.1 and were sterile filtered for long-term

storage. To prepare each stock solution, salts were weighed, added to a volumetric flask, dissolved and diluted to the appropriate volume with reagent-grade water. Stock solutions were stored in the refrigerator until use.

Table 3.1 Kreb's bicarbonate buffer Stock Solutions

Solution	Chemical	MW (g/mole)	500ml	200ml
1	$MgCl_2$-$6H_2O$	203.300	2.235	0.894
	$CaCl_2$-$2H_2O$	147.000	3.160	1.264
2	NaCl	58.440	66.600	26.640
	KCl	74.550	3.750	1.500
3	Na_2HPO_4	141.960	2.340	0.936
	NaH_2PO_4-H_2O	138.000	0.415	0.166
4	$NaHCO_3$	84.010	21.000	8.400

3.2.2 Preparation of Kreb's bicarbonate buffer from Stock Solutions

Fifty ml of each solution in the numbered order as depicted in Table 1 was added to a small volume of reagent grade water (about 200 ml). Each solution was dissolved prior to adding the next to prevent calcium salt precipitation. The mixture was diluted with reagent grade water to a final volume of 1 liter. Final salt concentrations in Kreb's bicarbonate buffer are shown below in Table 3.2.

Table 3.2 Kreb's bicarbonate buffer (pH 7.4)

Chemical	Final concentration (mM)
$MgCl_2$-$6H_2O$	1.10
$CaCl_2$-$2H_2O$	2.15
NaCl	113.96
KCl	5.03
Na_2HPO_4	1.65
NaH_2PO_4-H_2O	0.30
$NaHCO_3$	25.00

When mucosal buffer of pH 4 was used, the mucosal buffer pH was adjusted to 4.0 by adding a few drops of HCl into the mucosal buffer. Componenets of the buffer were not altered or removed since that would change the balanced solution composition across the mucosa and violate the basic principle of the Ussing chamber (Clarke, 2009).

3.2.3 Transport Study Buffers

3.2.3.1 Serosal (Basolateral) buffer preparation

For serosal buffer preparation, 40 mM unlabeled D-glucose was added to Kreb's bicarbonate buffer (pH 7.4). This concentration of D-glucose was used in our serosal buffer as it was shown to maintain tissue viability for the duration of our experiments by other previous permeability measurement studies (Grass and Sweetana, 1988; Jezyk et al., 1992; Grass and Sweetana, 1989; Sutton et al., 1992).

3.2.3.2 Mucosal (Apical) buffer preparation

Since glucose was used in serosal buffer, an equivalent 40 mM amount of unlabeled mannitol was added to the mucosal buffer. The mannitol equalizes the osmotic load between the mucosal and serosal buffers.

3.3 Animals

Animals were obtained from Charles River Canada Company, St. Constant, Quebec, Canada and were handled in accordance with the Principles of Animal Care set by the Canadian Federation of Biological Societies. All animal procedures were approved by the Animal Care Committee of the University of Saskatchewan and performed according to the guiding principles of the Canadian Council on Animal Care.

3.4 Gastric mucosa isolation from rats

Male Wistar rats (250-300 g) were anesthetized by injection of Equithesin (chloral hydrate 4.2% w/v, sodium pentobarbital 0.98% w/v, magnesium sulfate 2.12% w/v, ethanol 10% v/v, propylene glycol 40% v/v, and sterile water to a volume of 100 ml: 1 ml/250 g rat) through the intraperitoneal route. The abdominal cavity was entered by a medioventral incision. Retractors were placed on each side of the incision to hold the abdomen open. The animal was killed by removal of the heart. The stomach was removed by cutting its connections with the esophagus and duodenum, and was then placed in saline. The stomach was opened from the lesser curvature and then washed several times with saline until all stomach contents and blood were removed. The stomach was then stretched, mucosal side up, on the cork bottom of a plastic tray using pins to hold it in place. The tray was placed on ice to reduce the metabolic rate. The mucosa of the glandular portion of the stomach was carefully stripped from submucosa and serosa using a scalpel blade.

3.5 Ileal mucosa isolation from rats

A midline abdominal ventral incision was made immediately after rats were anesthetized by an intraperitoneal injection of Equithesin as described above for gastric mucosa isolation. Segments of distal ileum were removed, and rinsed with Kreb's Ringer bicarbonate solution to remove intestinal contents. Segments were classified as those with or without Peyer's patches by visual inspection. After segments were stripped of muscle layers, flat mucosal tissue was mounted in tissue holders in the Ussing chamber for electrophysiological measurements. As a control, an additional portion of the mucosa was frozen for later GAG extraction.

3.6 Mounting gastric or ileal mucosal layer in the Ussing chamber

The time between cutoff of blood supply and mounting of tissue in the Ussing chamber was less than 3 min. The exposed surface area of gastric or ileal mucosa with or without Peyer's patches was 2.5 cm^2. Both sides of the epithelium were perfused with Kreb's Ringer bicarbonate buffer of pH 7.4 or 4.0 on the mucosal side and 7.4 on the serosal side. The Ringer solution contained mannitol (40 mM) on the mucosal side and D-glucose (40 mM) on the serosal side. Total buffer volume of 1.5 ml on either side was maintained at 37°C and gassed with humidified 5% CO_2 and 95% O_2.

3.6.1 Vertical Diffusion Ussing chamber

The EVC 4000 voltage/current clamp (NaviCyte, Harvard Apparatus, Inc.) used for transport studies across mucosal segments of the gastrointestinal tract included 6 components: (1) six vertical diffusion Ussing chambers, each having a circular opening

where the mucosa was in contact with the buffer from the other side and acted as a partition between the two hemi-chambers thus creating two separate compartments: the mucosal compartment and the serosal compartment; (2) a 12-channel gas manifold; (3) a heat block; (4) 2 ports for voltage electrodes (one on each side), and 2 ports for the current electrodes. These electrodes allow the measurement of the electrical parameters of the tissue, including voltage potentials, trans-membrane electrical resistance, and short circuit current. The electrodes have a shaft diameter of 2.5 mm and a shaft length of 5.0 cm. The silver/silver chloride electrode uses a refillable glass barrel that contains silver chloride (AgCl)-saturated 3 M potassium chloride (KCl); and (5) instrumentation which measures both voltage and current.

3.6.2 Measurements of heparin movement across gastric or ileal mucosa in a vertical diffusion Ussing chamber

Immediately following separation from submucosa and serosa, gastric tissue or ileal mucosa was mounted on the pins of one hemi-chamber, and the matching hemi-chamber was attached to seal the diffusion apparatus. The tissue was trimmed if it protruded beyond the outer chamber sides.

The assembled chamber was placed in a block heater connected to a circulating water bath, which maintained a temperature of 37ºC. The reservoirs on each side of the mucosa were filled with warmed (37ºC) oxygenated Kreb's Ringer bicarbonate buffer of pH either 7.4 or 4.0. Buffers in the reservoirs were circulated by gas lift (95% O_2 / 5% CO_2), controlled by valves (Precision Instrument Design, Los Altos, CA). Buffers (1.5 ml /

hemi-chamber) were added to each side of the chamber simultaneously to prevent hydrostatic pressure effects.

Harvard/ Navicyte Micro-Reference voltage measuring electrodes (2.5 mm x 5.0 cm) were placed on either side of the mucosa as close as possible to reduce the magnitude of the series resistance of the solution. Electrodes used for passing current were placed in the rear of the chambers, as far as possible from the mucosa to ensure uniform current density across the mucosa. Voltage and current electrodes were connected to the amplifier. After eliminating asymmetries in the voltage measuring electrodes, the following basic electrical properties across the mucosa were measured at specific intervals: potential difference (PD) or voltage difference across the mucosa in mV, resistance (R) in Ω, and short circuit current (Isc) in μA (a measure of the net active ion transport across the mucosa).

Using the pulse generator, a current of 15 μAmps was passed across the mucosa and ΔV was recorded. The R was then calculated using Ohm's law:

$$R = PD_t I^{-1} \quad (1)$$

where PD_t is voltage difference across the mucosa at a specific time and I is the passed current across the mucosa of 15 μAmps.

Finally, PD_t was measured under the open circuit mode to be able to distinguish between passive and active transport mechanisms. Since R of the tissue is known, the short circuit current (Isc) can then be calculated:

$$Isc = PD_t R_t^{-1} \quad (2)$$

Under open circuit mode, the transmucosal current was clamped to zero. In other words, there was no net transmucosal current flow created by the spontaneous electrical potential (osmotic and electrochemical gradient) across the mucosa. thus, the movement of ions as measured by Isc in the Ussing chamber results from active transport.

The tissue was stabilized in buffer for 40 min with electrical measurements taken every five min. Heparin was then added to the mucosal buffer by adding 0.1 ml of the heparin stock solution to obtain a final concentration of 10 or 0.7 mg/ml. Electrical measurements were continued every two min for an additional 84 min. Mucosal and serosal buffers as well as mucosal tissues were then collected and frozen at -4ºC for later extraction and analysis.

3.7 Assessment of mucosal injury

Trypan blue was used to determine any existing minor damage in the isolated mucosa in some experiments. Gastric mucosa or ileal mucosal tissue without Peyer's patches was mounted in the Ussing chamber and UFH and/or LMWHs were added to the mucosal buffer at pH 4.0 or pH 7.4 respectively. The experiment was performed for 124 min as described above. Then, 100 μl of trypan blue stock solution (0.3 mg/ml) was added to the mucosal buffer. After 15 min, mucosal and serosal buffers were collected and absorbance was read at 590 nm. Serosal buffer without trypan blue was used as a control. The trypan blue concentration in samples was determined by comparing the absorbance to a reference curve prepared with known amounts of the dye. As well, gastric mucosa or ileal

mucosal tissue without Peyer's patches was intentionally punctured with a 25 gauge needle to cause injury. The electrical parameters, PD, R and Isc were determined with addition of heparin.

3.8 Heparin extraction from buffers

Mucosal and serosal buffers were dialyzed in distilled water for 48 h using molecular weight cut off (MWCO) 1000 dialysis tubing. The dialyzed buffers were then dried and analyzed for chemical heparin as well as heparin bioactivity by agarose gel electrophoresis and anti-factor Xa and anti-factor IIa activity respectively.

3.9 Heparin extraction from mucosal tissues

GAGs were extracted from treated and untreated mucosal tissue by a modified published method (Jaques, 1977). Mucosa was minced, defatted with acetone and isopropanol/petroleum ether (1:1), and digested by protease in 0.1M Tris buffer with 0.1M $CaCl_2$ at pH 8.0 at 37°C. Digests were purified by precipitating with 1%NaCl in acetone and methanol. The precipitates were then dried, dissolved in water, and analyzed.

3.10 Identification and chemical measurement of extracted heparin

Agarose gel electrophoresis was used to identify and measure heparin in extracts. Dried powders were dissolved in suitable volumes of water and applied to agarose gel slides, along with the administered heparin as a reference. Gels were fixed in 0.1% hexadecyltrimethylammonium bromide and air-dried. Slides were stained with 0.04% toluidine blue in 80% acetone, and background color was removed using 1% acetic acid.

Heparin was identified by electrophoretic migration as compared to reference material and amounts were determined by densitometry.

3.11 Measurement of anti-factor IIa (anti-IIa) and anti-factor Xa (anti-Xa) activity in buffers

Mucosal and serosal buffer extracts were desalted using QIAprep Spin M13 kit columns. Columns were washed twice with distilled water by centrifuging at 5000 rpm for 5 min. Dried powders from mucosal and serosal buffer extracts were dissolved in suitable volumes of distilled water, and were then added to the washed columns. After 15 min, columns were washed twice with distilled water, and were centrifuged at 5000 rpm for 5 min. The membrane of the spin column was then washed using a pipette with 200 μl of distilled water. The washes were collected and dried obtaining a desalted buffer extract. Mucosal buffer extracts were dissolved in 1 and 10 ml of distilled water, and serosal buffer extracts were dissolved in 100 μl of distilled water for the measurement of anti-IIa and anti-Xa activity.

3.11.1 Anti-IIa activity

Measurement of plasma anti-IIa activity is intended for the quantitative determination of therapeutic heparin in plasma. In this chromogenic assay method, when both thrombin and antithrombin III are present in excess, the rate of thrombin inhibition is directly proportional to the heparin concentration. Thus, the residual thrombin activity, measured with a thrombin-specific chromogenic substrate, is inversely proportional to the heparin concentration.

All reagents including thrombin reagent, antithrombin III, and thrombin substrate, were reconstituted as instructed by the manufacturer and were warmed to room temperature, 18-25ºC, before use. Human antithrombin III (25 µl) was added to a plastic test tube along with 25 µl of heparin standard or buffer extracts and 200 µl of buffer (50 mM Tris, 175 mM NaCl, and 7.5 mM Na2EDTA_2H2O, pH 8.4) with mixing and incubation at 37°C for 3 to 4 min. Human thrombin (100µl) was then added for 30 s to 200µl of the above mixture followed by addition of the chromogenic substrate S-2238 (200 µl) for 1min. Finally, 300 µl of acetic acid was added with mixing to terminate the reaction. The absorbance of the sample was read at 405 nm. Heparin concentrations in samples were determined by comparing the absorbance to a reference curve prepared with known amounts of heparin.

3.11.2 Anti-Xa activity

In this method, when both factor Xa and antithrombin III are present in excess, the rate of factor Xa inhibition is directly proportional to the heparin concentration. Thus the residual factor Xa activity, measured with a factor Xa-specific chromogenic substrate, is inversely proportional to the heparin concentration.

Reagents including bovine factor Xa, human antithrombin III, and factor Xa substrate were reconstituted as instructed by manufacturer. Human antithrombin III (200 µl) was added to a plastic test tube. Next, 25 µl of heparin standard or buffer extracts was added, mixed, and incubated at 37°C for 2 min. Bovine factor Xa (200 µl) was then added with mixing and incubated at 37°C for 1 min. Factor Xa substrate (200 µl) was added to the

same tube and incubated for exactly 5 min. Finally, 200 μl of acetic acid was added with mixing to terminate the reaction. The solution was read at absorbance of 405 nm. Heparin concentrations in samples were determined by comparing the absorbance to a reference curve prepared with known amounts of heparin.

3.12 Data analysis and statistical procedures

Electrical parameters, PD, R, and Isc, were normalized to the value taken just prior to heparin addition at 40 min. Changes in PD, R, and Isc were then determined by subtracting all recorded values at all other times during the experiment from the recorded value at 40 min. All data was expressed as mean ± standard error of the mean (SEM). Values were considered significant when $P<0.05$ throughout the study. Specifics regarding data analysis and statistical procedures used are described in each chapter.

Chapter 4: An *In Vitro* Study with an Ussing Chamber Showing That Unfractionated Heparin Crosses Rat Gastric Mucosa*

4.1 Abstract

Heparin, traditionally given parenterally, is used to treat and prevent thrombosis. Our previous results suggest that orally administered unfractionated heparin (UFH) is absorbed and has antithrombotic effects. However, there is little evidence indicating the site and mechanism of heparin absorption. Our aim was to determine if the stomach is an absorption site. Rat gastric mucosa was mounted in an Ussing chamber, and UFH was added to the mucosal buffer at pH 7.4. Potential difference (PD), resistance (R) and short circuit current (Isc) across the mucosa were determined comparing the mucosal to the serosal side. Mucosal and serosal buffers and tissue were analysed for chemical heparin and anticoagulant activity, anti- factor Xa (anti-Xa) and anti-factor IIa (anti-IIa) activity. The PD became more negative on UFH addition. Following a lag period, PD returned to the resting level. Changes in R were opposite to those in PD, while Isc did not change. Heparin was found in the serosal and mucosal buffer and tissue. Heparin in the serosal buffer had anti-Xa and anti-IIa activity. Decreasing the pH of the mucosal buffer to 4.0, decreased the lag period for PD. Decreasing the concentration of UFH resulted in less pronounced changes in PD and less heparin in the serosal buffer. Changes in PD suggest that heparin moves across the mucosa. Presence of heparin in the serosal buffer and mucosal tissue, indicate that heparin crosses rat gastric mucosa. A stable Isc indicates passive diffusion contributes to heparin movement. The stomach could be a site for oral heparin absorption.

*Bita Moazed and Linda M. Hiebert. The Journal of Pharmacology and Experimental Therapeutics, 2007;322 (1): 299-305.

4.2 Introduction

The commercial antithrombotic drug heparin is traditionally given by intravenous and subcutaneous routes due to the belief that it is not absorbed from the gastrointestinal tract and is ineffective when given orally (Canadian Pharmaceutical Association, 2005). This is based on the assumption that the high negative charge and molecular weight of unfractionated heparin (average molecular weight of 10 000 to 15 000 daltons) prevent its absorption (Money and York, 2001) and that heparin is destroyed by stomach acids (Dal Pozzo et al., 1989). Little or no change in plasma activated partial thromboplastin times (APTT), an assay commonly used to measure heparin in plasma, following oral heparin administration has also supported the presumption that heparin is not absorbed (Leone-Bay et al., 1998).

Results from our laboratory and others, however, have challenged the historical belief that heparin is ineffective when given by the oral route. Administration of heparin *in vivo,* by parenteral routes, as well as *in vitro* experiments showed that UFH and low molecular weight heparins (LMWHs) bind avidly to endothelium (Hiebert and Jaques, 1976a; Hiebert and Jaques, 1976b; Glimelius et al., 1978; Barzu et al., 1985; Hiebert et al., 1993; Hiebert et al., 2001). Our observations also showed that orally administered heparin was found with endothelium despite low plasma levels (Hiebert et al., 1993). As well, oral UFH and LMWHs significantly reduced thrombotic incidence in both rat jugular vein and carotid arterial models (Hiebert et al., 2001; Hiebert et al., 2000; Hiebert et al., 1996; Pinel et al., 2004). Furthermore, an increase in plasma anti-Xa activity and heparin in urine was observed in human subjects following oral administration (Hiebert et al.,

2005). Therefore, these results suggest that heparin is absorbed following oral administration, and that the oral route may be a feasible alternative for administering heparin.

The site of oral heparin absorption and the mechanism responsible, however, are not known. Previous studies on heparin absorption from the gastrointestinal tract examined the small intestine (Sue et al., 1976), the anatomically favored and major site of absorption for most compounds. Sue et al.,(1976) reported that when heparin was introduced into the gastrointestinal tract, increases in whole blood clotting time and plasma anti-Xa activity were observed. The anticoagulant effect was greater when heparin was placed in the stomach versus the small intestine. Furthermore, more chemical or [^{14}C] UFH was found in stomach tissue versus duodenum, jejunum, and ileum or colon tissue up to 24 hours following administration by stomach tube (Hiebert et al., 2000). These studies, therefore, suggest that the stomach may be a site for heparin absorption. Thus, the objective of the present study was to determine if orally administered heparin crosses rat gastric mucosa when mounted in an Ussing chamber. From these experiments, we conclude that heparin crosses rat gastric mucosa suggesting that the stomach may be a site for oral heparin absorption.

4.3 Methods

4.3.1 Chemicals

Bovine lung unfractionated heparin (156.2 Units/mg) was obtained from Scientific Protein Labs, Division of Viobin Corporation, Wisconsin, USA. For other materials used for this transport study please refer to section 3.1 of this thesis.

4.3.2 Gastric mucosa isolation from rats

Thirty four male Wistar rats weighing between 250 to 300 g were used in this study. Animals were handled according to the methods section 3.3 of this thesis.

4.3.3 Measurements of heparin movement across gastric mucosa using an Ussing chamber

Gastric mucosa of Wistar rats was isolated according to section 3.4 of this thesis. Heparin movement across the mucosa mounted in an Ussing chamber was performed according to the section 3.6.2 of this thesis.

4.3.4 Assessment of mucosal injury

Gastric mucosal tissue mounted in an Ussing chamber was tested for its integrity as is described in section 3.7 of this thesis.

4.3.5 Heparin extraction from buffers

Heparin was extracted from mucosal and serosal buffers as described in section 3.8 of this thesis.

4.3.6 Heparin extraction from mucosa

GAGs were extracted from treated and untreated mucosal tissue as described in section 3.9 of this thesis.

4.3.7 Identification and chemical measurement of extracted heparin

Agarose gel electrophoresis was used as described in section 3.10 of this thesis to identify and measure heparin in extracts.

4.3.8 Measurement of anti-factor IIa (anti-IIa) and anti-factor Xa (anti-Xa) activity in buffers

Please refer to section 3.11 of this thesis for a detailed description of how anti-Xa and anti-IIa activities were measured.

4.3.9 Data analysis and statistical procedures

All data are expressed as mean ± standard error of the mean (SEM). A one-tailed unpaired t-test was used to determine significant differences in the lag period before PD began to return to the resting level in different environments. A two-tailed t-test was used to measure differences in heparin concentrations in serosal buffers, experimental mucosa, and control tissue under different conditions, as well as to measure differences in PD increase upon heparin addition to the mucosal side of the Ussing chamber. A paired two-tailed t-test was used to measure differences in trypan blue concentrations between mucosal, and experimental and control serosal buffers. Values were considered significant at $P<0.05$.

4.4 Results

4.4.1 Electrical parameters of rat gastric mucosa following addition of UFH to the mucosal side at pH 7.4

To observe heparin movement across rat gastric mucosa under neutral conditions, as a control compared to the usual acidic environment of the stomach, the gastric mucosa was first placed in the Ussing chamber with buffers at pH 7.4. The electrical properties of the membrane were recorded every 5 min. Results are shown in Fig. 4.1. When UFH (10 mg/ml) was added to the buffer on the mucosal side of the mounted gastric mucosa after 40 min of stabilization, the PD became more negative, and the charge difference between the mucosal and serosal side of the membrane increased compared to PD prior to UFH addition (Fig 4.1A). After a lag period of 18.8 ± 4.7 min, the PD began to decrease. The PD achieved the previous resting level 39.3 ± 17.6 min later, after which the PD continued to change with time and became positive compared to the resting level. R of the mucosa followed a pattern similar to that seen for the PD (Fig. 4.1B). The R increased upon addition of UFH and then began to decrease reaching the resting level 23 ± 4.2 min later, following a lag period of 14.6 ± 4.3 min. Isc did not change after heparin addition (Fig. 4.1C). No change was observed in PD, R, or Isc, if buffer was added to the mucosal side instead of UFH (Fig. 4.1a, b, and c).

The presence of chemical heparin in mucosal and serosal buffers and experimental tissue was measured. In addition to the mucosal buffer, heparin was found in the serosal buffer (65.0 ± 20.6 μg/ml) as well as the mucosal tissue (51.9 ± 21.5 μg/g) when UFH was added to buffer at pH 7.4 (Table 4.1 and Fig. 4.2). Heparin was not found in mucosal

tissue obtained from the same stomach and not placed in the Ussing chamber. Based on recovery from the serosal buffer the rate of movement of heparin across the mucosal tissue was calculated to be 0.47 ± 0.10 μg/ cm^2/ min.

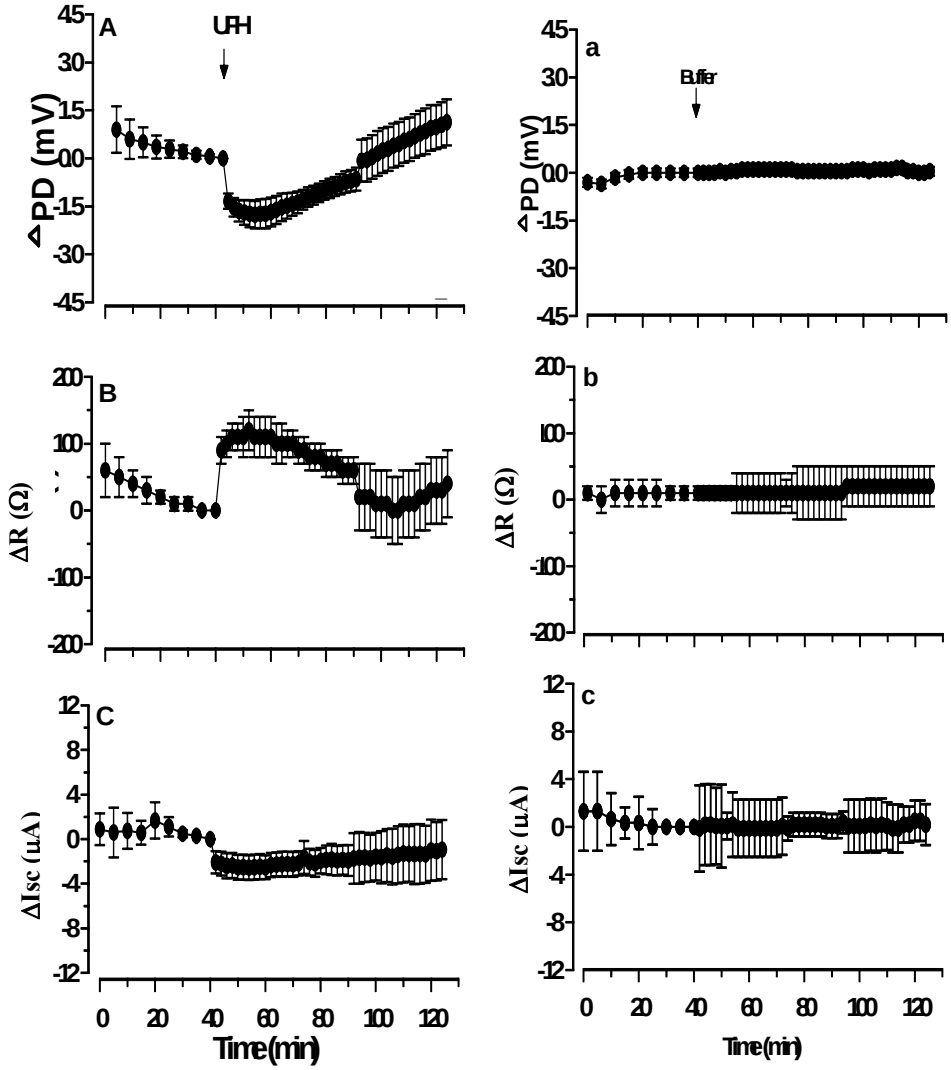


Figure 4.1 Changes in electrical parameters across rat gastric mucosa on heparin (10 mg/ml) or Kreb's buffer addition to the mucosal buffer. The potential difference (PD) became more negative when the mucosal side was compared to the serosal side immediately after addition of heparin to the mucosal buffer. PD returned to the resting level with time (A). Changes in resistance (R) followed the pattern of changes in PD (B). Short Circuit Current (Isc) did not change after heparin addition (C). Results are shown as mean ± SEM of 8 experiments. No change was observed in PD (a), R (b), or Isc (c) if buffer alone was added to the mucosal buffer. Results are shown as mean ± SEM of 6 experiments.

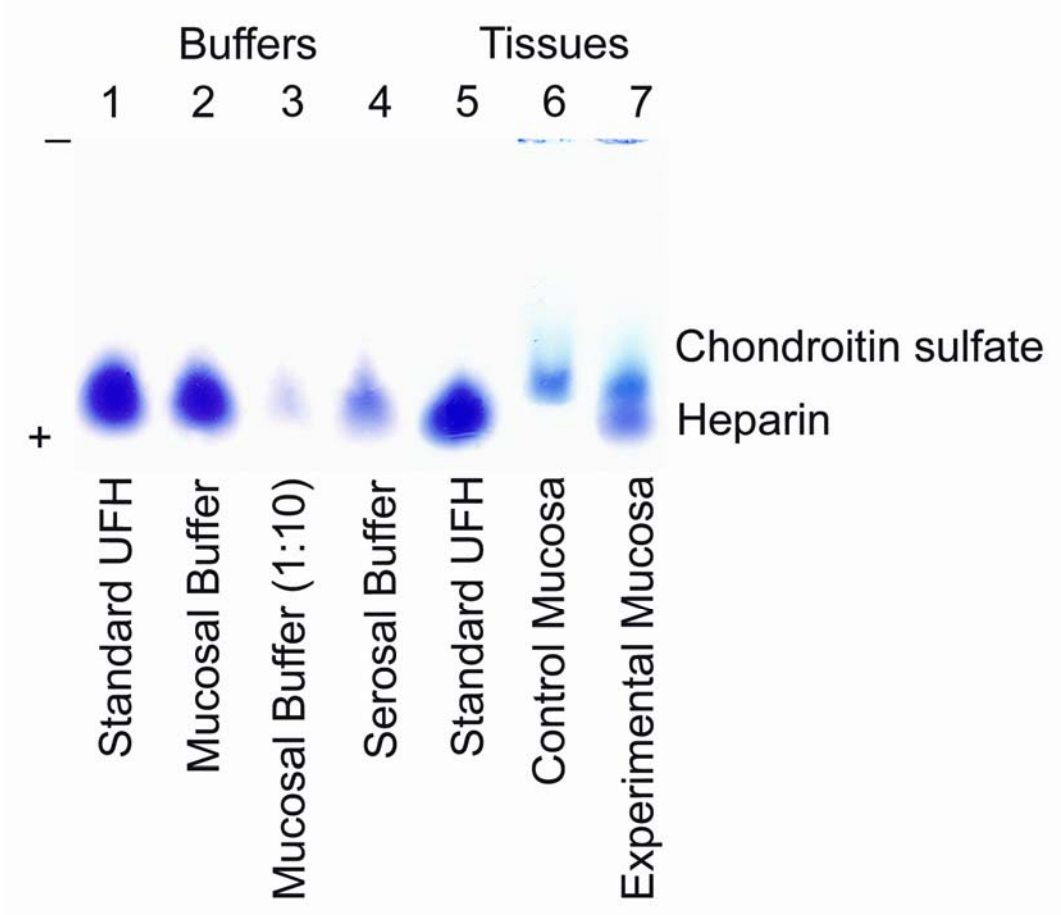


Figure 4.2 An electrophoretic gel showing heparin recovered from buffer and tissue following unfractionated heparin addition to the mucosal buffer. Using agarose gel electrophoresis, heparin was found in both the mucosal and serosal buffer as well as in experimental tissue 84 min after heparin addition to the mucosal buffer. Standard and recovered heparin samples were dissolved in measured amounts of distilled water and 2 µl was applied to each lane at the top of the gel, level with the negative sign. Lane 1, Standard unfractionated bovine lung heparin 2 mg/ml. Lane 2, Mucosal buffer extract dissolved in 1000 µl of water. Lane 3, Mucosal buffer extract dissolved in 10000 µl of water. Lane 4, Serosal buffer extract dissolved in 50 µl of water. Lane 5, Standard unfractionated bovine lung heparin 2 mg/ml. Lane 6, Control mucosal tissue extract where heparin was not added to the mucosal buffer, dissolved in 50 µl of water. Lane 7, Experimental mucosal extract dissolved in 50 µl of water. All material extracted from buffers is similar in migration and color to the original heparin. A blue band, moving slower than standard heparin, and likely chondroitin sulfate, is seen in the control mucosa (Lane 6). This band is seen in the experimental mucosa along with heparin (Lane 7).

Table 4.1 Heparin recovered from buffers and tissues following addition to mucosal buffer when rat gastric mucosa is placed in an Ussing chamber for 124 min

Treatment	Mucosal Buffer (µg)	Serosal Buffer (µg)	Control Mucosa (µg)	Experimental Mucosa (µg)
UFH (pH 7.4) 10 mg / ml, (n=6)	3917.7 ± 271.3	65.0 ± 20.6	0.0 ± 0.0	51.9 ± 21.5
UFH (pH 4) 10 mg / ml, (n=5)	2400.0 ± 597.0	111.3 ± 40.5	0.0 ± 0.0	26.9 ± 6.6
UFH (pH 4) 0.7 mg / ml, (n=5)	545.0 ± 237.2	18.3 ± 9.4	0.0 ± 0.0	15.0 ± 6.6
UFH (pH 4) 10 mg / ml, damaged mucosa, (n=4)	2500.0 ± 333.3	162.5 ± 8.3	0.0 ± 0.0	17.5 ± 2.0

4.4.2 Electrical parameters of rat gastric mucosa following addition of UFH to the mucosal side at pH 4.0

Since the stomach pH is acidic *in vivo*, electrical parameters of rat gastric mucosa were measured in our *in vitro* model with the mucosal buffer at pH 4.0, the average pH in rat stomach (Eastman and Miller, 1935). When UFH was added to the mucosal buffer at pH 4.0, PD increased as in the neutral condition (pH 7.4). After a lag period, the PD began to decrease to the previous resting level as shown in Fig. 4.3A. There was a trend toward a significant decrease in the lag period of 9.2 ± 2.8 min in the acidic environment compared to 18.8 ± 4.7 min in the neutral environment ($P< 0.06$, one-tailed t-test). PD reached the previous resting level 40.5 ± 7.9 min after the lag period in the acidic environment compared to 39.3 ± 17.6 min in the neutral environment. Isc changed little in the acidic environment following the addition of UFH (Fig. 4.3B). Heparin recovery was 111.3 ± 40.5 μg/ml from the serosal buffer when heparin was added to mucosal buffer at pH 4.0 compared to 65.0 ± 20.6 μg/ml in the neutral condition ($P< 0.3$, two-tailed t-test) (Table 4.1). Furthermore, 26.9 ± 6.6 μg/g heparin was recovered from the mucosal tissue when UFH was added to the mucosal buffer at pH 4.0 compared to 51.9 ± 21.5 μg/g in the neutral condition ($P=0.3$, two-tailed t-test) (Table 4.1). The rate of movement of UFH across the mucosal tissue at pH 4 was calculated to be 0.80 ± 0.19 μg/ cm^2/ min.

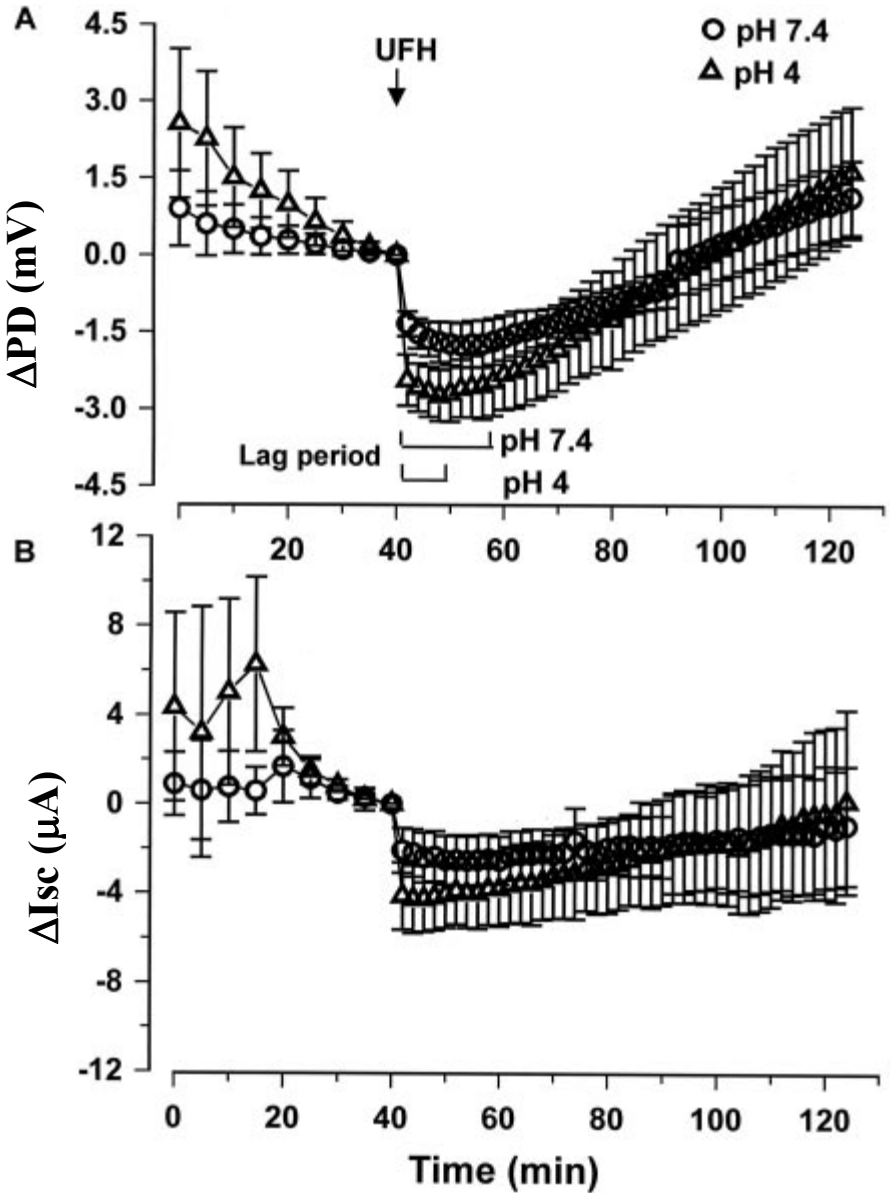


Figure 4.3 Effects of changing the pH of the mucosal buffer on the electrical parameters across rat gastric mucosa on addition of unfractionated heparin (10 mg / ml) to the mucosal buffer. Unfractionated heparin was added to the mucosal buffer at pH 4.0. Changes in potential difference (PD) and short circuit current (Isc) are compared to those when heparin is added at pH 7.4. The lag period before the PD began to rise was less at acidic pH versus neutral pH (A). Isc did not change after heparin addition (B). Results are shown as mean ± SEM of 6 experiments.

4.4.3 Biological activity of heparin

To determine if the heparin recovered in the serosal buffer had anticoagulant activity, anti-Xa activity and anti-IIa activity were measured in the desalted buffer extracts at pH 4. Anti-Xa and anti-IIa activity were found in both the mucosal and serosal buffer extracts. Heparin found in the serosal buffer extracts based on anti-Xa activity was 2.5 ± 0.3 μg/ml compared to 63.2 ± 1.5 μg/ml for the mucosal buffer, and based on anti-IIa activity was 2.6 ± 0.3 and 29.8 ± 3.1 μg/ml for the serosal and mucosal buffer respectively. The anti-Xa/IIa ratio was 2.08± 0.31 and 0.54 ± 0.11 for the mucosal and serosal buffer respectively.

4.4.4 Changes in electrical parameters of rat gastric mucosa following addition of UFH at different concentrations at pH 4.0

To determine the effect of heparin concentration on its movement through the gastric mucosa, heparin concentration was decreased and electrical properties of the tissue were measured. Decreasing the concentration of UFH from 10 to 0.7 mg/ml resulted in a change in PD that was less negative on addition of heparin to the mucosal buffer. The PD changed by -2.4 ± 0.5 mV when 10 mg/ml was added compared to -0.9 ± 0.1 mV when 0.7 mg/ml heparin was added to the mucosal buffer ($P< 0.02$, two-tailed t-test, Fig. 4.4A). The lag period was 25 ± 1.2 min before the PD began to decrease to the previous resting level. Isc did not change when 0.7 mg/ml UFH was added to the mucosal buffer (Fig. 4.4B). Heparin recovered from the serosal buffer (18.3 ± 9.4 μg/ml), when 0.7 mg/ml of heparin was added to the mucosal buffer, was significantly less compared to 111.3 ± 40.5 μg/ml when 10 mg/ml heparin was added ($P< 0.05$, two-tailed t-test) (Table 4.1). Rate of

movement of heparin across the mucosal tissue was calculated to be 0.13 ± 0.09 μg/ cm^2/ min.

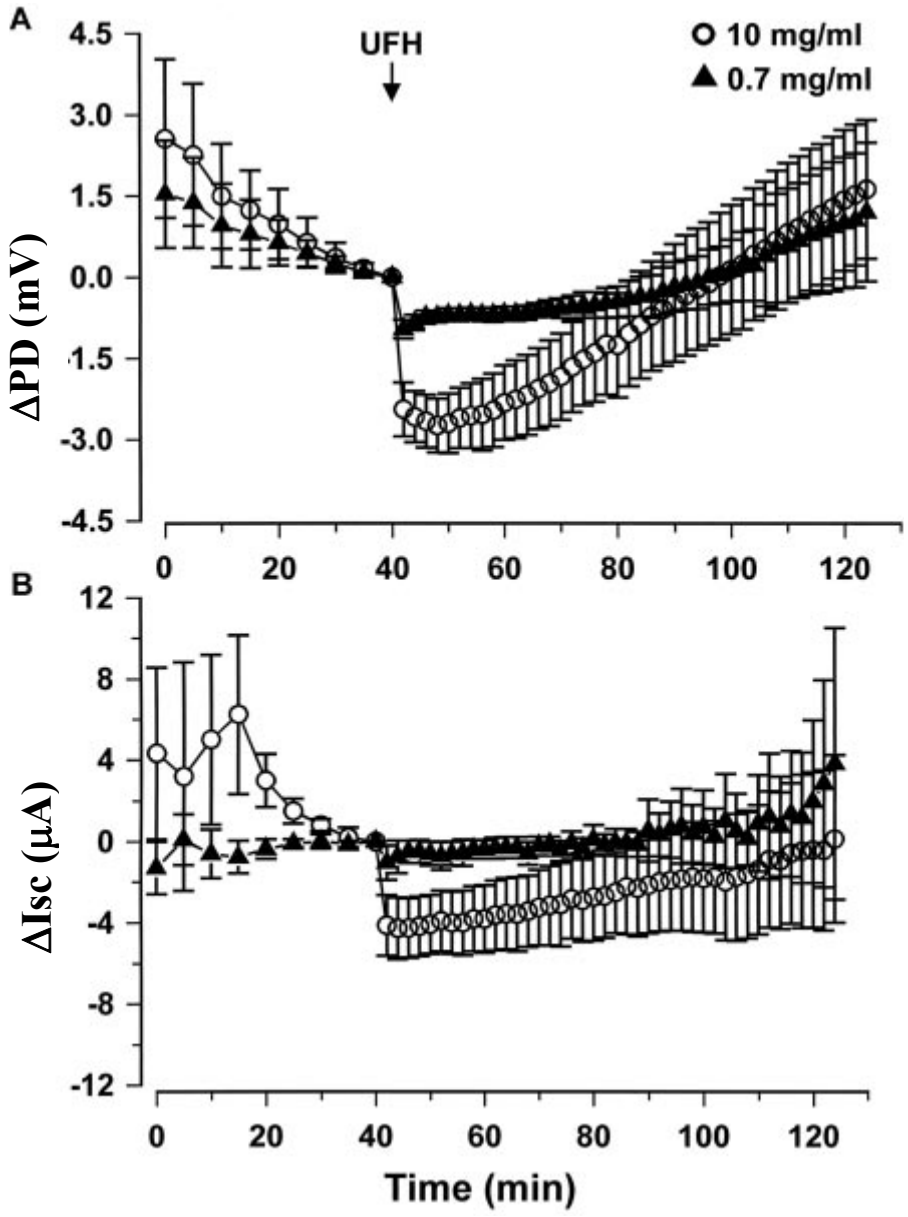


Figure 4.4 Effects of changing the concentration of unfractionated heparin in the mucosal buffer on the electrical properties across rat gastric mucosa. Decreasing the concentration of unfractionated heparin in the mucosal buffer results in less pronounced changes in potential difference (PD) (A). Short circuit current (Isc) did not change after heparin addition (B). Results are shown as mean ± SEM of 5 experiments.

4.4.5 Identification of mucosal damage

Trypan blue was used to detect any existing subtle damage in the isolated tissue sample tested. Mucosal and serosal buffers were collected 15 min after adding 100 μl of trypan blue stock solution (0.3 mg/ml) to the mucosal buffer following experiments lasting 124 min. The concentration of trypan blue in the mucosal and serosal buffers was determined and compared to control serosal buffer without trypan blue. The concentration of trypan blue in the mucosal buffer was significantly different than that of the serosal buffer (Table 4.2), $P< 0.0001$, two-tailed t-test. There was no difference in the concentration of trypan blue in the serosal buffer compared to the control buffer (Table 4.2) $P = 0.20$, two-tailed t-test.

4.4.6 Changes in electrical parameters of rat gastric mucosa following addition of UFH to damaged mucosa

To determine the electrical parameters when the mucosa was intentionally injured, the stomach mucosal preparation was damaged by puncture with a 25 gauge needle. When UFH (10 mg/ml) was added to the mucosal side of the damaged mucosa, the PD became negative but to a lesser degree compared to the intact mucosa. The PD reached -0.9 ± 0.3 mV when the mucosa was damaged compared to -1.75 ± 0.4 mV when it was intact. The PD began to decrease to its previous resting level immediately without a lag period. At 47 ± 4.7 min after drug addition, the PD reached a plateau without continuing to rise (Fig. 4.5A). The Isc decreased immediately after heparin addition but reached a plateau approximately 20 min after heparin addition (Fig. 4.5B). Heparin was recovered from serosal and mucosal buffers as well as tissues (Table 4.1). The amount of heparin

recovered from the serosal buffer with the mucosa damaged was 162.5 ± 8.3 μg/ml. This was comparable to 111.3 ± 40.5 μg/ml recovered from the serosal buffer of the intact mucosa (P< 0.3, two-tailed t-test). Rate of heparin movement across the mucosal tissue with intentional damage was calculated to be 1.16 ± 0.04 $\mu g/cm^2$/ min.

Table 4.2 Trypan blue recovered from buffers after addition to the mucosal buffer when rat stomach mucosa is placed in an Ussing chamber for 124 min

Buffers	Trypan blue (μg/ml)
Mucosal buffer	$40.00 \pm 4.65 \times E^{-8}$
***Experimental serosal buffer**	1.43 ± 0.16
****Control serosal buffer**	$1.35 \pm 7.07 \times E^{-3}$

* Serosal buffer obtained when trypan blue is added to the mucosal buffer.
** Serosal buffer obtained when trypan blue is not added to the mucosal buffer.

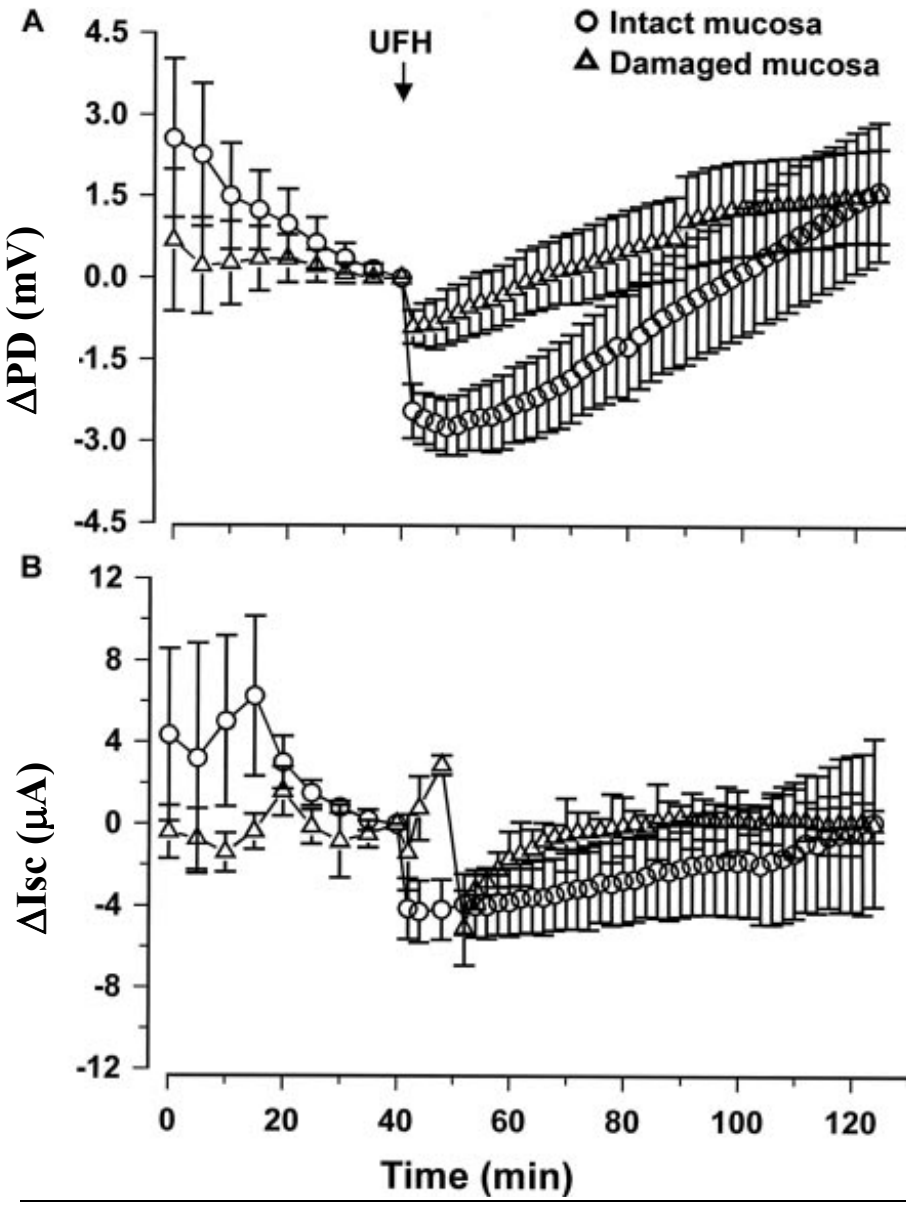


Figure 4.5 Effects of injury on electrical parameters across rat gastric mucosal tissue following addition of unfractionated heparin to the mucosal side. Damaging the mucosa resulted in less pronounced changes in potential difference (PD) (A) and variable changes in short circuit current (Isc) (B). Results are shown as mean ± SEM of 4 experiments.

4.5 Discussion

As a potent and safe drug, heparin has been widely used by parenteral administration for the prevention and treatment of thrombo-embolic disease. Although heparin is not considered effective when administered orally, results from our laboratory and others have shown evidence of oral heparin absorption (Hiebert et al., 1993; Hiebert et al., 2001; Hiebert et al., 2000; Hiebert et al., 1996; Hiebert et al., 2005; Engelberg, 1996). Heparin was recovered from aortic and vena caval endothelium following oral administration of unfractionated heparin to rats. If concentrations of heparin with endothelium in these vessels were applied to endothelium in the whole body, it was calculated that considerable amounts of the total heparin administered could be found with the endothelium (Jaques et al., 1991; Hiebert et al., 1993). Oral heparin was demonstrated to have antithrombotic activity in rat jugular vein (Hiebert et al., 2000; Hiebert et al., 2001), venous stasis (Costantini et al., 2000) and carotid arterial thrombosis models (Pinel et al., 2004), further indicating that heparins are absorbed following oral administration. Evidence of heparin absorption was also seen in humans (Engelberg, 1996; Hiebert et al., 2005). Little is known about the site of heparin absorption. Tissue distribution studies showed that orally administered heparin was found in gut and non-gut tissues. Considerable heparin was recovered from the stomach tissue, suggesting that the stomach may be a site of absorption (Hiebert et al., 2000), thus our present study was to determine if heparin crosses rat gastric mucosa. The glandular portion of the rat stomach, from which the mucosa was obtained, is similar to that of the human and has been used as a model for human disease (Ichikawa et al., 2000).

Heparin was found in the serosal buffer as well as mucosal tissue after heparin addition to the mucosal buffer of the Ussing chamber. Furthermore, the heparin recovered from the mucosal buffer had anti-Xa and anti-IIa activity. These findings clearly demonstrate that heparin crosses rat gastric mucosa and retains its anticoagulant activity. The anti-Xa/IIa ratio did not increase also indicating that heparin is not broken down when it crosses rat gastric mucosa, since longer heparin chains are needed for anti-IIa activity than anti-Xa activity (Boneu, 2000). The rate of transport was 70% of that when the gastric mucosa was intentionally damaged (0.80 $\pm$ 0.19 versus 1.16 $\pm$ 0.04 $\mu g/ cm^2/$ min) indicating that heparin easily crosses the mucosa. The Ussing chamber model differs from the *in vivo* situation by a number of factors including a small surface area and lack of an active circulation. If heparin was applied to the whole stomach, not only a surface 2.5 cm^2 in size, with an active circulation it is expected that a considerable amount of heparin could cross the stomach mucosa. Time could also be a factor. Our previous studies showed that heparin remains in the stomach in high concentrations at least 4 h after oral administration and could still be detected in the stomach after 24 h (Hiebert et al., 2000) a much longer time period than the 84 min used in this experiment.

Changes in PD across rat gastric mucosa also support heparin transport. When the mucosal side is compared to the serosal side, a change in PD is seen the moment heparin is added to the mucosal buffer, such that the mucosal buffer becomes more negative compared to the serosal buffer, and is likely due to the anionic charge on heparin. The PD remains negative and with time returns to the previous resting level and beyond suggesting that heparin binds to the tissue and moves across the mucosa to the serosal

side as supported by chemical measurement of heparin and anti-Xa and anti-IIa activity in the serosal buffer. These results are not an artifact of mucosal damage, since trypan blue dye added to the mucosal buffer was not found in the serosal buffer (Table 4.2), confirming the absence of any minor increases in permeability in the mucosal tissue. Furthermore, intentional damage of the mucosa caused a smaller change in PD, on addition of heparin, from that observed using undamaged mucosa, likely due to heparin moving freely from the mucosal to the serosal side.

Changes in the PD observed following heparin administration were also dose-dependent (Fig. 4.4). The PD became more negative and the lag period was shorter when 10 mg/ml compared to 0.7 mg/ml of heparin was added to the mucosal side. It is possible that the greater the heparin concentration, the easier its penetration through the mucosal tissue. This is supported by a decreased amount of heparin recovered from the serosal buffers when less heparin is added to the mucosal side.

The idea that heparin is absorbed in the stomach is in agreement with other findings. Sue et al. observed a much greater anticoagulant effect when heparin was placed in the stomach compared to the small intestine (Sue et al., 1976). Our recent *in vivo* studies in rats indicated that more heparin was found with endothelium when heparin was placed in the stomach compared to the small intestine (Hiebert et al., 2007).

Ionization of heparin may also affect its movement across the gastric mucosa. Placing heparin into an acidic mucosal buffer at pH 4, the average pH in rat stomach on a normal

diet (Eastman and Miller, 1935), reduced the lag period before the PD begins to return to baseline, compared to 7.4 (Fig. 4.3) . The reduced lag period suggests a more rapid drug movement at lower pH levels. This result is supported by the recovery of more chemical heparin from the serosal buffer when experiments are conducted at pH 4.0 compared to pH 7.4 (Table 4.1). The carboxylate groups of heparin have a pKa between 2 and 4 (Casu and Gennaro, 1975). Since an acidic environment may reduce ionization of weak acidic groups as seen in heparin, these results imply that ionization of heparin can be an important factor in determining the rate of absorption of heparin from the gastrointestinal tract. Our results agree with previous studies. Others have reported improvement in heparin absorption from the small intestine when the environment is made more acidic. When heparin was dissolved in diluted acids and added to the duodenum, whole blood clotting time was significantly increased compared to when heparin was dissolved in water (Sue et al., 1976). Instillation of buffers with heparin into dog duodenal loops, indicated that systemic anticoagulant effects were obtained at pH 4.0 but not pH 8.0 (Loomis, 1959).

Although this study does not deal with the mechanism responsible for heparin movement across gastric mucosa, observations of electrical changes support the idea that passive diffusion is at least partly responsible for heparin transport. The Isc did not change in the 84 min period after heparin addition to the mucosal buffer, suggesting passive diffusion since a change in Isc is indicative of active transport (Cooke and Dawson, 1978).

Furthermore, R of the tissue, an indicator of the tissue permeability, increases and becomes more positive when heparin is added to the mucosal side of the chamber. The R progressively decreases with time and may reflect the movement of heparin across the mucosa. It is likely that as heparin crosses the mucosa, the concentration on the mucosal side progressively decreases, thus decreasing the concentration gradient across the mucosa. This change in R suggests passive diffusion. However, we cannot rule out active transport as part of heparin movement through the gastric mucosa since R and PD continue to go beyond the previous resting level. Active transport processes may be activated when the concentration of heparin decreases helping its movement across the mucosa. Further studies are required to evaluate the exact contribution of passive and active transport in the movement of heparin across the stomach mucosa.

4.6 Conclusions

In conclusion, recovery of heparin in the serosal buffer and the mucosal tissue as well as anti-Xa and anti-IIa activity in the serosal buffer, support the idea of heparin movement across the rat gastric mucosa. Decrease in PD with time following an increase in PD upon UFH addition, also suggests heparin transport. Less pronounced changes in PD following decreasing concentrations of heparin added to the mucosal buffer, suggests heparin transport is concentration dependent. Furthermore, a decrease in the lag phase before the PD begins to return to the baseline with lower mucosal pH in the presence of heparin suggests that suppressing ionization may increase drug movement. Finally, indirect results from changes in R and Isc after heparin addition suggest that passive diffusion may play some role in the heparin movement across the mucosa. These results support

the idea that heparin may be absorbed from the stomach following oral administration. Further studies are required to understand how important gastric absorption of heparin may be in comparison to the small intestine. Moreover, considerable work is still required to evaluate mechanisms of heparin absorption from the stomach mucosa including the contribution of passive and active transport.

Chapter 5: Low Molecular Weight Heparins Cross Rat Gastric Mucosa Mounted in an Ussing Chamber*

5.1 Abstract

Low molecular weight heparins (LMWHs) are used subcutaneously for the management of thromboembolism despite evidence of their oral absorption. The site of LMWH absorption is unknown. Since previous studies suggest the stomach is important for absorption of unfractionated heparin, our aim was to determine if the stomach is a site for LMWH absorption. Gastric mucosa was mounted in a Vertical Diffusion Ussing Chamber, and the LMWHs, tinzaparin or reviparin, were added to the mucosal buffer at pH 7.4 or 4.0. Potential difference (PD), resistance and short circuit current (Isc) were measured across the mucosa. Buffers and tissues were analyzed for chemical LMWH and anti-factor Xa activity. The PD became more negative on LMWH addition comparing the mucosal side to the serosal. The PD returned to baseline following a lag period which was greater at pH 4.0 versus 7.4. Resistance changes were opposite to those for PD. Isc increased with time at pH 7.4 but not pH 4.0 which was most dramatic with reviparin. LMWHs were recovered from serosal buffer and tissue and had anti-factor Xa activity. Amounts found in serosal buffer and rate of movement was greater at pH 7.4 versus 4.0. Changes in PD, LMWH recovery and anti-factor Xa activity in serosal buffer suggest that LMWHs cross rat gastric mucosa. Changes in Isc suggest that active transport may depend on mucosal pH. Thus, LMWHs preferentially cross gastric mucosa under neutral conditions. Therefore the stomach, with an acidic environment, may not be the main site for LMWH absorption *in vivo*.

* Bita Moazed and Linda M. Hiebert. International Journal of Pharmacology, 2008;4(6): 431-442.

5.2 Introduction

Significant patient morbidity and mortality due to thrombosis is a major health concern in the Western world (Hirsh et al., 1995). Low molecular weight heparins (LMWHs) given by intermittent subcutaneous injection are safe and effective antithrombotics in either the in-hospital or out-of-hospital setting. Oral administration of LMWHs is not considered effective. This is due to studies showing little evidence of oral polyanion bioavailability measured by anti-Xa activity or activated partial thromboplastin time (APTT) (Iqbal et al., 2001; Lorentsen et al., 1989; Faaij et al., 1999; Salartash et al., 2000).

In opposition to these studies, findings from our laboratory and others have clearly demonstrated that low molecular weight polyanions such as dextran sulfate, sulodexide, pentosan polysulfate, sucrose octasulfate, and LMWHs were absorbed following oral administration (Jaques et al., 1991; Ofosu 1998; Nickel et al., 2000; Hiebert et al., 1999; Hiebert et al., 2000; Hiebert et al., 2001). Polyanions of low molecular weight, sucrose octasulfate and dextran sulfate were found associated with endothelium following oral administration despite little evidence in plasma (Jaques et al., 1991; Hiebert et al., 2002). Moreover, the LMWHs tinzaparin and reviparin, and dextran sulfate have antithrombotic activity in a rat jugular vein thrombosis model (Jaques et al., 1991; Hiebert et al., 2000; Hiebert et al., 2001).

Although evidence for oral absorption of LMWHs exists in the literature, there are no studies concerning the site of absorption. Tissue distribution studies of tinzaparin, following administration to rats by the oral route, show progression through the gut with

peak amounts of the drug recovered from stomach tissue and washes at 6-30 min of administration compared to 15-30, 30 min, 2 and 4 h, for duodenum, jejunum, ileum and colon respectively (Hiebert et al., 2004). The amount of tinzaparin in stomach at 15 min accounted for 46% of the drug administered versus 0.5% in colon at 4 h suggesting the stomach was an important distribution site. These findings agree with investigations of tissue distribution of [^{14}C]-labeled and cold sucrose octasulfate following oral administration in rats (Hiebert et al., 2002) where 84% of the dose administered was found in the stomach tissue at 6 min. Thus the stomach may be a site of absorption of LMWHs.

In our most recent *in vitro* study using a vertical diffusion Ussing chamber, we observed that the stomach could be a site for absorption of UFH following oral administration by indicating that bovine UFH moves across rat gastric mucosa from the mucosal side (lumen) to the serosal side (circulation) in both a dose- and pH-dependent manner (chapter 4 of this thesis). The main objective of this study was to investigate if LMWHs cross rat gastric mucosa, mounted in an Ussing chamber, and to better understand the site of absorption of LMWHs by manipulating the pH of the mucosal buffer.

5.3 Methods

5.3.1 Animals

Twenty-four male Wistar rats weighing between 250 and 300 g were used in the study. Animals were handled according to section 3.3 of methods of this thesis.

5.3.2 Isolation of Gastric Mucosa

For a detailed explanation on isolation of gastric mucosa, please refer to section 3.4 of this thesis.

5.3.3 Measurements of electrical parameters across mucosa using an Ussing chamber

A detailed description of how changes in electrical parameters were measured across the gastric mucosa mounted in an Ussing chamber is cited in section 3.6.2 of this thesis.

5.3.4 Assessment of mucosal injury

Please refer to the section 3.7 of this thesis for a detailed description.

5.3.5 LMWH extraction from buffers and tissue

GAGs were extracted from buffers and mucosal tissue as is described in sections 3.8 and 3.9 of this thesis.

5.3.6 Identification and chemical measurement of extracted LMWH

Agarose gel electrophoresis was used to identify and measure LMWH in extracts as described in section 3.10 of this thesis.

5.3.7 Measurement of anti-factor Xa activity in buffers

Please refer to the section 3.11 of this thesis for a detailed description of measurement of anti-Xa activity in mucosal and serosal buffer extracts.

5.3.8 Drugs and Chemicals

Tinzaparin (tinzaparin sodium, anti-Xa activity 90.7 IU/mg, peak maximum molecular mass of 5600 daltons) obtained from porcine mucosal heparin, was generously donated by Novo Nordisk, Denmark. Reviparin (reviparin sodium, Batch W 49522, average molecular mass of 4300 daltons, anti-Xa activity 130 IU/mg; anti-IIa activity 29 IU/mg) was from Knoll AG, Ludwigshafen, Germany. Both LMWHs were prepared as a stock solution of 150 mg/ml in Kreb's buffer. For other materials used in this study, please refer to section 3.1 of this thesis.

5.3.9 Statistics

All data are expressed as mean ± standard error of the mean (SEM). A one-tailed unpaired t-test was used to determine significant differences in the lag period before PD began to return to the resting level and to compare differences in the time taken for PD to reach the resting level after the lag period in different environments as well as to measure differences in PD increase upon heparin addition to the mucosal side of the Ussing chamber. A one-tailed t-test was also used to measure differences in heparin

concentrations in serosal buffers, and experimental mucosa, and to compare rates of movement across the mucosa under different conditions.

A two-tailed t-test was used to compare differences in LMWH concentrations in serosal and mucosal buffer extracts based on anti-Xa activity, and rates of movement of LMWHs across the mucosa. A paired two-tailed t-test was used to measure differences in trypan blue concentrations between mucosal, experimental and control serosal buffers. A two-tailed t-test, non-parametric Mann-Whitney test, was used to compare differences in Isc during the experimental period between groups. Differences in Isc were calculated by subtracting the first 5 values after LMWHs addition, obtained at 40-48min, from the last 5 values recorded, obtained at 116-124 min, during the experimental protocol. Values were considered significant at $P<0.05$.

5.4 Results

5.4.1 Addition of tinzaparin to mucosal buffer at pH 7.4

The gastric mucosa was placed in the Ussing chamber with buffers at pH 7.4 and electrical parameters were recorded every 5 min. Following tissue stabilization for 40 min, tinzaparin (10 mg/ml) was added to the mucosal buffer and electrical parameters were measured every 2 min for an additional 84 min. When tinzaparin was introduced into the mucosal buffer, the charge difference between the mucosal and serosal side of the membrane increased as the PD became negative compared to that prior to addition (Fig. 5.1A). After a lag period of 7.0 ± 1.2 min, the PD began to decrease. The PD achieved the previous resting level 35.0 ± 9.5 min later. The R of the mucosa followed a pattern opposite to that seen for the PD (Fig. 5.1B). The R increased upon addition of tinzaparin and following a lag period of 8.0 ± 1.4 min began to increase reaching the resting level 32.0 ± 2.5 min later. The Isc showed a slight increase of 2.7±0.5 μA throughout the experimental period (Fig. 5.1C). Placing buffer instead of tinzaparin into the mucosal side of the Ussing chamber did not cause any change in PD, R or Isc across the membrane (Fig. 5.1A, B, C).

At the completion of the experiment, tinzaparin was found in the serosal buffer (176.8 ± 75.7 μg/ml) and the experimental mucosal tissue (25.0 ± 1.5 μg/g) as well as in mucosal buffer (Table 5.1 and Fig. 5.2). Tinzaparin was not found in control mucosal tissue. Based on the recovery from the serosal buffer, the rate of movement of tinzaparin across the mucosal tissue was calculated to be 1.26 ± 0.54 $\mu g/cm^2/min$.

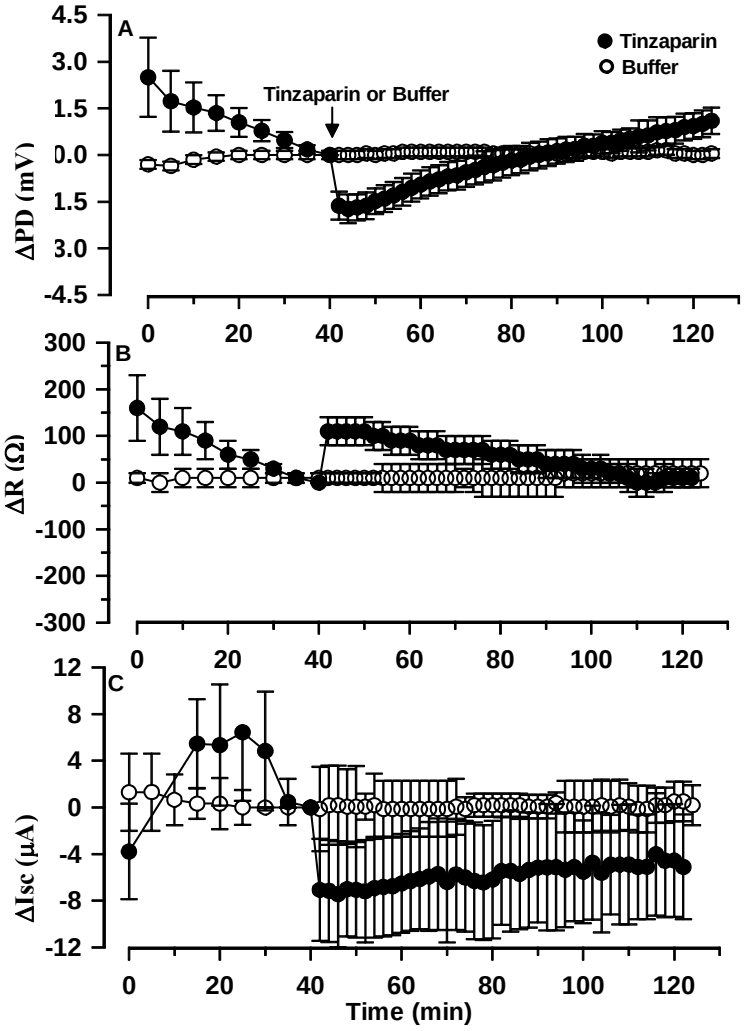


Figure 5.1 Changes in electrical parameters across rat gastric mucosa on tinzaparin (10 mg/ml) or Kreb's buffer addition to the mucosal buffer at neutral pH. The potential difference (PD) became more negative when the mucosal side was compared to the serosal side immediately after addition of tinzaparin to the mucosal buffer at pH 7.4. PD returned to the resting level with time (A). Changes in resistance (R) are similar to the pattern of changes in PD (B). Short Circuit Current (Isc) showed a slight but non significant increase during 84 min of the experiment (C). No change was observed in PD (A), R (B), or Isc (C) if buffer alone was added to the mucosal side. Results are shown as mean ± SEM of 7 experiments for tinzaparin and 3 experiments for buffer.

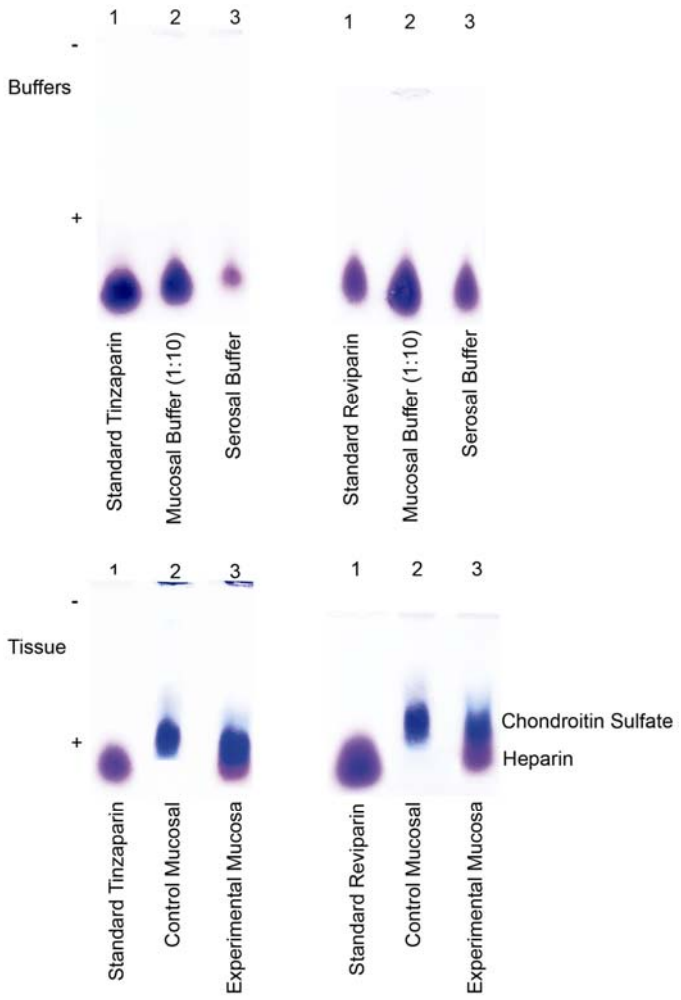


Figure 5.2 An electrophoretic gel showing heparin recovered from buffer and tissue following low molecular weight heparin addition to the mucosal buffer. Using agarose gel electrophoresis, low molecular weight heparins were found in both the mucosal and serosal buffer as well as in experimental tissue 84 min after their addition to the mucosal buffer. Standard and recovered heparin samples were dissolved in measured amounts of distilled water and 2 µl was applied to each lane (level with the negative sign). The upper gels show low molecular weight heparins (tinzaparin or reviparin) recovered from serosal buffer extracts: Lane 1, Standard tinzaparin or reviparin 2 mg/ml. Lane 2, Mucosal buffer extract dissolved in 10000 µl of water. Lane 3, Serosal buffer extract dissolved in 50 µl of water. The lower gels show low molecular weight heparins (tinzaparin or reviparin) recovered from the mucosal tissue: Lane 1, Standard tinzaparin or reviparin 2 mg/ml. Lane 2, Control tissue extract where buffer alone was added to the mucosal side, dissolved in 50 µl of water. Lane 3, Experimental mucosal extract dissolved in 50 µl of water. All material extracted from buffers is similar in migration and color to the original standard low molecular weight heparin. A blue band, moving slower than standard low molecular weight heparin, and likely chondroitin sulfate, is seen in the control mucosa (Lane 2). This band is seen in the experimental mucosa along with heparin (Lane 3).

Table 5.1 Tinzaparin and Reviparin recovered from buffers and tissues 84 minutes after addition to the mucosal buffer when rat gastric mucosa was placed in an Ussing chamber.

Treatment (10mg ml^{-1})	**Mucosal Buffer** (μg ml^{-1})	**Serosal Buffer** (μg ml^{-1})	**Control Mucosa** (μg g^{-1})	**Experimental Mucosa** (μg g^{-1})
Tinzaparin (pH 7.4, n=7)	2266.7 ± 584.2	176.8 ± 75.7	0.0 ± 0.0	25.0 ± 1.5
Tinzaparin (pH 4.0, n=6)	3250.0 ± 845.6	51.5 ± 2.5	0.0 ± 0.0	34.2 ± 5.2[a]
Reviparin (pH 7.4, n=4)	2875.0 ± 953.8	214.1± 70.6[b]	0.0±0.0	24.4 ± 1.8
Reviparin (pH 4.0, n=4)	3500.0 ± 333.3	47.5 ± 5.0	0.0 ± 0.0	35.0 ± 8.5

Results are shown as Mean±SEM. [a]Significantly greater than tinzaparin at pH 7.4, one-tailed-t-test. [b]Significantly greater than reviparin at pH 4.0, one-tailed t-test.

5.4.2 Addition of tinzaparin to mucosal buffer at pH 4.0

To mimic the *in vivo* pH of the stomach, rat gastric mucosa was placed in the Ussing chamber with mucosal buffer at pH 4.0, the average pH in rat stomach (Eastman and Miller, 1935). When tinzaparin was added to the mucosal buffer, PD increased and became more negative as in the neutral condition. After a lag period, the PD began to decrease to the previous resting level (Fig. 5.3A). There was a doubling of the lag period of 16.4 ± 0.5 min in the acidic environment compared to 7.0 ± 1.2 min in the neutral buffer that did not reach statistical significance (P=0.2, one-tailed t-test). PD reached the previous resting level 47.6 ± 11.4 min after the lag period in the acidic environment compared to 35.0 ± 9.5 min at pH 7.4 (P=0.2, one-tailed t-test). The Isc increased

throughout the experimental period (40-124 min) by 0.9 ± 0.5 μA at pH 4.0 compared to 2.7 ± 0.5 μA at pH 7.4 (P=0.06, two-tailed t-test, Mann-Whitney test) (Fig. 5.5b). Tinzaparin recovery from the serosal buffer was 51.5 ± 2.5 μg/ml when it was added to the mucosal buffer at pH 4.0, compared to 176.8 ± 75.7 μg/ml when added at pH 7.4 (P= 0.06, one-tailed t-test). Furthermore, 34.2 ± 5.2 μg/g tinzaparin was recovered from the mucosal tissue following addition to the mucosal buffer at pH 4.0 compared to 25.0 ± 1.5 μg/g when added to the mucosal buffer at pH 7.4 (P= 0.04, one-tailed t-test) (Table 5.1). The rate of movement of tinzaparin across the mucosal tissue at pH 4.0 was calculated to be 0.40 ± 0.02 μg/ cm^2/ min compared to 1.26 ± 0.54 μg/cm^2/min at neutral pH (P=0.06, one-tailed t-test) .

5.4.3 Anticoagulant activity of tinzaparin in buffers

To determine if tinzaparin recovered in the serosal buffer had anticoagulant activity, anti-Xa activity was measured in the desalted buffer extracts when added to mucosal buffer at pH 4.0. Anti-Xa activity was found both in the mucosal and serosal buffer extracts. Tinzaparin concentration based on anti-Xa activity was 13.6 ± 1.2 μg/ml in serosal compared to 133.2 ± 2.1 μg/ml in the mucosal buffer extracts.

5.4.4 Assessment of mucosal damage using trypan blue

Trypan blue was used to detect any minor damage to the mucosal tissue that may occur during the process of isolation. Mucosal and serosal buffers were collected 15 min after addition of 100 μl of trypan blue stock solution (0.3 mg/ml) to the mucosal buffer following experiments lasting 124 min. The concentration of trypan blue in the mucosal

buffer was significantly different than that of the serosal buffer, P< 0.0001, two-tailed t-test. There was no difference in the concentration of trypan blue in the serosal buffer compared to the control buffer (P = 0.20, two-tailed t-test) (Table 5.2).

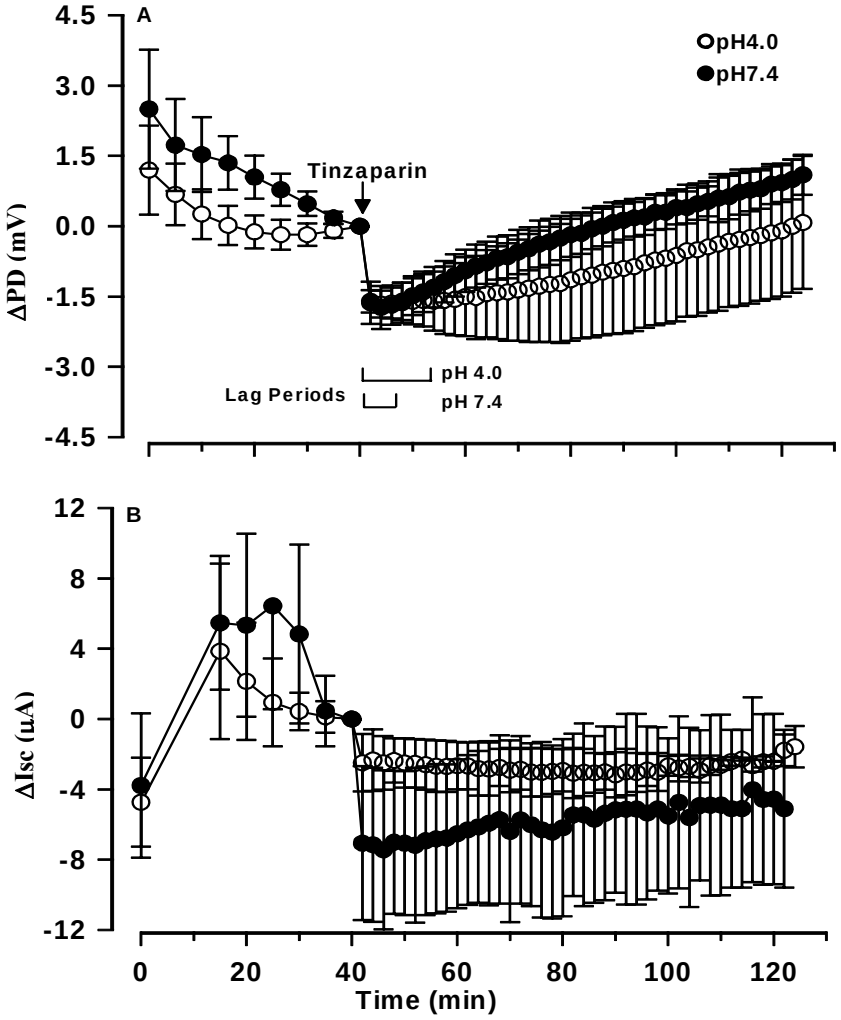


Figure 5.3 Changes in electrical parameters across rat gastric mucosa on addition of tinzaparin (10 mg / ml) to the mucosal buffer at acidic and neutral pH. Tinzaparin was added to the mucosal buffer at pH 4.0 or pH 7.4 and changes in potential difference (PD) and short circuit current (Isc) were compared. The lag period before the PD began to rise was longer at acidic versus neutral pH (A). Change in Isc was more obvious (40-124 min) after tinzaparin addition at acidic versus neutral pH (B). Results are shown as mean ± SEM of 6 experiments.

5.4.5 Addition of reviparin to mucosal buffer at pH 7.4 or pH 4.0

To observe if another LMWH caused changes in PD, R, or Isc across rat gastric mucosa, the mucosa was mounted in the Ussing chamber and reviparin was added to the mucosal buffer at pH 7.4 or pH 4.0. When reviparin was added, the PD became more negative, and the charge difference between the mucosal and serosal side of the membrane increased compared to that prior to reviparin addition (Fig. 5.4A). After a lag period, the PD began to decrease and achieved the previous resting level. There was a trend towards an increase in the lag period when reviparin was added to the mucosal buffer in the acidic environment versus the neutral environment where lag periods were 15.0 ± 2.8 versus 4.0 ± 0.0 min respectively (P= 0.06, one-tailed t-test). The PD reached its previous resting level at 36.7 ± 4.3 min when reviparin was added to the neutral mucosal buffer versus 46.0 ± 3.7 min later when added to the acidic mucosal buffer (P= 0.05, one-tailed t-test). The R of the mucosa followed a pattern similar to that seen for the PD. The R increased upon addition of reviparin and then began to decrease reaching the resting level 32.7 ± 3.6 min later at pH 7.4 compared to 44.5 ± 3.9 min later at pH 4.0 (P= 0.03, one-tailed t-test), following a lag period of 7.3 ± 2.9 min at pH 7.4 compared to15.0 ± 2.8 min at pH 4.0 (P=0.04, one-tailed t-test) (Results not shown). The Isc increased during the experimental period after reviparin addition with a much more dramatic increase at pH 7.4 (23.0 ± 12.4 μA) than pH 4.0 (0.5 ± 2.3 μA) (P=0.05, two-tailed t-test, Mann-Whitney test) (Fig. 5.4B).

Reviparin was recovered from both serosal buffer and the mucosal tissue. Significantly more reviparin was recovered from serosal buffer (214.1 ± 70.6 μg/ml) when added to

the mucosal buffer at pH 7.4 than at pH 4.0 (47.5 ± 5.0 μg/ml, P= 0.02, one-tailed t-test) (Table 5.1). Furthermore, 24.4 ± 1.8 μg/g reviparin was recovered from the mucosal tissue when added to the mucosal buffer at pH 7.4 compared to 35.0 ± 8.5 μg/g in the acidic environment (P= 0.1, one-tailed t-test) (Table 5.1). The calculated rate of movement of reviparin across the mucosal tissue was significantly greater (1.53 ± 0.51 μg/cm^2/min) at pH 7.4 compared to pH 4.0 (0.30 ± 0.06 μg/cm^2/min, P=0.02, one-tailed t-test).

Table 5.2 Trypan blue recovered from buffers 15 min after addition to the mucosal buffer when rat stomach mucosa was placed in an Ussing chamber for 124 min.

Buffers	Trypan blue (mg/ml)
Mucosal buffer	53.3 ± 5.7
***Experimental serosal buffer**	1.5 ± 0.1
****Control serosal buffer**	1.6 ± 0.3

Results are shown as mean ± SEM.
* Serosal buffer obtained when trypan blue is added to the mucosal buffer.
** Serosal buffer obtained when trypan blue is not added to the mucosal buffer.

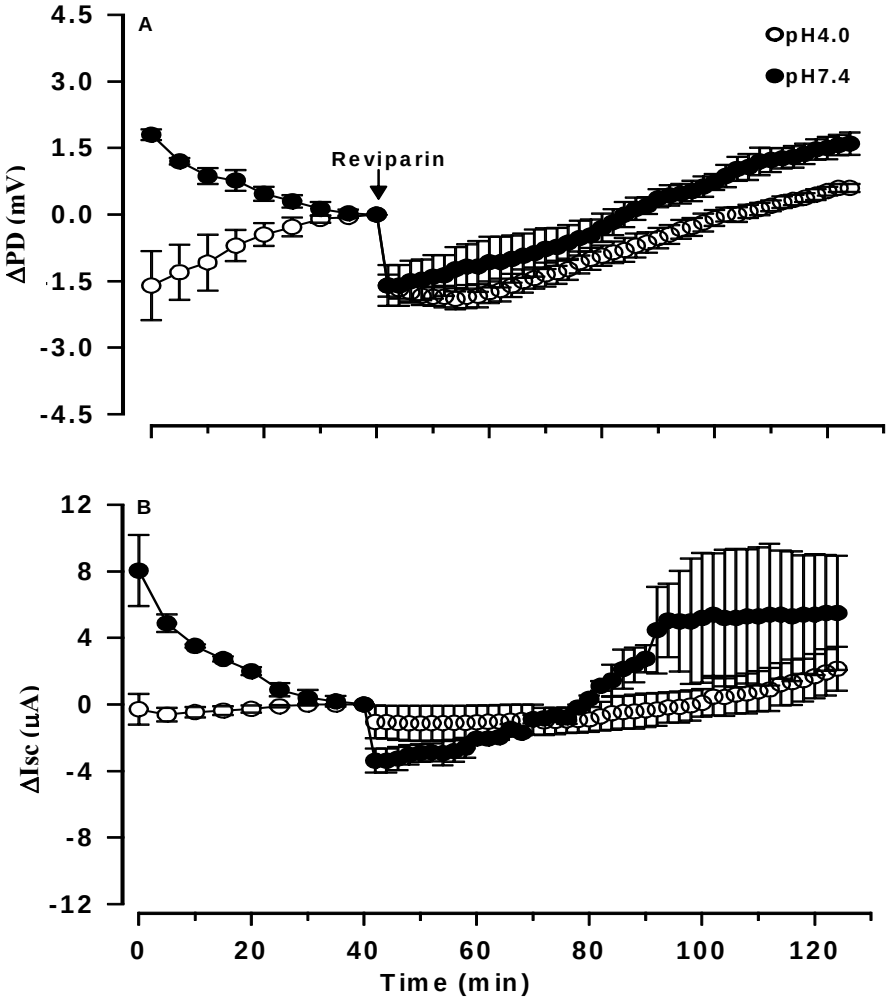


Figure 5.4 Changes in electrical parameters across rat gastric mucosa on addition of reviparin (10 mg / ml) to the mucosal buffer in neutral or acidic pH. Reviparin was added to the mucosal buffer at pH 4.0 or pH 7.4 and changes in potential difference (PD) and short circuit current (Isc) were compared. The lag period before the PD began to rise was longer at pH 4.0 versus pH 7.4 (A). Short Circuit Current (Isc) showed a dramatic increase at pH 7.4 (40-124 min) but only a slight increase at pH 4.0 (B). Results are shown as mean ± SEM of 4 experiments.

5.4.6 Anticoagulant activity of reviparin in serosal buffers

To determine if reviparin recovered in the serosal buffer had anticoagulant activity, anti-Xa activity was measured in the desalted buffer extracts when reviparin was added to mucosal buffer at pH 4.0. Anti-Xa activity was found both in the mucosal and serosal buffer extracts. Reviparin concentration found in the serosal buffer extracts based on anti-Xa activity was 14.1 ± 1.3 μg/ml compared to 132.0 ± 5.4 μg/ml in the mucosal buffer extracts.

5.4.7 Comparison of electrical parameters and recovery following addition of LMWHs at different pH

pH 7.4 Changes in PD across rat gastric mucosa following addition of reviparin to the mucosal buffer at pH 7.4 were compared to those after addition of tinzaparin to the mucosal buffer. Lag periods for PD were significantly less at 4.0 ± 0.0 min for reviparin compared to 7.0 ± 1.2 min for tinzaparin (P= 0.03, one-tailed t-test), and time to return to the previous baseline was 36.7 ± 4.3 min compared to 35.0 ± 9.5 min for reviparin and tinzaparin respectively (P= 0.4, one-tailed t-test) (Fig. 5.5a, and Table 5.3). There was a dramatic difference in changes in Isc throughout the experiment following addition of reviparin (23.0 ± 12.4 μA) compared to tinzaparin (2.7 ± 0.5 μA) in the mucosal buffer at pH 7.4 (P=0.06, two-tailed t-test, Mann-Whitney test) (Fig. 5.5c) where the Isc increased dramatically after addition of reviparin to the mucosal buffer, but showed only a small increase toward the end of the experiment after addition of tinzaparin. Recovery from the serosal buffer and the mucosal tissue was not different after addition of reviparin or tinzaparin. Reviparin recovery was 214.1 ± 70.6 μg/ml from the serosal buffer compared

to 176.8 ± 75.7 μg/ml after tinzaparin addition at pH 7.4 (P= 0.4, one-tailed t-test). Furthermore, 24.4 ± 1.8 μg/g reviparin was recovered from the mucosal tissue when added to the mucosal buffer at pH 7.4 compared to 25.0 ± 1.5 μg/g tinzaparin (P= 0.4, one-tailed t-test) (Table 5.1). The rate of movement of reviparin and tinzaparin across the mucosal tissue at pH 7.4 was calculated to be 1.5 ± 0.5 $\mu g/cm^2$/min and1.3 ± 0.5 $\mu g/cm^2$/min respectively (P=0.4, two-tailed t-test).

pH 4.0 Changes in the electrical parameters across rat gastric mucosa following addition of reviparin to the mucosal buffer at pH 4.0 were very similar to those of tinzaparin. Lag periods for PD were 15.0 ± 2.8 min versus 16.4 ± 0.5 min (P= 0.5, one-tailed t-test), and time to reach the previous resting level was 46.0 ± 3.7 min versus 47.6 ± 1.4 min for reviparin and tinzaparin respectively (P=0.5, one-tailed t-test) (Fig 5.5b). The Isc showed a small increase for both reviparin (0.5 ± 2.3 μA) and tinzaparin (0.9 ± 0.5 μA) (P=1.0, two-tailed t-test, Mann-Whitney test) (Fig. 5.5d). Recovery from the serosal buffers and the mucosal tissues were also very similar with 47.5 ± 5.0 μg/ml reviparin and 51.5 ± 3.2 μg/ml tinzaparin recovered from the serosal buffers (P=0.2, one-tailed t-test) and 35.0 ± 8.5 μg/g reviparin and 34.2 ± 5.2 μg/g tinzaparin recovered from the experimental mucosal tissues (P=0.5, one-tailed t-test) (Table 5.2). Moreover, concentrations of reviparin and tinzaparin in the mucosal and serosal buffer extracts based on anti-Xa activity were not different. Reviparin concentration found in the serosal buffer extracts based on anti-Xa activity was 14.1 ± 1.3 μg/ml compared to 13.6 ± 1.2 μg/ml for tinzaparin (P=0.7, two-tailed t-test). Reviparin concentration found in the mucosal buffer extracts based on anti-Xa activity was 132.0 ± 5.4 μg/ml compared to 133.2 ± 2.1 μg/ml

for tinzaparin (P=0.8, two-tailed t-test). The calculated rate of movement of reviparin (0.3 ± 0.1 μg/cm^2/min) and tinzaparin (0.4 ± 0.0 μg/cm^2/min) across the mucosal tissue at pH 4.0 did not differ (P=0.14, two-tailed t-test).

5.5 Discussion

Although antithrombotic activity of orally administered LMWHs in rat models has been thoroughly studied, the site of LMWH absorption is unknown. Our current results indicate that the stomach could be a possible absorption site. The LMWHs, tinzaparin and reviparin, were recovered from serosal buffer and mucosal tissue after addition to the mucosal side of gastric mucosa. Additionally, anticoagulant (anti-factor Xa) activity of LMWHs was maintained after crossing the mucosa.

Changes in electrical parameters also suggest that LMWHs cross gastric mucosa. The increase in PD negativity on addition of LMWHs to the mucosal buffer is likely due to the high negative charge of LMWHs making the mucosal side more negative than the serosal. The lag period, when PD does not change, can be explained as the time required for LMWHs to cross the mucosa. The PD then decreases in negativity as the serosal buffer contains more LMWHs. These changes were not artifacts of mucosal damage since trypan blue was not recovered from serosal buffer after its addition to mucosal buffer (Table 5.2).

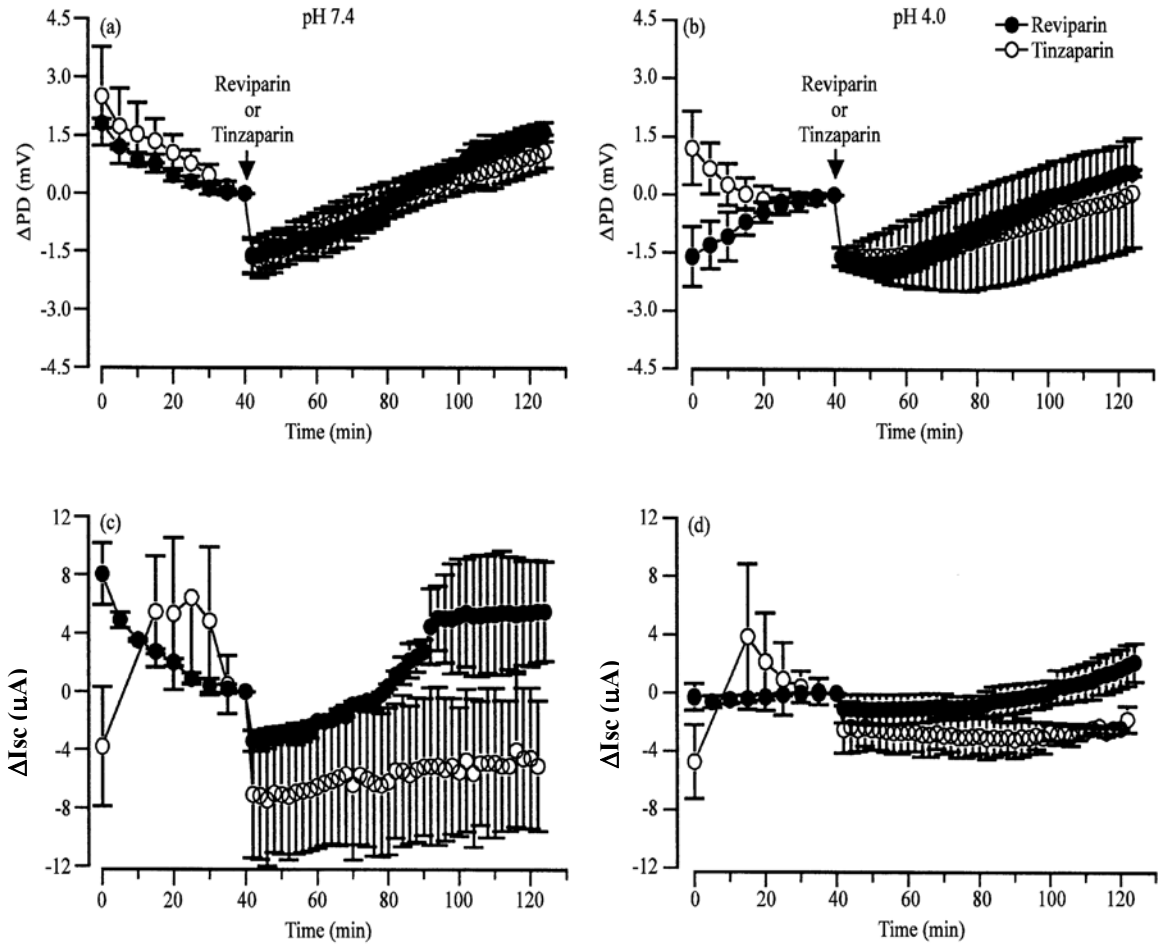


Figure 5.5 Comparison of changes in electrical parameters across rat gastric mucosa on addition of tinzaparin or reviparin (10 mg / ml) to the mucosal buffer of neutral or acidic pH. Rat gastric mucosa was mounted in the Ussing chamber with the mucosal buffer at pH 7.4 or pH 4.0, and changes in potential difference (PD) and short circuit current (Isc) after additon of tinzaparin were compared to those with reviparin addition. Changes in PD after addition of tinzaparin to the mucosal buffer at pH 7.4 (5A) or pH 4.0 (5a) were superimposed on those following reviparin addition. The Isc showed a dramatic increase at pH 7.4 after reviparin addition to the mucosal buffer versus a small increase for tinzaparin (5B). Unlike tinzaparin, a slight increase in Isc was observed at pH 4.0 for reviparin (5b). Results are shown as mean ± SEM of 4 experiments.

Table 5.3 Comparison of changes in electrical parameters (PD, R, and Isc) across rat gastric mucosa following addition of tinzaparin or reviparin to the mucosal buffer when mucosa is mounted in an Ussing chamber for 124 min.

pH 7.4		**Tinzaparin**	**Reviparin**
	Negativity (mV)	-1.8 ± 0.4	-1.6 ± 0.5
PD	Lag Period (min)	7.0 ± 1.2[a]	4.0 ± 0.0
	Time to reach resting level after lag period (min)	35.0 ± 9.5	36.7 ± 4.3[b]
R	Change in baseline after LMWH addition (Ω)	110 ± 30	110 ± 20
	Lag Period (min)	8.0 ± 1.4	7.3 ± 2.9
	Time to reach resting level after lag period (min)	32.0 ± 2.5	32.7± 3.6
Isc	Change in baseline (μA) (124min-40min)	2.7 ± 0.5	23.0 ± 12.4[b]
pH 4.0			
	Negativity (mV)	-2.3 ± 0.8	-2.0 ± 0.2
PD	Lag Period (min)	16.4 ± 0.5	15.0 ± 2.8
	Time to reach resting level after lag period (min)	47.6 ± 1.4	46.0 ± 3.7
R	Change in baseline after LMWH addition (Ω)	110 ± 35	130 ± 10
	Lag Period (min)	20.0 ± 9.3	20.0 ± 4.8
	Time to reach resting level after lag period (min)	51.6 ± 12.3	44.5 ± 3.9
Isc	Change in baseline (μA) (124min-40min)	0.9 ± 0.5	0.5 ± 2.3

Results are shown as mean ± SEM.
Potential Difference (PD), Resistance (R), and Short circuit current (Isc).
a. Significantly different than reviparin at pH 7.4
b. Significantly different than reviparin at pH 4.0

Absorption of LMWHs is supported by previous studies. Intragastric administration of tinzaparin and reviparin significantly reduced thrombotic incidence in a rat jugular vein model dose dependently 4 h after administration (Hiebert et al., 2000; Hiebert et al., 2001). Tissue distribution studies showed that oral tinzaparin and [^{14}C]-labeled and unlabeled sodium sucrose octasulfate were found in gut and non-gut tissues.

More drug was recovered from stomach tissue compared to lower gut levels and drug was found in plasma at 6 min (Hiebert et al., 2002; Hiebert et al., 2004). Lasker reported prolongation of blood clotting time after placing LMWH in the buccal cavity of mice (Lasker, 1975). Sue et al., also indicated absorption of LMWH (Sue et al., 1976). As well, LMWHs in drinking water returned systolic blood pressure of spontaneously hypertensive rats to normal (Vasdev et al., 1994).

Differences were seen between the LMWHs. A significantly shorter lag period for PD and a slightly faster rate of movement across the mucosa, based on recovery from serosal buffer (Table 5.1), was observed for reviparin compared to tinzaparin. The same significant decrease in thrombotic incidence was achieved at a lower oral dose of reviparin (0.025 mg/kg) compared to tinzaparin (0.1 mg/kg) in a rat jugular vein model (Hiebert et al., 2000; Hiebert et al., 2001). Each LMWH is chemically unique depending on the manufacturing process used. LMWHs have specific oligosaccharide composition and microstructural differences (Fareed et al., 2004a; Fareed et al., 2004b) that may influence drug interaction with gastric mucosa. Tinzaparin is of higher molecular weight (MW) than reviparin and has a 4,5 unsaturated uronic acid at its non reducing terminus

while reviparin has a 2,5 anhydro-D-mannose at the reducing terminus (Fareed et al., 2003). The decreased MW of reviparin versus tinzaparin may be a factor contributing to its faster movement through the mucosa. Sue et al., indicated that a decrease in MW facilitates heparin movement through intestinal membrane (Sue et al., 1976). Changes in electrical parameters indicate that both the lag period and the time required to reach the baseline for PD and R, are greater at pH 4.0 compared to pH 7.4 (Table 5.3). This suggests a slower movement of LMWHs across the gastric mucosa at lower pH, supported by decreased recovery of LMWHs in serosal buffer and a lower rate of movement across the mucosa at pH 4.0 than 7.4. This suggests that more LMWHs may be absorbed in the small intestine than the stomach. Since LMWHs have little capacity for protein binding, they may pass easily through the stomach lumen, entering the more basic duodenum.

Although this study was not designed to investigate the mechanism of absorption, our results suggest the involvement of both active transport and passive diffusion. The Isc increased over time at pH 7.4 but not pH 4.0. Since change in Isc is an indicator of active transport (Cooke and Dawson, 1978), our results suggest involvement of active transport and passive diffusion in movement of LMWHs across the mucosa at pH 7.4 and 4.0 respectively. Transporters for anionic compounds may become activated under neutral or more basic conditions. Changes in Isc are more dramatic for reviparin than tinzaparin at pH 7.4. This may be explained by separate transporters of varying numbers for anionic compounds of different MWs. Reviparin may have a greater affinity for a transporter than tinzaparin, or reviparin once bound to the receptor may interact with it in a more rapid

reversible manner. Changes in R also indicate involvement of passive transport. When the mucosal buffer LMWH concentrations are high, R is increased but the concentration gradient across the mucosa helps LMWH cross the membrane. As LMWH concentrations decrease, the concentration gradient across the mucosa decreases.

5.6 Conclusions

These results support earlier studies that LMWH is absorbed following oral administration. Results of this study suggest that LMWHs cross the stomach tissue, however movement is faster at neutral pH than under the natural acidic conditions found in the stomach. Although LMWH may be absorbed in the stomach, the intestine at neutral pH may be the preferred site of absorption. Further studies are required to investigate the mechanism of LMWHs movement across the gastrointestinal mucosa and to evaluate the role of passive and active transport in the process.

Chapter 6: The Movement of Heparins across Rat Gastric Mucosa is Dependent on Molecular Weight and pH*

6.1 Abstract

We recently measured the electrical parameters of rat gastric mucosa mounted in an Ussing Chamber to study heparin absorption. We showed that unfractionated heparin (UFH) and low molecular weight heparins (LMWHs) cross rat gastric mucosa in a pH-dependent manner. This article compares the movement of UFH and LMWHs through rat gastric mucosa and thus determines the effect of molecular weight. Potential difference (PD) increased on addition of heparins (10 mg/ml) to the mucosal buffer and, following a lag period, began to decrease eventually reaching its resting level. The lag period was significantly longer for UFH than LMWHs at pH 7.4 while at pH 4.0 it was longer for LMWHs. A slight increase in short circuit current (Isc) was seen for UFH at pH 4.0 while it increased significantly for LMWHs at pH 7.4. More UFH than LMWH was recovered from serosal buffer at pH 4.0 while more LMWH was recovered at pH 7.4. Changes in the lag period for PD and recovery from the serosal buffer suggest that LMWHs cross the gastric mucosa faster than UFH under neutral condition while UFH crosses faster under acidic conditions. Changes in Isc suggest that active transport is activated under acidic and neutral conditions for UFH and LMWHs respectively. This study suggests that decreasing the molecular weight of heparin, increases movement through the gastric mucosa under neutral but not acidic conditions. UFH may be absorbed more readily from the stomach while LMWHs may be better absorbed from the intestine.

* Bita Moazed and Linda M. Hiebert. Pharmaceutical Research, 2009;26(1):9751-9758.

6.2 Introduction

Heparins belong to a family of compounds called glycosaminoglycans (GAGs), which are derived from animal tissues. They are given as drugs of choice in the prevention and treatment of thrombo-embolic disorders by intravenous or subcutaneous routes and are believed to be ineffective when administered orally. Evidence of oral absorption of heparins has repeatedly been reported in the literature. Unfractionated heparin (UFH) and low molecular weight heparins (LMWHs) were found with endothelium following oral administration with little in plasma similar to that observed with parenteral administration (Hiebert et al., 1993; Hiebert et al., 2001; Jaques et al., 1991). Moreover, UFH and LMWHs were found to have antithrombotic effects after oral administration in a rat jugular vein (Jaques et al., 1991), venous stasis (Hiebert et al., 2004) and rat carotid arterial model (Pinel et al., 2004).

Evidence on the site and mechanism of oral heparin absorption was obtained from a few previous studies. Sue et al., (1976) introduced heparin into the gastrointestinal tract and found increases in whole blood clotting time and plasma anti-Xa activity with the anticoagulant effect being greater when heparin was placed in the stomach versus the small intestine. More chemical or [^{14}C] UFH was found in stomach tissue versus duodenum, jejunum, and ileum or colon tissue up to 24 hours following administration by stomach tube (Hiebert et al., 2000). Furthermore, more heparin was found in the endothelium when heparin was placed in the rat stomach with the pyloric sphincter tied versus the small intestine (Hiebert et al., 2007). These findings suggested that stomach may be a site for heparin absorption. Most recently, we showed using a vertical diffusion

Ussing chamber that UFH (chapter 4) and LMWHs (chapter 5) cross rat gastric mucosa and that movement is dependent on the pH of the environment.

The high negative charge and large molecular weight of heparins have been considered limiting factors to oral absorption (Money and York, 2001). Considering this, decreasing the molecular size may facilitate the passage of heparin through the gastric mucosal membrane. In support of this notion, orally administered LMWHs, tinzaparin and reviparin, have been shown to have antithrombotic activity at much lower doses than UFH in rat jugular vein model of thrombosis. Single dose of 0.025, 0.1, and 7.5 mg/kg resulted in 50% reduction in thrombosis incidence for reviparin, tinzaparin and bovine lung UFH, respectively (Hiebert et al., 2001; Hiebert et al., 2000; Hiebert et al., 1996; Pinel et al., 2004). Thus, the objective of the present study is to determine if LMWHs move across rat gastric mucosa, mounted in an Ussing chamber, faster than UFH.

6.3 Materials and Methods

6.3.1 Chemicals

Tinzaparin sodium stock solution (anti-Xa activity 90.7 IU/mg, peak maximum molecular mass of 5600 daltons) obtained from porcine mucosal heparin, was generously donated by Novo Nordisk, Denmark. Reviparin sodium stock solution (Batch W 49522, average molecular mass of 4300 daltons, anti-Xa activity 130 IU/mg; anti-IIa activity 29 IU/mg) was from Knoll AG, Ludwigshafen, Germany. Bovine lung unfractionated heparin (156.2 Units/mg) was obtained from Scientific Protein Labs, Division of Viobin Corporation, Wisconsin, USA. Other materials used for the study are described in section 3.1 of this thesis.

6.3.2 Gastric mucosa isolation from rats

Thirty four male Wistar rats (250-300 g) were obtained from Charles River Canada Company, St. Constant, Quebec, Canada, and were handled as described in section 3.3. Gastric mucosa was isolated as described in section 3.4.

6.3.3 Measurements of movement of heparins across gastric mucosa using an Ussing chamber

An EVC 4000 voltage/current clamp (NaviCyte, Harvard Apparatus, Inc.) was used for transport studies across the gastric mucosa. For detailed description please refer to the section 3.6 of this thesis.

6.3.4 Determination of heparins in buffers and tissues

Heparin in buffers and mucosal tissues was extracted as in methods, sections 3.8 and 3.9. Extracted heparin was identified and chemically measured as is described in section 3.10 of this thesis.

6.3.5 Data analysis and statistical procedures

All data are expressed as mean ± standard error of the mean (SEM). A one-tailed unpaired t-test was used to determine significant differences in the degree of negativity on addition of heparins, in the lag period before PD began to return to the resting level, and in the time period for PD to return to the baseline in different environments. A one-tailed t-test was also used to measure differences in heparin concentrations in serosal buffers and experimental mucosa under different conditions and in the rate of movement across gastric mucosa.

A one-tailed t-test was used to compare differences in Isc during the experimental period between groups. Differences in Isc were calculated by subtracting the average of the first 5 values obtained at 40-48 min following addition of heparins to the mucosal buffer in different environments, from the average of the last 5 values recorded at 116-124 min. Values were considered significant at $P<0.05$.

6.4 Results

6.4.1 Changes in electrical parameters of rat gastric mucosa following addition of UFH or LMWHs to the mucosal buffer at pH 7.4

Rat gastric mucosa was placed in the Ussing chamber bathed with mucosal buffer on the mucosal side and serosal buffer on the serosal side at pH 7.4 and electrical parameters of the membrane were recorded every 5 min. Changes in PD (mV) seen at the beginning of the experiment became negligible as the mucosal tissue was stabilized in the Ussing chamber for 40 min before drug addition. The PD increased and became more negative when heparins were added to the mucosal buffer by 1.6±0.3 mV, 1.8±0.4 mV and 1.6±0.5 mV upon addition of UFH, tinzaparin and reviparin respectively (Fig 6.1A, Fig. 6.1a, and Table 6.1). No difference was seen when LMWHs were compared to UFH (P=0.4, one-tailed t-test). There was a lag period during which the PD remained in the highly negative state and no changes were observed. The lag period was 18.8±4.7 min for UFH, 7.0±1.2 min for tinzaparin, 4.0±0.0 min for reviparin, and 5.7±0.9 min for LMWHs combined. The lag period was significantly greater for UFH versus LMWHs (P=0.005, one-tailed t-test). After the lag period, the PD began to decrease and reached its previous resting level at similar periods of time, 39.3±17.6, 35.0±9.5 and 36.7±4.3 min for UFH, tinzaparin and reviparin respectively (P=0.4, one-tailed t-test when LMWHs were compared to UFH).

The Isc did not change during the experimental period after UFH addition to the mucosal buffer at pH 7.4 while it did increase by 2.7±0.5 and 23.0 ± 12.4 μA after tinzaparin or reviparin addition respectively and by 11.4±6.2 μA for LMWHs combined. This Isc

change was significantly less for UFH when compared to values for LMWHs combined (P=0.04, one-tailed t-test) (Fig 6.1B, Fig. 6.1b, and Table 6.1).

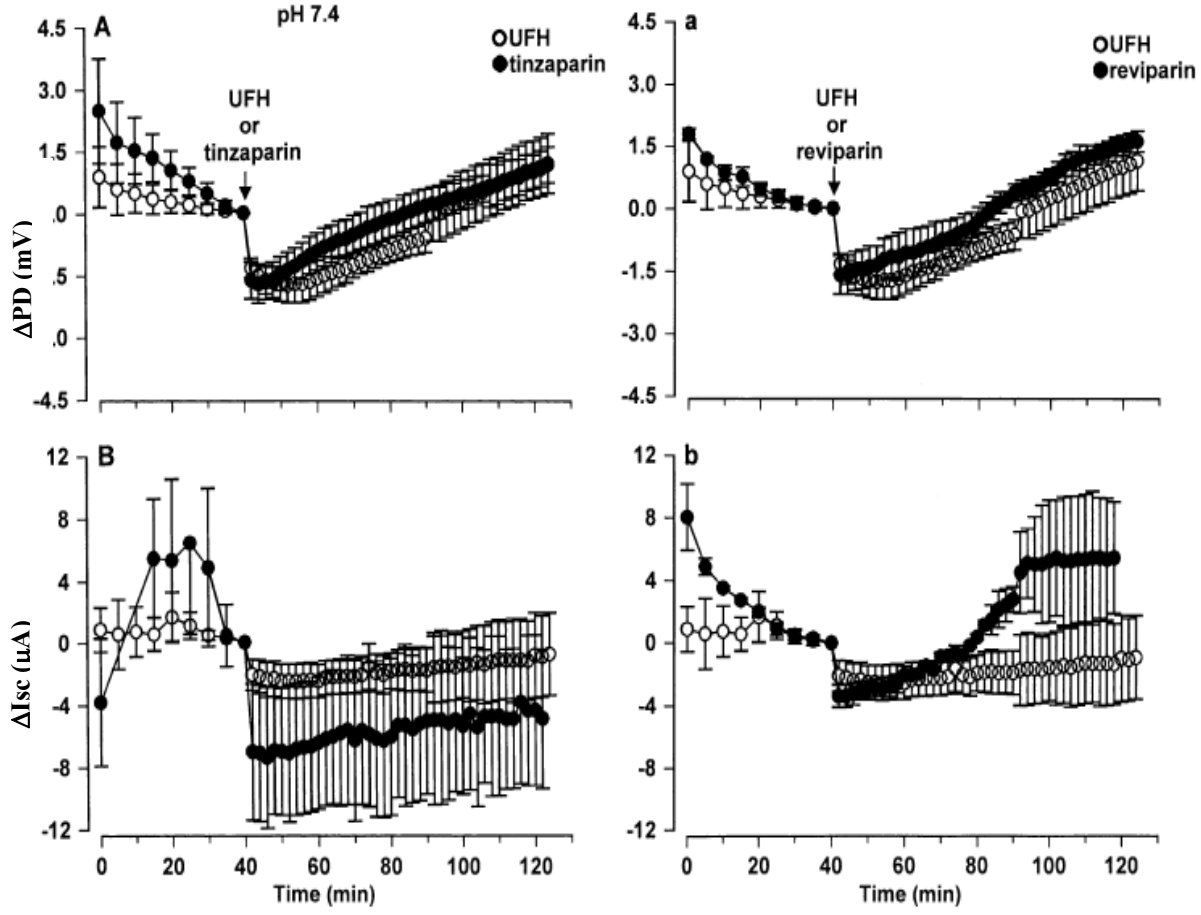


Figure 6.1 Changes in electrical parameters across rat gastric mucosa on addition of UFH or LMWHs (10 mg/ml) to the mucosal buffer at pH 7.4. The potential difference (PD) became more negative when the mucosal side was compared to the serosal side immediately after addition of heparins to the mucosal buffer. After a lag period, PD returned to the resting level with time (A and a). The lag period was significantly greater for UFH versus LMWHs. Short Circuit Current (Isc) did not change during the experimental period after UFH addition while it did increase significantly after tinzaparin or reviparin addition when LMWHs were compared to UFH (B and b). Results are shown as mean ± SEM of 8 experiments for UFH, 6 experiments for tinzaparin and reviparin.

Table 6.1 Changes in electrical parameters (PD, R, and Isc) across rat gastric mucosa in an Ussing chamber following addition of UFH or LMWHs to the mucosal buffer for 84 min

		Tinzaparin	Reviparin	LMWHs*	UFH
At pH 7.4					
Changes in PD	Negativity (mV	-1.8 ± 0.4	-1.6 ± 0.5	-1.7 ± 0.3	-1.6 ± 0.3
	Lag Period (min)	7.0 ± 1.2	4.0 ± 0.0	5.7 ± 0.9[a]	18.8 ± 4.7
	Time to reach resting level after lag period (min)	35.0 ± 9.5	36.7 ± 4.3	35.7 ± 5.0	39.3 ± 17.6
Changes in Isc	Change in baseline (μA) (124-40 min)	2.7 ± 0.5	23.0 ± 12.4	11.4 ± 6.2[b]	0.0 ± 0.0
At pH 4.0					
Changes in PD	Negativity (mV)	-2.3 ± 0.8	-2.0 ± 0.2	-2.2 ± 0.4	-2.2 ± 0.1
	Lag Period (min)	16.4 ± 0.5	15.0 ± 2.8	15.8 ± 5.3	9.2 ± 2.8
	Time to reach resting Level after lag period (min)	47.6 ± 1.4	46.0 ± 3.7	46.9 ± 5.9	40.5 ± 7.9
Changes in Isc	Change in baseline (μA) (124-40 min)	0.9 ± 0.5	0.5 ± 2.3	0.7 ± 1.0[a]	4.2 ± 1.6

∗ Results combined for tinzaparin and reviparin.
Potential Difference (PD), Resistance (R), Short circuit current (Isc).
a. Significantly less than UFH.
b. Significantly greater than UFH.

Heparins were extracted from serosal buffer and the mucosal tissue 84 min after addition of heparins to the mucosal buffer at pH 7.4 (Table 6.2). Recovery of UFH from serosal buffer at pH 7.4 was 65.0±20.6 μg and significantly less than that for LMWHs, 176.8±75.7 μg and 214.1±70.6 μg for tinzaparin and reviparin respectively (P=0.03, One-tailed t-test). Heparin recovered from mucosal tissue was similar for all three groups, 51.9±21.5, 25.0±1.5, and 24.4±1.8 μg for UFH, tinzaparin, and reviparin respectively (P=0.1, One-tailed t-test, when LMWHs were compared to UFH).

The rate of movement of heparins across the gastric mucosa based on the chemical recovery of heparins from the serosal buffer when the tissue was exposed to drugs for 84 min at pH 7.4 was calculated to be 0.5±0.1 μg/cm^2/min for UFH, 1.3±0.5 μg/cm^2/min for tinzaparin and 1.5±0.5 μg/cm^2/min for reviparin and 1.4±0.3 μg/cm^2/min for LMWHs combined (Table 6.2). There was a trend towards an increase in rate of movement across the mucosa for LMWHs versus UFH (P=0.05, one-tailed t-test).

Table 6.2 Recovery of heparins from buffers and tissues 84 min after addition of heparin to the mucosal buffer.

Treatments	Mucosal Buffer (μg)	Serosal Buffer (μg)	Control Mucosa (μg)	Experimental Mucosa (μg)	Rate[d] μg cm-^{2} min^{-1}
At pH 7.4					
UFH (n=6)	3917.7 ± 271.3	65.0 ± 20.6	0.0 ± 0.0	51.9 ± 21.5	0.5 ± 0.1
Tinzaparin (n=7)	2266.7 ± 584.2	176.8 ± 75.7	0.0 ± 0.0	25.0 ± 1.5	1.3 ± 0.5
Reviparin (n=4)	2875.0 ± 953.8	214.1 ± 70.6[a]	0.0 ± 0.0	24.4 ± 1.8	1.5 ± 0.5[a]
LMWHs[e] (n=11)	2510.0 ± 442.3	190.3 ± 48.1[a]	0.0 ± 0.0	24.8 ± 1.1[b]	1.4 ± 0.3[a]
At pH 4.0					
UFH (n=5)	2400.0 ± 597.0	111.3 ± 36.3	0.0 ± 0.0	26.9 ± 5.7	0.8 ± 0.1
Tinzaparin (n=6)	3250.0 ± 845.6	51.5 ± 2.3[c]	0.0 ± 0.0	34.2 ± 4.7	0.4 ± 0.0[b]
Reviparin (n=4)	3500.0 ± 333.3	47.5 ± 4.3	0.0 ± 0.0	35.0 ± 7.4	0.3 ± 0.0[b]
LMWHs[e] (n=10)	3350.0 ± 484.6	49.9 ± 2.2[b]	0.0 ± 0.0	34.5 ± 3.8	0.3 ± 0.0[b]

[a] Significantly greater than UFH, one-tailed t-test
[b] Significantly less than UFH, one-tailed t-test
[c] P=0.05, tinzaparin versus UFH, one-tailed t-test
[d] Rate of heparin movement across the gastric mucosa based on the chemical recovery of heparins from the serosal buffer
[e] Results for tinzaparin and reviparin combined

6.4.2 Changes in electrical parameters of rat gastric mucosa following addition of UFH or LMWHs to the mucosal buffer at pH 4.0. The electrical properties of the membrane were recorded when pH of the mucosal buffer was 4.0, the average pH of the stomach in rats fed a normal diet according to Eastman and Miller (1935). The same pattern of changes in PD was observed at pH 4.0 as noted at pH 7.4. Changes in PD increased and became more negative upon addition of heparins to the mucosal buffer after tissue stabilization in the Ussing chamber for 40 min compared to PD prior to drug addition (Fig. 6.2A, Fig. 6.2a, and Table 6.1). The PD became more negative by 2.2±0.1, 2.3±0.8 and 2.0±0.2 mV when UFH, tinzaparin or reviparin were added to the mucosal buffer respectively. The degree of negativity of PD was similar between groups and no difference was seen when LMWHs were compared to UFH (P=0.2, one-tailed t-test). After similar lag periods of 9.2±2.8 min for UFH, 16.4±0.5 min for tinzaparin and 15.0±2.8 min for reviparin (P=0.1, one-tailed t-test when UFH was compared to LMWHs), the PD began to decrease in negativity. There was no significant difference in the period of time it took for PD to reach its previous resting level when UFH was compared to LMWHs, 40.5±7.9 min for UFH, 47.6±1.4 min for tinzaparin and 46.0±3.7 min for reviparin (P=0.1, one-tailed t-test). The Isc increased by 4.2±1.6 μA in the acidic environment following addition of UFH to the mucosal buffer versus 0.9 ± 0.5 μA after tinzaparin addtion and 0.5 ± 2.3 μA after reviparin addition (P=0.05, One-tailed t-test when UFH was compared to LMWHs) (Fig. 6.2B, Fig. 6.2b, and Table 6.1).

Heparins were chemically recovered from serosal buffer and the mucosal tissue after addition of heparins to the mucosal buffer for 84 min at pH 4.0 (Table 6.2). Recovery of

UFH from the serosal buffer was 111.3±36.3 μg and significantly greater when compared to LMWHs, 51.5 ± 2.3 μg tinzaparin and 47.5 ± 4.3 μg reviparin (P=0.02, one-tailed t-test). Moreover, 26.9 ± 5.7 μg UFH was recovered from the mucosal tissue which was similar to LMWHs recovery, 34.2 ± 4.7 μg for tinzaparin and 35.0 ± 7.4 μg for reviparin (P=0.1, one-tailed t-test).

The rate of movement of heparins across the gastric mucosa based on their chemical recovery from serosal buffer when the tissue was exposed to drugs for 84 min at pH 4.0 was calculated to be 0.8 ± 0.1 μg/cm^2/min for UFH, 0.4 ± 0.0 μg/cm^2/min for tinzaparin, 0.3±0.0 μg/cm^2/min for reviparin, and 0.3 ± 0.0 μg/cm^2/min for LMWHs combined (Table 6.2). Rate of movement was significantly less for LMWHs combined compared to UFH (P <0.0001, one-tailed t-test).

6.5 Discussion

Heparins have been administered by intravenous and subcutaneous routes for more than 70 years. They are believed not to be effective when administered orally (Canadian Pharmaceutical Association, 2007). These assumptions are based on the observations that little or no change is seen in anticoagulant activity following oral administration of heparins (Dryjski et al., 1989) or heparins are highly charged or too large to be considered candidates for gastrointestinal absorption (Money and York, 2001). Based on these assumptions, considerable effort has been spent on increasing oral heparin absorption by addition of a simple organic chemical N -(8-(2-hydroxybenzoyl)amino)caprylate (SNAC) (Pineo et al., 2001), using adjuvants (Windsor

and Cronkeim, 1961), surfactants (Guarini and Ferrari, 1985), heparin-diamine complexes (Zoppetti et al., 1992), and combining them with biodegradable and non-biodegradable polycationic polymers (Jiao et al., 2002).

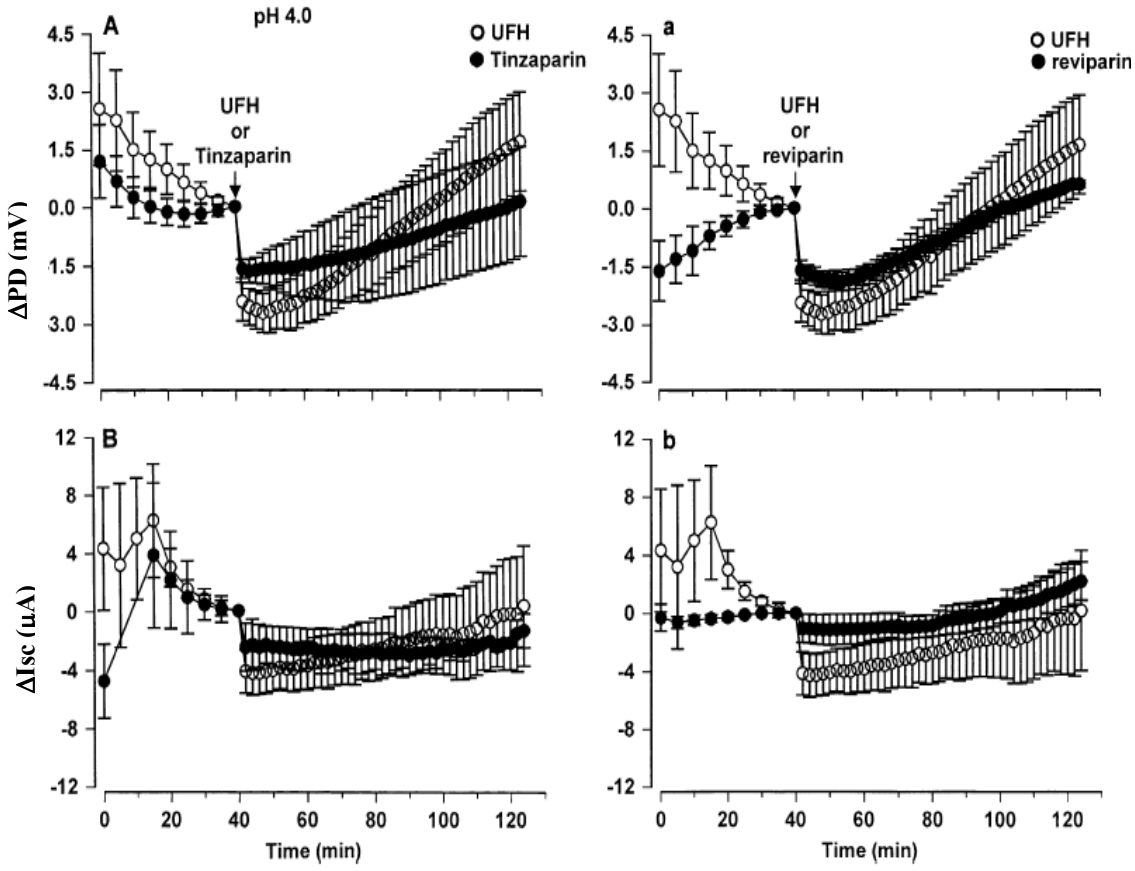


Figure 6.2 Changes in electrical parameters across rat gastric mucosa on addition of UFH or LMWHs (10 mg/ml) to the mucosal buffer at pH 4.0. The potential difference (PD) became more negative when heparins were added to the mucosal buffer. After a lag period, PD returned to the resting level with time (A and a). The lag period was similar for LMWHs versus UFH at pH 4.0. Short Circuit Current (Isc) increased during the experimental period when heparins were added to the mucosal buffer. Increase in Isc was similar when UFH was added to the mucosal buffer versus LMWHs (B and b). Results are shown as mean ± SEM of 6 experiments for UFH, 6 experiments for tinzaparin and reviparin.

Despite all these efforts, a great deal of evidence exists in the literature showing that heparins are effective when administered by the oral route without addition of other compounds or use of delivery agents. Both UFH and LMWH in drinking water given to spontaneously hypertensive rats returned systolic blood pressure to normal (Vasdev et al., 1994). Heparin was found with endothelium from the aorta and vena cava of rats given bovine UFH by stomach tube with little in plasma (Jaques et al., 1991; Hiebert et al., 1993). In addition, thrombosis was prevented by oral UFH and LMWHs in the rat jugular vein model (Hiebert et al., 1996; Hiebert et al., 2000; Hiebert et al., 2001), carotid artery model (Pinel et al., 2004), and venous stasis model (Costantini et al., 2000; Hiebert et al., 2004).

Although the effectiveness of orally administered heparins on thrombosis in the rat model has been thoroughly studied, there is little known about the site of heparin absorption. Tissue distribution studies showed that orally administered heparin was found in gut and non-gut tissues. Much was recovered from the stomach tissue (Hiebert et al., 2000). As well, more heparin is recovered from endothelium when UFH is placed in the stomach versus that of the duodenum for 15 min (Hiebert et al., 2007). These studies suggested that the stomach may be a site for heparin absorption when administered orally. Our recent *in vitro* studies using a vertical diffusion Ussing chamber with rat gastric mucosa showed that UFH and LMWHs cross rat gastric mucosa and that movement was dependent on the pH of the environment (Chapters 4 and 5). In the present study using this model we showed that movement across rat gastric mucosa is affected by molecular weight.

Changes in the electrical parameters following addition of UFH or LMWHs (tinzaparin or reviparin) suggested that heparins cross rat gastric mucosa. Changes in PD increased and became very negative the moment heparins were added to the mucosal buffer, when the mucosal side was compared to the serosal side (Fig. 6.1, Fig. 6.2, and Table 6.1). There was a lag period when the PD remained negative for some time after which it started to decrease and reached its previous resting level. Heparins were chemically recovered from the serosal buffer and the mucosal tissue.

Although changes observed in the electrical parameters of rat gastric mucosa after addition of UFH or LMWHs to the mucosal buffer followed the same pattern, there were some important differences (Table 6.1). UFH or LMWHs responded differently to pH changes in mucosal buffer. At pH 7.4, the lag period before PD began to return to previous resting level was significantly shorter for LMWHs compared to UFH. In contrast at pH 4.0, the lag period was significantly shorter for UFH compared to LMWHs. These results suggest that LMWHs may be better absorbed in a more basic environment while UFH may be better absorbed from an acidic environment. This is supported by chemical recovery of heparins from serosal buffers (Table 6.2). The LMWHs were found in significantly higher concentrations than UFH in serosal buffer at pH 7.4. However, the concentration of UFH in serosal buffer was significantly higher than that of LMWHs at pH 4.0. It is likely that prevention of ionization plays some role in facilitating the movement of UFH across the gastric mucosa under different environmental pH. As UFH is a very large and highly acidic molecule, an acidic environment may prevent ionization and thus facilitate movement through the gastric

mucosa. The LMWHs on the other hand, are smaller in size and therefore may be less affected by acidic conditions. Stomach absorption of UFH is also supported by the work of Sue et al., (1976) who showed significant increases in whole blood clotting time following administration of heparin dissolved in diluted acids.

When UFH or LMWHs are introduced into the mucosal buffer of the Ussing chamber, they interact with the mucosa and are then released into the serosal buffer. UFH has high nonspecific binding to proteins and cells (Hirsh, 1991: Barzu et al., 1985; Hiebert and Jaques, 1976). The UFH can bind to a variety of plasma proteins such as lipoproteins, fibrinogen, fibronectin, vitronectin and histidine-rich glycoproteins, as well as to proteins secreted by platelets like platelet factor 4 and von Willebrand factor and to endothelial cells (Hirsh, 1991). Thus, UFH interactions with components of mucosal membrane when added to the mucosal buffer is likely much more complicated than LMWHs. UFH may remain attached to the luminal mucosal membrane for some time before it can cross to the serosal side. Furthermore, some of the UFH that is internalized may remain associated with the mucosal cells for some time before it is released to the serosal side. This is supported by observations of Hiebert and McDuffie (1989) who found that UFH remained inside endothelial cells for 5 days after uptake as determined by the presence of toluidine blue stained metachromatic inclusions. LMWHs on the other hand, may pass through the mucosa easier since they have less protein binding capacity and smaller size. Our results imply that LMWHs are likely absorbed at a faster rate than UFH particularly in a neutral environment. This agrees with previous results showing that orally administered LMWHs were effective as anti-thrombotic drugs at lower doses than UFH

and thus may be absorbed faster than UFH. In the rat jugular vein thrombosis model, ED_{50}s of 7.5, 0.1 and 0.025 mg/kg were seen for UFH, tinzaparin and reviparin, respectively (Hiebert et al., 2000; Hiebert et al., 2001; Hiebert et al., 2002). Thrombosis was also prevented at 0.1 and 7.5 mg/kg for tinzaparin and bovine UFH respectively in a rat carotid artery model (Pinel et al., 2004).

The Isc results also support the concept that LMWHs are absorbed in the lower levels of the gut whereas the stomach could be a better site for UFH absorption (Tabel 6.1). Change in Isc, an indicator of active transport across the mucosal membrane (Cooke and Dawson, 1978), increased noticeably after addition of LMWHs to the mucosal buffer under neutral conditions. Contrary to this, Isc increased when UFH was added to the mucosal buffer at pH 4.0. The Isc results suggest that an active transport mechanism becomes activated and facilitates the transport of LMWHs and UFH across the mucosal membrane under neutral and acidic conditions respectively.

6.6 Conclusions

In conclusion, this study suggests that UFH or LMWHs require different environmental pH conditions for significant absorption. While acidic environment seems to assist in passage of UFH through the gastric mucosa, LMWHs are transported better in a basic environment. The LMWHs may pass through the gastric mucosa faster and have a higher concentration in the lower levels of the gut, since they have less protein binding activity and lower molecular weight compared to UFH. Further studies are needed to better understand the movement of heparins across the gut and the mechanisms involved.

Chapter 7: Transport of Unfractionated Heparin across Rat Ileal Mucosa With or Without Peyer's Patches in a Vertical Diffusion Ussing Chamber

7.1 Abstract

Various studies have shown that oral unfractionated heparin (UFH) is absorbed. Oral UFH binds avidly to endothelium and significantly reduces thrombosis in rats despite low plasma levels. However, the site and mechanism of UFH absorption is unknown. Our previous *in vitro* study suggested that the stomach may be a site for UFH absorption. The objective of this study was to investigate if UFH crosses rat ileal mucosa, and the effect of Peyer's patches. Rat ileal mucosa, with or without Peyer's patches, was mounted in an Ussing chamber. Mucosa was bathed in oxygenated Kreb's buffer containing mannitol (pH 7.4 or 4) on the mucosal side (lumen), and glucose (pH 7.4) on the serosal side (circulation). After 40 min, UFH (0.7-10 mg/ml) was added to the mucosal side. Potential difference (PD), resistance (R) and short circuit current (Isc) across the mucosal tissue were determined. Buffers and tissues were extracted and UFH content was measured by electrophoresis. The PD increased immediately on UFH addition in both tissues. Following a lag period, found to be shorter in the presence of patches, PD gradually began to return to the baseline. PD did not reach baseline in tissue without patches. The lag period decreased in tissue without patches at pH 4.0 compared to pH 7.4. Changes in R followed an opposite pattern. Isc changed most in tissue without Peyer's patches at pH 4.0. Heparin was recovered from serosal buffer. In conclusion, UFH moves across ileal mucosa. Peyer's patches increase rate of transport. Lowering pH increases transport only in tissue without Peyer's patches. Isc changes indicate active transport plays some role. Further studies are required to understand the mechanisms involved.

7.2 Introduction

The glycosaminoglycan, heparin, a widely used antithrombotic drug, is highly acidic, sulphated, and negatively charged. Heparin is used in the treatment of acute deep venous thrombosis and pulmonary embolism. It is also effective when used prophylactically in low doses to prevent the occurrence of venous thromboembolism in moderate risk patients. Heparin has traditionally been given parenterally, usually by intravenous infusion or subcutaneous injection (Canadian Pharmaceutical Association, 2009). It is believed that heparin is not absorbed when taken by mouth because of its large size and degradation by stomach acids (Money and York, 2001; Dal Pozzo et al., 1989). This historical belief has been challenged many times by our laboratory and other researchers (Hiebert et al., 1993; Hiebert et al., 2001; Hiebert et al., 2000; Hiebert et al., 1996; Pinel et al., 2004; Hiebert et al., 2005).

The lumen of the gastrointestinal (GI) tract contains absorption sites for a variety of macromolecules. Many particles and large molecules previously regarded as "nonabsorbable" are in fact absorbed. A number of studies have described the transport of intact macromolecules from the lumen across stomach mucosa (Curtis and Gall, 1992; Curtis et al., 1993) and small intestine (Heyman et al., 1982; Ramage et al., 1988; Kimm et al., 1996; Kimm et al., 1994; Warshaw et al., 1971). Thus, it is no longer tenable to regard the GI tract as an impenetrable "physical" barrier for large macromolecules. Results from our laboratory show that orally administered UFH is avidly bound to endothelium (Hiebert et al., 1993) and significantly reduces thrombosis in jugular vein and carotid arterial rat models despite low plasma heparin levels (Hiebert et al.,1996;

Hiebert et al., 2000; Pinel et al., 2004). Heparin absorption from the GI tract was also reported by Sue et al.,(1976) where increases in whole blood clotting time and plasma antifactor-Xa activity were observed following introduction into the GI tract. The site and mechanism of heparin absorption, however, is unknown. Our previous *in vitro* studies suggested that the stomach may be a site for UFH absorption (chapter 4 and chapter 6). However, it is not known if the small intestine contributes to heparin absorption.

The mucosal tissue of the small intestine is a site of intense immunological activity and is now regarded as an important immune organ. Peyer's patches (lymphoid follicles in the mucosa that extend to the submucosa), are especially differentiated to take up and transport antigenic macromolecules and microorganisms from the lumen across the epithelial barrier (Kernéis et al., 1997). Thus it is possible that UFH could be transported by this tissue. The objective of the present study was to investigate if UFH crosses rat ileal mucosa and to determine if the ileum could be involved in UFH absorption. In addition, we wished to determine if UFH movement across ileal mucosa was dependent on the presence of Peyer's patches by using ileal preparations with or without patches.

7.3 Materials and Methods

7.3.1 Animals

Thirty male Wistar rats weighing 250-300 g were used in this study. Please refer to section 3.3 of this thesis regarding animal use.

7.3.2 Drugs and Chemicals

Bovine lung UFH (156.2 units/mg) was obtained from Scientific Protein Labs, Division of Viboin corporation, Wisconsin, USA. For a list of other materials used in this present study, please refer to section 3.1 of this thesis.

7.3.3 Experimental protocol

Please refer to section 3.5 for a detailed experimental protocol.

7.3.4 Electrophysiological measurements

A comprehensive explanation of how electrical properties were measured across ileal mucosa mounted in an Ussing chamber are described in section 3.6.2.

7.3.5 Mucosal integrity

Please refer to section 3.7 of this thesis.

7.3.6 Heparin extraction from buffers and tissues

For a detailed description of heparin extraction from buffers and tissues please refer to sections 3.8 and 3.9.

7.3.7 Identification and chemical measurement of extracted heparin

Please refer to the section 3.10 of this thesis.

7.3.8 Measurement of anti-factor IIa and anti-factor Xa activities in buffers

Detailed descriptions of anti-factor IIa and anti-factor Xa activities are stated in section 3.11 of this thesis.

7.3.9 Analyses

All data are expressed as mean ± standard error of the mean (SEM). When different heparin concentrations and the effect on injury was studied in rat ileal mucosa without Peyer's patches at pH 7.4, a one-tailed unpaired t-test was used to compare differences in both PD and R lag period, in the time taken to reach baseline after the lag period, distance from baseline at 84 min as well as to measure changes on heparin addition to the mucosal buffer. As well, under these same conditions a one-tailed t-test was used to measure significant differences in Isc across rat ileal mucosa upon heparin addition to the mucosal buffer at pH 7.4. Differences in Isc were calculated by subtracting the average of first 5 values obtained between 40 to 48 min after heparin addition, from the average of last 5 values obtained between 116 to 124 min, during the experimental period. In addition, under these same conditions a one-tailed t-test was used to measure differences in heparin concentrations in mucosal and serosal buffers, and experimental mucosa and rates of movement.

When results were compared between tissue with and without Peyer's patches at the same mucosal buffer pH, a one-tailed unpaired t-test was used to determine changes in PD, R, or Isc and differences in heparin concentration in mucosal and serosal buffer and experimental mucosal tissue and rates of transport. When the effect of pH was studied, a two-tailed unpaired t-test was used to measure significant differences in these same parameters.

An unpaired two-tailed t-test was used to measure differences in trypan blue concentrations between mucosal, experimental and control serosal buffers. Values were considered significant at $P<0.05$.

7.4 Results

7.4.1 Changes in electrical parameters (PD, R, and Isc) across ileal mucosa not containing Peyer's patches (NPP) following UFH addition to mucosal buffer at pH 7.4

When UFH (10 mg/ml) was added to the mucosal buffer after 40 min of tissue stabilization, the PD became more negative by -1.5 ± 0.4 mV immediately on UFH addition (Fig. 7.1A, Table 7.1). Following a lag period of 20.4 ± 5.0 min, PD began to return to the baseline but did not reach it in the time period assigned to the experiment (84 min after UFH addition). When values were extrapolated, changes in PD were estimated to reach the baseline 92.6 ± 8.0 min after UFH addition. Difference in PD from the baseline at 84 min was -0.4 ± 0.3 mV (Table 7.1).

Changes in R followed an opposite pattern as in PD (Fig. 7.1B, Table 7.1). The R increased by 90 ± 30 Ω on UFH addition. The R decreased after a lag period of 31.0 ± 2.6 min. Time for changes in R to reach baseline was greater than the experimental period and was estimated to be 85.5 ± 12.7 min after UFH addition. Difference in R from the baseline at 84 min was 20 ± 20 Ω.

The Isc increased by 2.3 ± 1.3 μA throughout the experimental period (124-40 min, or time from addition of UFH until completion of the experiment) (Table 7.1, Fig. 7.1C). The Isc did not reach baseline during the experiment and was different than baseline by -0.3 ± 0.2 μA at 84 min after UFH addition. No changes were observed in electrical parameters across NPP ileal mucosa on buffer addition instead of UFH (Fig. 7.1a, b, c).

At 84 min after addition, UFH was recovered from buffers and tissues. Recovery was 1630.0 ± 183.4 μg from mucosal buffer, 80.0 ± 22.4 μg from serosal buffer, and 21.5 ± 3.7 μg from mucosal tissue (Table 7.2). Heparin was not found in mucosal control tissue. Based on recovery of UFH from serosal buffer, the calculated rate of UFH movement across the mucosal tissue was 0.6 ± 0.2 μg/1.5 ml/min/cm^2.

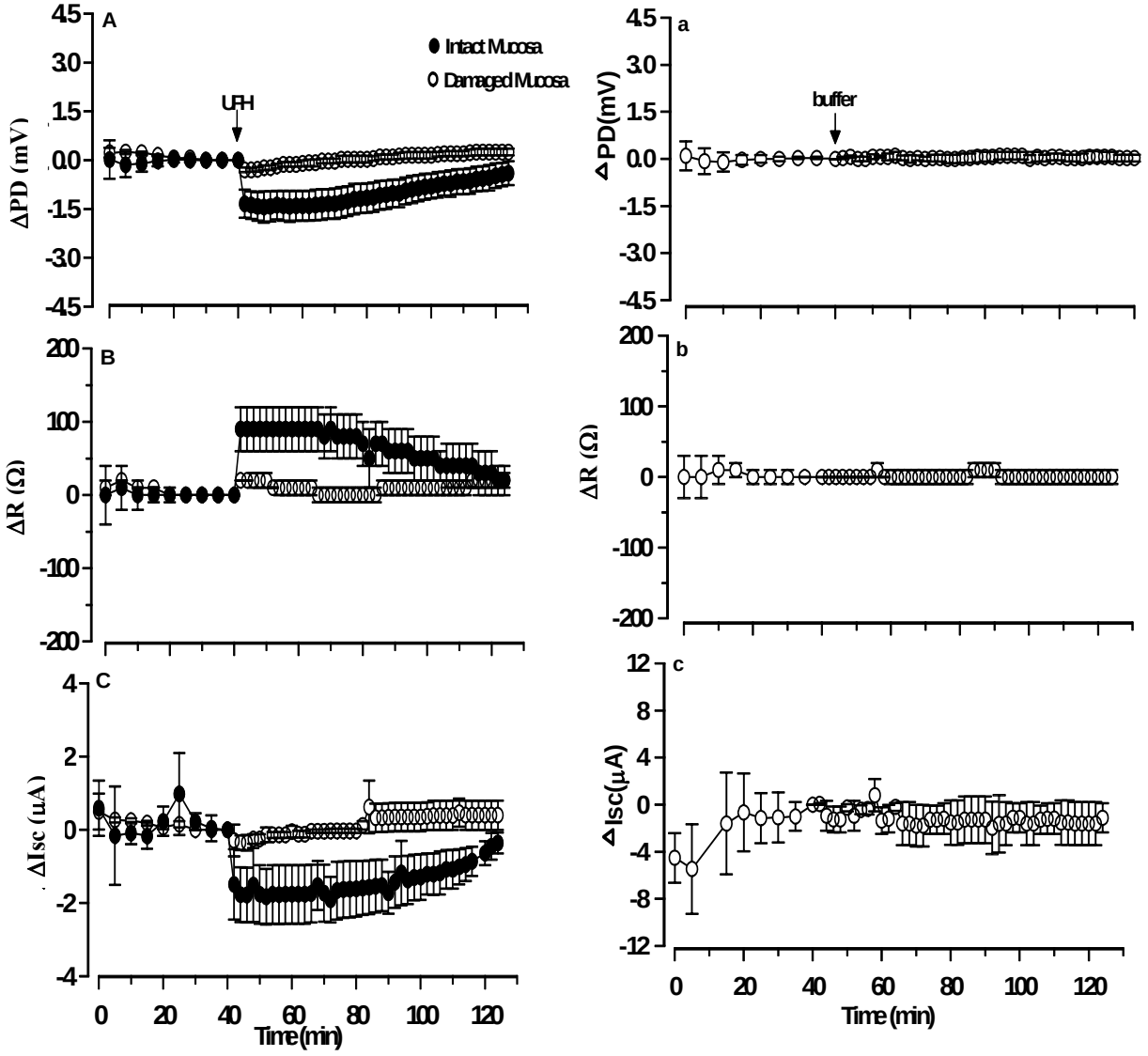


Figure 7.1 Changes in electrical parameters across intact and damaged rat ileal mucosa without Peyer's patches on addition of unfractionated heparin (UFH, 10 mg/ml) or Kreb's buffer to the mucosal buffer. UFH was added 40 min after mounting the tissue in an Ussing chamber and electrical parameters were recorded for the following 84 min. The potential difference (PD) increased immediately and became more negative when the mucosal side was compared to the serosal side on UFH addition to the mucosal buffer. Following a lag period, PD gradually began to return to the baseline. PD did not reach the baseline (A). Changes in resistance (R) and short circuit current (Isc) followed a similar pattern to PD (B, C). Damaging the mucosa resulted in less pronounced changes in PD (A), R (B), and in Isc (C). Results are shown as mean ± SEM. No change was observed in PD (a), R (b), or Isc (c) if buffer alone was added to the mucosal buffer. Results are shown as mean ± SEM.

Table 7.1 Comparison of changes in electrical parameters (PD, R, and Isc) across rat ileal mucosa without Peyer's patches following addition of UFH to the mucosal buffer under different conditions.

Changes in		pH 7.4 (10 mg/ml)	pH 7.4 (10 mg/ml, damaged)	pH 7.4 (0.7 mg/ml)
PD	Negativity (mV)	-1.5 ± 0.4	-0.3 ± 0.1[a]	-0.7 ± 0.1
	Lag Period (min)	20.4 ± 5.0	7.5 ± 1.7[a]	25.0 ± 1.9
	Time to reach baseline after UFH addition (min)	92.6 ± 8.0[*]	30.5 ± 10.9[a]	118.5 ± 17.5[*]
	Difference in PD from baseline at 84 min (mV)	-0.4 ± 0.3	+0.3 ± 0.1	-0.1 ± 0.1
R	Change in baseline after UFH addition (Ω)	90 ± 30	20 ± 10	40 ± 10[a]
	Lag Period (min)	31.0 ± 2.6	9.0 ± 2.4[a]	29.5 ± 2.4
	Time to reach baseline after UFH addition (min)	85.5 ± 12.7[*]	26.5 ± 9.5[a]	94.5 ± 9.5[*]
	Difference in R from baseline at 84 min (Ω)	20 ± 20	20 ± 10	10 ± 0.0
Isc	Change in baseline (μA)**	2.3 ± 1.3	0.7 ± 0.6	0.3 ± 0.1
	Difference in Isc from baseline at 84 min (μA)	-0.3 ± 0.2	+0.2 ± 0.1	0.0 ± 0.0

Mean ± SEM.

* PD, R and Isc did not reach baseline in the time period assigned to the experiment after UFH addition. Values were obtained by extrapolating the curve until it crossed the baseline in each experiment.

** Determined from time of addition of UFH until completion of the experiment.

a-Significantly less than pH 7.4 undamaged, one-tailed t-test.

PD, Potential Difference; R, Resistance; Isc, Short circuit current

Table 7.2 Heparin recovered from buffers and tissues following addition to mucosal buffer at pH 7.4 or 4.0, when rat ileal mucosa with or without Peyer's patches is placed in an Ussing chamber for 124 min.

Treatment	Mucosal Buffer (μg)	Serosal Buffer (μg)	Control Mucosa (μg)	Experimental Mucosa (μg)	Rate μg/1.5ml/min/cm^2
Without patches					
10 mg/ml UFH, pH 7.4 (n=6)	1630.0 ± 183.4	80.0 ± 22.4	0.0 ± 0.0	21.5 ± 3.7	0.6 ± 0.2
10 mg/ml UFH, pH 7.4 damaged mucosa (n=4)	1300.0 ± 104.6	190.0 ± 19.0[b]	0.0 ± 0.0	19.0 ± 2.1	1.4 ± 0.1[b]
0.7 mg/ml UFH, pH 7.4 (n=4)	1340.0 ± 111.0	50.0 ± 15.3	0.0 ± 0.0	16.5 ± 3.4	0.4 ± 0.2
10 mg/ml UFH, pH 4.0 (n=6)	1260.0 ± 99.1	220.0 ± 28.5[b]	0.0 ± 0.0	19.0 ± 2.3	1.6 ± 0.2[b]
With patches					
10 mg/ml UFH, pH 7.4 (n=6)	1140.0 ± 125.5[a]	225.0 ± 41.5[b]	0.0 ± 0.0	13.5 ± 1.1[a]	1.6 ± 0.3[b]
10 mg/ml UFH, pH 4.0 (n=6)	1100.0 ± 79.1	245.0 ± 10.5	0.0 ± 0.0	12.5 ± 1.3[c]	1.8 ± 0.1

Mean ± SEM.
a- Significantly less than pH 7.4 in undamaged ileum without Peyer's patches.
b- Significantly greater than pH 7.4 in undamaged ileum without Peyer's patches.
c- Significantly less than pH 4.0 in undamaged ileum without Peyer's patches.

7.4.2 Anticoagulant activity of heparin

Anti-Xa activity and anti-IIa activity were measured in the desalted buffer extracts at pH 7.4 to determine if the recovered UFH in the serosal buffer retained its anticoagulant activity. Anti-Xa and anti-IIa activity were found in both the mucosal and serosal buffer extracts. Heparin found in the serosal buffer extracts based on anti-Xa activity was 3.7 ± 0.2 μg/ml compared to 72.6 ± 3.7 μg/ml for the mucosal buffer, and based on anti-IIa activity was 2.6 ± 0.1 and 26.6 ± 1.3 μg/ml for the serosal and mucosal buffer respectively. The anti-Xa/IIa ratio was 2.8 ± 0.1 and 1.4 ± 0.1 for the mucosal and serosal buffer respectively.

7.4.3 Proof of mucosal integrity

When trypan blue dye was added to mucosal buffer at pH 7.4, at 84 min after UFH addition and the experiment was run for an additional 15 min, there was little appearance of the dye in serosal buffer (Table 7.3) which was not significantly different than control serosal buffer (P=0.53). When mucosa was intentionally damaged, the PD became negative by -0.3 ± 0.1 mV after UFH addition to mucosal buffer compared to -1.5 ± 0.4 mV in intact tissue (P=0.02) (Table 7.1, Fig. 7.1A). The PD returned to baseline 30.5 ± 10.9 min after UFH addition following a lag period of 7.5 ± 1.7 min which was significantly faster than 92.6 ± 8.0 min for reaching baseline (P=0.001) and a lag period of 20.4 ± 5.0 min (P=0.03) when tissue was intact. Difference in PD from baseline at 84 min was +0.3 ± 0.1 mV for damaged and -0.4 ± 0.3 mV for intact tissue (P=0.07).

Changes in R increased by 20 ± 10 Ω upon UFH addition when tissue was damaged compared to 90 ± 30 Ω in intact tissue (P=0.09) (Table 7.1, Fig. 7.1B). The lag period was significantly shortened to 9.0 ± 2.4 min from 31.0 ± 2.6 min for damaged and intact tissue respectively (P=0.0004). There was also a significant decrease in time for changes in R to reach baseline which was 26.5 ± 9.5 min for damaged compared to 85.5 ± 12.7 min for intact tissue (P=0.005). Difference in R from baseline at 84 min was 20 ± 10 Ω for damaged and 20 ± 20 Ω for intact tissue respectively (P=0.09).

The Isc increased throughout the experimental period by 0.7 ± 0.6 μA for damaged compared to 2.3 ± 1.3 μA for the intact (P=0.2) (Fig. 7.1C, Table 7.1). Difference in Isc from baseline at 84 min was +0.2 ± 0.1 μA for damaged and -0.3 ± 0.2 μA for intact tissue (P=0.09).

Heparin recovery from mucosal buffer was 1300.0 ± 104.6 μg for damaged and 1630.0 ± 183.4 μg for the intact (P=0.06, Table 7.2). Significantly more UFH was recovered in serosal buffer of the damaged tissue, 190.0 ± 19.0 μg, compared to 80.0 ± 22.4 μg for the intact (P=0.002). Heparin recovered from experimental mucosa was similar for damaged versus intact tissue, 19.0 ± 2.1 μg compared to 21.5 ± 3.7 μg (P=0.3). Rate of UFH movement across damaged mucosa was significantly greater, 1.4 ± 0.1 μg/1.5 ml/min/cm^2, compared to 0.6 ± 0.2 μg/1.5 ml/min/cm^2 when tissue was intact (P=0.002).

Table 7.3 Trypan blue recovered from buffers after addition to the mucosal buffer for 15 min when rat ileal mucosa without Peyer's patches is placed in an Ussing chamber for 124 min

Buffers	Trypan blue (μg/ml)
Mucosal buffer	28.4 ± 3.5
***Experimental serosal buffer**	3.2 ± 2.0
****Control serosal buffer**	2.0 ± 0.9

* Serosal buffer obtained when trypan blue is added to the mucosal buffer.
** Serosal buffer obtained when trypan blue is not added to the mucosal buffer.

7.4.4 Effects of decreasing UFH concentration

When a lower concentration of UFH (0.7 versus 10 mg/ml) was added to the mucosal buffer, the PD became more negative immediately by -0.7 ± 0.1 mV which was not significantly different than -1.5 ± 0.4 mV at 10 mg/ml (P=0.08). The lag period of 25.0 ± 1.9 min at 0.7 mg/ml was similar to 20.4 ± 5.0 min at 10 mg/ml of UFH (P=0.2). The estimated PD at 84 min was 118.5 ± 17.5 min at 0.7 mg/ml compared to 92.6 ± 8.0 min at 10 mg/ml UFH (P=0.1). Difference in PD from baseline at 84 min was -0.1 ± 0.1 mV at 0.7 mg/ml, similar to -0.4 ± 0.3 mV at 10 mg/ml UFH (P=0.2).

The R increased by 40 ± 10 Ω upon UFH addition at 0.7 mg/ml compared to 90 ± 30 Ω at 10 mg/ml UFH (P=0.07). The lag period for R was 29.5 ± 2.4 min and 31.0 ± 2.6 min at 0.7 mg/ml and 10 mg/ml of UFH respectively (P=0.3). The estimated time to reach baseline after UFH addition was 94.5 ± 9.5 min at 0.7 mg/ml and 85.5 ± 12.7 min at 10

mg/ml UFH (P=0.3). Difference in baseline at 84 min for R was 10 ± 0.0 Ω at 0.7 mg/ml and 20 ± 20 Ω at 10 mg/ml UFH (P=0.4).

The Isc showed a slight increase of 0.3 ± 0.1 μA throughout the experimental period at 0.7 mg/ml compared to 2.3 ± 1.3 μA at 10 mg/ml (P=0.1) (Table 7.1). The Isc was at baseline at 84 min at 0.7 mg/ml compared to below the baseline by -0.3 ± 0.2 μA at 10 mg/ml (P=0.07).

Heparin recovery from mucosal buffer was 1340.0 ± 111.0 μg at 0.7 mg/ml compared to 1630.0 ± 183.4 μg at 10 mg/ml (P=0.08) (Table 7.2). There was a trend towards less UFH recovery from serosal buffer, 50.0 ± 15.3 μg, at 0.7 mg/ml compared to 80.0 ± 22.4 μg at 10 mg/ml (P=0.1), and for mucosal tissue, 16.5 ± 3.4 μg, at 0.7 mg/ml compared to 21.5 ± 3.7 μg at 10 mg/ml (P=0.2). Rate of heparin movement across mucosa was 0.4 ± 0.2 μg/1.5 ml/min/cm^2 at 0.7 mg/ml compared to 0.6 ± 0.2 μg/1.5 ml/min/cm^2 at 10 mg/ml UFH (P=0.1).

7.4.5 Effect of acidity of the mucosal buffer

Upon UFH addition at pH 4.0, PD increased in negativity by -1.2 ± 0.1 mV that was similar to -1.5 ± 0.4 mV at pH 7.4 (P=0.5) (Table 7.4). There was a trend toward a significant decrease in lag period which was 10.0 ± 1.1 min at pH 4.0 versus 20.4 ± 5.0 min at pH 7.4 (P=0.08). The PD reached baseline 77.2 ± 2.8 min after UFH addition at pH 4.0 compared to the extrapolated value of 92.6 ± 8.0 min at pH 7.4 (P=0.1). The

difference in PD from baseline at 84 min was 0.0 ± 0.0 mV at pH 4.0 compared to -0.4 ± 0.3 mV at pH 7.4 (P=0.2).

The R increased by 60 ± 10 Ω at pH 4.0 which was no different than 90 ± 30 Ω at pH 7.4 (P=0.6). The lag period significantly decreased to 12.8 ± 2.6 min at pH 4.0 from 31.0 ± 2.6 min at pH 7.4 (P=0.0018). The R reached baseline 77.2 ± 2.8 min and 85.5 ± 12.7 min after UFH addition at pH 4.0 and pH 7.4 respectively (P=0.6). At 84 min, R was below baseline by -70 ± 10 Ω at pH 4.0, compared to 20 ± 20 Ω above the baseline at pH 7.4 (P=1). The Isc increased by 5.4 ± 3.1 μA throughout the experimental period at pH 4.0 compared to 2.3 ± 1.3 μA at pH 7.4 (P=0.4) (Table 7.4). Difference in Isc from baseline at 84 min was +3.5 ± 2.2 μA at pH 4.0 compared to -0.3 ± 0.2 μA at pH 7.4 (P=0.16).

Heparin recovery from mucosal buffer decreased to 1260.0 ± 99.1 μg at pH 4.0 from 1630.0 ± 183.4 μg at pH 7.4 (P=0.08) (Table 7.2). Interestingly, significantly more UFH was recovered from serosal buffer at pH 4.0, 220.0 ± 28.5 μg, compared to 80.0 ± 22.4 μg at pH 7.4 (P=0.0025). Similar amounts of heparin were recovered from mucosa with 19.0 ± 2.3 μg at pH 4.0 compared to 21.5 ± 3.7 μg at pH 7.4 (P=0.6). Calculated rate of UFH movement across mucosa was significantly greater, 1.6 ± 0.2 μg/1.5 ml/min/cm^2, at pH 4.0 compared to 0.6 ± 0.2 μg/1.5 ml/min/cm^2 at pH 7.4 (P=0.0025).

Table 7.4 Comparison of changes in electrical parameters (PD, R, and Isc) across rat ileal mucosa with or without Peyer's patches following addition of UFH to the mucosal buffer at pH 7.4 or 4.0.

pH 7.4		**Ileum** (with Patches)	**Ileum** (without Patches)
PD	Negativity (mV)	-0.6 ± 0.4	-1.5 ± 0.4
	Lag Period (min)	11.0 ± 5.1	20.4 ± 5.0
	Time to reach baseline after UFH addition (min)	26.0 ± 12.9[c]	92.6 ± 8.0
	Difference in PD from baseline at 84 min (mV)	+1.9 ± 0.5[c]	-0.4 ± 0.3
R	Change in baseline after UFH addition (Ω)	30 ± 20	90 ± 30
	Lag Period (min)	10.5 ± 5.3[c]	31.0 ± 2.6
	Time to reach baseline after UFH addition (min)	28.0 ± 14.7[c]	85.5 ± 12.7
	Difference in R from baseline at 84 min (Ω)	-100 ± 40[c]	20 ± 20
Isc	Change in baseline∗ (μA)	1.1 ± 0.5	2.3 ± 1.3
	Difference in Isc from baseline at 84 min (μA)	+0.9 ± 0.5[c]	-0.3 ± 0.2
pH 4.0		**Ileum** (with Patches)	**Ileum** (without Patches)
PD	Negativity (mV)	-0.02 ± 0.02[c]	-1.2 ± 0.1
	Lag Period (min)	8.4 ± 3.1	10.0 ± 1.1
	Time to reach baseline after UFH addition (min)	8.4 ± 3.1[c]	77.2 ± 2.8
	Difference in PD from baseline at 84 min (mV)	+0.6± 0.1[bc]	0.0 ± 0.0
R	Change in baseline after UFH addition (Ω)	30 ± 10[c]	60 ± 10
	Lag Period (min)	12.0 ± 4.4	12.8 ± 2.6[a]
	Time to reach baseline after UFH addition (min)	14.0 ± 5.5[c]	77.2 ± 2.8
	Difference in R from baseline at 84 min (Ω)	-90 ± 10	-70 ± 10
Isc	Change in baseline∗ (μA)	2.3 ± 1.0	5.4 ± 2.7
	Difference in Isc from baseline at 84 min (μA)	+2.2 ± 0.1	+3.5 ± 2.2

Mean ± SEM.
∗ Determined from time of addtion of UFH until completion of the experiment.
a- Significantly different than mucosal buffer at pH 7.4 in ileum without Peyer's patches.
b- Significantly different than mucosal buffer at pH 7.4 in ileum with Peyer's patches.
c- Significantly different than ileum without Peyer's patches.
PD, Potential Difference; R, Resistance; Isc, Short circuit current.

7.4.6 Comparison of electrical parameters across ileal mucosa containing Peyer's patches (PP) when mucosal pH is 7.4 or 4.0

When UFH was added to mucosal buffer at pH 4.0, there was a small increase in PD negativity by -0.02 ± 0.02 mV compared to -0.6 ± 0.4 mV at pH 7.4 (P=0.12) (Table 7.4, Fig 7.2A, Fig 7.2B). The lag period was 8.4 ± 3.1 min at pH 4.0 compared to 11.0 ± 5.1 min at pH 7.4 (P=0.6). The PD was at the baseline by 8.4 ± 3.1 min after UFH addition at pH 4.0 compared to 26.0 ± 12.9 min at pH 7.4 (P=0.18). The PD was significantly different than baseline by +0.6 ± 0.1 mV at pH 4.0 compared to +1.9 ± 0.5 mV at pH 7.4 (P=0.04).

There was an increase in R by 30 ± 10 Ω upon UFH addition at pH 4.0 which was no different than 30 ± 20 Ω at pH 7.4. The lag period of 12.0 ± 4.4 min at pH 4.0 was similar to 10.5 ± 5.3 min at pH 7.4 (P=0.8). Time to reach baseline was 14.0 ± 5.5 min at pH 4.0 and 28.0 ± 14.7 min at pH 7.4 (P=0.4). The R moved below baseline and had a similar value of -90 ± 10 Ω at pH 4.0 and of -100 ± 40 Ω at 7.4 (P=1.0). The Isc increased by 2.3 ± 1.1 μA throughout the experimental period at pH 4.0 compared to 1.1 ± 0.5 μA at pH 7.4 (P=0.4). The Isc was greater than baseline at 84 min at pH 4.0 by +2.2 ± 0.1 μA, compared to +0.9 ± 0.5 μA at pH 7.4 which was near significance (P=0.06).

Heparin recovery from mucosal and serosal buffer was similar at pH 4.0 compared to pH 7.4, and was 1100.0 ± 79.1 μg and 1140.0 ± 125.5 μg respectively for mucosal buffer (P=0.8) and 245.0 ± 10.5 μg and 225.0 ± 41.5 μg respectively for serosal buffer (P=0.6)

(Table 7.2). Heparin recovery from PP mucosal tissue was also similar for pH 4.0 and 7.4, at 12.5 ± 1.3 μg and at 13.5 ± 1.1 μg respectively (P=0.6). The calculated rate of heparin movement across the mucosa was also similar, 1.8 ± 0.1 μg/1.5 ml/min/cm^2 at pH 4.0 and 1.6 ± 0.3 μg/1.5 ml/min/cm^2 at pH 7.4 (P=0.6).

7.4.7 Comparison of electrical parameters across rat ileal mucosa with (PP) or without Peyer's patches (NPP)

At pH 7.4

When 10 mg/ml UFH was added to the mucosal side of the chamber, there was a trend towards a decreased negative change in PD, -0.6 ± 0.4 mV for PP mucosa compared to -1.5 ± 0.4 mV for NPP mucosa (P=0.08) (Table 7.4, Fig. 7.2). There was a trend towards a decrease in the lag period which was 11.0 ± 5.1 min in the presence of patches compared to 20.4 ± 5.0 min for NPP mucosa (P=0.1). The PD reached baseline 26.0 ± 12.9 min after UFH addition for PP mucosa which was significantly shorter than the estimated value of 92.6 ± 8.0 min for NPP tissue (P=0.001). The PD continued above baseline by +1.9 ± 0.5 mV at 84 min for PP mucosa which was significantly greater than the difference from baseline of -0.4 ± 0.3 mV at 84 min for NPP mucosa (P=0.003).

Changes in R were opposite to PD. Increase in R was 30 ± 20 Ω for PP and 90 ± 30 Ω for NPP mucosa respectively (P=0.2). The lag period was significantly shortened to 10.5 ± 5.3 min for PP compared to 31.0 ± 2.6 min for NPP mucosa (P=0.007).

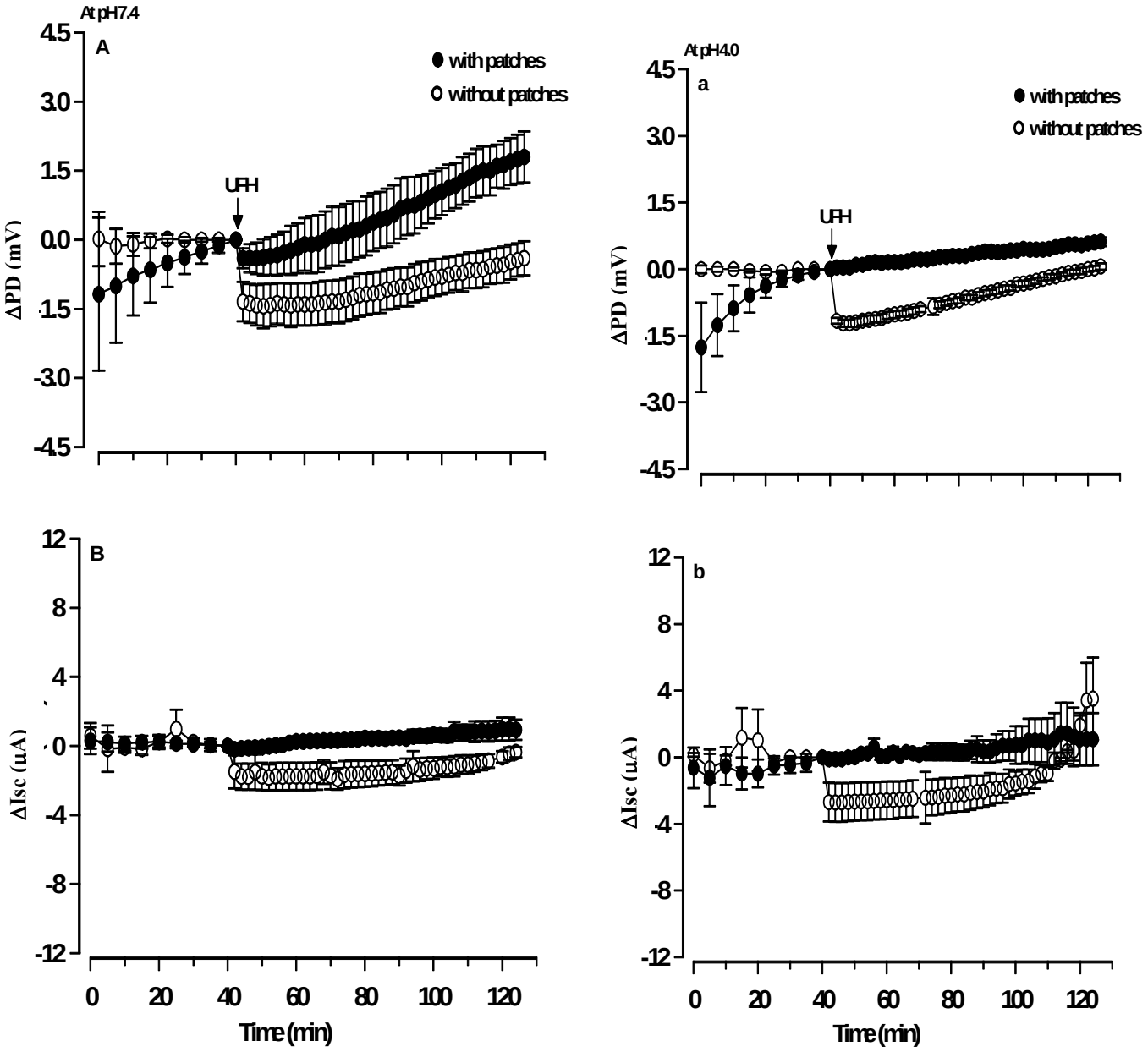


Figure 7.2 Effects of changing the pH of the mucosal buffer on the electrical parameters across rat ileal mucosa with or without Peyer's patches on addition of unfractionated heparin (UFH, 10 mg/ml) to the mucosal buffer. UFH was added to the mucosal buffer at pH 7.4 or pH 4.0. Changes in potential difference (PD) and short circuit current (Isc) across the tissue without Peyer's patches are compared to those when the tissue contains Peyer's patches. At pH 7.4, PD returned to the baseline at a faster rate and the lag period is shorter in tissues with Peyer's patches compared to tissues without Peyer's patches (A and a). Isc changed little after heparin addition in both tissues (B). Results are shown as mean ± SEM.

The R reached baseline 28.0 ± 14.7 min after UFH addition for PP which was significantly shorter than 85.5 ± 12.7 min for NPP tissue (P=0.01) at 84 min. The R continued below baseline by and was -100 ± 40 Ω for PP compared to the difference from baseline of 20 ± 20 Ω for NPP tissue (P=0.01). The Isc increased by 1.1 ± 0.5 μA throughout the experimental period in the presence of patches compared to 2.3 ± 1.3 μA in NPP tissue (P=0.2) (Table 7.4). The Isc was greater than the baseline by +0.9 ± 0.5 μA at 84 min for PP tissue which differed significantly from -0.3 ± 0.2 μA below baseline for NPP tissue (P=0.03, Fig. 7.2).

Heparin recovery from mucosal buffer in the presence of patches was significantly less than when patches were absent, 1140.0 ± 125.5 μg compared to 1630.0 ± 183.4 μg respectively (P=0.02), while more UFH was recovered from serosal buffer at 225.0 ± 41.5 μg for PP tissue compared to 80.0 ± 22.4 μg for NPP tissue (P=0.004). Moreover, significantly less UFH, 13.5 ± 1.1 μg, was recovered from PP mucosa compared to 21.5 ± 3.7 μg from NPP mucosa (P=0.02). Rate of UFH movement across PP mucosa of 1.6 ± 0.3 μg/1.5 ml/min/cm^2 was significantly greater than 0.6 ± 0.2 μg/1.5 ml/min/cm^2 for NPP tissue (P=0.004).

<u>At pH 4.0</u>

When patches were present in ileal mucosa, change in PD negativity was significantly decreased to -0.02 ± 0.02 mV from -1.2 ± 0.1 mV in the absence of patches on addition of 10 mg/ml UFH to mucosal buffer at pH 4.0 (P<0.0001) (Table 7.4, Fig. 7.2). The lag period was similar for PP and NPP mucosa, 8.4 ± 3.1 min and 10.0 ± 1.1 min respectively

(P=0.3). The PD reached baseline immeditately after UFH addition when patches were present, 8.4 ± 3.1 min, compared to 77.2 ± 2.8 min in the absence of patches (P<0.0001). At 84 min, PD was above baseline by +0.6 ± 0.1 mV for PP but at baseline for NPP tissue (P=0.0003).

Increase in R from baseline was significantly less, 30 ± 10 Ω for PP mucosa compared to 60 ± 10 Ω for NPP tissue (P<0.0001). There were comparable lag periods of 12.0 ± 4.4 min and 12.8 ± 2.6 min for PP and NPP mucosa respectively (P=0.4). The R reached baseline in a significantly shorter time period, 14.0 ± 5.5 min, after UFH addition in the presence of patches compared to 77.2 ± 2.8 min in NPP tissue (P<0.0001). At 84 min, R was below the baseline by 90 ± 10 Ω for PP and by 70 ± 10 Ω for NPP mucosa (P=0.1). The Isc increased by 2.3 ± 1.0 μA throughout the experimental period in the presence of patches compared to an increase of 5.4 ± 2.7 μA in NPP tissue (P=0.2) (Table 7.4). At 84 min, Isc was greater than baseline by +2.2 ± 0.1 μA for PP which was similar to +3.5 ± 2.2 μA for NPP mucosa (P=0.3).

Heparin recovery from mucosal buffer was similar for PP and NPP mucosa, 1100.0 ± 79.1 μg and 1260.0 ± 99.1 μg respectively (P=0.1). Similar amounts of heparin were also recovered in serosal buffer for PP and NPP mucosa, 245.0 ± 10.5 μg and 220.0 ± 28.5 μg respectively (P=0.2). Significantly less UFH, 12.5 ± 1.3 μg, was recovered from PP tissue compared to 19.0 ± 2.3 μg from NPP tissue (P=0.01). Rate of heparin movement across

mucosa was similar for PP and NPP tissue, 1.8 ± 0.1 μg/1.5 ml/min/cm^2 and 1.6 ± 0.2 μg/1.5 ml/min/cm^2 respectively (P=0.2).

7.5 Discussion

Unfractionated heparin (UFH), a highly acidic and negatively charged GAG, is widely used intravenously as an antithrombotic drug (Kim et al., 2006; Money and York 2001). We and others have challenged the belief that UFH is ineffective when given orally. UFH entered the circulation after oral administration and was recovered from aortic and vena caval endothelium with less than 1% found in plasma (Jaques et al., 1991; Hiebert et al., 1993). Oral UFH significantly reduced thrombotic incidence in both rat jugular vein and carotid arterial models in a dose-dependent manner although plasma heparin concentrations were low (Hiebert et al., 2001; Hiebert et al., 2000; Hiebert et al., 1996; Pinel et al., 2004). An increase in plasma anti-Xa activity and heparin in urine was observed in human subjects following oral administration (Hiebert et al., 2005). A single dose of 20,000 units or 20,000 units of UFH given twice weekly to humans increased the anticoagulant activity significantly (Engelberg 1995). The site and mechanism of heparin absorption is unknown.

Our previous *in vitro* study suggested that the stomach could be a site for UFH absorption (Chapter 4). It is not known if the small intestine is also involved in heparin absorption. Costantini et al., (2000) administered a radioiodinate derivative of UFH intraduodenally to rats and measured increased radioactivity immediately in plasma and on the surface of aortic and vena caval segements. The same authors also showed in a stasis-induced

venous thrombosis model, that UFH administered intraduodenally to rats prevents thrombus formation in a dose-dependent manner.

The lumen of the intestine contains a variety of macromolecules. Minute absorption of intraluminal proteins by the intestine of adult animals is believed to play a key role in inducing sensitization to food proteins ingested orally (Andre et al., 1975; Carini, 1987; Challacombe and Tomasi, 1980; Dannaeus et al., 1979; Fallstrom et al., 1984; Richman et al., 1978). In fact, a number of previous studies have described the existence of pathways for the uptake of intact macromolecular proteins from the lumen across the mucosa of the small intestine (Heyman et al., 1982; Ramage et al., 1988; Kimm et al., 1996; Kimm et al., 1994; Warshaw et al., 1971). In the jejunum, mucosal to serosal macromolecular transport is a saturable process that is both microtubule and energy dependent (Heyman et al., 1982; Kimm et al., 1994) and is regulated by the enteric nervous system acting via muscarinic pathways (Kimm et al., 1994). Nitric oxide has also been shown to play a role in the regulation of jejunal macromolecular uptake (Kimm et al., 1996). Likewise, in the ileum, mucosal to serosal macromolecular uptake has been shown to be both microtubule and energy dependent (Marcon-Genty et al., 1989) and under neural regulation acting through muscarinic receptors (Bijlsma et al., 1996).

Furthermore, experiments using the adenylate cyclase activator forskolin suggest that cAMP plays a role in the regulation of ileal macromolecular uptake (Bijlsma et al., 1996). Studies have demonstrated the *in vivo* uptake of intact human growth hormone across rat colon (Moore et al., 1986) and the bioactive arginine vasopressin has been detected in

rabbit plasma following injection into ligated colonic loops (Lundin and Vilhardt, 1986). The bacterial chemotactic peptide *N-formyl-methionyl-leucyl-phenylalanine* (fMLP) has also been shown to be absorbed intact across rat colon (Ferry et al., 1989). In addition, transport of intact macromolecules across the proximal and distal large intestine was demonstrated to be a saturable process. Furthermore, intact bovine serum albumin (BSA) transport in the proximal colon is an energy dependent process that utilizes microtubules and is regulated by the enteric nervous system (Hardin et al., 1999).

This present study confirms that the small intestine is capable of sampling luminal macromolecules and further extends this sampling process to large carbohydrates like UFH. Segments of the ileal mucosa with Peyer's patches (PP) or without Peyer's patches (NPP), were mounted in a vertical diffiusion Ussing chamber and electrical properties across the membrane were measured on addition of 10 mg/ml UFH to the mucosal buffer. Changes in PD across the NPP ileal mucosa upon UFH addition at pH 7.4, when mucosal side was compared to the serosal, suggest UFH movement across the NPP membrane and could be explained in terms of the structure and molecular weight of heparin. As UFH is a very negatively charged polyelectrolyte, the PD across the ileal membrane which is created by the difference in charge between the mucosal and serosal sides, becomes very negative upon UFH addition. A lag period is observed which may be due in part to the large molecular weight and high degree of ionization of UFH at pH 7.4. Upon addition to the mucosal buffer, UFH likely binds to the apical ileal epithelial surface and is internalized by intestinal epithelial cells before it is released from the basolateral membrane. Enterocyte's have a specialized apical surface, the brush border, which

continually forms endocytotic vesicles at the microvillus base and in doing so, proteins are taken up in both membrane-bound and soluble fractions (Hemmings 1981). An adsorptive endocytosis after binding of molecules to the cell membrane has been considered to be relevant for the uptake and transfer of possible immunogenic food proteins (Wall and Maack, 1985).

Existence of a lag period is also supported by observations that heparin binding proteins are present on the surfaces of many cell types, including epithelial cells. Previous work has shown that heparin-like molecules are taken up directly by cells and carried to the peri-nuclear (e.g. smooth muscle cells; Castellot et al., 1985a) or intra-nuclear location (e.g. hepatocytes; Ishihara et al., 1986). Flint et al., (1994) has shown uptake of labelled [^{3}H] heparin into the cytoplasm of both epithelial and mesenchymal intestinal cell cultures, followed by localisation around the nucleus.

As UFH is transported across the mucosa, the mucosal side becomes increasingly less negative compared to the serosal side and the PD moves towards the baseline. Furthermore with time, positively charged molecules on the serosal side may be attracted by highly negatively charged UFH on the mucosal side neutralizing charge difference between the two sides of the membrane. Interestingly, there is evidence in the literature of jejunal serosal to mucosal macromolecular protein transport which was, as for mucosal to serosal uptake, both saturable, and energy and microtubule dependent (Kimm et al., 1997). Movement of UFH across the NPP ileal mucosa is supported by recovery of chemical heparin from the serosal buffer. Heparin was trapped in the experimental

mucosal tissue at 84 min further indicating interaction of UFH with components of the ileal mucosal membrane.

Movement of heparin across the ileal membrane may be dose-dependent since changes in PD on 0.7 mg/ml addition of UFH were observed to a lesser degree compared to 10 mg/ml UFH and time to reach baseline increased although differences did not reach significance. Accordingly a trend toward less UFH recovery from the serosal buffer and mucosal tissue supports this finding.

The PD changes did not occur when UFH was absent from the mucosal chamber. When permeability of the mucosa was assessed with a low-molecular weight probe (Trypan Blue) at 124 min, an equal amount of TB crossed both experimental and control serosal buffers, indicating that mucosal permeability was constant further confirming no mucosal disruption and tissue viability. Moreover, when mucosa was damaged by a needle, the pattern of change in PD was different than when it was intact. Negativity increased and lag period decreased significantly on UFH addition, while PD reached to and continued above baseline in damaged tissue. These results are expected as the tear created in the tissue eases the movement of UFH across the membrane. Recovery of larger amounts of heparin in serosal buffer of damaged compared to intact mucosa and less UFH trapped inside the damaged compared to intact mucosa support the above findings. Heparin movement across the NPP ileal mucosa is supported by previous work showing that protein macromolecules like horseradish peroxidase (mol wt, 40,000) traverse piglet

jejunum intestinal tissue not containing Peyer's patches (Keljo and Hamilton, 1983; Cornell et al., 1971).

Our results suggest that an acidic mucosal environment eases the passage of heparin through NPP ileal mucosa. The lag period for PD on UFH addition decreased significantly to half that seen in neutral conditions and PD reached baseline at 84 min. Similar results were obtained in rat gastric mucosa (Chapter 4). Decreasing the pH of the mucosal buffer to 4.0 from 7.4, decreased the lag period for PD. Since UFH is a very acidic molecule, our findings agree with the pH-partition theory stating that a drug will cross the intestinal epithelium in its non-ionized form. The pH-dependent changes in PD and therefore in drug permeation observed in our study were likely due to changes in the degree of UFH ionization in different environments and not due to pH-dependent changes in the properties or integrity of the membrane. Ready penetration of undissociated drug molecules but impediment of the transport of the ionized molecule across the ileal epithelium has been shown by others. Schanker et al., (1958), perfused rat small intestine with acidic and basic drug solutions, and revealed a relationship between the degree of ionization and the rate of drug absorption. Weaker acids and bases were readily absorbed; stronger, highly ionized acids and bases were more slowly absorbed; and completely ionized acids and bases were hardly absorbed. Hogben et al., (1959) showed in rats that raising intestinal pH increased and decreased absorption of bases and acids respectively. Loomis (1959) placed heparin in dog duodenal loops and showed its absorption when administered in a citrate-phosphate solution of pH 4.0 but not at pH 8.0 or from physiological saline solution. Results of the present study are in agreement with that of

Loomis as transport of UFH across the ileal mucosa decreased when mucosal pH rose to 7.4 from 4.0. Heparin transport from a solution of low pH might be attributed to decreased ionization of carboxyl groups in UFH. Since the PKa of heparin carboxyl groups range from 2-4 (Casu and Gennaro, 1975), the degree of ionization and therefore the net charge of the molecule likely will be reduced by the low pH environment.

This study shows that Peyer's patches, aggregations of lymphoid tissue usually found in the ileum, facilitate heparin movement across ileal mucosa at pH 7.4. When Peyer's patches were present, the PD negativity and lag period on UFH addition decreased to almost half of that seen in NPP tissue at pH 7.4 not at pH 4.0. Moreover, PD reached baseline and became more positive than baseline at 84 min. These results are supported by significantly greater and lesser amounts of chemical heparin recovered from serosal and mucosal buffer respectively in PP compared to NPP tissue at pH 7.4 but not at pH 4.0, and a greater calculated rate of transport across mucosa in PP tissue at pH 7.4 but not pH 4.0. The role of Peyer's patches in macromolecular transport across the intestinal mucosa was also shown by other investigators. Keljo and Hamilton (1983), using an Ussing chamber, showed that the mean horseradish peroxidase (mol wt, 40,000) transport rate across piglet jejunum segments with a patch was increased threefold compared with control (non-patch) tissue. Soni et al., 2006 mounted freshly excised rat, ovine and bovine ileal PP and NPP tissues in a modified horizontal polyethylene diffusion chamber and showed that the uptake of horseradish peroxidase and *S. Typhimurium* across the membranes was a rapid process occurring more readily in tissue with PP than without in all species.

7.6 Conclusions

UFH moves across the ileal mucosa and this transport is concentration dependent. Peyer's patches help UFH movement across the ileal mucosa at pH 7.4. Lowering pH increases the rate of UFH transport only in tissue without patches. Changes in R suggest that passive diffusion plays a role in UFH transport across the mucosa. However, Isc changes indicate that active transport is involved especially in mucosa without patches at pH 4.0. We conclude that UFH is transported across ileal mucosa under physiological conditions (pH 7.4), the presence of Peyer's patches increases the rate of UFH transport across the ileal mucosa that supports the concept that Peyer's patches serve a macromolecular antigen sampling function in the gut.

Chapter 8: Transport of Unfractionated Heparin across Rat Gastric Mucosa: Does it Depend on Active Transport?

8.1 Abstract

Our previous studies indicate that heparins are absorbed and effective when given orally. Results suggest the stomach may be an absorption site for unfractionated heparin (UFH) however the mechanism(s) involved is not known. Our objectives were to assess the mechanisms of UFH transport across rat gastric mucosa. Intact mucosa was mounted in an Ussing chamber with Kreb's buffer containing mannitol (pH 7.4 or 4) on the mucosal side, and glucose (pH 7.4) on the serosal side. After 40 min of equilibration, UFH (10 mg/ml) was added to mucosal and/or serosal buffers with and without addition of inhibitors, sodium fluoride (0.1 mg/ml), colchicine (1 μM), or amiloride (1 mg/ml). Potential difference (PD), mucosal vs serosal side, and short circuit current (Isc) were measured for an additional 84 min. Change in Isc indicates active transport. After an initial increase in negativity, immediately on UFH addition to only the mucosal buffer, the Isc increased with time at pH 4.0 but not 7.4 with changes in the 84 min period of 4.2 ± 1.5 μA and 0.0 ± 0.0 μA respectively. When UFH was added to both buffers, Isc increased by 15.5 ± 5.3 μA at pH 4 and by -0.3 ± -0.3 μA at pH 7.4. There was no immediate change in PD after UFH addition to both buffers however, PD increased progressively with time at pH 4 but not pH 7.4 with respective changes of 3.6 ± 0.5 mV and -0.1 ± 0.5 mV throughout the 84 min period. In the presence of sodium fluoride, colchicine, or amiloride, the PD became more negative on UFH addition at both pH 7.4 and 4. The PD returned to baseline with time at pH 7.4; 64.6 ± 4.4, 30.7 ± 13.1 and 52.8 ± 2.3 min for sodium fluoride, colchicine, and amiloride respectively; but not pH 4 (all > 84 min). Results suggest that UFH transport across gastric mucosa is an active process

involving metabolic energy expenditure, cytoplasmic tubule formation, and sodium-coupled systems. Passive diffusion may play a major role in UFH transport at pH 7.4 more likely seen in intestine than stomach.

8.2 Introduction

Unfractionated heparin (UFH) is a naturally occuring, very highly polyanionic glycosaminoglycan. The polyanionic nature of heparin is due to sulphate and carboxyl groups which are attached to the core carbohydrate structure. UFH has a variety of uses including the treatment of thrombosis. Heparin is considered not effective orally (Kim et al., 2006), since it is believed to be degraded by stomach acids (Dal Pozzo et al., 1989); there is little or no change in anticoagulant activity following oral administration (Leone-Bay et al., 1998); and it has high negative charge and molecular weight (average 10 000 to 15 000 daltons) (Money and York, 2001).

However, considerable research by our group and others leave little doubt that UFH is absorbed and effective. These findings are 1) although less than 1% of administered heparin was found in plasma, heparin was recovered from endothelial cells lining blood vessels, after giving heparin to rats by stomach tube (Jaques et al., 1991; Hiebert et al., 1993); 2) tissue distribution studies in rats at 24 hours after administration of ^{14}C and cold heparin showed the presence of chemical heparin and radioactivity in all tissues including liver, kidney, heart, lung, bone marrow, muscle, etc., consistent with the idea that the drug was distributed to endothelium (Hiebert et al., 2000); 3) in a rat jugular vein model, a dose-dependent decrease in thrombosis incidence was observed with oral UFH (Jaques

et al., 1991; Hiebert et al., 1996). Thrombosis was also prevented by oral heparin in a rat carotid arterial model (Pinel et al., 2004)**;** 4) a small but significant increase in anticoagulant (anti-Xa) activity and the presence of heparin in urine up to the 5th day, was observed after drinking 1000 units/kg of UFH in humans (Hiebert et al., 2005); 5) most recently, we showed, using a vertical diffusion Ussing chamber, that UFH (Chapter 4) crosses rat gastric mucosa and that movement is dependent on the pH of the environment such that UFH may be better absorbed from the stomach at pH 4.0 than pH 7.4.

Having established that UFH does cross rat gastric mucosa, it is pertinent to consider what mechanism(s) are involved in this process. To date most information on macromolecular gastric absorption involves mechanisms by which proteins are transported across the gastrointestinal tract. Gastric mucosa may represent an important primary site for sampling the luminal contents by the gastrointestinal immune system (Curtis and Gall, 1992). Both clinical and animal studies (Reimann and Lewin, 1988; Catto-Smith, 1989) have demonstrated that antigen applied to the gastric mucosal surface can initiate a type I hypersensitivity reaction in susceptible subjects.

Studies in the stomach (Curtis and Gall, 1992; Curtis et al., 1993) and different regions of the small bowel (Heyman et al., 1982; Kimm et al., 1996; Marcon-Genty et al., 1989) showed that intact antigen uptake is an energy-dependent process that uses the cellular microtubular network. The sampling mechanism in the stomach, when compared to the intestine, transports a larger percentage of the total protein intact, but degrades a smaller percentage. This is physiologically reasonable and convincing since the primary purpose

of gastric sampling is not to absorb nutrients, as in the small bowel, but to provide the immune system with traces of antigenically intact protein.

In our present *in vitro* study, using a vertical diffusion Ussing chamber, we studied how UFH crosses rat gastric mucosa under different environmental pH conditions (pH 4.0 and 7.4). Our results show that both passive transport and/or active transport are likely involved. However, most evidence favors the latter route dominant at pH 4.0, the average pH in rat stomach (Eastman and Miller, 1935), although the process is a complex one likely involving metabolic energy expenditure, cytoplasmic tubule formation, and sodium-coupled systems.

8.3 Methods

8.3.1 Animals

Fourty-eight animals were obtained from Charles River Canada Company, St. Constant, Quebec, Canada, and were handled as described in section 3.3 of this thesis.

8.3.2 Chemicals

Bovine lung unfractionated heparin (UFH, 156.2 units/mg) was obtained from Scientific Protein Labs, Division of Viobin Corporation, Wisconsin, USA. Sodium Fluoride (NaF), colchicine and amilioride were purchased from Sigma-Aldrich, ON, Canada. For a list of other materials used in this transport study please refer to section 3.1.

8.3.3 Gastric mucosa isolation from rats

Rat gastric mucosa was isolated as described in section 3.4 of this thesis.

8.3.4 Ussing chamber technique for measurement of heparin movement across rat gastric mucosa

We used an EVC 4000 voltage/current clamp (NaviCyte, Harvard Apparatus, Inc.) for our transport study across the gastric mucosa from rats as is explained in detail in section 3.6.

8.3.5 Experimental procedures

Electrical parameters [potential difference (PD), resistance (R), and short circuit current (Isc)] across rat gastric mucosa were recorded following addition of UFH (10 mg/ml) to mucosal buffer at pH 4.0 and compared to when UFH was added to both mucosal and serosal buffers. In addition, UFH was added to the mucosal buffer, and pharmacological inhibitors; sodium fluoride (NaF, 0.1 mg/ml), colchicine (1μM), or amilioride (1mg/ml) were added to both mucosal and serosal buffer when mucosal buffer was either at pH 7.4 or pH 4.0, and serosal buffer was always at pH 7.4.

8.3.6 Data analysis and statistical procedures

Recordings of electrical parameters when pharmacological inhibitors were present in both mucosal and serosal buffer and UFH was present in the mucosal buffer were compared to when UFH was only present in mucosal buffer. As well, electrical parameters of gastric mucosa following different drug formulations at pH 7.4 were compared to the same respective set of data at pH 4.0.

A two-tailed unpaired t-test was used to measure differences in PD increase upon drug additions at different pHs to the mucosal and/or serosal side of the Ussing chamber as well as change from baseline at the end of the experiment (124 min). A one-tailed unpaired t-test was used to determine significant differences in the lag period before PD began to return to the resting level, as well as the time taken for PD to reach baseline after the lag period after different drug additions. Differences in Isc were calculated by subtracting the first 5 values obtained every 2 min after drug addition from the last 5 values recorded upto 124 min. A two-tailed unpaired t-test was used to compare differences in Isc from baseline during 84 min of the experiment. All data are expressed as mean ± standard error of the mean (SEM). All values were considered significant at $P<0.05$.

8.4 Results

8.4.1 Electrical parameters of rat gastric mucosa at pH 7.4

8.4.1.1 Following addition of UFH to mucosal buffer

Rat gastric mucosa was stabilized in the Ussing chamber for 40 min before drug addition and the electrical properties of the membrane were recorded. When UFH (10 mg/ml) was added to the mucosal buffer, the PD became more negative by -1.6 ± 0.3 mV (Fig.8.1A and Table 8.1) when the mucosal side was compared to the serosal. After a lag period of 18.8 ± 4.9 min, the PD became more positive reaching the resting level 39.3 ± 17.6 min later, after which the PD became positive compared to the resting level at 124 min by 0.5 ± 0.8 mV. The R of the mucosa followed a pattern opposite to that of PD (Fig. 8.1B). The

R increased by 120 ± 30 Ω upon UFH addition to mucosal buffer, then decreased following a lag period of 14.6 ± 4.3 min and reached resting level 23 ± 4.2 min later. The R was 40 ± 50 Ω below baseline at 124 min. Isc did not increase throughout the experimental period after heparin addition and was less than the baseline by -0.3 ± 1.6 μA at 124 min (Fig. 8.1C and Table 8.1).

8.4.1.2 Following addition of UFH to mucosal and serosal buffer

To investigate the role of passive and/or active transport in heparin movement across rat gastric mucosa under neutral conditions, UFH (10 mg/ml) was added to both the mucosal and serosal buffers. Electrical parameters were compared to those when the same concentration of UFH was added only to the mucosal side (Fig. 8.1 and Table 8.1). When UFH was added to both the mucosal and serosal buffers, there was no increase in PD negativity as was seen upon UFH addition only to the mucosal buffer, which was -1.6 ± 0.3 mV (P=0.007, two-tailed t-test) (Fig. 8.1A, and Table 8.1). The PD, however, became slightly more positive (0.4 ± 0.3 mV) on UFH addition to both buffers when compared to that prior to UFH addition. A lag period was not observed and PD was at baseline by 7.0 ± 1.0 min after UFH addition where it remained for the rest of the experimental period, and was only different from baseline by -0.1 ± 0.5 mV at 124 min. These changes were significantly different for lag period of 18.8 ± 4.9 min (P=0.02, one-tailed t-test), but not for time to reach baseline of 39.3 ± 17.6 min (P=0.09, one-tailed t-test), and change from baseline by 0.5 ± 0.8 mV at 124 min (P=0.06, two-tailed t-test) when UFH was added only to the mucosal side.

The R of the mucosa followed a pattern opposite to that seen for the PD upon UFH addition to both sides of the membrane (Fig. 8.1B). There was a decrease in R of 30 ± 20 Ω, a lag period was not observed, and R returned to its resting level 8.0 ± 0.0 min after UFH addition where it remained for the rest of the experimental period. These changes were significantly less than a lag period of 14.6 ± 4.3 min (P=0.05, one-tailed t-test), but not for time to reach resting level of 23 ± 4.2 min (P=0.3, one-tailed t-test) after which R continued to decrease and was 40 ± 50 Ω below baseline at the end of the experiment when UFH was added only to the mucosal side (P=0.5, two-tailed t-test). The Isc did not increase during the 84 min of the experimental period and was less than the baseline by -0.3 ± 0.3 μA at 124 min (Fig. 8.1C). This was not different than -0.3 ± 1.6 μA below the baseline seen on UFH addition to only the mucosal side (P=0.3, two-tailed t-test).

8.4.1.3 Following addition of UFH to mucosal buffer and NaF to mucosal and serosal buffers

Sodium Fluoride (NaF, 0.1 mg/ml) was added to both the mucosal and serosal buffers along with UFH addition to mucosal buffer to investigate the role of active transport in the movement of UFH through gastric mucosa. PD increased in negativity by -1.3 ± 0.1 mV that was comparable to -1.6 ± 0.3 mV upon UFH addition to only the mucosal side (P=0.4). After a lag period of 15.6 ± 4.3 min, similar to a lag period of 18.8 ± 4.9 min when NaF was not present (P=0.3), the PD decreased to its previous resting level at 64.8 ± 4.4 min and was less than the baseline by -0.08 ± 0.1 mV at 124 min which was not different than change from baseline by 0.5 ± 0.8 mV at 124 min (P=0.5) in the absence of NaF (Fig. 8.2A). There was a significant increase in time to reach the resting level in the

presence of NaF compared to 39.3 ± 17.6 min upon UFH addition to mucosal side without NaF (P=0.04).

Isc changed by a small amount from baseline (0.2 ± 0.0 μA) during the experimental period in the presence of NaF which was significantly different from -0.3 ± 1.6 μA seen in the absence of NaF (P=0.02) (Table 8.1, Fig. 8.2 B).

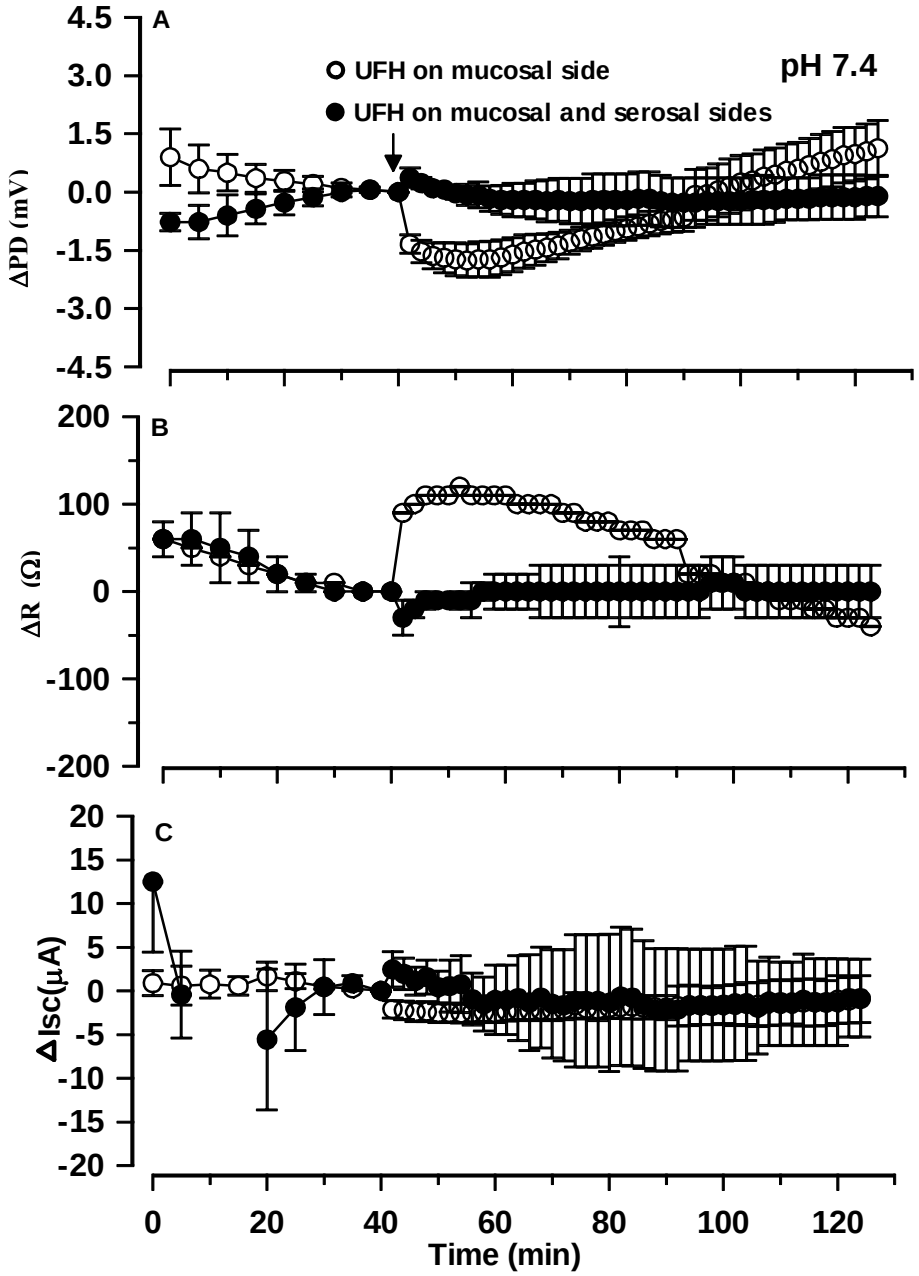


Figure 8.1 Changes in electrical parameters across rat gastric mucosa on heparin (10 mg/ml) addition to either the mucosal side or mucosal and serosal sides. The potential difference (PD) became more negative when the mucosal side was compared to the serosal side immediately after addition of heparin to only the mucosal side not both. PD returned to the resting level with time (A). Changes in resistance (R) followed the pattern of changes in PD (B). Short circuit current (Isc) did not increase after heparin addition (C). Results are shown as mean ± SEM of 5 and 3 experiments when UFH was added to only the mucosal side or both mucosal and serosal sides respectively.

Table 8.1 Changes in electrical parameters (PD and Isc) across rat gastric mucosa mounted in an Ussing chamber following addition of UFH (10 mg/ml) and/or different inhibitors to the mucosal buffer.

pH 7.4		UFH (M)	UFH (M,S)	UFH (M) + NaF (M,S)	UFH (M) + Col. (M,S)	UFH (M) + Ami. (M,S)
	Negativity (mV)	-1.6 ± 0.3	0.0 ± 0.0[a]	-1.3 ± 0.1	-0.8 ± 0.0	-1.7 ± 0.1
Δ PD	Lag Period (min)	18.8 ± 4.9	0.0 ± 0.0[a]	15.6 ± 4.3	14.5 ± 2.5	18.8 ± 1.5
	Time to reach baseline after lag period (min)	39.3 ± 17.6	7.0 ± 1.0	64.8 ± 4.4[a]	0.7 ± 13.1	52.8 ± 2.3
	Change from baseline at 124 min (mV)	0.5 ± 0.8	-0.1 ± 0.5	-0.1 ± 0.1	1.5 ± 1.4	0.3 ± 0.0
Δ Isc	Change in baseline (μA) (124-40 min)	-0.3 ± 1.6	-0.3 ± 0.3	0.2 ± 0.0[a]	-0.1 ± 0.7	-0.4 ± 1.1
pH 4.0						
	Negativity (mV)	-2.2 ± 0.1	-0.1 ± 0.1[a]	-1.4 ± 0.1[a]	-1.4 ± 0.1[ab]	-1.5 ± 0.1[a]
Δ PD	Lag Period (min)	8.5 ± 2.9	4.7 ± 1.2	8.9 ± 0.9[b]	24.5 ± 2.0[ab]	6.4 ± 0.8[b]
	Time to reach baseline after lag period (min)	40.2 ± 6.0	4.7 ± 0.5[a]	>124[ab]	> 124[ab]	> 124[ab]
	Change from baseline at 124 min (mV)	2.1 ± 1.4	3.6 ± 0.5[b]	-0.9 ± 0.1[ab]	-0.5 ± 0.1	-0.3 ± 0.0[b]
Δ Isc	Change in baseline (μA) (124-42 min)	4.3 ± 1.6[b]	15.5 ± 5.3	3.5 ± 1.8	4.0 ± 1.8	3.7 ± 1.6

a-Significantly different than UFH addition only to the mucosal side.
b-Significantly different than pH 7.4.
PD, Potential Difference; Isc, Short circuit current; UFH, Unfractionated Heparin; M, Mucosal buffer; S, Serosal buffer; NaF, sodium fluoride (0.1 mg/ml); Col., Colchicine (1 μM); Ami., Amiloride (1 mg/ml).

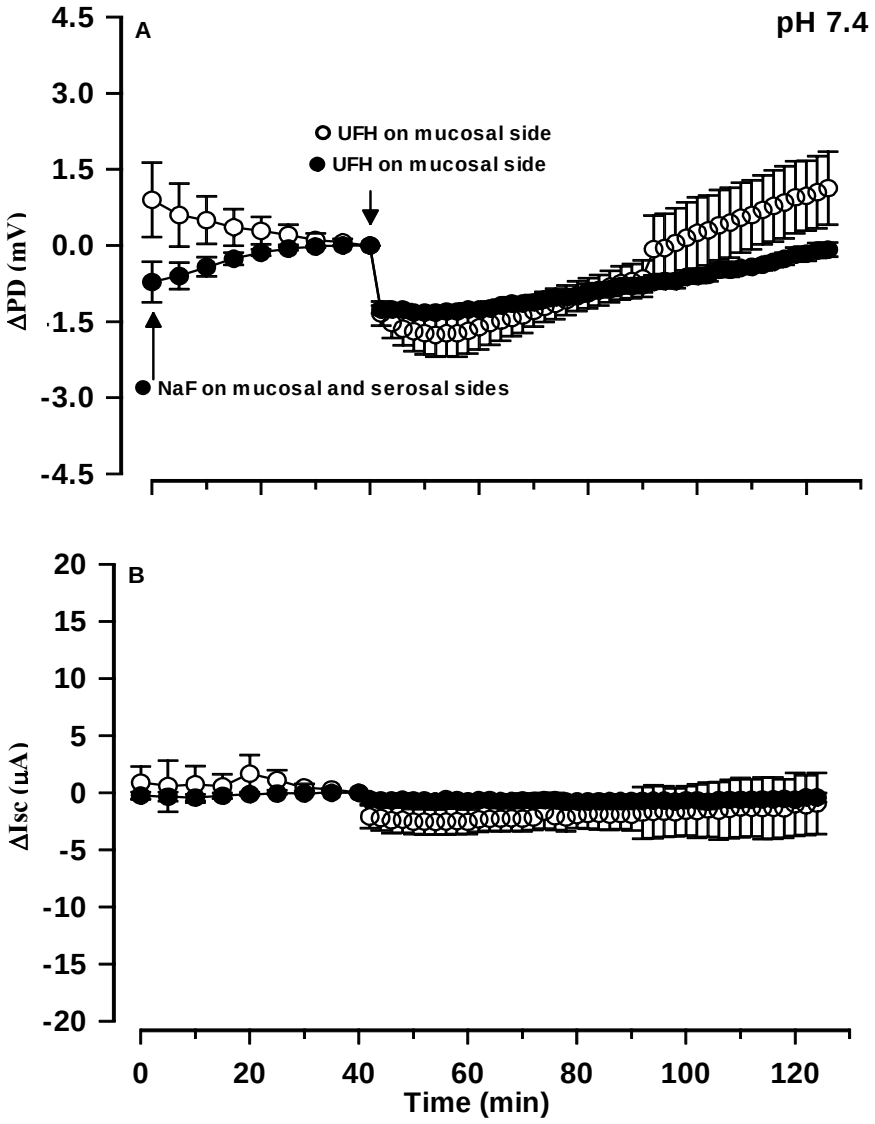


Figure 8.2 Changes in electrical parameters across rat gastric mucosa on heparin (10 mg/ml) addition to the mucosal side with or without sodium fluoride (NaF). Unfractionated heparin was added to the mucosal side at pH 7.4. Changes in potential difference (PD) and short circuit current (Isc) are compared to those when NaF is added to the mucosal and serosal sides. The PD became more negative and there was comparable lag periods before PD began to rise to its baseline in the presence or absence of NaF (A). However, it took a significantly longer time to reach baseline in the presence of NaF. Isc increased by a small amount in the presence of NaF but not in its absence (B). Results are shown as mean ± SEM of 5 and 5 experiments in the presence or absence of NaF respectively.

8.4.1.4 Following addition of UFH to mucosal buffer and colchicine to mucosal and serosal buffers

To observe if endocytosis plays a part in movement of UFH across the gastric mucosa, colchicine (1 μM) was added to both mucosal and serosal buffers along with UFH (10 mg/ml) to mucosal buffer and electrical properties were recorded. In the presence of colchicine, the pattern of changes in electrical parameters (PD, R, and Isc) across the mucosa was similar to addition of UFH alone (Table 8.1, Fig. 8.3). When colchicine was present, PD increased in negativity by -0.8 ± 0.0 mV compared to -1.6 ± 0.3 mV without colchicine (P=0.1). After a lag period of 14.5 ± 2.5 min in the presence of colchicine, which was similar to 18.8 ± 4.9 min without colchicine (P=0.3), PD decreased to its previous resting level (Fig. 8.3A**)**. The PD reached its resting level 30.7 ± 13.1 min after the lag period and moved above baseline by 1.5 ± 1.4 mV at 124 min when colchicine was present. This is compared to reaching the baseline 39.3 ± 17.6 min after the lag period (P=0.3) and a difference from baseline by 0.5 ± 0.8 mV upon UFH addition without colchicine (P=0.5).

When UFH was added in the presence of colchicine, Isc did not increase during the 84 min of the experimental period and was less than the baseline by -0.1 ± 0.7 μA at 124 min (Table 8.1, Fig. 8.3 B). This was similar to -0.3 ± 1.6 μA that was seen with UFH addition to only the mucosal side (P=0.6).

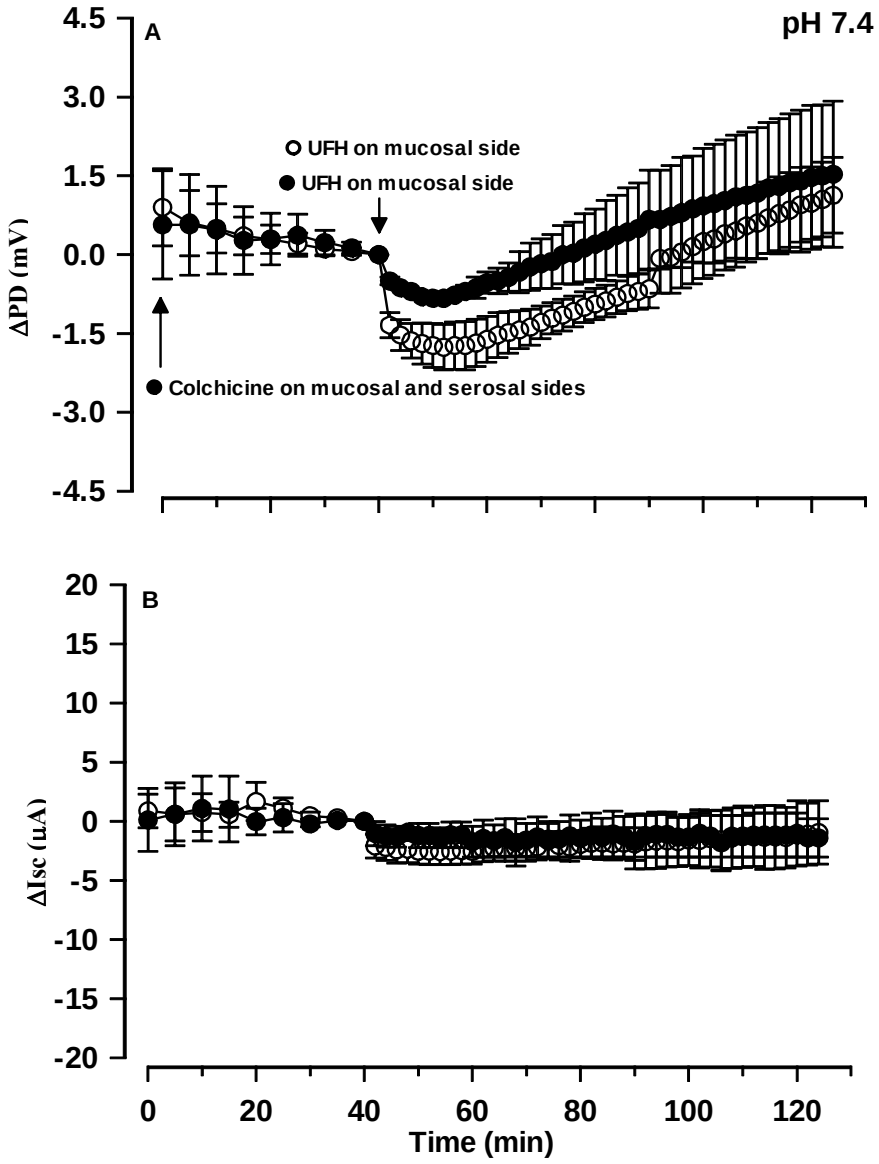


Figure 8.3 Changes in electrical parameters across rat gastric mucosa on heparin (10 mg/ml) addition to the mucosal side with or without colchicine. Unfractionated heparin was added to the mucosal side at pH 7.4. Changes in potential difference (PD) and short circuit current (Isc) are compared to those when colchicine was added to the mucosal and serosal sides. There was a trend towards a smaller increase in the negativity of PD, a slightly shorter lag period and a faster movement towards reaching baseline in the presence of colchicine but differences were not significant (A). Isc did not increase in the presence or absence of colchicine (B). Results are shown as mean ± SEM of 5 and 3 experiments in the presence or absence of colchicine respectively.

8.4.1.5 Following addition of UFH to mucosal buffer and amiloride to mucosal and serosal buffers

To observe if sodium- coupled channels are involved in transport of sodium heparin (UFH) across the gastric mucosa, amiloride (1 mg/ml) was added to mucosal and serosal buffers along with UFH (10 mg/ml) in mucosal buffer. Changes in electrical properties of the membrane are shown in Table 8.1 and Fig. 8.4. The pattern of electrical changes was the same as when UFH was added without amiloride. The PD increased in negativity by -1.7 ± 0.1 mV similar to -1.6 ± 0.3 mV with UFH addition alone (P=0.9). Lag periods were 18.8 ± 1.5 min and 18.8 ± 4.9 min with and without amiloride respectively (P=0.5) (Fig. 8.4A). The PD returned to resting level 52.8 ± 2.3 min after the lag period and moved above baseline by 0.3 ± 0.0 mV at 124 min with amiloride addition. These changes are similar to reaching baseline 39.3 ± 17.6 min after the lag period (P=0.1) and moving above baseline by 0.5 ± 0.8 mV on UFH addition alone (P=0.8).

When UFH was added in the presence of amilioride, Isc did not increase during the 84 min experimental period and was less than baseline by -0.4 ± 1.1 μA at 124 min (Table 8.1, Fig. 8.4 B). This was similar to -0.3 ± 1.6 μA seen with UFH addition to only the mucosal side (P=0.5).

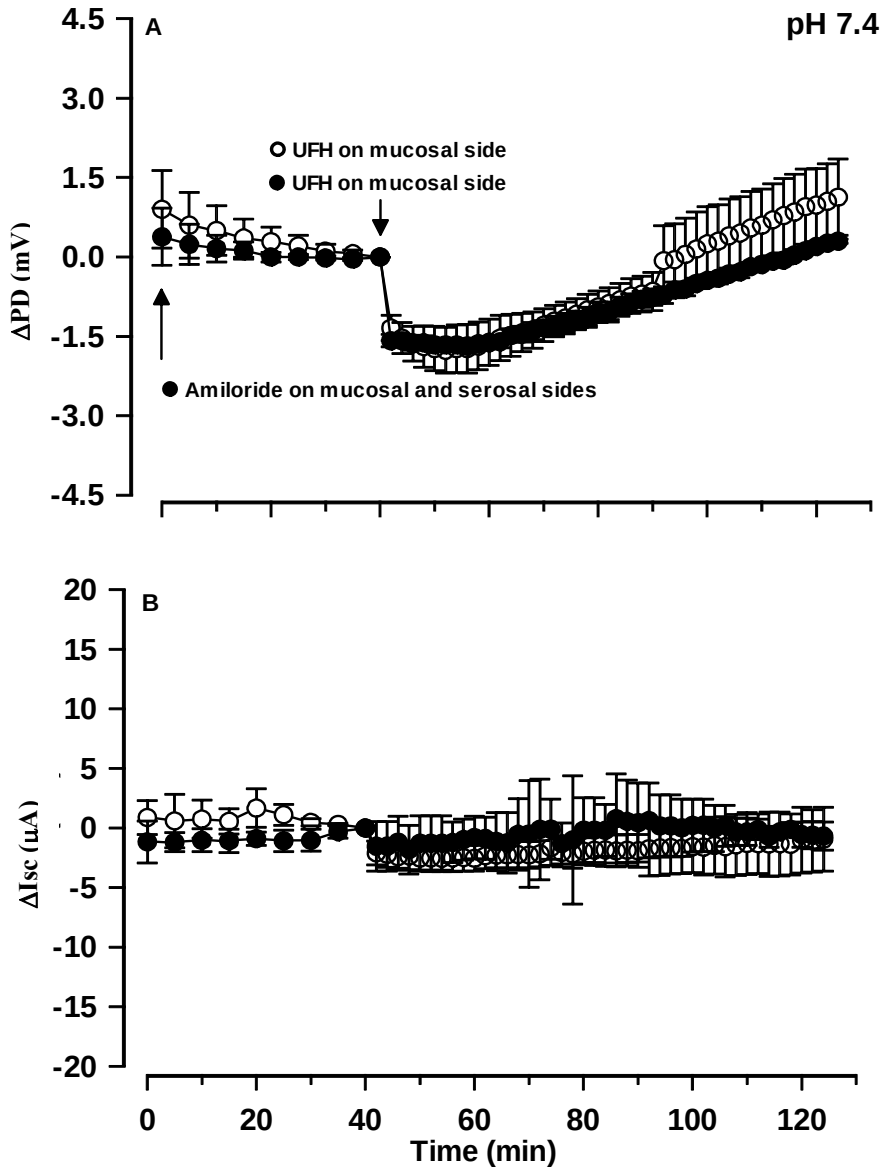


Figure 8.4 Changes in electrical parameters across rat gastric mucosa on heparin (10 mg/ml) addition to the mucosal side with or without amiloride. Unfractionated heparin was added to the mucosal side at pH 7.4. Changes in potential difference (PD) and short circuit current (Isc) are compared to those when amiloride was added to the mucosal and serosal sides. The negativity of PD and lag periods before PD began to rise to baseline and, the time to reach baseline in the presence or absence of amiloride were similar (A). Isc did not increase in the presence or absence of amiloride (B). Results are shown as mean ± SEM of 5 and 5 experiments in the presence or absence of amiloride respectively.

8.4.2 Electrical parameters of rat gastric mucosa at pH 4.0

8.4.2.1 Following addition of UFH to mucosal buffer

When UFH (10 mg/ml) was added to the mucosal buffer at pH 4.0, the average pH in rat stomach (Eastman and Miller, 1935), the PD became more negative by -2.2 ± 0.1 mV (Fig.8.5a and Table 8.1) when the mucosal side was compared to the serosal. After a lag period of 8.5 ± 2.9 min, the PD became more positive reaching the resting level 40.2 ± 6.0 min later, after which the PD became positive compared to the resting level at 124 min by 2.1 ± 1.4 mV. The R of the mucosa followed a pattern opposite to that of PD. The R increased upon UFH addition to mucosal buffer by 160 ± 40 Ω, decreased following a lag period of 8.0 ± 2.6 min and reached resting level 39.0 ± 7.2 min later. The R was 70 ± 80 Ω below baseline at 124 min. Isc increased throughout the experimental period after heparin addition and was above the baseline by 4.3 ± 1.9 μA at 124 min (Fig. 8.5b and Table 8.1).

8.4.2.2 Following addition of UFH to mucosal and serosal buffer

UFH (10 mg/ml) was added to both mucosal and serosal buffers to investigate the role of passive and/or active transport under acidic conditions (Fig. 8.5, Table 8.1). On UFH addition to both buffers, PD increased in negativity by -0.1 ± 0.1 mV compared to -2.2 ± 0.1 mV on UFH addition to only the mucosal side ($P<0.0001$, Fig. 8.5a). A lag period of 4.7 ± 1.2 min was observed for PD upon UFH addition to both sides of the membrane where PD was at baseline. The PD became more positive with time and was above baseline by 3.6 ± 0.5 mV at 124 min. These results are compared to a lag period of 8.5 ±

2.9 min (P=0.06) and time to reach resting level of 40.2 ± 6.0 min (P=0.001) after which PD continued to become more positive than the resting level by 2.1 ± 1.4 mV (P=0.3) when UFH was added to only the mucosal side. The Isc changed by 15.5 ± 5.3 μA during the experimental period on UFH addition to both mucosal and serosal buffers at pH 4.0 which was not significantly greater than 4.2 ± 1.5 μA on UFH addition only to the mucosal buffer (P=0.2, Fig.8.5b).

8.4.2.3 Following addition of UFH to mucosal buffer and NaF to mucosal and serosal buffers

Membrane electrical properties were recorded when NaF (0.1 mg/ml) was added to both the mucosal and serosal buffers along with UFH addition to mucosal buffer at pH 4.0. The PD increased in negativity by -1.4 ± 0.1 mV compared to -2.2 ± 0.1 mV upon UFH addition only to the mucosal side (P=0.001). After a lag period of 8.9 ± 0.9 min in the presence of NaF, which was similar to 8.5 ± 2.9 min in the absence of NaF, the PD began to decrease (P=0.4, Fig. 8.6 a). However, PD reached a plateau and did not reach its resting level in the time period assigned to the experiment (84 min). The PD was less than baseline by -0.9 ± 0.1 mV at 124 min. These results are compared to 40.2 ± 6.0 min for time for PD to reach baseline after the lag period in the absence of NaF (P<0.0001) after which PD became more positive than resting level by 2.1 ± 1.4 mV at 124 min (P=0.01). In the presence of NaF at pH 4.0, Isc increased by 3.5 ± 1.8 μA during the experimental period compared to 4.2 ± 1.5 μA in its absence (P=0.8, Fig. 8.6b).

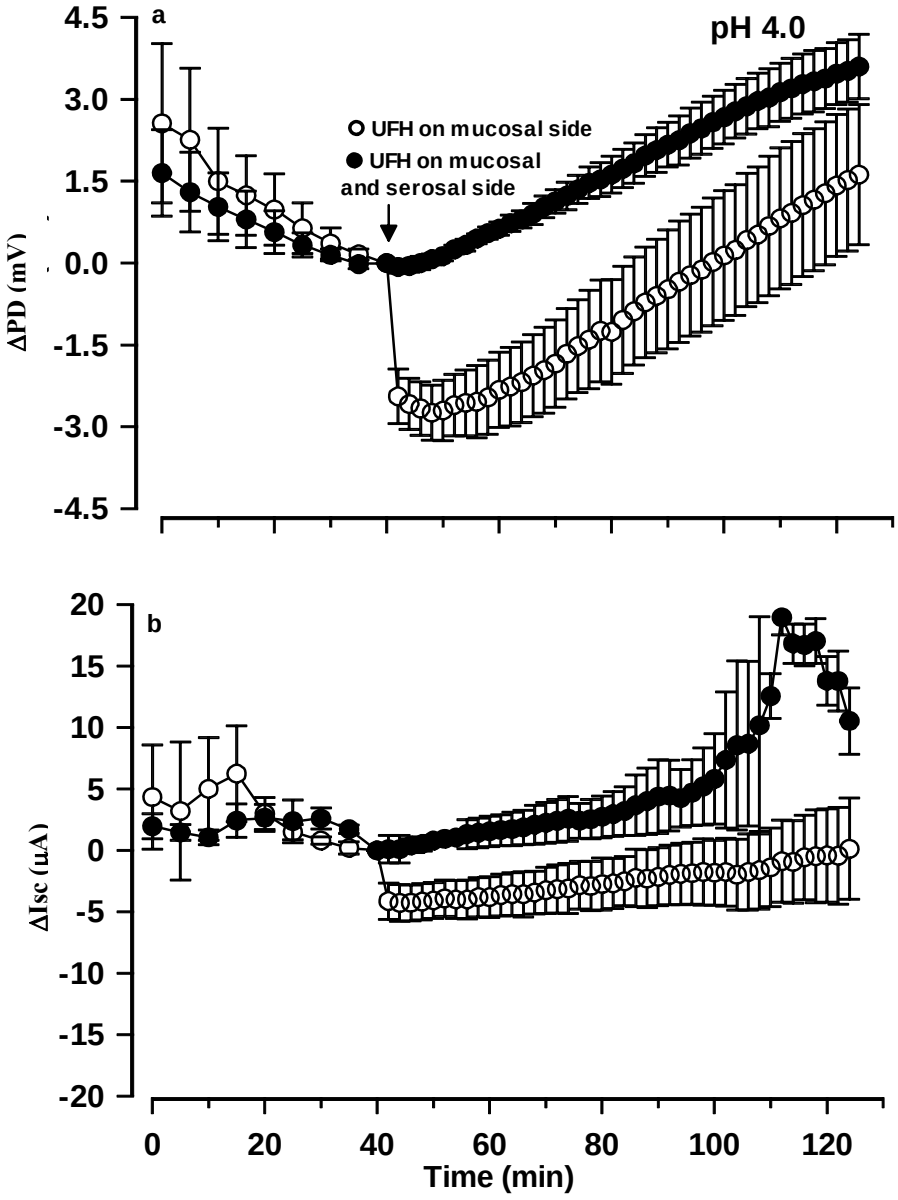


Figure 8.5 Changes in electrical parameters across rat gastric mucosa on heparin (10 mg/ml) addition to either the mucosal side or mucosal and serosal side at pH 4.0. A great and small increase in negativity of potential difference (PD) was observed immediately after addition of UFH to only the mucosal side or to both sides of the membrane respectively. PD returned to baseline and continued to become more positive than baseline during 84 min (A). Short circuit current (Isc) showed small and significant increase after heparin addition to only mucosal or both sides of the membrane respectively (C). Results are shown as mean ± SEM of 5 and 6 experiments when UFH was added to only the mucosal side or both mucosal and serosal sides respectively.

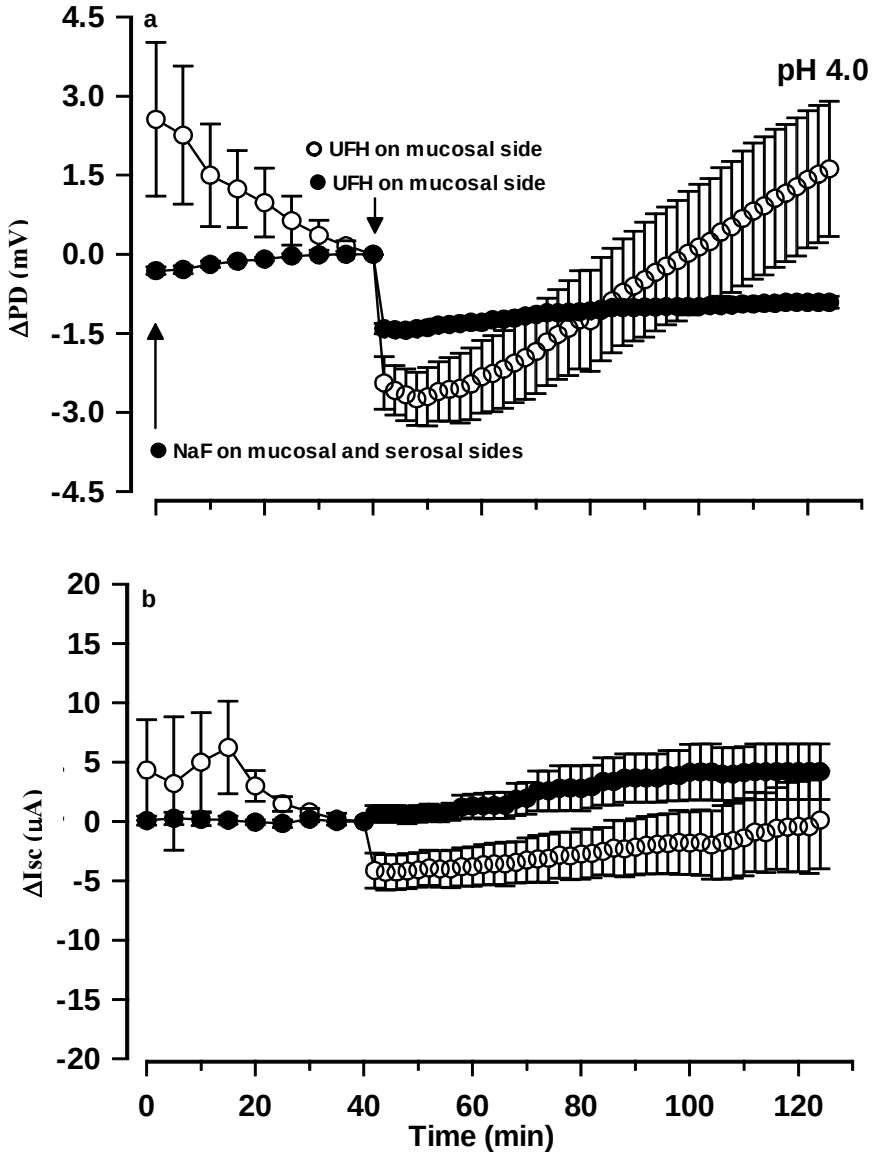


Figure 8.6 Changes in electrical parameters across rat gastric mucosa on heparin (10 mg/ml) addition to the mucosal side with or without sodium fluoride (NaF) at pH 4.0. Changes in potential difference (PD) and short circuit current (Isc) are compared to those when NaF is added to the mucosal and serosal sides. The PD became more negative and there was comparable lag periods before PD began to rise to its baseline in the presence or absence of NaF (A). However in the presence of NaF, PD did not reach the baseline for the duration of the experiment. Isc increased from baseline and was similar in the presence or absence of NaF (B). Results are shown as mean ± SEM of 7 and 5 experiments in the presence or absence of NaF respectively.

8.4.2.4 Following addition of UFH to mucosal buffer and colchicine to mucosal and serosal buffers

Colchicine (1 μM) was added to both mucosal and serosal buffers along with UFH (10 mg/ml) addition to the mucosal buffer, and membrane electrical properties were recorded and compared to results after UFH addition to only the mucosal buffer at pH 4.0 (Fig. 8.7, Table 8.1). When colchicine was present, PD increased in negativity by -1.4 ± 0.1 mV on UFH addition compared to -2.2 ± 0.1 mV on UFH addition to only the mucosal side (P=0.003, Fig. 8.7a). There was a significant increase in the lag period of 24.5 ± 2.0 min in the presence of colchicine, compared to 8.5 ± 2.9 min in its absence (P=0.001). In the presence of colchicine, PD did not reach baseline in the time assigned to the experiment and was less than baseline by -0.5 ± 0.1 mV at 124 min compared to reaching the resting level 40.2 ± 6.0 min after the lag period (P=0.0007) and moving above baseline by 2.1 ± 1.4 mV (P=0.1) when UFH was present in the mucosal buffer only.

When colchicine was present, Isc increased by 4.0 ± 1.8 μA from baseline at 124 min at pH 4.0 that was similar to a change of 4.2 ± 1.5 μA in its absence (P=0.9, Fig. 8.7b).

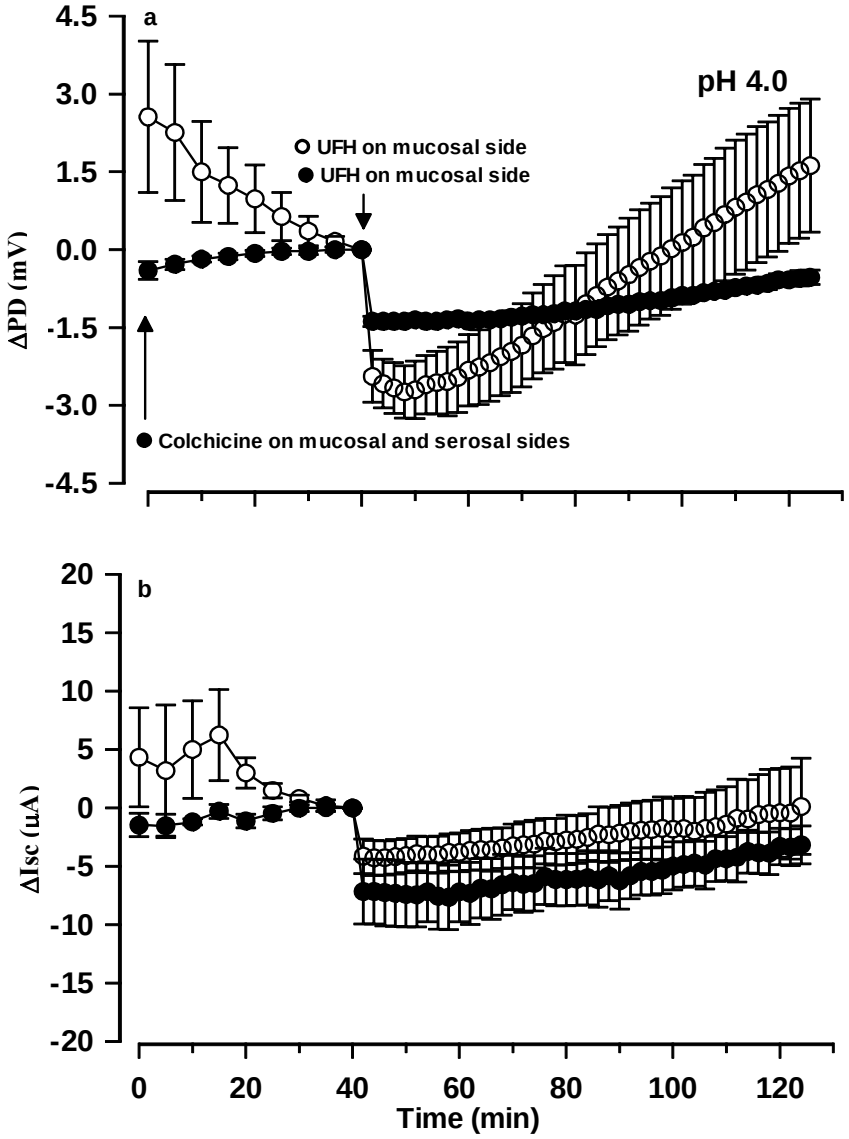


Figure 8.7 Changes in electrical parameters across rat gastric mucosa on heparin (10 mg/ml) addition to the mucosal side with or without colchicine at pH 4.0.
Changes in potential difference (PD) and short circuit current (Isc) are compared to those when colchicine was added to the mucosal and serosal sides. There was a significantly smaller increase in the negativity of PD, a significantly longer lag period for PD after which PD did not reach the baseline during the experimental period in the presence of colchicine (A). Increase in Isc was similar in the presence or absence of colchicine (B). Results are shown as mean ± SEM of 4 and 5 experiments in the presence or absence of colchicine respectively.

8.4.2.5 Following addition of UFH to mucosal buffer and amiloride to mucosal and serosal buffers

Amiloride (1 mg/ml) was added to mucosal and serosal buffers with UFH (10 mg/ml) addition to the mucosal buffer. Membrane electrical properties were recorded and compared to those following UFH (10 mg/ml) addition to only the mucosal buffer (Fig. 8.8, Table 8.1). In the presence of amiloride, PD increased in negativity by -1.5 ± 0.1 mV on UFH addition compared to -2.2 ± 0.1 mV in its absence (P=0.002, Fig. 8.8a). There was a lag period of 6.4 ± 0.8 min in the presence of amiloride compared to 8.5 ± 2.9 min in its absence (P=0.2). The PD did not reach baseline by the end of the experiment and was less than baseline by -0.3 ± 0.0 mV at 124 min in the presence of amiloride. This is compared to reaching the baseline 40.2 ± 6.0 min after lag period (P=0.0002) and final values above baseline by 2.1 ± 1.4 mV when amiloride was not present (P=0.08).

The Isc increased by 3.7 ± 1.6 μA at 124 min when amiloride was present that was similar to 4.2 ± 1.5 μA during the experimental period when it was absent from mucosal and serosal buffers (P=0.6, Fig. 8.8b).

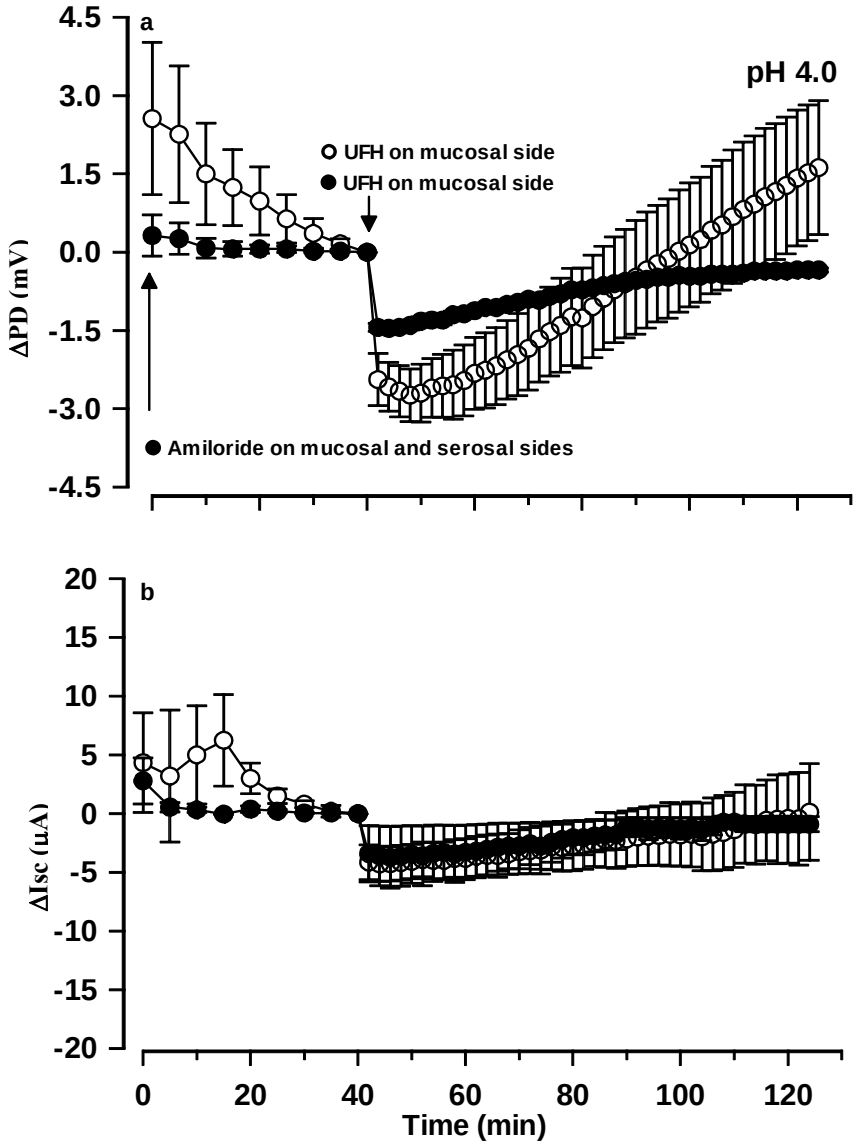


Figure 8.8 Changes in electrical parameters across rat gastric mucosa on heparin (10 mg/ml) addition to the mucosal side with or without amiloride at pH 4.0.
Changes in potential difference (PD) and short circuit current (Isc) are compared to those when amiloride was added to the mucosal and serosal sides. The PD became significantly more negative and there was comparable lag periods before PD began to rise to its baseline in the presence or absence of amiloride (A). However, PD did not reach the baseline for the duration of the experiment in the presence of amiloride. Increase in Isc was similar between the two groups (B). Results are shown as mean ± SEM of 5 and 5 experiments in the presence or absence of amiloride respectively.

8.4.3 Comparison of electrical parameters of rat gastric mucosa at pH 4.0 or pH 7.4

8.4.3.1 Following addition of UFH to mucosal buffer

When UFH was added to the mucosal buffer at pH 7.4 or pH 4.0, PD became more negative (Table 8.1). PD negativity increased by -2.2 ± 0.1 mV in acidic environment compared to -1.6 ± 0.3 mV in the neutral condition (P=0.2). There was a trend towards a significantly shorter lag period of 8.5 ± 2.9 min in the acidic environment, compared to 18.8 ± 4.9 min in the neutral environment (P=0.06). The PD reached its resting level 40.2 ± 6.0 min after the lag period in the acidic environment that was similar to 39.3 ± 17.6 min in the neutral environment (P=0.4, one-tailed t-test). Changes in R followed the same pattern as in PD. The Isc increased by 4.2 ± 1.5 μA during 84 min in the acidic environment. However, there was no increase in Isc from baseline on UFH addition to mucosal buffer in the neutral environment and Isc was less than baseline by -0.3 ± 1.6 μA at 124 min (P=0.001, Table 8.1).

8.4.3.2 Following addition of UFH to mucosal and serosal buffer

On UFH addition to both buffers, PD showed a small increase in negativity by -0.1 ± 0.1 mV at pH 4.0, and became more positive by 0.4 ± 0.3 mV at pH 7.4 (P=0.08, Table 8.1). A lag period of 4.7 ± 1.2 min was observed where PD was at its resting level in the acidic environment. However, a lag period was not observed for PD in the neutral environment (P=0.1) and PD was at its resting level by 7.0 ± 1.0 min (P=0.07) after UFH addition where it remained for the rest of the experimental period. The PD became more positive with time and was above baseline by 3.6 ± 0.5 mV at 124 min at pH 4.0. This is compared to PD being below baseline by -0.1 ± 0.5 mV at 124 min (P=0.002).

The Isc showed a dramatic change from baseline during the experimental period (15.5 ± 5.3 μA) at pH 4.0 that showed a trend towards a difference (-0.3 ± 0.3 μA at pH 7.4, P=0.06).

8.4.3.3 Following addition of UFH to mucosal buffer and NaF to mucosal and serosal buffers

The PD increased in negativity by -1.4 ± 0.1 mV and -1.3 ± 0.1 mV in the presence of NaF at pH 4.0 and 7.4 respectively (P=0.4, Table 8.1). There was a significantly shorter lag period of 8.9 ± 0.9 min at pH 4.0 compared to 15.6 ± 4.3 min at pH 7.4 (P=0.04). Time for PD to reach the baseline was greater than 124 min assigned to the experiment and PD was lower than the baseline by -0.9 ± 0.1 mV at pH 4.0. This is compared to PD reaching the baseline 64.8 ± 4.4 min after the lag period (P=0.006) and being lower than baseline by -0.1 ± 0.1 mV at pH 7.4 (P=0.0002). In the presence of NaF at pH 4.0, Isc increased by 3.5 ± 1.8 μA during the experimental period which showed a trend to be greater than an increase from baseline of 0.2 ± 0.0 μA at pH 7.4 (P=0.06).

8.4.3.4 Following addition of UFH to mucosal buffer and colchicine to mucosal and serosal buffers

In the presence of colchicine, PD increased in negativity by -1.4 ± 0.1 mV and -0.8 ± 0.0 mV at pH 4.0 and 7.4 respectively (P=0.004, Table 8.1). There was a lag period of 24.5 ± 2.0 min at pH 4.0 which was significantly greater than 14.5 ± 2.5 min at pH 7.4 (P=0.005). Time for PD to reach its baseline was greater than 124 min and PD was lower than the baseline by -0.5 ± 0.1 mV at pH 4.0. This is compared to PD reaching its

baseline 30.7 ± 13.1 min after the lag period (P=0.002) and being above baseline by 1.5 ± 1.4 mV at pH 7.4 (P=0.05). In the presence of colchicine at pH 4.0, Isc increased by 4.0 ± 1.8 μA during the experimental period which was greater than -0.1 ± 0.7 μA at pH 7.4 (P=0.08).

8.4.3.5 Following addition of UFH to mucosal buffer and amiloride to mucosal and serosal buffers

In the presence of amiloride, PD increased in negativity by -1.5 ± 0.1 mV and -1.7 ± 0.1 mV at pH 4.0 and 7.4 respectively (P=0.1, Table 8.1). There was a lag period of 6.4 ± 0.8 min at pH 4.0 which was significantly shorter than 18.8 ± 1.5 min at pH 7.4 (P<0.0001). The PD did not reach baseline and was lower than baseline by -0.3 ± 0.0 mV at pH 4.0 compared to PD reaching baseline 52.8 ± 2.3 min after the lag period (P<0.0001) and being above baseline by 0.3 ± 0.0 mV at pH 7.4 (P<0.0001). Isc increased by 3.7 ± 1.6 μA during the experimental period at pH 4.0 in the presence of amiloride which showed a trend to be significantly greater than -0.4 ± 1.1 μA at pH 7.4 (P=0.1).

8.5 Disscussion

Our results were obtained after careful evaluation of changes in electrical properties [potential difference (PD), resistance (R) and, short circuit current (Isc)] of the gastric mucosa mounted in a vertical diffusion Ussing chamber apparatus after UFH was added to the mucosal buffer once the tissue was stabilized for 40 min. UFH crosses rat gastric mucosa when added to the mucosal buffer in a pH-dependent manner. Changes in PD after UFH addition at pH 7.4 or 4.0 followed a similar pattern including increase in

negativity, a lag period with a trend towards a significant decrease at pH 4.0 compared to 7.4, and time to reach baseline after the lag period that was similar at both pHs. After an initial increase in negativity immediately on UFH addition, the Isc (an indicator of active transport), increased with time at pH 4.0 but not pH 7.4 suggesting involvement of active and passive mechanisms of transport in UFH movement across the gastric mucosa at pH 4.0 and 7.4 respectively.

Since the definition of active transport is when a substance moves against or without an existing electrochemical gradient, UFH was added to both mucosal and serosal sides of the membrane and electrical properties were recorded. When the mucosal side was compared to the serosal, no change in PD was seen the moment heparin was added to both mucosal and serosal buffers, such that the mucosal buffer did not become more negative compared to the serosal, and is likely due to the equal anionic charges added to the mucosal and serosal side. The PD remained negative and with time returned to the previous resting level and above at pH 4.0 but not pH 7.4 confirming that active and passive transports plays dominant roles in heparin transport at pH 4.0 and pH 7.4 respectively. This is supported by a dramatic increase in Isc at pH 4.0 but not pH 7.4. Metabolical and pharmacological inhibitors were used in an attempt to determine the pathway and cellular mechanisms involved in gastric UFH uptake. Our results showed that UFH transport by gastric mucosa at pH 4 is an energy-dependent process (NaF experiments) and uses the cellular microtubular network (colchicine experiments) as well as sodium-coupled transport systems (amiloride experiments). Inability of changes in PD to reach the baseline after UFH addition at pH 4.0 when NaF, colchicine or amiloride are

present indicates that UFH transport across the gastric epithelium occurs largely by an active process. After an initial increase in PD negativity on UFH addition in the presence of NaF, likely due to the anionic charge on heparin, the PD returned to the baseline with time at pH 7.4 but not at pH 4.0. As well, minor versus significant changes in Isc from baseline at 124 min on UFH addition in the presence of NaF at different mucosal pH's are indicative of passive versus active transport playing major roles in UFH movement at pH 7.4 and pH 4.0 respectively. Inability of changes in PD to reach baseline at experiment completion and change in Isc observed following UFH administration in the presence of colchicine or amiloride at pH 4.0 but not pH 7.4 are also in agreement with the above results (Table 8.1). These findings describe the possible existence of a passive component to UFH transport present at pH 7.4 which are near conditions in the intestine but not the acidic conditions found in the stomach.

Studies on the mechanism of UFH transport across gastric tissue are scarce. Our results however, are in agreement with others studying the mechanism(s) of transport of macromolecules such as proteins across the gastrointestinal tract. Curtis and Gall (1992) showed that in the stomach uptake of intact bovine serum albumin (BSA) involves an active, energy dependent mechanism that utilizes the microtubular network and is regulated by cyclooxygenase metabolites (Curtis et al., 1993). Moreover, many studies in the intestine have repeatedly shown that proteins, in both intact and degraded forms, cross different regions of the intestinal epithelium. Fluxes for horse radish peroxidase (HRP, 40 KDa) in adult rabbit jejunum (Heyman et al., 1982) and ileum (Isolauri et al., 1990) and in piglet jejunum (Keljo et al., 1985; Keljo and Hamilton, 1983), for intact β-

lactoglobulin (18.4 KDa) in rabbit ileum (Isolauri et al., 1990; Marcon-Genty et al., 1989), and for intact bovine serum albumin (BSA, 66.2 KDa) across rat jejunum have been shown. Accumulation of intact protein on the serosal surface represented only a small percentage of the total protein in the intestine. In contrast, in the stomach a larger proportion of the total protein crossing rat gastric mucosa remained intact. Mucosal transport of intact macromolecules in the intestine also appears to occur via an active transcellular route that uses a microtubular endocytotic pathway and is regulated by sodium channels since the nerve blocker tetrodotoxin, which binds noncompetetively to electrogenic sodium channels, significantly reduced antigen uptake (Kimm et al., 1994). Transport of degraded peptides however, appeared to occur via a separate cellular pathway. Although changes in PD across the mucosa after addition of colchicine or amiloride followed the same pattern at pH 4.0, there still was an important difference (Table 8.1).

When colchicine was present, the lag period for PD was significantly longer on UFH addition compared to when amiloride was added, likely indicative of a biphasic active characteristic of UFH transport across the gastric membrane at pH 4. It is possible that transepithelial transport of the UFH macromolecule is initiated by a constitutively activated, energy consuming endocytotic mechanism present at the gastric epithelial membrane. It is then likely that some sodium-coupled systems are activated later, further facilitating the movement of UFH through the gastric mucosa. Accordingly, some studies in the intestine suggested the involvement of more than a single mechanism for the uptake of intact proteins (Kimm et al., 1994; Walker et al., 1972).

Studies in the small intestine have shown enterocytes to uptake and transport proteins across the mucosa (Walker, 1981; Walker, 1987). The enterocytes have a specialized apical surface, the brush border that continually forms endocytotic vesicles at the microvillus base. In doing so, proteins are taken up in both membrane-bound and soluble fractions (Hemmings, 1981). As endocytotic vesicles move through the cell, they fuse with lysosomal vesicles and the protein inside is degraded before expulsion at the basolateral membrane (Walker, 1981). Protein that escapes degradation in the transmural passage may do so by incomplete breakdown after lysosomal fusion (Stahl and Schwartz; 1986) or by uptake through a separate nondegradative pathway (Heyman et al., 1982). The results presented in our study implicate the presence of a sampling mechanism in the stomach for UFH similar to that seen for proteins described in the small intestine. Few cells in the gastric mucosa possess an apical brush border. Ultrastructural studies described that the majority of cells with this specialization are endocrine cells (Ito, 1981), with one exception the "Tuft cell" (Isomaki, 1973). This cell closely resembles the mature small bowel enterocyte, but no function has yet been ascribed to it. The Tuft cell may play a role in macromolecular transport including carbohydrates like UFH across the gastric mucosa.

Active transport occurs through the cells and is primarily dependent upon the operation of so-called ion pumps or "ATPase" (primary active transport). These pumps create chemical or electrochemical driving forces that cause the passive movement of a single substrate (passive transport via carriers or channels) or the coupled movement of two or more substances (secondary active transport via carriers). Therefore, for carriers or

channels to contribute to active transepithelial transport their function must be coordinated with that of a pump. The carriers or channels can be in series (e.g. apical Na^+-glucose cotransporter or amiloride-sensitive sodium channels with the basolateral Na^+, K^+, ATPase in Na^+-absorptive gastric epithelium) or in parallel with a pump. Jackson and Norris (1985) proposed a model describing Na^+ transport system in rat gastric mucosa *in vitro*. They considered the system to include three components: two rate-limiting, amiloride-sensitive entry mechanisms at the apical membrane, one conductive channel in which Na^+ movement varies with PD, and an electrically neutral mechanism, possibly a Na^+-H^+ exchanger, in which the movement of Na^+ is independent of PD, and a pump process at the basolateral membrane in which the Na^+ movement is independent of PD, but which is electrogenic, and which may be represent a constant-current source. So, it is likely that the entry of UFH across the apical gastric membrane is a secondary active transport mechanism that is indirectly coupled to the consumption of ATP by Na^+, K^+, ATPase pump located at the basolateral membrane in series with the Na^+ channel in the apical membrane. Therefore operation of Na^+, K^+, ATPase, by sending three Na^+ cation out and two K^+ into the cell, produces a transmembrane Na^+ gradient that is used as driving force by Na^+ channel at the apical membrane to passively transport UFH across the gastric membrane.

The results of this study suggest that UFH movement across the gastric mucosa occurs by an active transcellular process at pH 4.0. The role of paracellular permeability in UFH transport was investigated by calculating resistance (R), an indicator of the tissue permeability. The pattern of changes in R is always opposite to that of PD at pH 4.0 or

pH 7.4. The R increased on UFH addition to the mucosal buffer, likely since UFH is a large relatively impermeable anionic molecule. The R then progressively decreased with time and likely reflected UFH movement across the mucosa. As heparin gradually moves from the mucosal to the serosal side, its concentration on the mucosal side progressively decreases. The measured R of the membrane decreases as UFH moves across the membrane since large anionic UFH is likely present on both sides of the membrane. This change in resistance is accompanied by no change in Isc at pH 7.4 suggesting that movement across the membrane is passive.

Our results suggest that an active transcellular pathway involving metabolic energy expenditure, cytoplasmic tubule formation, and sodium coupled systems seem a dominant route for UFH transport across the gastric mucosa, although some passive diffusion may also be involved. Our results are in contrast to those of Atisook and Madara (1991) in the intestine that suggested tight junctions are permeable to small oligopeptides but selectively exclude larger macromolecules. They noted permeation of an 11-aminoacid hemepeptide (MP-11, molecular weight 1,900) through tight junctions but exclusion of HRP (molecular weight 40,000). In accordance to their work, Curtis and Gall (1992) then assumed a commonality of tight junction function along the gastrointestinal tract and excluded the paracellular pathway for the transport of BSA (molecular weight, 66,200) across the rat gastric mucosa.

8.6 Conclusions

In summary, our results demonstrate that the stomach is capable of sampling large carbohydrates in the lumen. Heparin is absorbed following oral administration, and the acidic stomach pH likely favors active transport of heparin across gastric mucosa *in vivo*. Macromolecular transport occurs via an energy-dependent transcellular process that likely uses the microtubular network of the cell as well as sodium coupled systems in apical and basolateral membranes.

9.0 General Discussion and Conclusions

Intravenously administered unfractionated heparin (UFH) is the antithrombotic drug of choice in hospitalized patients since it is believed not to be absorbed when taken orally. Numerous studies over several years showed convincing results concerning heparin absorption following oral route of administration. This evidence was discussed in previous chapters. Studies regarding the site of oral heparin absorption, however, are scarce. Our goal in this study was to investigate where in the gastrointestinal tract oral heparin is absorbed and by what mechanism. Although intestine is primarily considered the anatomical site of absorption for most materials (Thomson et al., 2003[a]; Thomson et al., 2003[b]), we began our study using the glandular portion of rat gastric mucosa (Ichikawa et al., 2000). Stomach tissue was chosen since tissue distribution studies recovered considerable amount of heparin from stomach tissue in contrast to other parts of the gut and non-gut tissues (Hiebert et al., 2000). In addition, more heparin was found on endothelium 15 minutes following placing heparin in the stomach than when heparin was injected into the duodenum when the rat pyloric sphincter was tied (Hiebert et al., 2007).

We have chosen to use the *vertical diffusion Ussing chamber* to study the movement of heparin across the gastrointestinal tract. Since the gastrointestinal epithelia are polar in nature, our studies require the separation of the apical and basolateral side of the gastric mucosa, which is possible when using the Ussing chamber. The Ussing chamber has previously been found useful for transport and drug absorption studies using excised tissue and monolayers of cells grown on filters (Ayalon et al., 1982; Yamashita et al.,

1997; Wallon et al., 2005). Furthermore, it has proved to be effective for screening and predicting drug absorption in humans (Yamashita et al., 1997; Watanabe et al., 2004).
It is unlikely that the site of heparin absorption can be determined by histochemistry. Traditionally, histochemical localization of heparins has been achieved by using metachromatic dyes such as toluidine blue and alcian blue, which change from blue to purple in the presence of heparins. This works better for UFH than LMWHs.

Unfortunately LMWHs, because of a shorter chain length, do not produce the same degree of metachromasia as seen with UFH. In addition, gastrointestinal tissues contain other endogenous components that react with metachromatic dyes such as chondroitin sulphate and heparan sulphate which confound the interpretation especially with a compound which is only weakly metachromatic such as LMWH. Regarding histochemistry with use of antibodies, it is difficult to prepare an antibody for heparin. The very few antibodies that are prepared for heparin, also cross react with heparan sulphate, which are endogenously present in gastrointestinal tissue.

Regarding use of radioisotopes, because heparin is highly negatively charged and contain counter ions, radiolabelled heparin including tritium are quite unstable and clear localization of heparin using autoradiography is difficult. The labelled atom may just as likely be part of the counter ion as attached to the core heparin molecule. A ^{14}C UFH, with heparin attached to the core can be produced with very weak labelling. This does not work well for LMWHs since the chain length is shorter and even weaker labelling occurs than that seen with UFH. Previous studies used radiolabeled heparin that either contained

labile ^{35}S groups that were chemically or metabolically lost or transferred (Linhardt 1992; Larsen et. al., 1986a; Kazatchkine et. al., 1979; Levy and Petracek 1962; Lloyd et. al., 1971; Lloyd et. al., 1968) or ^{3}H reduced end groups that resulted in a product chemically different from pharmaceutical heparin (Halton et. al., 1980; Watanabe et. al., 1982). Because of these difficulties, we have adopted the *in vitro* method using the Ussing chamber to study the site of oral heparins absorption and the mechanism responsible.

For data presentation, electrical parameters, PD, R, and Isc, were normalized to the value taken just prior to heparin addition at 40 min. Changes in PD, R, and Isc were then determined by subtracting all recorded values at all other times during the experiment from the recorded value at 40 min. This normalization allowed the expression of changes in PD, R, and Isc after heparin addition. Control experiments without addition of heparins or pharmacological inhibitors to the mucosal and/or serosal compartments were done to assess tissue viability and stability over the duration of the experiment as well as eliminating the possibility of using data from damaged tissue. In order to compare values to those of other investigators using similar tissue, electrical parameters, before normalization, including PD, R, and Isc were measured. These actual values are summerized in Table 9.1 immediately after mounting the tissue and every 20 min thereafter. Immediately after mounting, PD and Isc were high. In the first 40 min of the experiment however, electrical parameters rapidly stabilized. From this point on there was little change in PD, Isc and R. These actual electrical measurements in rat gastric mucosa were in agreement with those of other investigators (Curtis and Gall, 1992; Curtis et al., 1993). The maintenance of PD and R values indicate that the tissue remained viable

throughout the experimental period. However, our measurements of PD did not match those obtained by the Miller group (Miller et al., 1994). Miller et al., (1994) reported a PD of -32 ± 2.2 mV for several hours which was reduced to -3.3 ± 1.4 mV upon mucosal exposure to 0.6 M NaCl. This descrepancy could be due to using a buffer which was chemically different than our Kreb's bicarbonate buffer solution. Moreover, in studies by the Miller group, the concentration of mannitol and glucose on the mucosal and serosal sides of the membrane was not the same.

Tabel 9.1. Summary of actual tissue electrical parameters.

Parameters	Time (min)						
	0	20	40	60	80	100	120
PD (mV)	-1.8 ± 0.5	-1.5 ± 0.5	-1.5 ± 0.5	-1.4 ± 0.5	-1.5 ± 0.5	-1.4 ± 0.5	-1.4 ± 0.5
R (Ohm)	140.0 ± 67.5	138.3 ± 69.1	135.0 ± 70.4	135.0 ± 71.6	135.0 ± 70.1	135.0 ± 74.1	133.3 ± 72.3
Isc ($\mu A/cm^2$)	6.9± 1.9	5.8 ± 1.4	6.8 ± 2.4	5.1 ± 1.6	5.4 ± 1.5	5.2 ± 1.4	5.4 ± 1.3

Values are mean ± SEM of 4 experiments. Potential difference, PD; Resistance, R; and Short circuit current, Isc.

In the present study, we used 40 mM unlabeled D-glucose in the serosal solution to help maintain tissue viability. At the same time, 40 mM mannitol was added to the mucosal side to provide an equivalent osmotic load between serosal and mucosal buffer solutions. We used a glucose concentration at 40 mM, which is greater than physiological glucose, to account for a larger tissue surface area of 2.5 cm^2 and smaller buffer volume of 1.5 ml bathing serosal side when compared with other drug permeability studies. These concentrations have been used in other studies with the Ussing chamber (Atisook et al., 1990; Grass and Sweetana, 1988; Grass and Sweetana, 1989; Jezyk et al., 1992). It has been shown that the mucosal glucose but not mannitol concentrations elicits decreases in transepithelial resistance by altering tight juntion structure and function (Atisook et al.,

1990; Madara and Pappenheimer, 1987). Perfusion studies showed that at 25mM mucosal glucose, all glucose absorption was transcellular. However, at mucosal glucose concentrations of 125 mM, almost 30% of glucose absorption occurred paracellularly (Atisook et al., 1990; Madara and Pappenheimer, 1987).

Although the concentration of 40 mM serosal glucose, used here, is considerably higher than isotonic concentrations there was no evidence of mucosal damage since potential difference and resistance were maintined during the 124 minute peiod (Table 9.1). The membrane was not permeable to trypan blue following completion of the experiments. Glucose concentrations of 10-20 mM and higher have been routingly added to the serosal solution by many laboratories to provide an energy source to the Ussing chamber-mounted tissue (Atisook et al., 1990; Grass and Sweetana, 1988; Grass and Sweetana, 1989; Jezyk et al., 1992). The gut mucosa is often exposed to hyperosmolar concentrations with the ingestion of food (Fordtran and Ward Locklear, 1966). Back leak of serosal glucose through epithelium is not sufficient to activate the process by which tissue resistance decreases (Atisook et al., 1990). More recently lower concentrations of glucose (10 – 12 mM) have been used in serosal buffer and are likely glucose concentrations of choice in future experiments.

Our findings showed that the stomach tissue at acidic pH is capable of transporting UFH from the luminal compartment to the serosal (chapter 4) via an active mechanism (chapter 8). We followed changes in PD across stomach tissue when UFH was introduced in the mucosal buffer, similar to inside the stomach, mounted in an Ussing chamber after 40

min of tissue stabilization. PD across the mucosa is created by the movement of ions from the mucosal to the serosal compartment or vise versa. The ionic composition of the mucosal and serosal buffers in this study was the same. Therefore, when UFH was added to the mucosal buffer, increase in PD negativity was observed which after a lag period decreased to the previous baseline. The pattern of changes in PD and other electrical parameters across the membrane on UFH addition to the mucosal buffer and the possible reasons for such behaviors have repeatedly been explained in detail in previous chapters (4 and 8) and are indicative of UFH movement across the gastric mucosa.

UFH movement across gastric mucosa was further confirmed by the presence of heparin in serosal buffer and the experimental mucosal tissue. Moreover, measurements of UFH anti-Xa and anti-IIa anticoagulant activities showed that UFH retains its anticoagulant activities while crossing the mucosal tissue intact. The integrity of UFH crossing the mucosa was supported by the lack of increase in the ratio of anti-Xa/IIa activities. Longer heparin chains are needed for anti-IIa activity than anti-Xa activity of UFH (Boneu, 2000). The possibility that large macromolecules can pass the mucosal barrier are in agreement with other clinical and animal studies (Reimann and Lewin, 1988; Catto-Smith, 1989) demonstrating initiation of a type I hypersensitivity reaction in susceptible subjects following antigen application to the gastric mucosal surface.

Use of pharmacological inhibitors (chapter 8) provided further evidence that the gastric mucosa, at acidic pH 4, samples macromolecular UFH from the mucosal to the serosal compartment via an active energy-dependent mechanism of transport using the cellular

microtubular network as well as sodium-coupled transport systems. Increase in PD negativity on UFH addition to the mucosal buffer did not reach baseline with time in the presence of an inhibitor of cellular ATP production (Varma et al., 2007); sodium fluoride (NaF, 0.1 mg/ml), an inhibitor of microtubular polymerization and endocytosis (Stollman et al., 2005); colchicine (1 μM), or an inhibitor of sodium channels (Kellenberger et al., 2003); amiloride (1 mg/ml). These changes in PD were supported by dramatic increases in short circuit current (Isc) from baseline at 124 min at pH 4.0. On the other hand, UFH is likely to be transported across the intestinal mucosa by passive diffusion (through the cell membrane) and/or paracellular pathway (through tight junctions) not seen in the stomach. This is supported by the ability of changes in PD to reach baseline at the end of the experiment on UFH addition to the mucosal buffer in the presence of pharmacological inhibitors like NaF in the mucosal and serosal buffers (chapter 8). As well, Isc did not change from baseline during the experiment at pH 7.4.

Changes in resistance of the tissue (R) as discussed in detail in chapter 4 was opposite to the pattern of changes in PD and indicate a role for passive diffusion in the transport of UFH. However our findings indicate that active transport is the dominant mechanism for transport of UFH across the gastric mucosa, which agrees with description of the gastric mucosa as a “tight epithelium”.

Our findings showed that decreasing the molecular size does not increase the rate of heparin transport across the gastric mucosa under acidic pH conditions. However, making the environment inside the stomach more basic facilitates the transport of LMWHs

compared to UFH. This was supported by significantly greater recovery of LMWHs than UFH from serosal buffer at pH 7.4. Moreover, significantly less LMWHs than UFH was trapped inside the experimental mucosal tissue at pH 7.4. Thus, while the basic environment appears to help passage of LMWHs through the gastric mucosa, UFH is transported better in an acidic environment.

Under physiological conditions the pattern of changes in PD on UFH addition to the mucosal buffer was similar between rat ileal mucosa containing Peyer's patches at pH 7.4 and gastric mucosa at pH 4.0 mounted in the Ussing chamber. There were increases in PD negativity of -0.6 ± 0.4 mV and -2.2 ± 0.1 mV on UFH addition to the mucosal buffer for ileum and stomach tissue respectively. There was a lag period before PD began to decrease to its baseline of 11.0 ± 5.1 min for ileum that was similar to 9.2±2.8 min for stomach. However, time to reach baseline after the lag period was significantly shorter when ileum was mounted in the Ussing chamber (26.0 ± 12.9 min) compared to the stomach tissue (40.5±7.9 min). This indicates faster movement of UFH through ileal mucosa than gastric tissue supported by significantly more UFH recovery from the serosal buffer of the mounted ileum (225.0 ± 41.5 μg) than the stomach tissue (111.3 ± 36 μg). Interestingly, less UFH was trapped in the experimental mucosal tissue (13.5 ± 1.1 μg) of the mounted ileum than the stomach (26.9±5.7 μg). Further evidence of faster movement of UFH through the ileal mucosa was obtained by measurement of rate of transport. Under physiological conditions ileum transported UFH at a rate of 1.6 ± 0.3 μg/1.5 ml/min/cm^2 at pH 7.4 versus 0.8±0.1 μg/1.5 ml/min/cm^2 by stomach tissue at pH 4.0.

Our results likely reflect the leakier nature of the intestinal epithelium versus tight gastric tissue. This is indicated by comparison of Isc measurements across ileal and stomach tissue. On UFH addition to the mucosal buffer, Isc showed a small increase from baseline by 1.1 ± 0.5 mA at 124 min with ileal tissue mounted in the Ussing chamber. This is compared to a dramatic increase in Isc of 4.2±1.5 mA from baseline when stomach was mounted. Based on these findings we can conclude that under physiological conditions, when the pH of the intestine is more basic and there are patches present versus highly acidic condition of the stomach with no patches, there may be a substantial passive component of UFH transport in the intestine that is not seen in the stomach.

UFH is a highly acidic carbohydrate. The carboxylate groups of heparin have a pKa between 2 and 4 (Casu and Gennaro, 1975) and thus one may expect heparin to be transported under acidic conditions present in the stomach faster than in the basic environment of the intestine. We were able to show that acidic mucosal pH helps the movement of UFH through gastric mucosa as well as ileal mucosa without patches. However, the importance of Peyer's patches in the intestine should not be underestimated. There was a lag period of 20.4 ± 5.0 min for PD in the absence of patches which was shortened to 11.0 ± 5.1 min in the presence of patches in ileum. Moreover, time to reach baseline was 92.6 ± 8.0 min in the absence of patches which was significantly different than 26.0 ± 12.9 min in the presence of patches. The importance of Peyer's patches in transport of UFH was also supported by the chemical recovery of heparin. In the absence of patches there was 80.0 ± 22.4 μg heparin recovered from the

serosal buffer and 21.5 ± 3.7 μg was trapped inside the tissue. However, when patches were present, a significantly greater amount of UFH, 225.0 ± 41.5 μg, was recovered from the serosal buffer and significantly less heparin of 13.5 ± 1.1 μg was trapped inside the tissue. Moreover, the rate of transport of UFH across the ileal mucosa without and with Peyer's patches was 0.6 ± 0.2 μg/1.5 ml/min/cm^2 and 1.6 ± 0.3 μg/1.5 ml/min/cm^2 respectively.

These patches are aggregations of the lymphoid tissue in the intestine and constantly are sampling antigens. If we consider the large number of patches present especially in the ileum as well as their nature of constant sampling and taking up materials from the luminal to the serosal compartment, one can appreciate their likely huge contribution to the UFH transport across the ileal mucosa despite the basic nature of the environment. As well, there are other factors that possibly contribute to more absorption of UFH across the ileal tissue versus stomach that include the larger surface area of the intestine and the longer period of time that food stays in the intestine than the stomach.

Our study implies that both ileum and stomach transport UFH in an intact form since there was no increase in the ratio of anti-Xa/IIa activities between mucosal and serosal buffers in each tissue. However, it is likely that a greater percentage of the total UFH is transported in a degraded form in ileum than in the stomach and a larger percentage is transported intact in stomach tissue. This conclusion is based on a larger ratio of anti-Xa/IIa activities in the serosal buffer of the mounted ileum compared to stomach although the ratio in mucosal buffer is more similar between ileum and the stomach. This

agrees with other investigators studying transport of proteins across the gastrointestinal tissue (Heyman et al., 1982; Isolauri et al., 1990; Keljo et al., 1985; Keljo and Hamilton, 1983; Marcon-Genty et al., 1989; Curtis et al., 1993; Curtis and Gall 1992). It was found that a smaller versus larger proportion of the total protein accumulated intact on the serosal surface of the intestine versus stomach.

In summary, rat gastrointestinal mucosa is capable of sampling luminal macromolecular carbohydrates from the mucosal to the serosal compartment. This process in the stomach occurs via an active energy-dependent mechanism that uses microtubular network of the cell as well as sodium coupled systems. The mechanisms by which heparin is transported across the ileal mucosa have not been investigated in this study. However, this study provided evidence of larger amounts of heparin being absorbed from the ileal mucosa than the stomach tissue under physiological conditions. The stomach tissue may be an initial site of absorption of orally administered heparin. When heparin is delivered to the intestine particularly the ileum, it likely is absorbed in larger quatities.

The study provides strong evidence of heparin absorption following oral administration and implies that the oral route may be a feasible alternative for administering heparin. Such oral heparin formulations would be extremely convenient and safe from the patient's point of view and insures adherence of patients to continued therapy as it facilitates the treatment in an outpatient setting. This likely defines a better health care alternative.

10.0 Future considerations

10.1 Vertical Diffusion Chamber Studies

Our results provided evidence that LMWHs are absorbed from the stomach mounted in a vertical diffusion Ussing chamber following oral administration. However movement is faster at neutral pH than under the acidic conditions found in the stomach. Further studies using the same model are required to understand how important gastric absorption of LMWHs may be in comparison to the small intestine. Moreover, considerable work is still required to evaluate mechanisms of absorption of oral LMWH including the contribution of passive and active transport from the stomach mucosa in contrast to the intestine.

We also investigated the importance of intestinal absorption of UFH particularly from the ileum compared to absorption from the gastric mucosa. We know about the predominant active transport mechanism of UFH absorption in the stomach under physiological acidic conditions. However, how heparin is transported across the ileal mucosa is unknown. Further studies using the same model are required to understand the mechanism(s) of UFH transport across the ileal mucosa with or without Peyer's patches under different pH conditions.

It is also interesting to know the possible involvement of the large intestine in the absorption of oral UFH or LMWHs. Colon tissue contains a number of Peyer's patches which may be involved in the absorption of oral heparins. As well, the involvement of

other parts of the small intestine such as duodenum and jejunum in the absorption of oral heparins should be investigated.

10.2 *In Vivo* Perfusion Studies

The Ussing chamber model that we used for our transport studies has some limitations as discussed earlier. A small surface area about 2.5-cm^2 of the gastrointestinal mucosa fits in our chamber which is significantly smaller than the whole stomach or intestine *in vivo*. There is no an active circulation to and from the tissue and therefore the time of tissue survival in the chamber is limited compared to *in vivo* situation. Removal of heparin from the serosal side by the circulation could also increase absorption rate. If heparin could be applied to the whole gastrointestinal tissue *in vivo* where there is an active circulation and an indefinite period of time for absorption to take place, a considerable amount of heparin may cross the mucosa. Oral heparin absorption could also be studied in an *in vivo* perfusion model. UFH, LMWH, or saline can be administered by stomach tube to rats and blood and endothelial cells collected by sequential perfusion with saline and collagenase and tested for chemical heparin as well as anticoagulant activities.

As well, a fluorescein-labeled UFH, fluorescein-labeled LMWH or saline can be administered to rats by stomach tube and, sections of stomach and intestinal tissue be examined by fluorescent microscopy for chemical heparin.

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