THE UNIVERSITY OF CALGARY

MACROMOLECULAR TRANSPORT IN THE STOMACH

by

Glenn Curtis

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The undersigned certify that they have read, and recommended to the Faculty of Graduate Studies for acceptance , a thesis entitled, "Macromolecular Transport in the Stomach" submitted by Glenn H. Curtis in partial fulfillment of the requirements for the degree of Master of Science.

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Abstract

We have previously demonstrated that the stomach is capable of mounting a Type I hypersensitivity reaction to luminal antigen challenge. These findings imply that movement of antigenically intact macromolecules occurs across the gastric mucosa. Rat gastric mucosa was mounted in Ussing chambers, and BSA (0.5 mg/ml) and ^{125}I-BSA (10 μCi) added to mucosal fluids. After equilibration, serosal fluids were sampled for two 30 minute periods, and fluxes of immunologically intact BSA (determined by an enzyme linked immunosorbent assay) and total BSA (^{125}I-BSA) calculated under basal conditions and in the presence of NaF, colchicine and 4^{o} C. Additional experiments examined macromolecular permeability in tissues sensitized to egg albumin by adding egg albumin (10 μg/ml) to both serosal and mucosal chambers. Immunologically intact BSA (21.3±4.5 ng/cm^2/30min) crossed the gastric mucosa as approximately one quarter of the total BSA flux (78.2±7.5 ng/cm^2/30min). The uptake of immunologically intact BSA was significantly reduced by NaF, an inhibitor of ATP production and endocytosis, colchicine, which inhibits polymerization of cytoskeletal microtubules, and at 4^{o} C, which acts as a general metabolic inhibitor. The transmural passage of antigen was not significantly altered by IgE mediated anaphylaxis. These findings indicate that intact protein antigens cross the gastric mucosa. This appears to be an active energy dependent mechanism that utilizes the microtubular network.

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My Mom & Dad.....who not only provided me with support and inspiration, but also with gas money!

My babe.....the Libster!!!!!!!

"There is a theory which states that if ever anyone discovers what the universe is for and why it is here, it will instantly disappear and be replaced with something even more bizarre and inexplicable."

"There is another theory which states that this has already happened!"

Douglas Adams

List of Abbreviations

ATP	adenosine triphosphate
BSA	bovine serum albumin
EA	chicken egg albumin
G	conductance
HRP	horse radish peroxidase
IgE	immunoglobulin E
IgG	immunoglobulin G
I_{sc}	short-circuit current
PBS	phosphate buffered saline
Pd	potential difference
RMCP II	rat mast cell protease II
TBS	tris buffered saline.

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I. INTRODUCTION

The absorption of macromolecules has been extensively examined in the intestine (Walker 1981, Walker 1987), but the permeability of the gastric mucosa to antigenically intact proteins has been relatively overlooked. This oversight may be due to the traditionally accepted view that the stomach is an organ of primarily secretory function, with little or no absorptive capacity.

We recently demonstrated that the stomach plays a potentially important role in the pathogenesis of food allergies (Catto-Smith 1989). When a luminal antigen is perfused in vivo through the stomach of a rat sensitized to that antigen, there is an increase in gastric acid secretion, and a delay in gastric emptying (Catto-Smith 1989). Teleologically, these responses are prudent, as increasing acid secretion and delaying emptying will maximize antigen breakdown before entry into the bowel. A corollary to these findings is that protein antigens may be crossing the gastric epithelium immunologically intact and interacting with immune cells in the lamina propria where the reaction is triggered. The aim of the present study was to:

1. quantify the passage protein (both immunologically intact as well as degraded) across the rat gastric mucosa in vitro,

2. determine the pathway and mechanisms of protein uptake by altering cellular metabolic and cytoskeletal function, and

3. determine if the uptake of intact protein is altered during an allergic reaction.

II. BACKGROUND

A. INTESTINAL AND GASTRIC IMMUNOLOGICAL REACTIONS

Food allergy is a frequently invoked but controversial clinical diagnosis (Bock 1980). It is described as is a form of food intolerance in which there is an abnormal immunological reaction to food. One mechanism where by this occurs is the immediate or Type I immunoglobulin E (IgE) dependent hypersensitivity reaction (Gleich 1980). In this reaction, primary exposure to a food protein triggers the immune system to produce IgE antibodies directed against dietary protein epitopes. The Fc portion of these immunoglobulins bind to mast cell Fcε receptors with high affinity ($K=10^{-9}M$, Conrad 1975). On subsequent exposure to the antigen, IgE antibodies on the surface of mast cells are cross linked, and degranulation of the mast cell is triggered (Ishizaka 1984).

Experimental work in this field has shown that rats sensitized to egg albumin (EA) develop an intestinal IgE mediated anaphylactic reaction on subsequent intraluminal exposure to EA. To date, acute antigen challenge has been linked with alterations in morphology and water and electrolyte transport (Perdue 1984a, 1984b, 1986). These abnormalities were associated with mucosal mast cell degranulation and mediator release (Perdue 1984a, 1984b, 1985, 1986). Additional recent work has demonstrated that rat mast cell protease II (RMCP II), a protease unique to the mucosal mast cell, is also released during anaphylactic reactions (Patrick 1988). When this protease is found in the serum, it serves as a specific

marker of mucosal mast cell degranulation (Patrick 1988, Miller 1983).

New data has shown that in addition to roles in food storage and breakdown, the stomach is capable of mediating immunological events. Catto-Smith et al (1989) demonstrated that when the stomach of a rat sensitized to egg albumin (EA) was perfused in vivo, gastric acid secretion increased significantly when EA was added to the perfusate. Non-sensitized, sham-treated rats showed no significant alteration in acid secretion with the addition of EA to the perfusate. This response was specific for the sensitizing protein, as no potentiation of acid secretion was seen after the addition of bovine serum albumin (BSA). In a separate series of experiments, ^{51}Cr was instilled into the stomach of conscious rats, and the rate of movement into the intestine measured. When EA was installed with the radioactive marker, sensitized animals showed a significant reduction in gastric emptying compared to controls. Evidence was provided that this response was an IgE/mast cell mediated Type I (immediate) hypersensitivity reaction. The acid secretory response to EA challenge was duplicated in naive animals that received immune serum from sensitized animals. Heat treatment of immune serum to 56° C for 30 min prior to transfer denatures IgE (Augustin 1979), and when performed prior to immune serum transfer, abolished the acid response. Mucosal mast cell activation in the gastric mucosa of sensitized/challenged animals was demonstrated by histological evidence of degranulation, increased luminal histamine and a significant increase in serum levels of RMCP II.

One clinical report in the literature has examined gastric mucosal reactions

in patients with food allergies (Reimann 1988). Thirty patients with proven allergies and 20 healthy controls had allergens applied directly to their gastric mucosa and monitored with an endoscope. All ectopic individuals developed pathological reactions that included swelling and hemorrhagic lesions. These appeared 30 min to 2 hrs following antigen application. Biopsies were removed before and after allergen addition, and examined for mast cell number and histamine content. Control volunteers showed no alteration in these parameters, but allergic individuals had significant reductions in both mast cell numbers and tissue histamine content, both indicators of mast cell degranulation.

The mucosal mast cells that mediate Type I hypersensitivity reactions in the stomach are located in the gastric lamina propria. In order for luminal antigen to reach and activate this population of cells, it must first penetrate the gastric epithelium while remaining immunologically intact. The finding that mast cell activation can be triggered by a luminal challenge indicates that intact antigen is crossing the gastric epithelium. This has not yet been described in the stomach <u>in vitro</u>.

B. MECHANISMS OF MACROMOLECULAR UPTAKE

The gastrointestinal epithelium has an important protective role in preventing the passage of a variety of potentially damaging acids, enzymes, undigested foods, bacteria, and bacterial byproducts to underlying tissues. Despite this, the uptake of intact proteins from the lumen of the small intestine has been well documented under both normal and pathological conditions (Walker 1981, Walker 1987).

By conventional wisdom, protein uptake in the intestine occurs by active mechanisms at two sites. The first and largest tissue involved in antigen sampling is the normal gut epithelium. The enterocyte is equipped with a specialized apical surface (the brush border) designed for absorption. Proteins may bind to receptors located on the brush border and move to the bases of the microvilli where endocytosis occurs (Stahl 1985, Walker 1981, Hemmings 1981). As pits of brush border membrane invaginate, and endocytotic vesicles are formed, proteins remaining in solution may also be taken up (Hemmings 1981). Once internalized, endocytotic vesicles fuse with lysosomes, and proteins are degraded before expulsion at the baso-lateral surface (Walker 1981, Walker 1987). In this process, some protein escapes breakdown and crosses intact. This may result from incomplete degradation in the lysosomal vesicle (Stahl 1985), or from a small percentage of protein crossing by a separate non-degradative pathway (Heyman 1982). In vitro studies in adult rabbit jejunum and ileum have estimated that 1% to 6% of all protein crossing the mucosa remains antigenically intact, and the

remainder is degraded (Heyman 1982, Isolauri 1989, Marcon-Genty 1989).

The other tissue that actively samples proteins in the small bowel is the specialized epithelium containing membranous epithelial cells or "M" cells overlying intestinal lymphoid nodules. This structure, termed a Peyer's patch may represent an important mechanism for the sampling and monitoring of luminal contents by lymphoid tissues (Owen 1977). Three studies have reported conflicting results on the ability of this tissue to transport intact protein relative to the intestine. Keljo et al (1983, 1985) reported 2.5 and 3 fold increases in intact protein transport in intestinal segments containing Peyer's patches compared to segments without patches in piglet jejunum, while Isolauri et al (1990) showed that tissue containing Peyer's patches transported intact protein at an identical rate to normal intestine in the rabbit ileum.

In addition to the traditional pathways described above, several new ideas are emerging in the intestinal macromolecular transport literature. To date, the paracellular movement of ions through tight junctions is well described. Intercellular channels are negatively charged and hence cation selective (Madara 1989), and also that this pathway is carefully regulated (Madara 1988). Paracellular movement of macromolecules however, is a new area of speculation. Exactly how large negatively charged proteins move through this channel remains to be defined. Madara (1987) showed that when glucose or amino acids were instilled into the intestinal lumen, so called "tight junctions" physically opened as the enterocyte actomyosin ring contracted. This conclusion was based on several observations.

The addition of luminal nutrients caused a marked reduction in tissue resistance, attributed by the authors to junction opening. Additionally, transmission electron microscopy showed close apposition of tight junctions in fasted animals, and easily visualized dilatations in the area of apposition when either glucose or amino acids were present in the lumen. Speculating somewhat, spontaneous opening and closing of tight junctions may be a significant part of the intact antigen uptake pathway, whereas proteins taken up through enterocytes may be completely degraded. The macromolecular literature on paracellular protein movement is more anecdotal than quantitative and mechanistic. To date, ultrastructural studies have yielded conflicting results on macromolecular movement through tight junctions. The passage of HRP through tight junctions has been confirmed in the rat ileal mucosa while undergoing cholinergic stimulation (Phillips 1987), and shown not to occur in the hamster small intestine either with or without luminal glucose (Atisook 1991).

Finally, there are two very non-specific mechanisms that may play minor roles in the movement of macromolecules across the mucosa. The first comes from recent evidence showing that all cell types in the normal gastric mucosa and intestine can accumulate water-soluble markers not permeable to plasma membranes. This is postulated to occur from spontaneous wounding and resealing of the cells' apical membranes, creating temporary holes (McNeil 1989). Additionally, cell extrusion zones may play a minor role in the transmural uptake of macromolecules, despite recent evidence showing that the macromolecular

barrier is largely conserved at these sites (Madara 1990).

The mechanism(s) of macromolecular uptake in the stomach remains speculative at this point. There is no gastric equivalent of the Peyer's Patch, and few cell types in the gastric mucosa possess an apical brush border. The majority of cells with this specialization are endocrine cells that possess a small apical microvillus and a broad basal surface containing numerous granules (Ito 1981). One exception is the Tuft cell of the rat gastric corpus (Isomaki 1973). It has a strongly developed microvillus membrane, a cyto-canicular network and an enzyme pattern similar to that of the gut enterocyte, and a lack of basal secretory granules. To date, descriptions of this cell have been based solely on morphology, and no functional studies have verified a role in active nutrient uptake.

III. DEVELOPMENT OF EXPERIMENTAL PROTOCOL

This chapter supplements the methods described in Section IV, and provides additional background on the preliminary experiments that led to the final BSA transport protocol. The goal of these experiments was to develop a system for measuring the movement of BSA across the rat gastric mucosa in vitro. Briefly, rat gastric mucosa with the muscularis externae removed was mounted in modified Ussing chambers. A protein (bovine serum albumin: BSA) was included with mucosal bathing solutions. Serosal fluids were removed periodically, and assayed for BSA. Preliminary work focused on establishing a sensitive, reproducible and accurate assay for measuring BSA and then adapting a Ussing chamber apparatus to maximize BSA transport and detectability. Integrating the assay buffer systems and non-specific protein blockers with those used in the Ussing chamber experiments became an important factor in experimental accuracy. Finally, tissue viability in the system had to be assessed and the sampling periods adjusted such that experiments concluded before tissue degradation occurred.

A. BOVINE SERUM ALBUMIN (BSA) ELISA

In order to measure immunologically intact protein, an enzyme linked immunosorbent assay (ELISA) was developed. We began with a generic 2 step "sandwich" ELISA protocol, and adapted it to measure BSA. As is schematically represented in Figure 1, 96 well ELISA plates (Dynatech Immulon 2) were coated with rabbit anti-BSA IgG (Cappel 0202-0342) diluted 1:500 in phosphate buffered saline (PBS) containing 0.05% tween 20 (Sigma P-1379) and blocked with 10% egg albumin (Fischer A-388) in PBS/tween. Undiluted samples as well as BSA standards of known concentration were added (50 µl per well) and incubated for 1.5 h. After washing with PBS/tween, rabbit anti-BSA IgG horse radish peroxidase (HRP) conjugate (Cappel 3202-0342) diluted 1:500 in PBS/tween was added and incubated for 1.5 h. Following a final wash in PBS/tween, the peroxidase colour reaction substrate (O-phenylenediamine and H_2O_2) was added. Optical density (O.D.) of the wells was read using a Titretrek Multiscan plate reader at 490 nm, and from these values a standard curve was generated plotting the log of BSA concentration against O.D. (Figure 2). A best fit (least squares) linear regression equation was calculated for the linear portion of the standard curve (Figure 2), and sample O.D.'s were converted into BSA concentrations using this equation. The linear portion of the curve extends over 1.25 log units, and the lower limit of detectability was 10 ng/ml of BSA. All unknowns assayed with optical densities less than that of the 10 ng/ml standard are reported as having 0 ng/ml BSA.

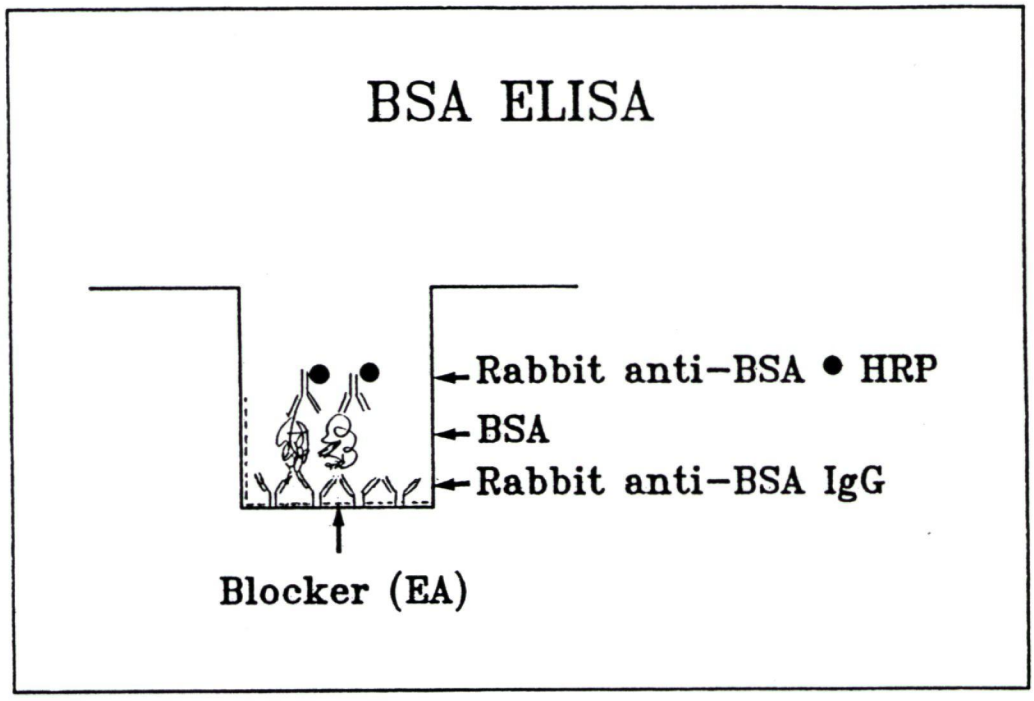

Figure 1. Cross-section through an ELISA plate well.

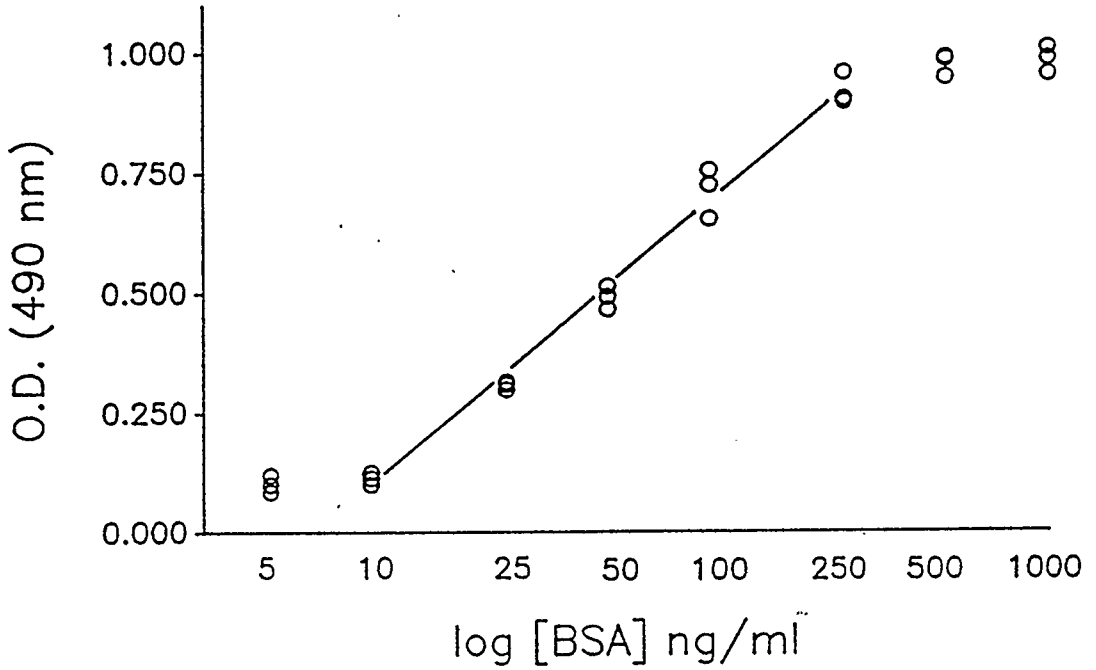

Figure 2. A representative BSA ELISA standard curve. The log of BSA concentration is plotted against optical density for triplicate BSA standards. The linear regression has been plotted for the linear portion of the curve.

After establishing a reproducible standard curve, the accuracy of the assay in our system remained to be determined. This was a particular concern as the buffers and blocking proteins used in the assay protocol including the standard curve, were different than those found in samples collected from the Ussing chambers. These differences are summarized in Table 1.

Table 1. Composition of ELISA assay and Ussing chamber buffers.

ASSAY BUFFERS	USSING CHAMBER BUFFERS
phosphate buffered saline	Kreb's buffer
(mono and dibasic salt/phosphate buffered)	(phosphate/bicarbonate buffered)
pH 7.2	pH 7.4
0.05% Tween 20	10 mM glucose
	oxygenated
	Ca, Mg
	50 μg/ml gelatin

In a preliminary series of experiments, standard curves were established wherein identical concentrations of BSA were diluted in each buffer. A representative experiment (Figure 3) shows that standards diluted in assay buffers had optical densities (O.D.) approximately 25% greater than those of corresponding samples diluted in the Ussing chamber buffers.

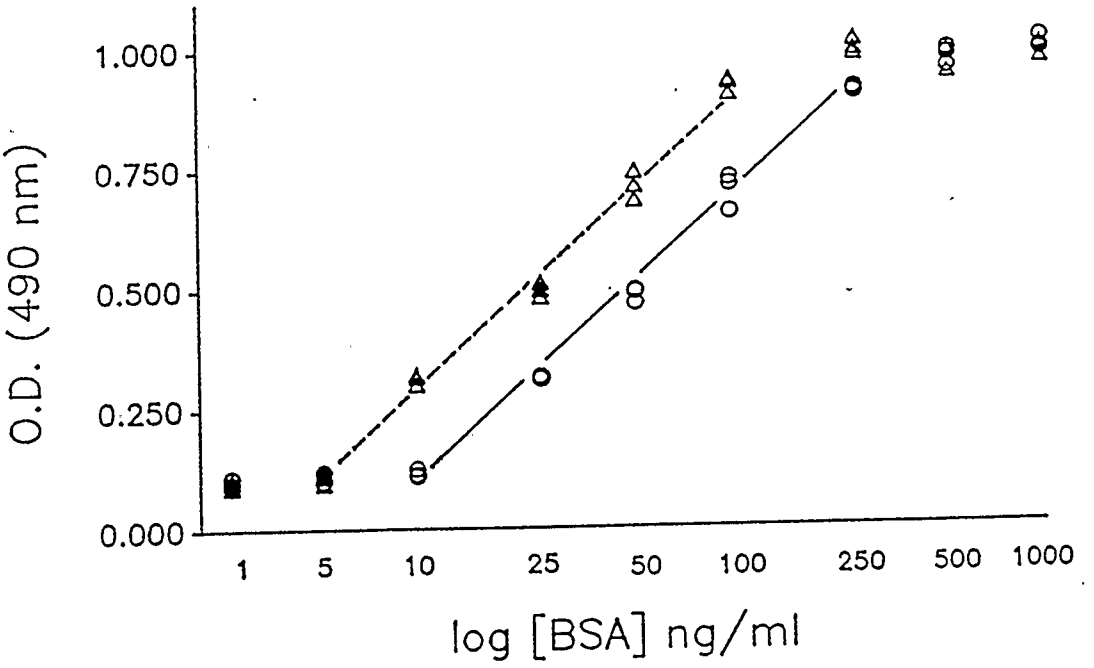

Figure 3. The effect of changing buffer composition on sample optical density. Samples diluted in the assay buffer (△--△) are compared with samples diluted in Ussing chamber buffer (O—O).

Another series of experiments were therefore performed to determine the cause of this shift. Standard curves diluted in Ussing chamber buffer (oxygenated Kreb's buffer containing 10 mM glucose, 50 μg/ml gelatin, pH 7.4) were compared to standard curves whose buffer composition was modified from the Ussing chamber recipe to resemble the assay buffer one variable at a time. The results are summarized in Table 2.

Table 2. The effect of exchanging, adding or removing Ussing chamber buffer ingredients on the standard curve.

EXPERIMENT	EFFECT ON STANDARD CURVE
exchange buffers	no effect
change buffer pH	no effect
remove glucose	no effect
remove oxygenation	no effect
remove Ca, Mg	no effect
remove gelatin	shift to right
add tween 20	shift to left

These experiments showed that tween 20 was responsible for the differences seen when standard curves were diluted in the two different buffers. While tween 20 increased the sensitivity of the assay, it could not be added to Ussing chamber fluids for tissue viability reasons. Therefore, subsequent standard curves did not include this ingredient. Additionally, it was noted that when gelatin, the non-specific protein blocker present in Ussing chamber fluids was removed, assay sensitivity was further decreased. In all subsequent experiments, assay accuracy was ensured by diluting assay standards in identical solutions to those found in the experimental samples (oxygenated Kreb's buffer containing 10 mM glucose, 50 μg/ml gelatin, pH 7.4).

Each experiment took two days to complete. The first day, tissues were mounted, and samples were collected. The assay was performed the next day. It was therefore necessary to examine how best to store the samples overnight to optimize BSA immunoreactivity. A series of test samples of known BSA concentration (50 ng/ml) were treated as follows and then assayed together on a single ELISA plate.

1. sample prepared fresh immediately prior to assaying,
2. sample flash frozen, stored at -70°C overnight, and thawed immediately prior to use,
3. sample stored at 4°C overnight and warmed to room temperature immediately prior to use, and
4. sample stored at 4°C for 48 h and warmed to room temperature immediately prior to use.

The results are shown in Table 3.

Table 3. The effect of sample storage on immunoreactivity. * $p<0.05$ compared to fresh sample. Results are mean ± S.E.

Sample Treatment	Time Until Assayed (h)	Reported [BSA] (ng/ml)
1. fresh	0	50.7±0.9
2. frozen	24	41.1±2.4*
3. 4°C	24	49.5±1.3
4. 4°C	48	16.8±5.7*

In all subsequent experiments, the samples were stored at 4° C for a maximum of 24 h from the time of collection until assayed.

B. USSING CHAMBER EXPERIMENTS

When this series of experiments was started, our laboratory had a well established Ussing chamber protocol designed for the study of ion and sugar transport. Our standard Ussing chamber exposes 0.4 cm^2 of tissue to 10 ml of fluid on each of the mucosal and serosal sides. The fluids are maintained at 37°C and oxygenated (95% O_2 and 5% CO_2) while circulating through glass water jackets. Once a reliable assay system had been established, the first experiment was to put a known concentration of BSA (50 ng/ml) into the chambers without tissue present and determine the recovery rate in samples collected every 30 min for 2 h. No detectable BSA was recovered from any chamber at the end of the first 30 min period (n=8), indicating that adsorption of BSA to the glass water jacket was a likely problem. Similar observations were made when a non-specific protein blocker (gelatin 50 μg/ml) and/or a silicone coating (Sigmacote) was added. The equipment was redesigned based on the following criteria:

1. material must be resistant to BSA adsorption,
2. chamber fluid volume must be small to minimize dilution of any BSA crossing,
3. maximize transporting surface area of tissue.

The adsorptive capacities of three materials were compared; glass, teflon, and lucite. Preliminary studies showed that adsorption was temperature dependent, so all tests were performed at 37°C by once again adding 50 ng/ml BSA with or without 50 μg/ml gelatin to the vessels. Solutions were sampled every 30 min for two h, and BSA recovery assessed as described above. These tests showed lucite to be the material of choice. New chambers were therefore designed and built from lucite. They exposed 1.2 cm^2 of tissue to 3 ml of fluid on each side. This yielded a theoretical BSA concentration increase of 10 fold over the standard design (0.4 cm^2 x 10 ml). It must be noted that without this amplification, transmural movement of BSA would have been below the limits of detectability of the assay system for the duration of the 2 hr experiment.

C. IN VITRO PROTOCOL

The initial objective was to measure the passage of immunologically intact BSA (measured by ELISA) from the mucosal compartment where BSA was in high concentration (0.5 mg/ml) to the serosal compartment *with several caveats*. First, trauma to the tissue during stripping and mounting had to be minimal, and second, tissue degradation over the two h experimental period was a concern. A number of parameters were examined to assess these questions. In the first series of experiments, light microscopy was performed on stripped tissues to confirm that the muscularis externae was consistently removed and to verify the integrity of the remaining mucosal sheet. Immediately after stripping, tissues were placed in Carnoy's fixative. The next day, samples were removed, dehydrated through graded alcohols, and embedded in plastic resin (Polysciences 17324). Two micron sections were cut and stained with Lee's methylene blue-basic fuchsin (Bennett 1976). As is shown in representative photomicrographs (Figure 4), stripping the stomach consistently removed the muscularis externa, but did not compromise the muscularis mucosae or the mucosa.

The first assessment of tissue function in vitro involved monitoring the electrical properties of the mucosa. For the duration of the experiment, the spontaneous transepithelial potential difference (Pd) was measured. The tissue was clamped at zero volts by continuous application of an appropriate short-circuit current (I_{sc}) using an automatic voltage clamp apparatus (DVC-1000; World

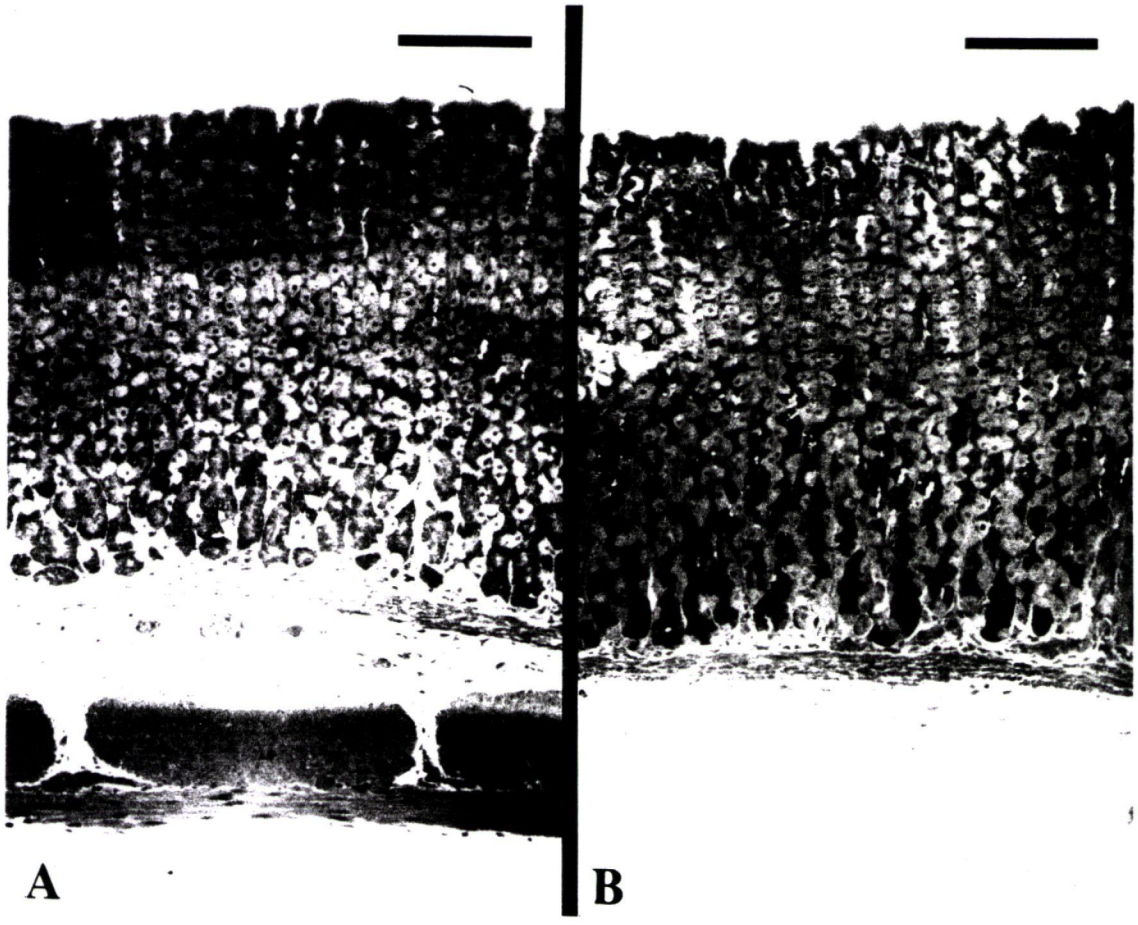

Figure 4. Photomicrographs of rat gastric corpus (A) before and (B) after removal of the muscularis externa. Note the complete removal of the muscularis externa, and that the mucosa and muscularis mucosae are not damaged. Bar represents 100 μm.

Precision Instruments). At all sampling times (0, 30, 60, 90, 120 min after mounting), the I_{sc} was removed for 5-10 s, and tissue opened Pd recorded. Tissue conductance (G; mS/cm^2) was calculated from Pd (mV) and I_{sc} ($\mu A/cm^2$) using Ohm's law (Clarkson 1964). Conductance is the inverse of resistance, and an indicator of net ionic movement across the tissue. Increased conductance was indicative of elevated ionic movement, possibly reflecting tissue breakdown. The first group of experiments showed that tissues damaged in stripping or mounting displaying high conductances (50-100+ mS/cm^2). These were easily spotted both visually and electrically, and not included. Examination of tissue degradation was a more complicated problem. A representative I_{sc} tracing is shown in Figure 5, and mean electrical results are summarized in Table 4.

Immediately after mounting, I_{sc} and Pd were low and conductances were relatively high. In the first 25 minutes, I_{sc} and Pd rapidly rose and conductances stabilized. From this point on, there was a gradual decline in I_{sc} and Pd, and an increase in conductance. By 120 min, conductance values had increased to parallel those seen at time 0. This drift was indicative of tissue degradation, but these results alone were difficult to interpret, so additional parameters were examined.

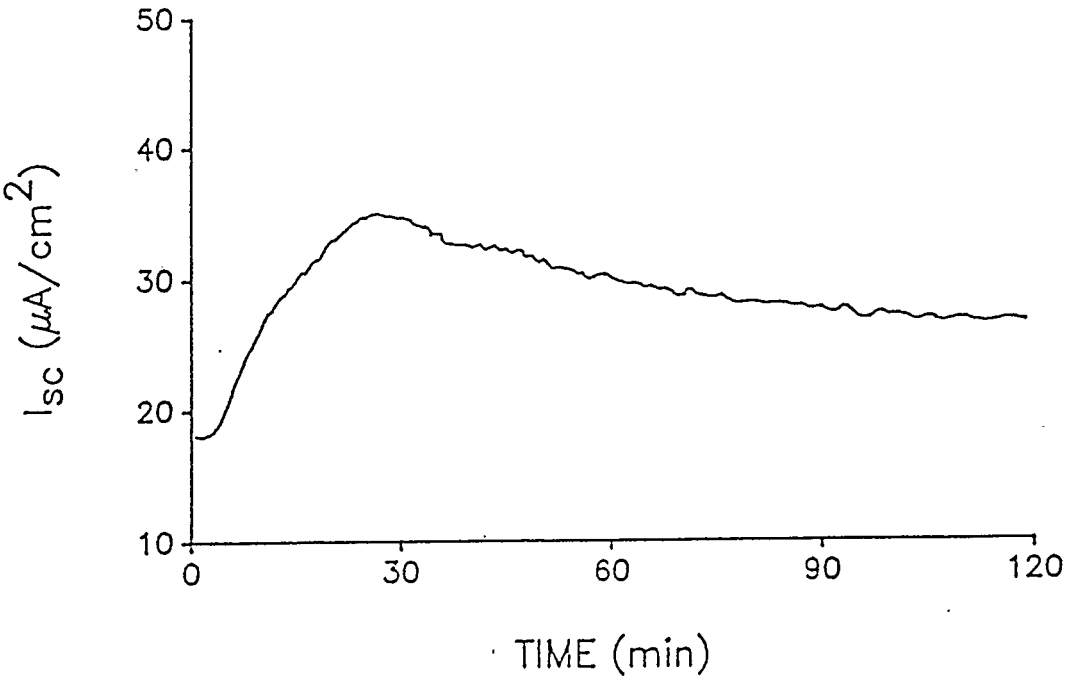

Figure 5. A representative short-circuit current (I_{sc}) tracing.

Table 4. Mean ± S.E. (n=7) 30 min short-circuit current (I_{sc}) values, potential differences (Pd), and conductances (G).

Time (min)	0	30	60	90	120
I_{sc} ($\mu A/cm^2$)	18.9±1.8	33.2±4.0	32.5±5.0	30.2±5.0	28.0±4.5
Pd (mV)	-1.5±0.3	-3.5±.7	-3.3±0.5	-2.7±0.6	-2.2±0.5
G (mS/cm^2)	15.5±2.2	11.7±2.0	12.6±2.2	13.8±2.1	15.4±2.2

Further evidence of tissue degradation was obtained from measuring the mucosal to serosal movement of antigenically intact BSA (Figures 6 & 7). Figure 6 shows a linear increase in serosal BSA concentration up to 90 min with a loss of linearity in the 90-120 min period. When this data was transformed into individual 30 min fluxes (Figure 7), several features became apparent. First, BSA transport reached steady state conditions in the middle two periods ($p=0.93$; 30-60 min to 60-90 min period). This occurs when two adjacent periods have equal fluxes, and indicates that there is no appreciable difference in tissue function or viability with respect to BSA transport over these periods. Without establishing steady state conditions, flux data is uninterpretable. Second, there was a significant rise in the last 30 min flux. There are two possible explanations for this increase. Although unlikely, there may have been an increase in the rate of active sampling in the fourth period. More likely, tissue degradation was becoming a significant factor by introducing an additional passive component to BSA transport. To assess this, a series of experiments were performed using a "non-specific" marker of gastric permeability, ^{51}Cr-EDTA (ethylenediamenetetraacetic acid, ICN 17226). This small lipophobic molecule (M.W. 360) has previously been used to measure intestinal (Ramage 1988, Behrens 1987) and gastric (Kvietys 1990) mucosal permeability. By adding 10 μCi of ^{51}Cr-EDTA to normal mucosal bathing fluids and sampling at standard 30 min intervals, we were able to calculate the amount crossing in each period as a percentage of the total mucosal radioactivity present (Figure 8).

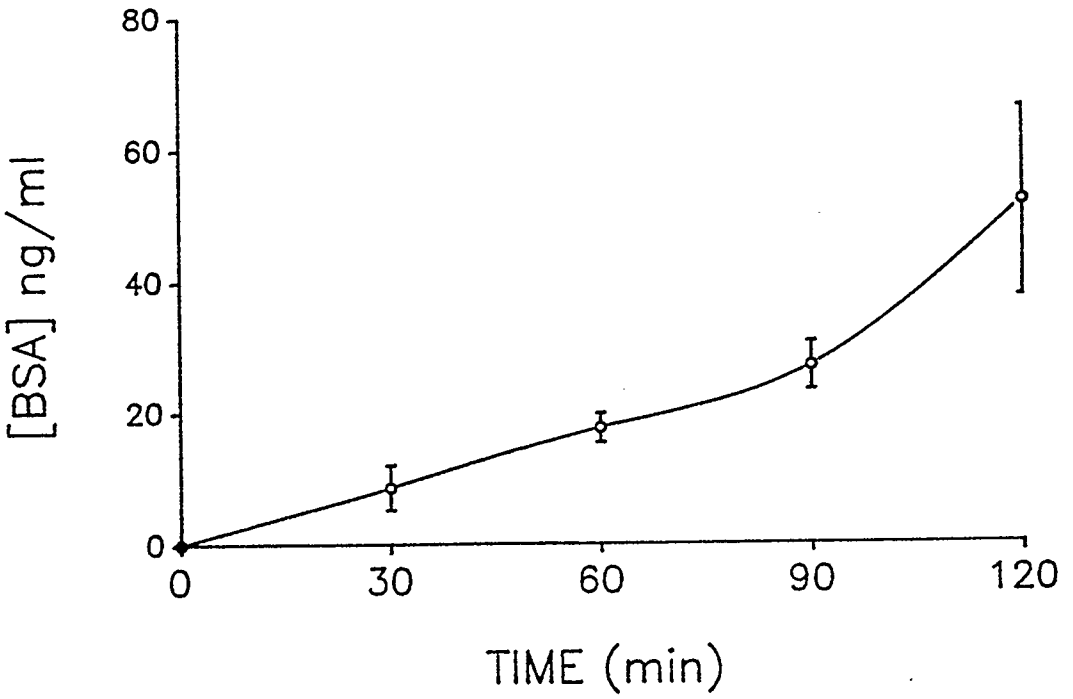

Figure 6. Serosal concentration of intact BSA as assessed by ELISA every 30 min for 2 h (n=7). Note the upturn of BSA concentration in the 90-120 min period. Results are mean ± S.E.

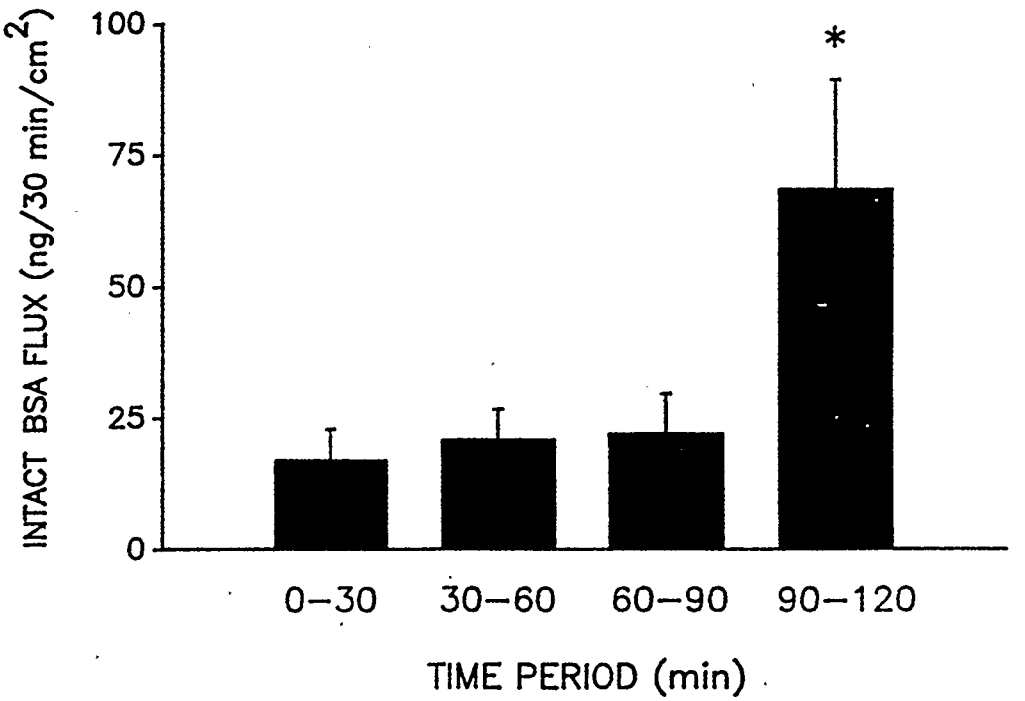

Figure 7. Intact BSA 30 min fluxes expressed as mean ± S.E. calculated from ELISA data (n=7). Note the similar magnitude of fluxes in the middle two periods, and the significant increase in the final period. * $p<0.05$ compared to all other periods.

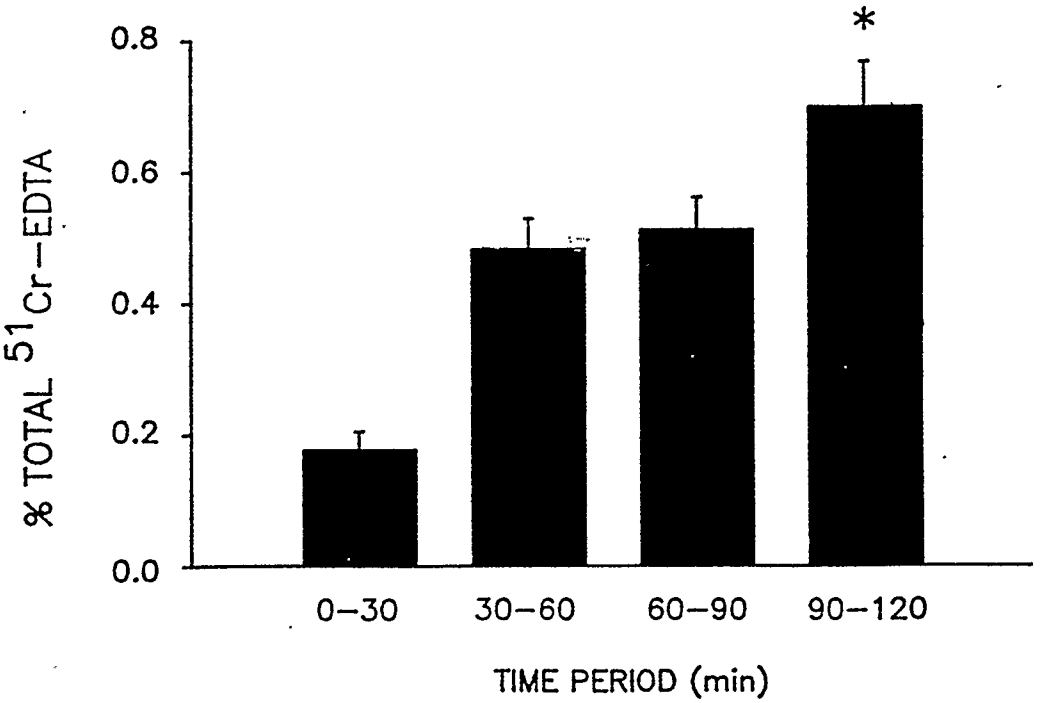

Figure 8. Percent of total ^{51}Cr-EDTA crossing in each 30 min period (n=8). Note that movement of EDTA was in steady state for the middle two periods, and significantly increased in the final period. * $p<0.05$ compared to all other periods. Results are mean ± S.E.

Reflecting the experiments with BSA movement across the mucosa, ^{51}Cr-EDTA movement was in steady state for the middle two periods ($p=0.37$; 30-60 min to 60-90 min period), and significantly increased in the final period. Additionally, it was possible to separate total EDTA movement (Figure 8) into energy dependent and energy independent components by metabolically inhibiting the tissue with 2 mM NaF (Figure 9). In this way, it was possible to not only assess overall non-specific permeability changes, but also to determine which hypotheses pertaining to the last period increase was correct.

Figure 9 shows that the active component of EDTA transport remained constant over the last three periods, and that it was the passive component of EDTA transport that significantly increased the overall EDTA flux seen in the last period. From these results, it became apparent that tissue degradation was a significant factor after 90 min, and that the increase seen in intact BSA transport during this final period resulted from an overall increase in permeability, and not from an upregulation of active transport. The fourth period was therefore excluded in subsequent experiments on the grounds that it included a significant in vitro artefact.

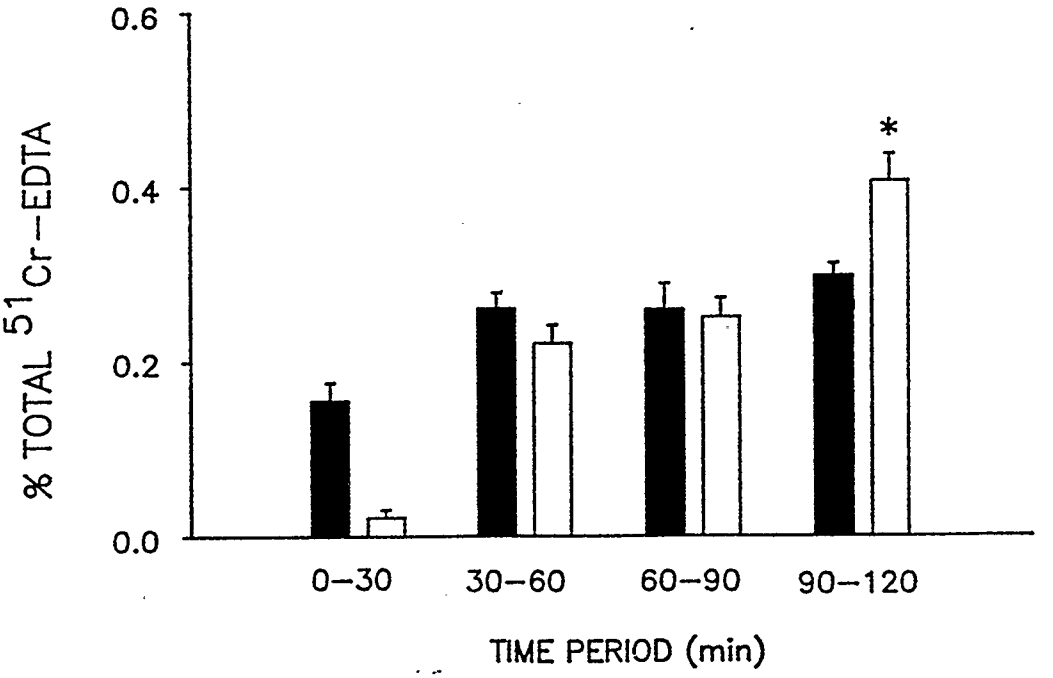

Figure 9. Active (■) and passive (□) components of ^{51}Cr-EDTA uptake (n=8). In addition to experiments measuring total ^{51}Cr-EDTA movement (Figure 8), experiments were performed with the metabolic inhibitor NaF. The passive data depicted above represent residual ^{51}Cr-EDTA movement in NaF experiments, and the active component was computed by subtracting passive ^{51}Cr-EDTA movement from the total movement (Figure 8) for each period. Note that the active component of EDTA uptake is similar for the last three periods, but that the passive component of uptake increases significantly in the last period. * $p<0.05$ compared to passive transport in all other periods. Results are mean ± S.E.

IV. METHODS AND MATERIALS

A. ANIMAL MODEL

Two groups of non-fasted Hooded Lister rats weighing 150-200 g were studied: unmanipulated controls and animals sensitized to chicken egg albumin (EA). From birth to the point of study, rats were maintained on a diet free of EA and bovine serum albumin (BSA). Rat sensitization followed a previously established protocol (Perdue 1984a). Briefly, an outbred strain of rat (the Hooded Lister) was selected due to its ability to develop an IgE response to protein antigens. The protein, egg albumin (EA; Sigma grade V, A-5503) was bound to an adjuvant, and minute quantities were administered by intraperitoneal injection. The adjuvant chosen (aluminum potassium sulfate, Fischer A-601) is an immunostimulant that selectively drives the immune response toward IgE production (Jarrett 1978). Animals were studied 14 days after sensitization when IgE titers peaked. On the day of study blood was taken for determination of anti-EA IgE titers by passive cutaneous anaphylaxis (Ovary 1964), and a minimum titer of 1/64 was considered acceptable. Animal care and procedures followed the guidelines of the Canadian Council of Animal Care.

B. EXPERIMENTAL PROTOCOL

Immediately after cervical dislocation, the stomach was removed, opened along the lesser curvature, and flushed with Kreb's solution. The opened stomach was pinned out mucosa down and immersed in oxygenated Kreb's buffer. Kreb's buffer contained 115 mM NaCl, 8 mM KCl, 1.25 mM $CaCl_2$, 1.2 mM $MgCl_2$, 2 mM KH_2PO_4, and 25 mM $NaHCO_3$. To separate the mucosa from the muscularis, Kreb's buffer was injected under the muscularis along the edges of the tissue. This allowed the muscle layer to be removed as a sheet. Stripped corpus was mounted in short-circuited lucite Ussing chambers that exposed 1.2 cm^2 of tissue to 3 ml of Kreb's solution bubbled with 95% O_2 and 5% CO_2 on each side. Temperature was maintained at 37° C with the exception of one series of experiments which examined the effect of 4° C on macromolecular uptake. The mucosal solution contained 10 mM mannitol, 0.5 mg/ml BSA (Sigma A-9647), and in experiments assessing the total movement of BSA, 10 μCi ^{125}I-BSA (ICN 68031). Serosal solutions contained 10 mM glucose and 50μg/ml gelatin (a non-specific protein blocker; Bio-rad 170-6537). To monitor tissue viability, the spontaneous transepithelial potential difference (Pd) was measured for the duration of the experiment. The tissue was clamped at zero volts (short-circuited) as previously described. In experiments examining pharmacological inhibition of macromolecular uptake, tissue was preincubated with the drug during the stripping process (approximately 10 min) and exposed to the drug on both serosal and mucosal

surfaces for the duration of the Ussing chamber experiment. The effect of 2 mM NaF (Sigma S-1504) and 10 mM colchicine (Sigma C-9754) was examined. To induce gastric anaphylaxis in sensitized animals, EA (Sigma A-5503; 1 μg/ml) was added to both mucosal and serosal chambers.

Transmucosal movement of BSA was assessed over three 30 min periods; a 30 min equilibration period followed by two 30 min periods in which fluxes of both intact and total BSA passage were calculated. Samples, 200 μl, were obtained from the serosal reservoir (the "cold" chamber) at 30 min intervals starting immediately after the chambers were set up, and from the mucosal chamber (the "hot" chamber) at the start and end of the experiment. Samples for measurement of immunologically intact BSA were maintained at 4^{o} C for a maximum of 24 h until assayed by ELISA for BSA content. Samples for measurement of total mucosal to serosal BSA movement (immunologically intact + degraded BSA) were assayed for ^{125}I-BSA by scintillation spectrometry, and fluxes were calculated for individual 30 min periods, based on the difference in cold side counts per min over the 30 min period (C_2-C_1), the concentration of BSA present in the hot chamber, the chamber volume, the sampling time, the tissue area, and the hot side counts per min as follows:

$$\frac{(C_2\text{-}C_1 \text{ cpm}) * \text{concentration BSA ng/ml} * \text{chamber volume ml}}{\text{total hot counts cpm} * 30 \text{ min} * \text{tissue area cm}^2}$$

Fluxes are expressed as ng/30 min/cm^2. In order to determine the percentage of

BSA degraded in transmural passage, values obtained from ELISA experiments were converted from concentrations to 30 min fluxes (expressed as ng/30 min/cm^2). In this way, the mean of each 30 min ELISA flux (*intact* BSA flux) could be directly compared to the mean of each 30 min ^{125}I-BSA flux (*total* BSA flux), and a percentage BSA degradation during transmural passage calculated for each 30 min period as follows:

$$\% \text{ DEGRADED} = (\text{TOTAL} - \text{INTACT}) / \text{TOTAL} * 100$$

Control experiments without the addition of BSA to the mucosal compartment were done to eliminate the possibility of our having obtained a false positive in the ELISA from chamber contamination with BSA or antibody cross-reactivity with other proteins present. In experiments examining the effect of gastric anaphylaxis on BSA permeability, 500 µl of additional chamber fluid was collected at 90 min and frozen until assayed for RMCP II by ELISA as previously described (Miller 1983).

C. SDS-PAGE, IMMUNOBLOTTING and AUTORADIOGRAPHY:

To confirm that BSA measured by ELISA was intact after transport across the mucosa, serosal samples were collected at 90 min and an immunoblot performed. Samples were separated on a 10% non-denaturing linear gradient

polyacrylamide gels (Laemmli 1970, Hedrick 1968). Molecular weight markers (Sigma MW-ND-500) ranging from 14.2 kDa to 132 kDa, freshly prepared BSA, and experimental samples were run concurrently. Gels were electrophoretically transferred onto nitrocellulose paper (0.2 μm pore size) using a BioRad transblotting apparatus. Transblotts were performed overnight at 4° C using a constant 30 volts. Ninety min before stopping the procedure, the power was increased to 70 volts. For immunoblotting, nitrocellulose sheets were blocked with 10% EA (Fischer A-388) in tris buffered saline (TBS) containing 0.05% tween 20 (Sigma P-1379) for 2 h. Next, the transblot was incubated with rabbit anti-BSA HRP conjugate (Cappel 3202-0342 diluted 1:250 in TBS/tween) for 1.5 h. Following three 10 min washes, the HRP colour reaction was performed using a commercial kit (BioRad 170-6431).

To verify that the radiolabel used to assess total BSA movement (^{125}I) remained coupled to BSA after 90 min in the mucosal chamber, mucosal ("hot" chamber) samples were collected at the conclusion of the experiment. As described above, experimental samples were separated on 10% non-denaturing linear gradient polyacrylamide gels concurrently with molecular weight markers and freshly prepared ^{125}I-BSA. In order to visualize ^{125}I, the gel was autoradiographed for 2 h with Kodak X-OMAT film.

D. STATISTICS

Results were analyzed by a Student's *t* test where applicable, and by a one-way analysis of variance (ANOVA) with post hoc comparison by Newman-Keuls if more than two groups were examined. Differences were considered different at $p < 0.05$ unless otherwise stated. All results are expressed as mean ± S.E.

V. RESULTS

BSA transport data for normal stomach tissue are summarized in Figure 10. All negative control experiments (without mucosal BSA; n=5) failed to show any evidence of BSA transport, indicating that there was no non-specific contamination of the chamber apparatus by BSA or other proteins that are recognized by the antibodies used in the BSA ELISA. When the mucosal chamber contained BSA (n=7), there was a consistent linear accumulation of BSA in the serosal compartment in all experiments over the initial 30 minute equilibration period and in both subsequent flux periods. Converting the ELISA results into individual 30 min fluxes allowed quantitative comparison between the two flux periods (Figure 11). BSA fluxes were stable in the 30-60 min and 60-90 min periods indicating "steady state conditions". The average flux for these two periods was 21.3±4.5 ng/30 min/cm^2. To visualize antigenically intact BSA after mucosal passage an immunoblot was performed on serosal samples that were collected at 90 min and for comparison, on freshly prepared samples containing BSA (Figure 12). Antigenically intact BSA in freshly prepared samples and in 90 min serosal samples was visualized as a single band at 66.2 kDa.

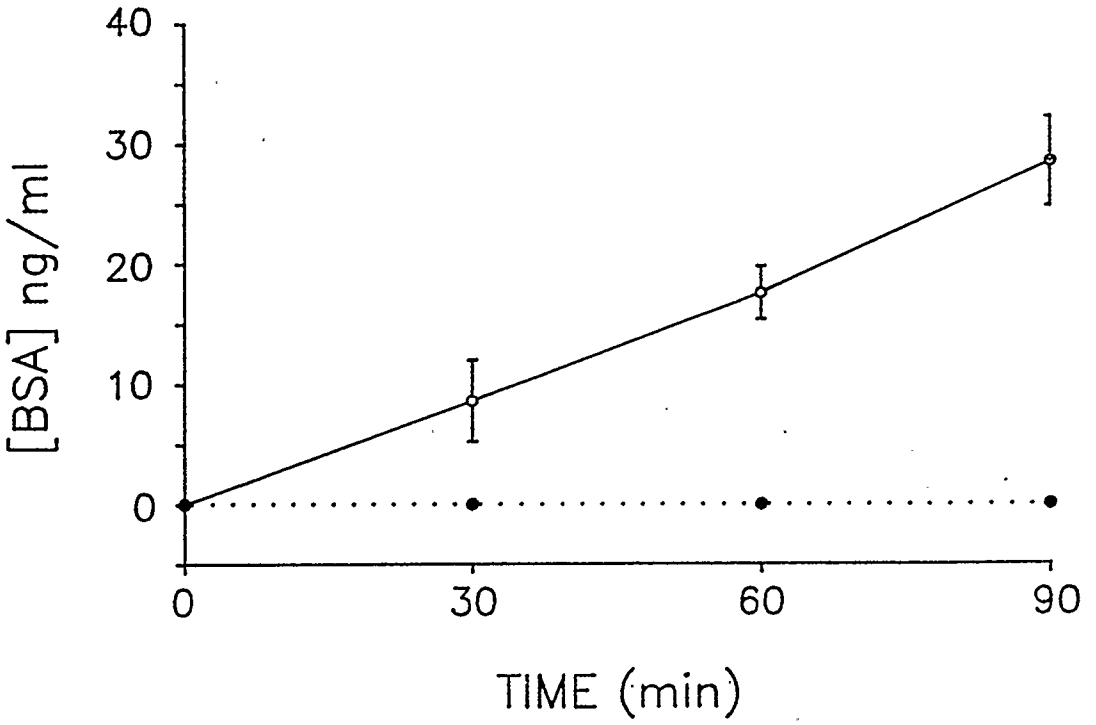

Figure 10. Accumulation of antigenically intact BSA in the serosal compartment (o—o). BSA concentration, measured by ELISA, is plotted as mean ± S.E. over 90 min. Negative controls are experiments without the addition of BSA to mucosal fluids (●···●).

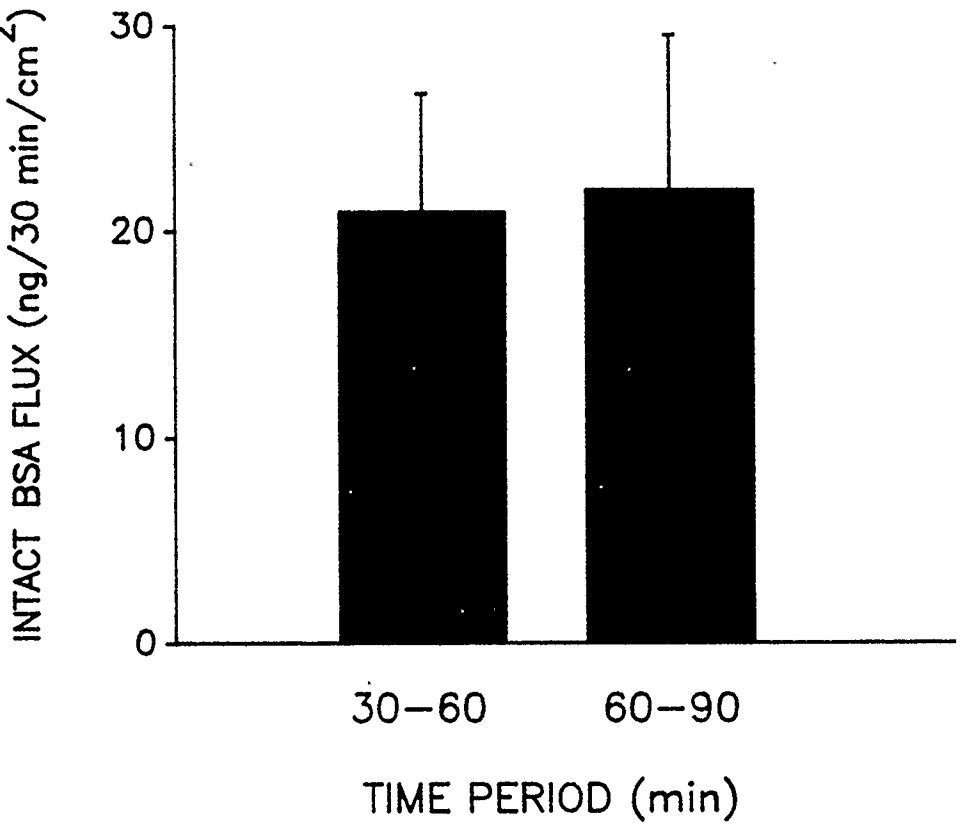

Figure 11. Serosal accumulation of intact BSA measured by ELISA is plotted as individual 30 min fluxes (ng/30 min/cm^2). Results are mean ± S.E.

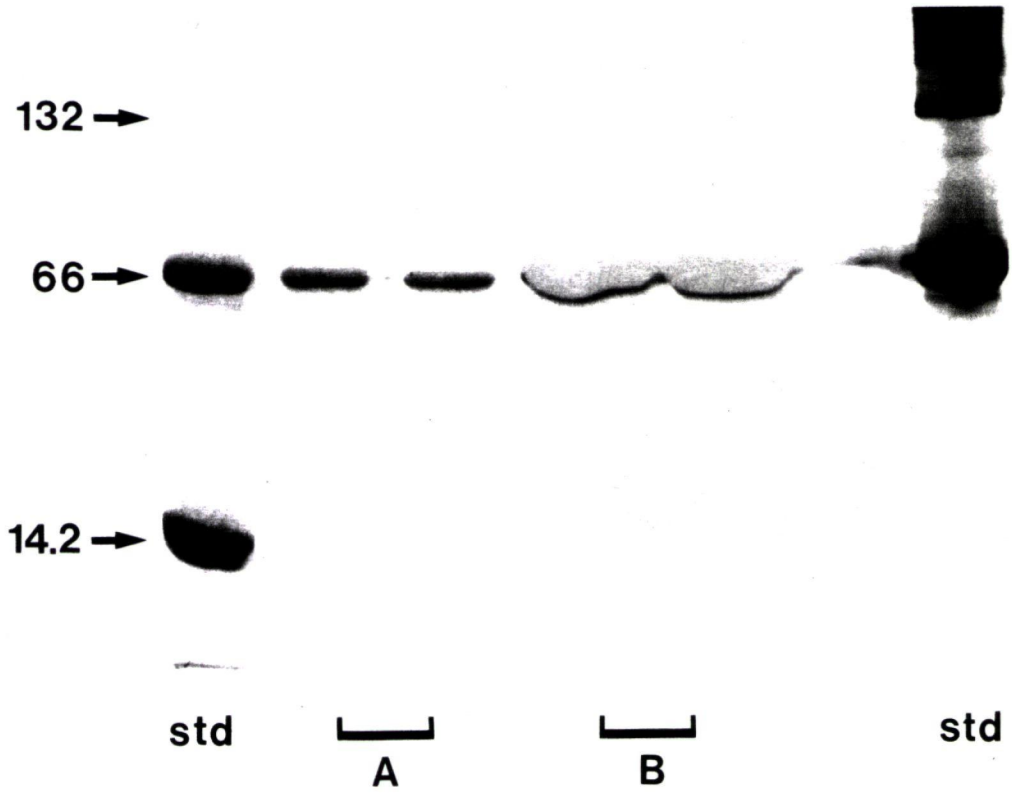

Figure 12. Immunoblot of BSA collected (A) fresh (immediately after preparation) and (B) from the serosal chamber after 90 min. Immunologically intact BSA is visualized as a 66.2 kDa band in both fresh and 90 min serosal samples.

Total BSA movement across the gastric mucosa is shown in Figure 13. Total fluxes, measured by ^{125}I-BSA addition to mucosal bathing fluids achieved "steady state conditions" in the two flux periods. The average flux for these periods was 78.2±7.5 ng/30 min/cm^2. To ensure that the radiolabel was still associated with BSA after 90 min in the mucosal chamber, mucosal fluids were gathered at the completion of the experiment, run on a non-denaturing gel, and autoradiographed. Figure 14 shows that all radioactivity in the chamber was visualized as a band of similar molecular weight to the BSA standard. No free ^{125}I was seen at the ion front, or in association with smaller molecular weight proteins. Comparison of total BSA movement to transport of antigenically intact BSA is shown in Figure 15. When averages were calculated for the two flux periods, transport of antigenically intact BSA represented 27% of the total movement of BSA across the gastric mucosa.

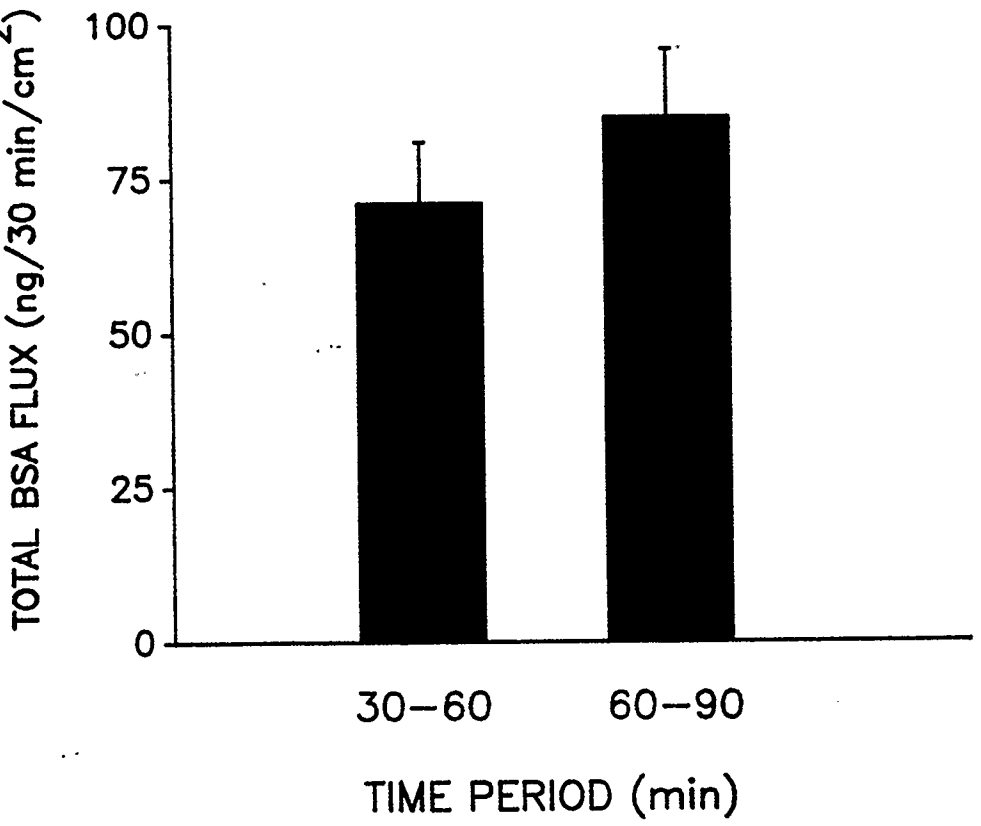

Figure 13. Total transmural movement of BSA (both intact and degraded) measured by the passage of radiolabelled BSA across the gastric mucosa. Fluxes are in ng/30 min/cm^2, mean ± S.E.

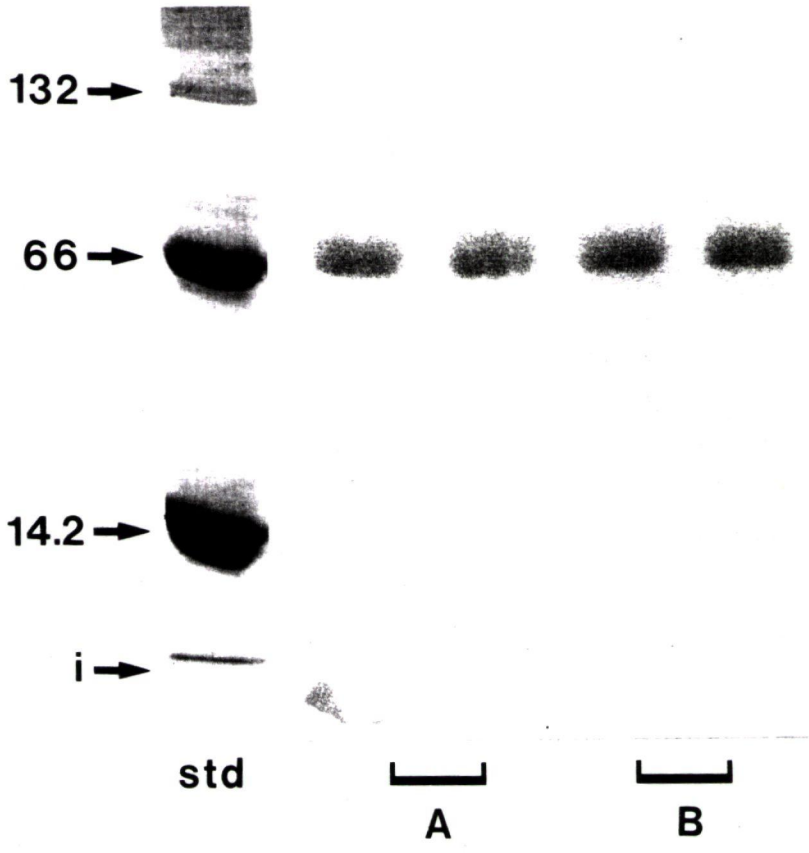

Figure 14. Autoradiograph of fresh ^{125}I-BSA (A) and samples collected from the mucosal ("hot") chamber after 90 min (B). Radioactivity remained associated with a 66 kDa protein (BSA) for the duration of the experiment. No free radioactivity was seen at the ion front (i), or in association with lower molecular weight proteins.

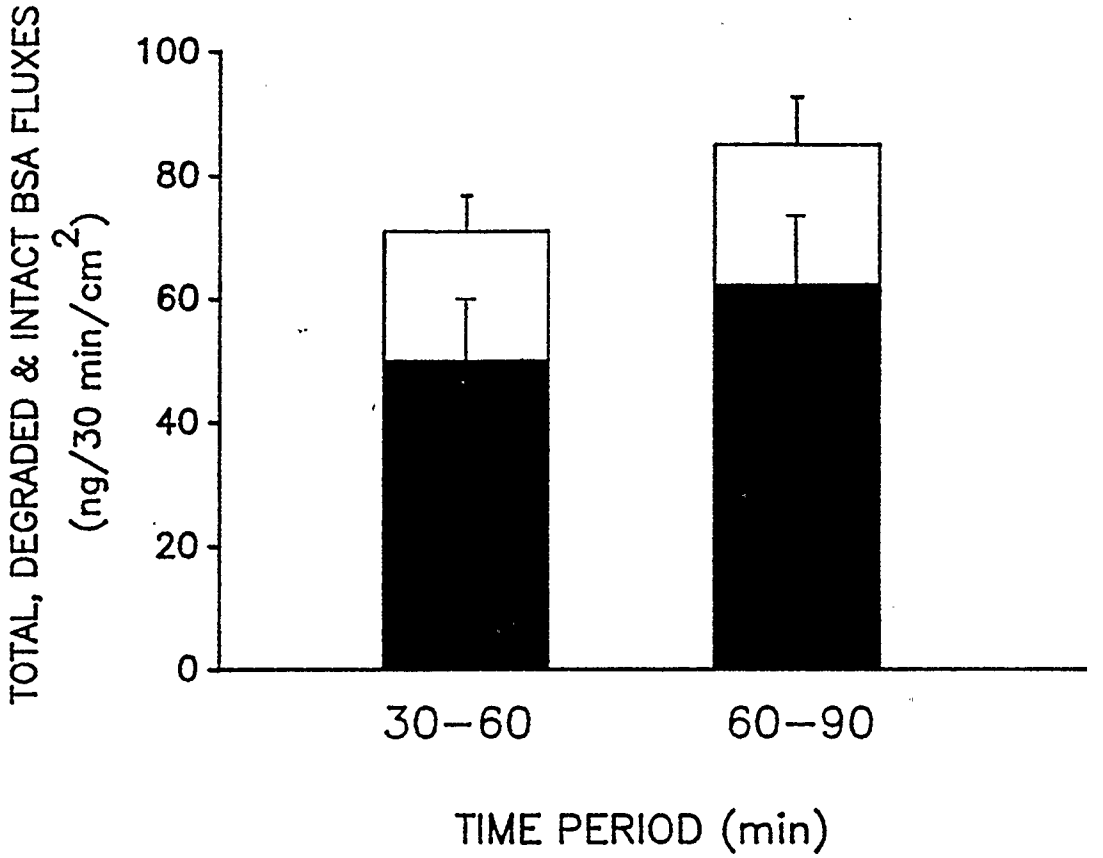

Figure 15. The total movement of BSA (□ + ■) is divided into intact (□) and degraded (■) BSA fluxes (ng/30 min/cm^2). Results are mean ± S.E.

To elucidate the cellular mechanisms involved in the transport of intact BSA, BSA fluxes were measured during pharmacologic and metabolic inhibition of tissue function (Figure 16). Gastric macromolecular uptake at 4^{o} C (n=6), a temperature which provides general metabolic inhibition and effectively abolishes endocytosis (Silverstein 1977, Keljo 1983) significantly inhibited transport of intact BSA. NaF (2mM) which inhibits endocytosis by lowering cellular ATP levels (Silverstein 1977, Keljo 1983) also markedly reduced transport of intact BSA (n=6). In addition, NaF (n=8) significantly inhibited the total BSA flux determined by ^{125}I-BSA movement (Figure 17). Colchicine (10mM) which prevents polymerization of microtubules and impairs the movement of endocytosed material from apical to basal membranes (Silverstein 1977, Keljo 1983) significantly reduced the passage of intact BSA into the serosal compartment (n=7) in both flux periods (Figure 16).

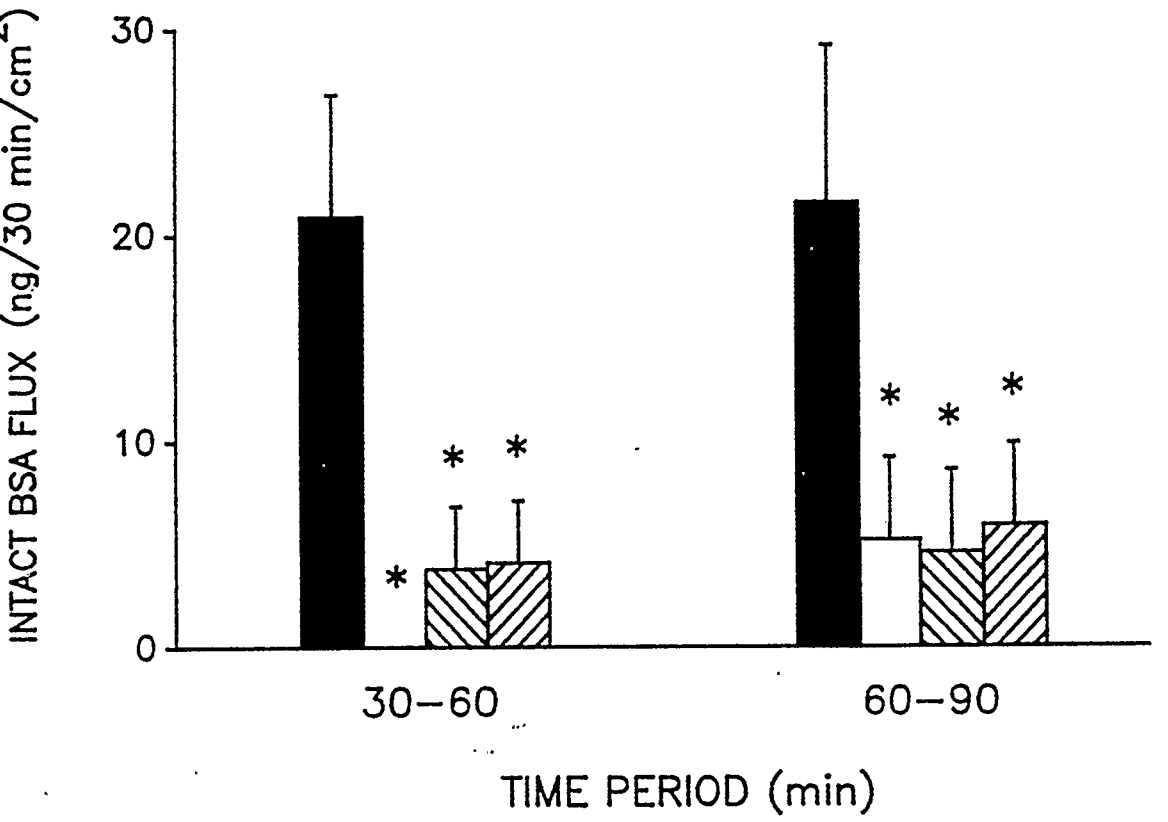

Figure 16. Pharmacological and metabolic inhibition of intact BSA movement across the gastric mucosa. Control tissues (■) compared to tissues incubated at 4° C (□), with 2mM NaF (▨), and with 10 mM colchicine (▨). All ELISA data is expressed as individual 30 min fluxes in ng/30 min/cm^2. Mean ± S.E.; * $p < 0.05$ compared to control.

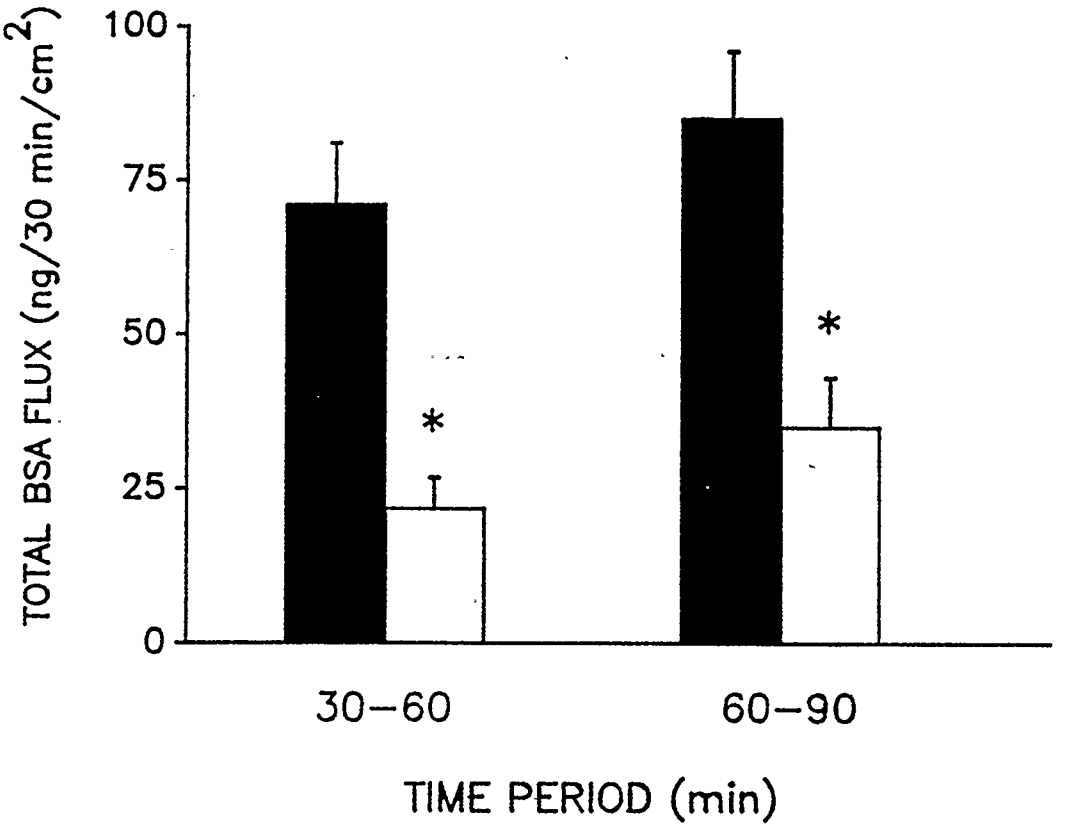

Figure 17. Pharmacological inhibition of total BSA movement. Control tissues (■) are compared to tissues incubated with 2 mM NaF (□). Fluxes are in ng/30 min/cm^2, mean ± S.E. * $p < 0.05$ compared to control.

Anaphylaxis did not alter macromolecular transport in the stomach. As is shown in Figure 18, there was no significant alteration in the uptake of intact BSA in sensitized-challenged tissues (n=9) compared to that of control tissues (n=7). To confirm that a Type I hypersensitivity reaction and mucosal mast cell degranulation had taken place, RMCP II was assayed in the mucosal chamber. RMCP II was significantly ($p=0.037$) increased in mucosal solutions from sensitized-challenged tissue (53.5±12.3 ng/ml, n=7) compared to control tissues (19.7±0.8 ng/ml, n=5).

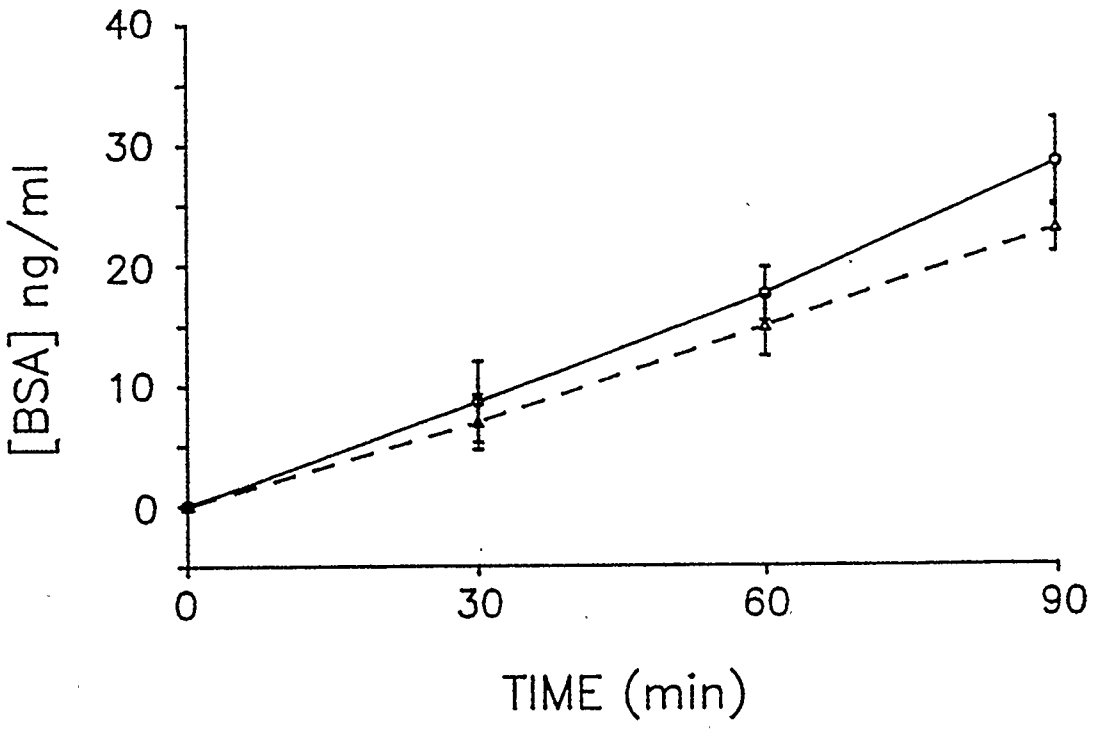

Figure 18. The effect of gastric anaphylaxis on macromolecular transport. Serosal concentration of intact BSA for controls (O—O) and sensitized-challenged tissues (Δ- - -Δ) is plotted as mean ± S.E. against time.

VI. DISCUSSION

This study provides evidence that the gastric mucosa is capable of actively sampling luminal protein antigens, and further, that some of this protein remains antigenically intact after transmural passage. These results were obtained after careful evaluation of tissue stability and viability in the modified Ussing chamber apparatus. Histological examination of stripped tissues showed a consistent, clean separation of the muscularis externa from the submucosa, and no evidence of mucosal or muscularis mucosae disruption. After mounting, tissues stabilized electrically during the 30 min equilibration period. From 30 min on, there was a gradual decline in conductance, necessitating further experiments to evaluate viability. When permeability of the mucosa was assessed with a low molecular weight probe molecule (^{51}Cr-EDTA), an equal amount crossed in both flux periods, indicating that non-specific mucosal permeability was constant. Additionally, specific studies of both intact and total BSA uptake found transmural transport to be in "steady state conditions" for the two flux periods, providing functional evidence of tissue stability and viability over the 30-60 and 60-90 min periods.

Experiments examined the movement of both antigenically intact as well as total (intact plus degraded) protein across the gastric mucosa in vitro. While in steady state transporting conditions, an intact BSA flux that averaged 21 ng/30 min/cm2 was measured. The uptake of intact protein made up a significant proportion (27%) of the all BSA movement across the mucosa (average total BSA

flux 78 ng/30 min/cm^2). This data confirms the in vivo observations made by Hatz et al (1990) concerning the ability of intact protein to cross the gastric mucosa. Anesthetized mice had their pylori clamped and were given ovalbumin orogastrically. After 30 min, immunologically intact ovalbumin was detected in low levels (21-25ng/ml) in serum by ELISA.

Macromolecular transport in the intestine has been examined in vitro in a number of previous studies. Although none of these reports used an identical experimental system to this study, an approximation of the amount of protein moved across the intestinal mucosa can be obtained for comparison purposes. Heyman (1982) reported intact horse radish peroxidase (HRP, M.W. 40 kDa) fluxes of 1 ng/30 min/cm^2 in adult rabbit jejunum. Studies of adult rabbit ileum have reported, intact HRP fluxes of 4 ng/30 min/cm^2 (Isolauri 1989) and intact β-lactoglobulin (M.W. 18.4 kDa) fluxes of 2.5 (Isolauri 1989) and 180 ng/30 min/cm2 (Marcon-Genty 1989). Two additional studies in piglet jejunum estimated intact HRP fluxes of 237 ng/30 min/cm^2 (Keljo 1983), and 228 ng/30 min/cm^2 (Keljo 1985). The intact protein transport data reported in this study falls into the broad spectrum of values obtained from the intestinal literature, and is marginally higher than the majority of studies reporting values of 1-4 ng/30 min/cm^2. These same intestinal studies also examined the total movement of protein and computed the percentage of total protein crossing that remained intact. Contrasting the results reported in this study where the intact flux represents a significant proportion (27%) of the total, the intestine degrades the majority of protein crossing. Four percent

of total HRP crossing the adult rabbit jejunum remains intact (Heyman 1982). The adult rabbit ileum was shown to pass 1% (Isolauri 1989) and 6% (Marcon-Genty 1989) of total β-lactoglobulin intact, and 1% of total HRP intact (Isolauri 1989). The sampling mechanism in the stomach transports less protein in total, but concurrently degrades a smaller percentage so that a similar net amount crosses intact. If the primary purpose of gastric sampling is to provide the immune system with traces of antigenically intact luminal protein rather than to absorb nutrients as in the small bowel, the stoichiometry of uptake described makes physiological sense.

Metabolic and pharmacologic inhibitors were used to determine the pathway and cellular mechanisms involved in gastric macromolecular sampling. These studies showed that antigen uptake is an active, energy dependent process (NaF and 4°C experiments) and utilizes the cellular microtubular network (colchicine experiments). Inhibition in all of these experiments was consistently in the vicinity of 75-100% of control, indicating that BSA transport across the epithelium occurs largely by an active process. This is consistent with classical textbook descriptions of the gastric mucosa as a "tight epithelium", and contrasts the results seen in similar experiments performed in the intestine, where inhibition was variable and frequently in the vicinity of 50% (Keljo 1983, Heyman 1982, Marcon-Genty 1989). This implies that there may be a substantial passive component of antigen uptake in the intestine that is not seen in the stomach. As the movement of intact BSA was largely NaF sensitive (energy dependent) and accountable for one quarter of the

total BSA movement, we expected to see at least a 25% reduction in total BSA movement with NaF addition. The reduction seen was larger than this, indicating that in addition to the uptake of intact protein, there is an energy dependent component in the transport of degraded protein.

These results suggest that protein movement across the gastric mucosa occurs by an active transcellular process. Studies in the small intestine have shown the enterocyte to have an important role in protein uptake and transport across the mucosa (Walker 1981, Walker 1987). The enterocytes' specialized apical surface, the brush border, 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 prior to expulsion at the basolateral membrane (Walker 1981). Protein that escapes degradation in transmural passage may do so by incomplete breakdown after lysosomal fusion (Stahl 1985) or by uptake through a separate non-degradative pathway (Heyman 1982). The results presented in this study implicate the presence of a sampling mechanism similar to that described in the small bowel. Few cells in the gastric mucosa however, possess an apical brush border. The majority of cells with this specialization are endocrine cells (Ito 1981), but one exception called the Tuft cell has been described in ultrastructural studies (Isomaki 1973). This cell closely resembles the mature small bowel enterocyte, but no function has yet been ascribed to it. Further work is required to examine the role of the Tuft cell in

macromolecular transport.

When the uptake of a bystander antigen (BSA) was examined in sensitized-challenged tissues, there was no increase in macromolecular transport. This finding occurred despite RMCP II data confirming mast cell degranulation. This differs from a wealth of literature examining bystander antigen uptake during anaphylactic reactions in the gut (Bloch 1981, Turner 1988). This may point to a unique role for the Type 1 hypersensitivity reaction in the stomach. The relationship between histamine release, and subsequent gastric acid secretion has been well documented (Debas 1987), but the physiological stimulus triggering such a release has not. The transport of intact antigen into the gastric lamina propria and submucosa may provide the catalyst for activation of localized mast cells via surface bound IgE in ectopic individuals. This seems teleologically prudent in view of the responses that mast cell activation in the stomach initiates. In an in vivo rat model, perfusion of the gastric lumen with an antigen the animals had previously been sensitized to resulted in increased gastric acid output and delayed gastric emptying (Catto-Smith 1989). These responses are likely aimed at maximizing antigen breakdown before entry into the small bowel.

Using an in vivo mouse model where ovalbumin was instilled into a stomach following pyloric ligation (Hatz 1990), serum ovalbumin concentration was shown to increase significantly by triggering a gastric anaphylactic reaction with either intravenous or orogastric di-DNP-lysine in animals previously immunized to that compound. This differs from the more "physiological" role ascribed to antigen

sampling and mast cell activation above. The differences seen may result from an artificial amplification of the anaphylactic reaction in the di-DNP-lysine model, where the sensitization process alone increases mucosal mast cell numbers by 75% in the stomach. Alternatively, our model eliminates any tissue damage that may occur secondary to the increase in vascular permeability seen in anaphylactic reactions in vivo. The contribution of localized edema to the disruption of the mucosal barrier is unknown, and absent in vitro.

These experiments demonstrate that the stomach is capable of sampling luminal protein antigens and that a portion of protein crossing the mucosa remains immunologically intact. Macromolecular transport occurs via an energy dependent process that utilizes the microtubular network. Uptake of intact protein was not altered by gastric anaphylaxis. The site of antigen uptake in the gastric mucosa is unknown, but uptake may be restricted to one specific cell type. Further studies will be carried out to immunohistochemically localize this cell.

VII. FUTURE DIRECTIONS OF RESEARCH

The experiments described in this thesis established the technique whereby macromolecular transport across an epithelium could by measured in vitro, and went on to characterize the movement of both antigenically intact as well as degraded protein across a novel tissue; the gastric mucosa. A great deal of work remains to be done in these systems. Further experiments might involve:

1. Identifying at a histological and ultrastructural level, the pathway of antigen uptake in the gastric mucosa, and determining whether there is a specific cell type in the mucosa involved in antigen uptake,

2. Identifying at a mechanistic level how luminal proteins move through or around antigen sampling cells in the mucosa, and the mechanism whereby some protein is degraded while other is conserved antigenically, and

3. Assessing macromolecular transport in the gastric mucosa in other pathophysiological conditions. As an example, a well described model of gastric injury in the rat results from administration of non-steroidal anti-inflammatory drugs (NSAIDs) such as indomethacin. The effect of NSAIDS on gastric protein sampling is unknown.

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