Advances in Drug Delivery Systems, 4

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Proceedings of the Fourth International Symposium on Recent Advances in Drug Delivery Systems, Salt Lake City, UT, U.S.A., February 21–24, 1989

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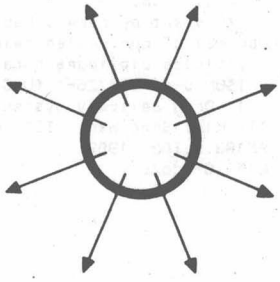

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This set of papers has been published as a special issue of *Journal of Controlled Release*, Vol. 11, issues 1–3

ELSEVIER Amsterdam — Oxford — New York — Tokyo 1990

ELSEVIER SCIENCE PUBLISHERS B.V.
Sara Burgerhartstraat 25
P.O. Box 211, 1000 AE Amsterdam, The Netherlands

Distributors for the United States and Canada:

ELSEVIER SCIENCE PUBLISHING COMPANY INC.
655, Avenue of the Americas
New York, NY 10010, U.S.A.

Library of Congress Cataloging-in-Publication Data

International Symposium on Recent Advances in Drug Delivery Systems (4th : 1989 : Salt Lake City, Utah)
Advances in drug delivery systems, 4 : proceedings of the Fourth International Symposium on Recent Advances in Drug Delivery Systems, February 21-24, 1989, Salt Lake City, UT, U.S.A. / edited by James M. Anderson, Sung Wan Kim, and Kristine Knutson.
p. cm.
"This set of papers has been published as a special issue of Journal of controlled release, vol. 11, issues 1-3."
Includes bibliographical references.
ISBN 0-444-88225-1 (U.S.)
1. Drug delivery systems--Congresses. I. Anderson, James M. II. Kim, Sung Wan. III. Knutson, Kristine. IV. Title.
RS199.5.I58 1989
615'.6--dc20 89-71521
CIP

ISBN 0-444-88225-1

This book is printed on acid-free paper.

Printed in The Netherlands

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PREFACE

The Fourth International Symposium on Recent Advances in Drug Delivery Systems was held in Salt Lake City, Utah, from February 21 through 24, 1989. This symposium was organized to provide a setting in which scientists could gather to discuss recent advances and state-of-the-art technology in drug delivery systems. Speakers were chosen on the basis of their research efforts, which were felt to be important to the future development of drug delivery systems. Speakers included pharmaceutical scientists, polymer chemists, bioengineers, pharmacologists, and physicians whose efforts were considered to be at the leading edge of the research and development of drug delivery systems.

Perspectives of drug delivery system research and development have changed markedly over the six-year period since the first International Symposium on Recent Advances in Drug Delivery Systems. Focus on biological mechanisms and interactions important to drug delivery systems has increased. This changing emphasis is reflected in the content of this symposium. The symposium was divided into five major areas to provide perspective and focus in the research, development, and clinical application of drug delivery systems. These areas were: transport mechanisms across membranes, proteins and macromolecular drug delivery, new developments in drug delivery, pharmacokinetics and pharmacodynamics in controlled drug delivery, as well as targeting and site-specific delivery.

The Proceedings of the Fourth International Symposium on Recent Advances in Drug Delivery Systems contains 30 papers which were generated from the topics presented at the symposium. The interdisciplinary and multifaceted nature of drug delivery systems is obvious from the breadth of topics presented in the Proceedings.

The editors extend their appreciation to the sponsors of the symposium: Baxter Healthcare Corp., Ciba-Geigy Corp., E.I. DuPont de Nemours and Company Inc., Riker Laboratories Inc., Theratech, Inc., and Upjohn Company.

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The editors also acknowledge the efforts of and extend their appreciation to Dr. Jorge Heller and Professor Jan Feijen for their assistance in generating these proceedings. Together, we were responsible for the review and editing of the papers contained in these Proceedings.

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CONTENTS

I. Transport Mechanisms Across Membranes

BIOPHYSICAL MODEL APPROACHES TO MECHANISTIC TRANSEPITHELIAL STUDIES OF PEPTIDES*

N.F.H. Ho, J.S. Day, C.L. Barsuhn, P.S. Burton and T.J. Raub**

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Key words: peptide absorption; Peyer's patch; buccal absorption; intestinal absorption; absorption mechanisms; Caco-2 cells

Systematic studies were conducted to understand the physicochemical and biophysical mechanisms governing the membrane transport of peptides. These studies focused primarily on the Peyer's patch of the small intestine, particularly the M cells, with respect to the vesicular transport mechanisms, and also the buccal, intestinal and Caco-2 (human-derived colon carcinoma cell) membrane systems with respect to the passive diffusional mechanism of peptides. The development of quantitatively sensitive experimental methodologies was required and, in conjunction with the specific membrane system, was initially assessed with non-peptide compounds of well-known active or passive transport properties.

*Using a miniature closed perfusion cell system positioned over the large Peyer's patch of the rabbit coupled with cannulation of the mesenteric blood and lymph vasculatures, adsorptive endocytosis uptake and concurrent appearance kinetics in the blood and lymph were followed with cationic poly(*d*-lysine) (PDL, 55 kDa) conjugated with [^{14}C]formaldehyde. The influx of PDL by the apical membrane was 100-fold larger than the efflux into blood; none was detected in lymph. A mixture of metabolic inhibitors, 2-deoxyglucose and Na azide, caused partial inhibition of endocytosis. By using a fluorescein isothiocyanate–PDL conjugate and fluorescence microscopy, PDL was found to be localized predominantly on the apical membrane surface of all intestinal epithelia. It also was found to be accumulated intracellularly by epithelia, most likely M-cells, that occupy the dome region of the Peyer's patch and by cells within the lymphoid follicles. The results suggested that PDL was trapped by the cells and lymphoid follicles and that the rate-determining step in the appearance of PDL in the mesenteric lymph is the migration of lymphocytes from the lymph space associated with M-cells.*

*Transport studies were conducted to determine structure–passive absorption relationships of small model peptides using the buccal, intestinal mucosal and/or Caco-2 cell monolayer membranes. In the course of buccal absorption studies, amino acids and their BOC-derivatives, and ancillary non-peptide compounds were included to aid in data interpretation. Based on the amphoteric peptide series, (*D*-Phe)$_n$Gly, and the neutral series, Ac(*D*-Phe)$_n$NH$_2$, where n = 1, 2 or 3, terminal charges on zwitterionic peptides have a negative effect on membrane permeability even though*

*Paper presented at the Fourth International Symposium on Recent Advances in Drug Delivery Systems, Salt Lake City, UT, U.S.A., February 21–24, 1989.

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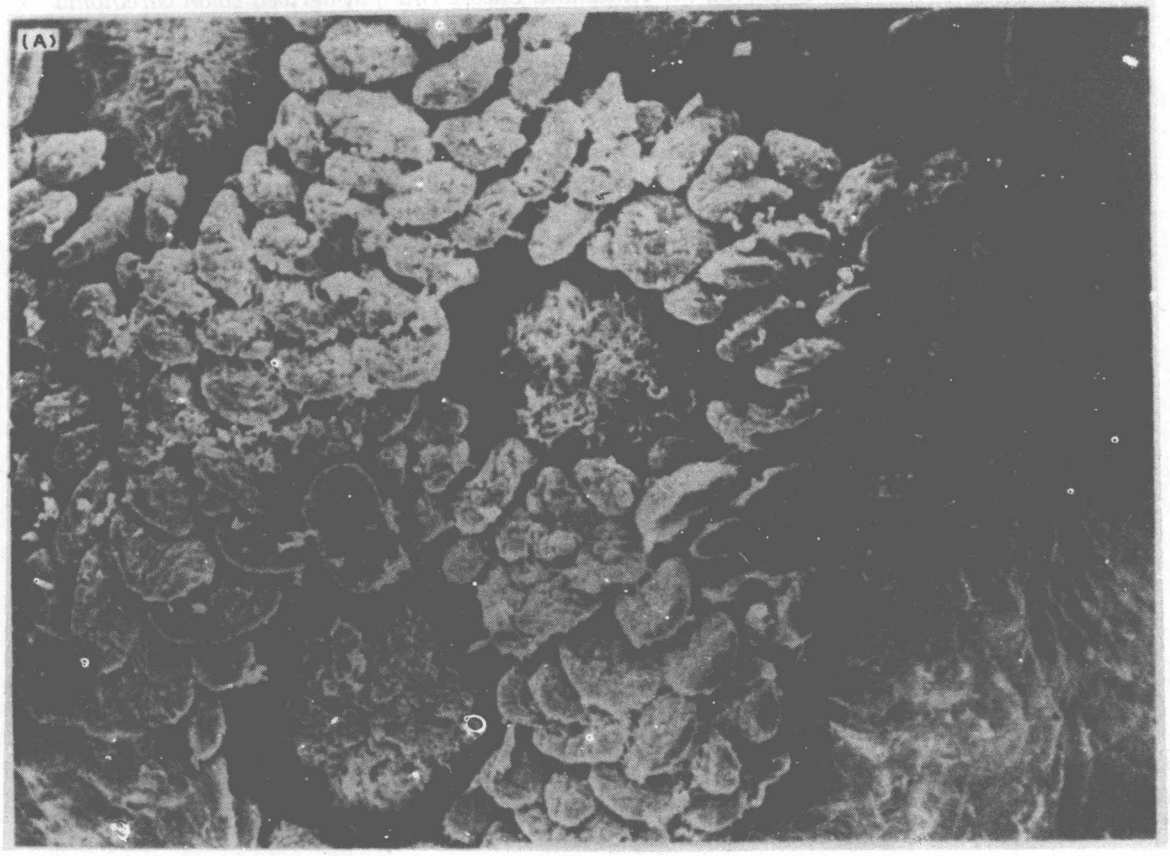
the effective partition coefficient in n-octanol/water is relatively high. Although the partition coefficient is increased by incremental additions of D-*Phe, the membrane permeability tends to decrease. This may be related not only to molecular size but also to the number of solvated amide bonds. Partition coefficients by the n-octanol/water scale appear to be poor predictors of membrane absorption of peptides.*

INTRODUCTION

Biologically active peptides and proteins are of increasing pharmaceutical interest with the advent of biotechnological efforts. They are generally not well absorbed on oral administration. On the whole, our basic understanding of transport mechanisms across membranes in conjunction with the structural and physicochemical properties of the peptide and protein molecules requires systematic approaches.

In the transepithelial delivery of peptides and proteins, we have focused on the membranes of the small intestine, buccal and nasal cavities, and cultured cell monolayer models. quantitatively sensitive experimental systems were de-



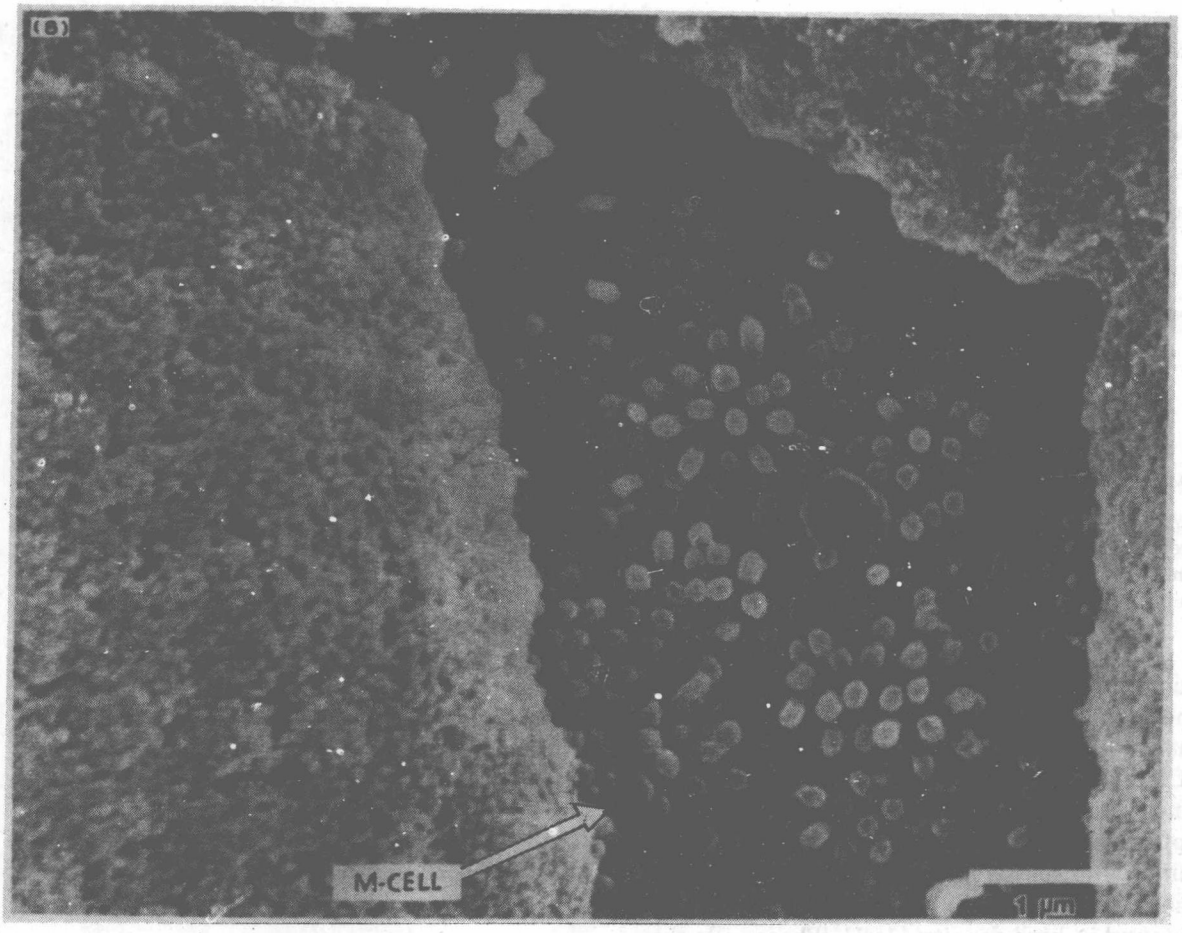


Fig. 1. Scanning electron micrographs of Peyer's patch in rabbit jejunum. Figure 1A shows topology of villi of columnar enterocytes and dome region containing M-cells. Figure 1B shows sparse population of truncated microvilli of M-cells and dense glycocalyx structures of adjacent columnar enterocytes. Courtesy of M.J. Cho.

signed to understand the physicochemical and biophysical mechanisms governing the transport of model peptides. The M-cells of Peyer's patches in the small intestine are emphasized with respect to vesicular mechanisms, and the buccal, small intestinal and Caco-2 (human adenocarcinoma cells of the colon) cell monolayer membranes with respect to passive diffusional mechanisms of peptide transport.

TRANSEPITHELIAL TRANSPORT RELATED TO PEYER'S PATCHES

Characteristics of Peyer's patches and M-cells

Peyer's patches are distinct morphological regions found in the jejunum, ileum and appendix of animals, including the human. Their distribution, size and number in the small intestine vary among animal species. In rats they appear as small white spots distributed numer-

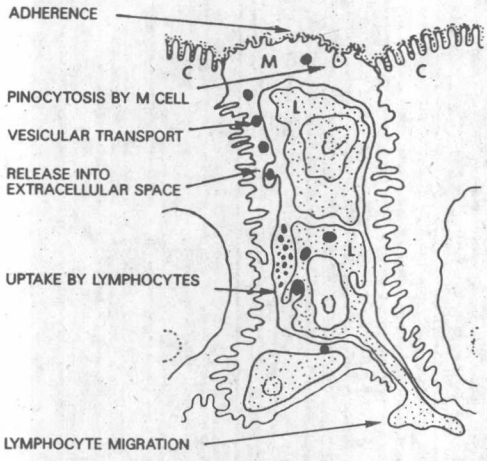


Fig. 2. Schematic diagram of the internalization of proteinaceous substances by M-cells and vesicular transport to lymphocytes in the lymph pocket. Diagram reproduced from Ref. [20].

ously throughout the small intestine, whereas they are located primarily in the ileum of humans [1]. The composition of the Peyer's patch of follicle-associated epithelium (FAE) is distinctive in a number of respects. They contain apparently normal columnar absorptive cells, but few mucus-secreting goblet cells. Further, the morphologically unique microfold or M-cell is found in high density [1–3]. These cells are thought to serve the specialized function of macromolecular uptake from the intestinal lumen and transport to the lymphatic system [4–7]. In marked contrast to normal absorptive cells, this uptake and transport is not accompanied by digestive proteolysis [7]. Because of these properties it is thought that the M-cell serves as an immunological sampling mechanism for the intestinal mucosa. The cells allow the passage of antigens, viruses and microorganisms through the intestinal epithelial barrier and into the underlying lymph space occupied by lymphocytes and macrophages where they are trapped and neutralized [8–11]. However, the containment of toxic agents may not be perfect for reasons unknown.

Morphologically, the M-cell appears to be well suited for its sampling role (Fig. 1). The surface is sparsely populated by truncated microvilli, making it readily accessible to macromolecules, viruses and colloidal particles. There exist low levels of alkaline phosphatase and other membrane-bound enzymes at the brush border, suggesting the lack of an extensive enzymatic system for digestion and absorption of nutrients. The membrane is active in both fluid-phase pinocytosis and adsorptive endocytosis. In the cytoplasm, numerous membrane vesicles are observed near the apical surface. The M-cells have a paucity of lysosomes, enabling proteins to pass virtually intact. Other striking features are the anatomical association of the lymphocytes with the M-cells and the close proximity of the lymphoid follicles to the intestinal lumen (Fig. 2). Characteristically, the basolateral surfaces of the cells are deeply invaginated to form a pocket accommodating a cluster of lymphoid cells and macrophages [12]. Thus, protein-containing vesicles need not traverse the length of the cell, but undergo exocytosis to resident lymphocytes only a few micrometers from the apical membrane.

Transport studies: Review of the literature

Research employing *in vivo* incubation of model markers in ligated intestinal segments

TABLE 1

Vesicular uptake of various substances by M-cells in various animal species

Animal	Marker	Reference
Guinea pig	India ink	13
Mouse	Carbon black; latex spheres, 2 μm	14
Rabbit	*E. coli*	15
Mouse	Reovirus 1	16
Rabbit	Cationized ferritin	2
Rabbit	Lectin-ferritin conjugate; cationized ferritin	7
Rabbit	IgA	17,18
Rabbit	Lectin-HRP[a] and fluorescein conjugates	19
Mouse	HRP	20

[a]HRP is horseradish peroxidase.

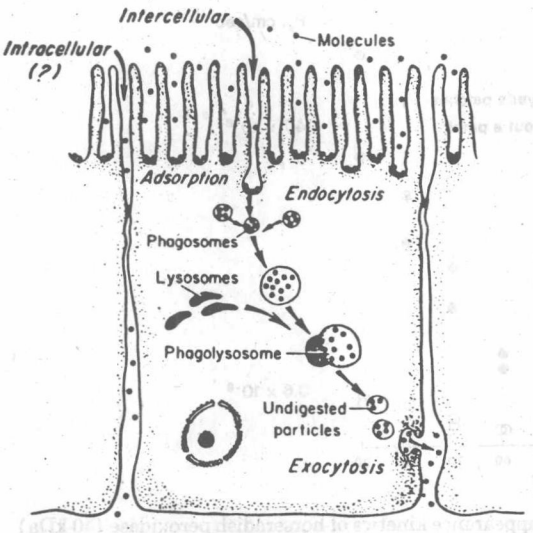


Fig. 3. Schematic diagram of the internalization of proteinaceous substances and intracellular transport of vesicles in absorptive columnar enterocytes. Diagram reproduced from Ref. [6].

TABLE 2

In vitro transport kinetics across Peyer's patches and normal epithelium using Ussing chambers[a]

Animal	Marker	Reference
Piglet	HRP	21
Rabbit	HRP	22
Rabbit	RU-41740[b] glycoprotein mixture of 350 and 95 kDa subunits	23

[a]Excised intestinal specimens are used after muscle and visceral layers are stripped off.
[b]RU-41740 is an immune modulator.

followed by electron microscopic or autoradiographic examination of tissue preparations have provided a fundamental understanding of the vesicular transport mechanism and membrane surface properties of M-cells. Representative results of such studies using various substances ranging from proteins, lectins, colloidal solids, viruses, and microorganisms in various animal species are listed in Table 1. Such studies have given us invaluable insights to various transport processes involving membrane surface adsorption, membrane vesicle internationalization, intracellular trafficking, exocytosis, and uptake by lymphoid follicles and macrophages (Fig. 2). However, in absorptive columnar enterocytes, macromolecules are internalized in the crypts of microvilli and the endosomes are sequestered by the lysosomes, where the macromolecules are enzymatically degraded for the most part. Subsequently, the material exits the basolateral membrane into the microvasculature (Fig. 3).

With respect to mechanism, cationized ferritin, horseradish peroxidase and lectins are taken up by adsorptive endocytosis, while native ferritin is taken up by fluid-phase pinocytosis. While these tend to be non-specific processes, recent studies of the M-cell specific binding and internalization of the immunoglobin IgA suggests a receptor-mediated process may be involved [17]. Similarly, reovirus type 1 but not type 3 bind exclusively to M-cell surfaces, again consistent with M-cell specific recognition sites [16]. Kinetically, these types of studies only give a qualitative estimate of the internalization and transport rates. It has been shown that the transcytosis and lymphocyte uptake is achieved within one hour [20]. However, the rate-determining step in finding the permeant marker in the lymphatic system appears to be the migration of the permeant containing lymphocytes from the M-cell into the mesenteric lymph circulation. This process can be very slow. In a study examining the uptake of latex beads by Peyer's patches, a significant number of the absorbed beads were still found in resident macrophages underlying the M-cell six weeks after cessation of the latex feeding [14].

A more quantitative approach to follow the transport kinetics of proteinaceous substances involves the use of an excised intestinal segment from which the underlying muscle and visceral layers are carefully stripped off and the mucosal tissue is mounted between a conven-

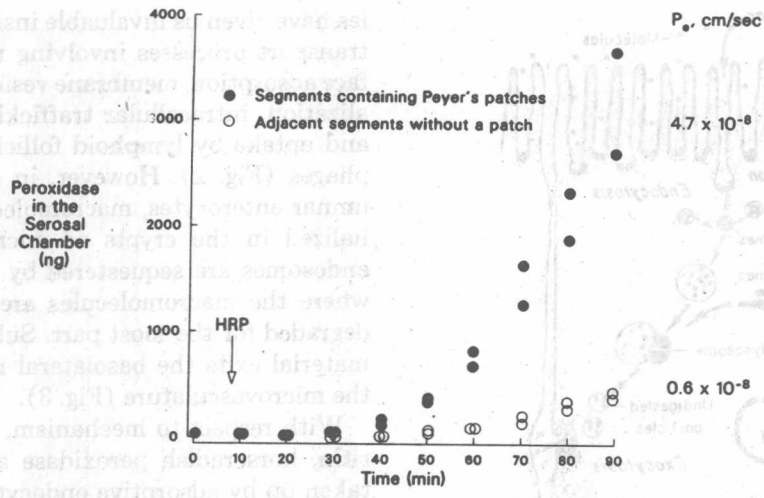


Fig. 4. Influence of Peyer's patch and normal mucosal tissues on the appearance kinetics of horseradish peroxidase (40 kDa) across piglet jejunum preparations mounted in a Ussing chamber. Plot reproduced from Ref. [21].

tional two-compartment diffusion cell (Table 2). Here, the transport properties of tissues containing Peyer's patches and adjacent normal mucosa can be compared. An example of such studies is the work of Keljo and Hamilton [21], which is shown in Fig. 4. Horseradish peroxidase (HRP) is seen to be transported across piglet jejunal segments containing Peyer's patches more rapidly in the intact state than adjacent segments of normal mucosa. Enzymatic breakdown of HRP occurred more extensively in patch-free epithelia than in patch-containing epithelia. The apparent lag time to steady state was 40 min. Transport across the patch was inhibited by sodium fluoride, a potent inhibitor of endocytosis.

In taking advantage of the availability of this data, we estimated the effective mass transfer coefficient using the expression:

$$P_e = \frac{1}{AC_D(O)}\left(\frac{\Delta M}{\Delta t}\right) \qquad (1)$$

where P_e is the effective permeability coefficient; A is the apparent surface area; $C_D(O)$ is the initial concentration in the donor compartment; and M is the mass of HRP in the receiver compartment at the time t. The P_e value of 4.7×10^{-8} cm/s for the tissue containing Peyer's patches is about 7-fold larger than the value of 0.6×10^{-8} cm/s for the patch-free tissue. The magnitude of the P_e is indicative of a membrane-controlled situation. Since the Peyer's patch is composed of M-cells and columnar enterocytes, it is not possible to delineate the relative contributions of transport pathways and mechanisms of the paracellular route, M-cells and normal enterocytes, as well as intracellular metabolism, to the effective mass transfer coefficient without more extensive studies. To further complicate the situation, the protein must also traverse the multilayer of cells in these *in vitro* tissue preparations. However, this study indicates that the intact HRP found in the receiver of the Ussing chambers must be that fraction which escapes uptake by lymphocytes associated with the M-cells.

Current transport studies of the Peyer's patch

To quantify and understand the absorption kinetics of Peyer's patches as compared to normal intestinal tissue in the rabbit *in situ*, we

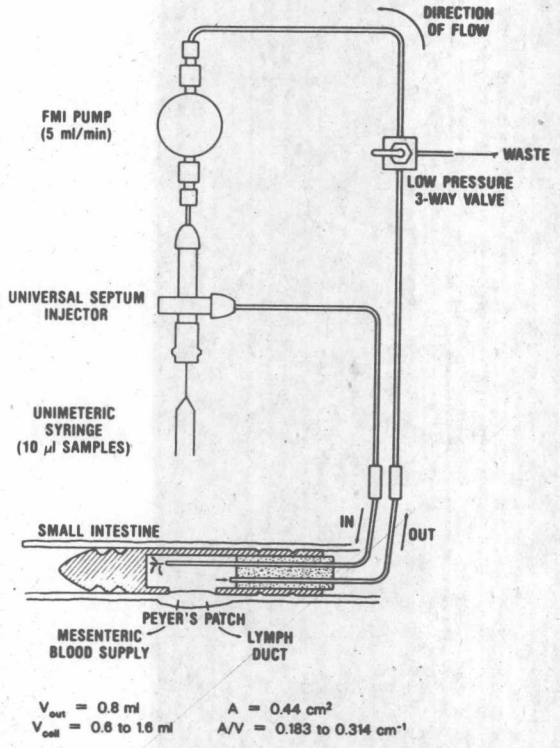


Fig. 5. Schematic diagram of miniature closed-perfusion cell system to follow disappearance kinetics in Peyer's patch and normal mucosa of the rabbit's small intestine. Cannulation of mesenteric lymph duct and mesenteric venous vasculature permits appearance kinetic determinations.

developed a miniature, closed perfusion system (Fig. 5). The system consists of a specially designed glass cell which is inserted into the intestine of the anesthetized rabbit (Fig. 6) and whose elliptically shaped opening covers a defined area of the Peyer's patch or normal intestine, a reciprocating pump, teflon tubing, and a universal septum injector for the withdrawal of microliter samples for assay. The closed perfusion setup allows one to continuously follow the uptake kinetics by the intestinal cells. After cannulation of both the mesenteric venous vasculature draining the intestinal segment of interest and the mesenteric lymph duct, the continuous sampling of blood and lymph permits the determination of the appearance kinetics of the permeants. The blood loss is replaced by intravenous administration of Ringer's solution. The rabbit is a particularly good animal model since the Peyer's patches are large (elliptical in shape, 0.75×1.5 cm) and the epithelial tissue is hardy. It has been estimated that the M-cells constitute about 30 to 50% of the total membrane area of the rabbit's patch taken as a planar surface. In following the permeation kinetics, one recognizes that the overall kinetics reflect transport across both M-cells and columnar enterocytes. Efforts to quantitatively delineate the kinetics of each pathway will be challenging and will require clever use of strategies.

The advantages of the experimental method are four-fold. First, it recognizes realistically that mass transport from the intestinal lumen to the mesenteric blood and lymphatic systems takes place across a single layer of absorptive cells. Secondly, one can follow the disappearance of permeants in the intestinal lumen and concurrent appearance of the solute and metabolites in the mesenteric vasculature with time, and also account for mass balance. Thirdly, the local tissue of interest can be prepared for examination by electron microscopy, autoradiographic, fluorescence and histochemical techniques at the termination of the kinetic study. Fourthly, the transport kinetics of the patch and adjacent patch-free epithelia can be readily studied and compared.

To evaluate the intestinal perfusion system and characterize various properties of the mucosal membranes of the Peyer's patch as compared to normal intestinal mucosa in both the jejunum and ileum, we initially followed the uptake kinetics using a variety of radiolabeled solutes as model compounds and calculated the permeability coefficients of the aqueous boundary layer and the membrane. Salicylic and n-octanoic acids were employed to determine the magnitude of the aqueous boundary layer barrier; *d*-glucose and taurocholic acid were used to characterize active transport mechanisms.

Both normal mucosa and Peyer's patches in the jejunum and ileum absorbed *d*-glucose effi-