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Uptake of taurocholate and the intrinsic factor-cobalamin complex occurs predominantly in ileal villous cells

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DEDICATION

TO MY DEAR PARENTS

HE FOR HIS INSISTENCE THAT I STUDY MEDICINE

SHE FOR NOT DISSENTING

ACKNOWLEDGMENT

Writing the acknowledgments section of this thesis was, without question, the most pleasurable aspect of the work since it afforded me the opportunity to put in print the gratitude that I was unable to express adequately by other means to the many individuals who made this work possible.

It will be well nigh impossible to adequately thank my thesis suppervisor and mentor, Cyrus R. Kapadia, M.D., Assistant Professor of Medicine, and I shall not attempt to do so. Suffice it to say that he played a paramount role in every single aspect of this work.

The name of Robert M. Donaldson, M.D., Professor of Medicine, Yale University School of Medicine and Chief of Medical Services, West Haven Veterans Administration Hospital, more aptly belongs on the title page as a co-supervisor of this work. In the unusual modesty so characteristic of him, Dr. Donaldson edited his name out from the final draft. I couldn't disagree with him more. Fortunately, this section was written late enough to escape his editorial axe, and I have seized the opportunity to express to him my sincerest appreciation for suggesting the project, providing the laboratory facilities and for making available to me his immense experience in this field. I am especially grateful for allowing me to quote from his yet-to-be published review article on cobalamin transport.

Miss Kathy McManus was of invaluable help in initiating me to the laboratory and assisting in all aspects of the experimental work. Miss Nana Frema Busia helped with proofreading and typing of the rough draft. The final draft was typed by Mrs. Annie Cooper-Brown. I am extremely grateful to this wonderful trio.

Finally, I wish to acknowledge the continued support and encouragement of my dear parents, to whom this thesis is dedicated. Needless to say, the decision they took more than twenty six years ago culminated in this thesis.

TABLE OF CONTENTS

TABLE OF CONTENTS (continued)

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS USED

IF : Intrinsic Factor

Cbl : Cobalamin

TC : Taurocholate

EDTA : Ethylene Diamine Trichloroactetate

KRB : Krebs-Ringers-Bicarbonate buffer solution

UPTAKE OF TAUROCHOLATE AND THE INTRINSIC FACTOR-COBALAMIN COMPLEX OCCURS PREDOMINANTLY IN ILEAL VILLOUS CELLS

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ABSTRACT

Both the intrinsic factor (IF) mediated uptake of cobalamin (Cbl) and the active transport of conjugated bile salts occur predominantly in the distal ileum; however, it is unclear which cell type (villous, crypt, or both) possesses the apparatus specific for these transport processes. To resolve this, we measured the uptake of free and IF-bound ^{57}Co-labeled cyanocobalamin (^{57}CoCbl) and of ^{3}H-labeled taurocholate (^{3}H-TC) by isolated guinea pig crypt and villous cells prepared by a modification of the method of Weiser. Cell preparations were checked for purity by measuring levels of thymidine kinase (as a crypt cell marker) and of sucrase and alkaline phosphatase (as villous cell markers). Cell viability was established by the steady rate of incorporation of tritium-labeled leucine into protein by both villous and crypt cell preparations. Incubation of ileal villous cells with IF-bound ^{57}CoCbl at 37°C for 30 minutes resulted in a level of uptake 40 times greater than incubation with free Cbl under the same conditions. However, IF-mediated uptake was absent with either ileal crypt cells or with jejunal crypt or villous cells. Similar results were obtained

when crypt and villous cell preparations were incubated with ^{3}H-TC for 5 minutes. Here, ileal villous cell uptake was 41-fold, 35-fold, and 39-fold more than the levels for ileal crypt, jejunal villous, and jejunal crypt cell preparations respectively. These results suggest that the differentiated ileal villous cell, but not the undifferentiated ileal crypt cell, possesses the apparatus for the uptake of conjugated bile salts and the intrinsic factor-cobalamin complex. Jejunal enterocytes, differentiated or undifferentiated, lack this capacity.

CHAPTER 1

BACKGROUND INFORMATION; OBJECTIVES

1.1 Intestinal Epithelial Cell Renewal and Differentiation

In order to augment its absorptive surface area, the small intestinal mucosa is organized into 0.5 - 1.5 mm-long finger-like projections called villi. Opening into the bases of these villi are 400 µm-long simple tubular glands which form the crypts of Lieberkühn. The (simple columnar) epithelium that lines the villi continues into the upper haves of the walls of the crypts as well, but the lower halves of the crypt cells are lined by highly undifferentiated cells which show vigorous mitotic activity. This anatomical fact (of the localization of proliferative cells to the crypts) is a long established one (1,2). Equally well established is the existence of a gradient of defferentiation from crypt cells up the villus surface (3). This gradient is both a morphological as well as a functional one since during its migration the crypt cell acquires many of the specialized enzymes required for digestion (4,5) whilst losing its DNA (6)-, RNA (7)-, and cholesterol (8)- synthesizing enzymes. Autoradiographic methods involving the in vivo injection of radioactively-labelled thymidine have been used to confirm that in laboratory animals it takes the newly-formed crypt cell 24-76 hours to reach full maturity at the villus tip, where it desquamates into the intestinal lumen. Lipkin and Bell (9) have reviewed this renewal process extensively.

Inspite of the above knowledge, the actual factors that determine whether a given crypt cell remains in its undifferentiated form in the crypt

and proliferates or migrates upward and differentiates are not known. Carnie et al (10,11) have advanced the theory that all crypt cells migrate up the villus and are either proliferative (P) cells, which are capable of dividing but incapable of differentiating, or non-proliferative (Q) cells which can differentiate but cannot divide. Division of a P cell may produce two P cells or two Q cells; however, for reasons as yet unclear but probably related to local environmental influences (9), the division of a P cell deep in the crypts produces only P cells. During migration up the crypt, an increasing fraction of P-cell dividions yields Q cells until, at the uppermost level of the crypt, Q cells are formed exclusively from P-cell divisions. At this level, proliferation ceases and differentiation is accelerated.

1.2 Objectives of the Present Work

While the phenomenon of crypt cell differentiation is of general interest in cellular biology, it takes on added relevance in the field of gastrointestinal physiology because of its potential implications in the understanding of human health and disease states. It has been a long-held belief of many gastrointestinal physiologists that in general the absorptive functions of the intestine are performed exclusively by the mature villous cell whereas the secretory functions are the mainstay of the crypt cells. Two of the better-studied absorptive functions of the intestine are the intrinsic factor (IF) mediated uptake of cobalamin (Cbl) and the active transport of conjugated bile salts. Both of these transport processes have been shown to reside predominantly in the ileum but it is unclear whether it is the differentiated villous cell or the undifferentiated crypt cell that

has the capacity for such transport. We were therefore interested in measuring the uptake of free and IF-bound ^{57}Co-labeled cyanocobalamin (^{57}CoCbl) and of ^{3}H-labeled taurocholate (^{3}H-TC) by isolated guinea pig crypt and villous cells. To do this, we utilized a modification of the method of Weiser (23) to obtain pure preparations of crypt and villous cells. Purity of cell preparations was confirmed by assays of enzymes specific for one or the other cell type. Cell viability was established by the steady rate of incorporation of tritium-labeled leucine into protein by both villous and crypt cell preparations. Finally, the uptake studies were repeated with jejunal enterocytes to confirm the absence of uptake of IF-Cbl or TC in these cells.

1.3 Methods for the Preparation of Isolated Crypt and Villous Cells

Various and sundry methods for the preparation of isolated crypt and villous cells have been devised. These may be broadly categorized into mechanical and biochemical methods. The mechanical methods include that of Dietschy and Siperstein (8) in which isolated crypt and villous cells were obtained simply by scraping the intestinal mucosa with a glass plate, first lightly (to produce the more superficial villous cells), then more forcefully (to yield the crypt cells). More refined versions of the scraping method have been used by Imondi et al (12) in their "intestinal planning apparatus" which was capable of successive 125μ cuttings because of an incorporated micrometer, and by Das and Gray (13) using a cryostat and a dissecting microscope. Harrison and Webster (14) and Sjostrand (15) obtained pure preparations of crypt and villous cells by mechanical vibration of everted intestine in appropriate buffer solutions.

The biochemical methods include enzymatic digestion with trypsin (16, 17), trypsin-pancreatin (18), or lysozyme (19). A difficulty inherent in these protease digestion methods is that long incubation times (or high enzyme concentrations) result in cells of low viability whereas short incubation times (or low enzyme concentrations) give poor yield; since the optimum enzyme concentration and incubation time are highly variable, any studies that utilize cells thus prepared are bound to yield inconsistent results. To circumvent this difficulty, recent techniques of intestinal mucosal cell fractionation utilize incubation in calcium-binding agents such as citrate (20, 21) or EDTA (22, 23). We used Merchant and Heller's (24) modification of the EDTA-incubation method of Weiser (23) to dissociate guinea-pig intestinal cells into pure crypt and villous cell preparations for use in the binding studies to be described in Chapter 2.

1.4 Biochemical Changes Accompanying Crypt to Villous Maturation

As has already been mentioned, an inevitable concomitant of crypt cell differentiation is the loss of certain biochemical markers and the acquisition of others. The enzymes of the intestinal epithelium may be classified into three groups on the basis of the ratios of their activities in crypt cells and in villous cells (25). In the first group (see Table 1.1), the ratio of the enzymatic activity in crypt cells to that in villous cells is substantially greater than unity. This group comprizes the enzymes associated with pyrimidine and pyrimidine nucleotide biosynthesis. By contrast, in group 3 the ratios of enzyme activities in crypt cells to villous cells if much less than one. The brush border enzymes — the disaccharidases, oligopeptidases, and alkaline phosphatase — belong to this group. Finally,

TABLE 1.1

	Ratio of enzymatic activity in crypt cells: villous cells	Reference
Group 1		
Thymidine Kinase	50	26
Aspartate carbamoyltransferase	2.3	27
Uridine Kinase	1.7	28
Group 2		
Acid B-galactosidase	0.86	29
B-glucuronidase	0.88	30
Acid phosphatase	0.83	31
Group 3		
Maltase	0.17	32
Lactase	0.25	29
Invertase (sucrase)	0.11	29
Dipeptidase	0.43	33
Alkaline phosphatase	0.49	34

Table showing the changes in enzymes levels accompanying crypt cell to villous cell maturation.

enzymes constituting group 2 have similar activities in crypt and villous cells, that is, the ratio of their activities in these two cell types approaches unity.

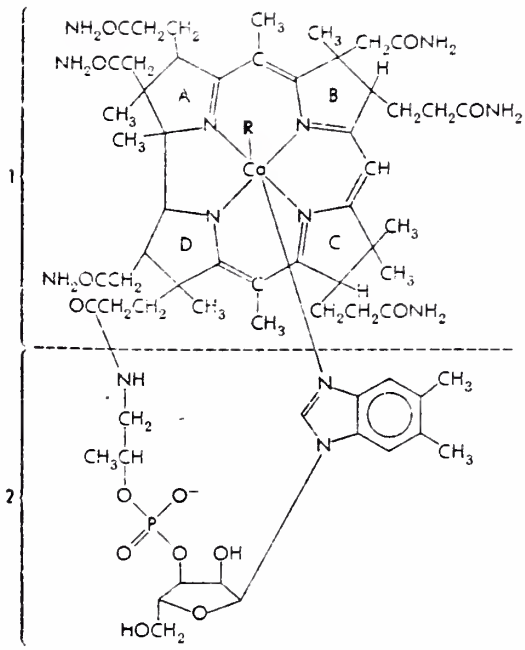
It follows from Table 1.1 that Group 1 enzymes may be used as markers for the crypt cell and Group 3 enzymes as markers for the villous cell preparation. This fact provides a method for checking the consistency of cell separations. We chose to assay thymidine kinase (as a crypt cell marker), sucrase, alkaline phosphatase (as villous cell markers), and acid phosphatase. The acid phosphatase assay was done as a further check on the purity of cell preparations since previous work has shown that the ratio of enzymatic activity is close to 0.85.

1.5 Review of Cobalamin Transport

The term 'cobalamin' refers to a family of natural and semi-synthetic compounds all of which consist of two major portions — a planar group (the corrin nucleus) and a "nucleotide" moiety lying perpendicular to it (fig. 1.1).

In vivo, the cobalamins exist predominantly as hydroxycobalamin, methylcobalamin, and adenosylcobalamin. This fact notwithstanding, the term cyanocobalamin pervades the literature largely because of its historical position as the first of the cobalamins to be isolated. It is now known that the cyanide moiety is an artifact resulting from the use of charcoal in the original isolation procedures. In this work, the term "cobalamin" is used to mean "cyanocobalamin", the substance most often used in research on cobalamin transport.

FIGURE 1.1



VITAMIN B_{12} CONGENERS

Permissive Name	R Group
Cyanocobalamin (Vitamin B_{12})	—CN
Hydroxocobalamin	—OH
Methylcobalamin	$—CH_3$
5'-Deoxyadenosyl-cobalamin	—5'-Deoxyadenosyl

Figure 1.1: The structural formula of the cobalamin molecule.

The three major portions of the molecule are:

1. A planar group or corrin nucleus - a porphyrin-like ring structure with four reduced pyrrole rings (designated A to D) linked to a central cobalt atom and extensively substituted with methyl, acetamide, and propionamide residues.

2. A 5,6-dimethylbenzimidazolyl nucleotide, which links almost at right angles to the corrin nucleus with bonds to the cobalt atom and to the propionate side chain of the D ring.

3. A variable R group - the most important of which is found in the stable compounds cyanocobalamin and hydroxocobalamin and the active coenzymes methylcobalamin and 5-deoxyadenosylcobalamin. While there are a number of other cobalamin derivatives in nature, formed by covalent binding of various ligands to the cobalt atom, these are of no apparent value to man.

(Reproduced from Goodman and Gilman (95)).

The cobalamin molecule is a relatively large molecule (molecular weight of 1300-1400 daltons) which possesses an appreciable water solubility because of the polar side groups on the corrin ring (fig. 1.1). The above properties of cobalamin preclude its efficient absorption by any simple, passive, transepithelial process. For one thing, the epithelial barriers that have to be traversed have an "effective pore size" many orders of magnitude smaller than the smallest diameter of the cobalamin molecule. Secondly, the relative hydrophobicity of the cobalamin molecule makes it incompatible with the lipid bilayer of the cell membrane. Little wonder, then, that a complex and highly specific transport process involving three different binding proteins has evolved for the assimilation and transport of this vitamin. The know cobalamin-binding proteins are intrinsic factor, transcobalamin II, and the R proteins. Whilst a knowledge of the role of intrinsic factor only is pertinent for the understanding of this work, the functions of the R proteins and transcobalamin II will be reviewed briefly for the sake of completeness.

The R Proteins:

This family of glycoproteins, so named because of their rapid electrophoretic migration (41), are found in granulocytes (as transcobalamin III), plasma (as transcobalamin I) and in many glandular secretions (saliva, gastric juice, bile, milk, tears) (42). In man they have a molecular weight of 56,000 to 66,000 daltons and a carbohydrate content of 33-40% (43). It appears that the R proteins function mainly as storage proteins for cobalamin. Indeed, most of the circulating endogenous cobalamin is bound to transcobalamin I. Thus it is conceivable that transcobalamin I binds cobalamin

for a slow, sustained delivery to tissues which then either use the cobalamin or excrete it. Consistent with this role for TC I is its very slow turnover rate (of several days). TC III on the other hand has an extremely rapid turnover rate (half life in plasma of the order of several minutes) and its role, if any, in the storage of cobalamin is less clear. As suggested by Carmel and Herbert (47) and by Scott et al (48), plasma TC III maybe an artifact resulting from its inevitable release from granulocytes after blood has been drawn.

Transcobalamin II:

Unlike intrinsic factor and the R proteins (which are glycoproteins), transcobalamin II is a polypeptide. It is found in plasma as a globulin of molecular weight 38,000 daltons (43). Most TC II is probably synthesized by the liver but minor amounts may be synthesized by the kidney and the intestine as well (44, 45). TC II synthesis by mouse monocytes has also been reported (46). Compared to TC I, circulating TC II has a fairly rapid turnover rate (half-life of one to a few hours) and is found at a much lower concentration in plasma than TC I.

Intrinsic Factor

Intrinsic factor is a globular glycoprotein of molecular weight 45,000 and a carbohydrate content of about 15%. In most animal species, including man, it is synthesized predominantly by the parietal (oxyntic) cells of the gastric mucosa and secreted as part of gastric juice (49, 50). In a few species, localization of intrinsic factor to the chief cell (rat, mouse) or the mucus cell (hog) has been demonstrated (49). Regardless of the site of synthesis, intrinsic factor secretion is stimulated by H_2-receptor

agonists (51, 52, 53) and cholinergic agents (53) and inhibited by H_2-receptor antagonists (54).

It has been inferred from the maximum amount of Cbl that can be bound to IF that each molecule of IF binds only one molecule of Cbl (35). The affinity of Cbl for the IF binding site is so great that binding occurs instantaneously (41, 36). Once bound, the IF-Cbl complex becomes significantly more resistant to proteolytic destruction than IF (37).

Sequence of Events in Cobalamin Uptake

Uptake of Cbl may occur by two distinctly different processes depending on the quantity of Cbl ingested. When physiologic amounts of Cbl are ingested, uptake occurs primarily by the IF-mediated process to be described below. With larger quantities of Cbl, as may exist in an in vitro incubation of Cbl with isolated cells, a non-specific, non-IF dependent diffusion mechanism may contribute to the measured uptake. For this reason, studies on IF-Cbl uptake must include control incubations with free Cbl; levels of uptake in the latter are subtracted from the IF-Cbl incubations to obtain the true amount of uptake ascribable to the IF-mediated mechanism.

The orderly sequence of events involved in Cbl absorption was first proposed by Cooper and Castle (38) in 1960. Subsequent work by other investigators has established the correctness of most of the steps in the original proposal of Cooper and Castle. These events may be summarized as follows: 1) dietary Cbl is released from food protein complexes by acid gastric juice (38); 2) the released Cbl binds to salivary and gastric R proteins present in the stomach (39); 3) the Cbl-R protein complex reaches the proximal small intestine where pancreatic proteases rapidly degrade the R proteins with

the concomitant release of Cbl (39); 4) binding of Cbl with IF to form a macromolecular complex which, unlike the R proteins, is resistant to digestion by pancreatic proteases (37, 39); 5) the IF-Cbl complex binds to membrane receptors located in the distal ileal mucosa; there is evidence (40, 55, 56) that the site of the IF molecule which binds to the ileal receptor is different from that which bears the Cbl molecule; 6) the Cbl molecule is absorbed into the ileal mucosal cell and released into the portal circulation.

Binding of the IF-Cbl complex to the ileal receptor (step 5, above) requires calcium ions and a pH greater than 5.6. It has been postulated (57, 43) that Ca^{++} ions link certain critical anions on the IF molecule and the receptor to form "salt bridges" that maintain the IF molecule in an appropriate configuration for transfer of the Cbl molecule.

While the seqence of Cbl uptake presented above is that currently held by most investigators to be correct, it must be mentioned that there is some evidence that an alternative mechanism of cobalamin absorption may exist. Steinberg et al (93) and Toskes et al (94) have presented evidence that in patients with pancreatic insufficiency, the failure to degrade R proteins (step 3) is unimportant in explaining the pathogenesis of Cbl malabsorption in these patients.

Little is known about the transepithelial cell transport of Cbl once it is bound to the ileal mucosa. However, since this phase of the assimilation of Cbl is beyond the scope of this work, it will suffice to mention that there is some indirect evidence (58, 59, 60) that as Cbl leaves the ileal serosal surface, it is bound to an ileal pool of TC II which is distinct from circulating TC II. The newly absorbed Cbl enters the systemic circulation

where the Cbl is probably transferred to the systemic pool of TC II and thence to surface membrane receptors present on all cells.

1.6 Review of Bile Acid Transport in the Small Intestine

The earliest definitive work on the absorption of bile acids appears to have been that of Schiff (61) who demonstrated that these substances were readily absorbed by the small intestine but not by other regions of the gastrointestinal tract. Tappeiner (62), and later Frölicher (63) subsequently showed that bile acid absorption occured much more readily in the distal small intestine than the proximal small intestine.

A large number of studies on the transepithelial absorption and secretion of bile acids have appeared in the literature over the past several decades (64, 65, 66, 67, 68). These studies have established with certainty that in most species, including man, bile acid absorption occurs predominantly in the distal ileum and to a lesser but measurable extent in the proximal small bowel. In a few species, including the hen (69, 70, 71, 72), however, bile acid uptake by the duodenum and jejunum has been shown to far exceed that in the ileum. This fact led to the suggestion that bile acid absorption may occur by two distinct mechanisms — a passive transport process and an active transport process — and that one or the other process may predominate at different sites of the enterohepatic circulation depending on the species and certain other factors to be mentioned below.

Lack and Weiner (73), using everted gut sac preparations of four equal segments obtained from the entire length of the small intestine, demonstrated that bile acid uptake occurs by a passive process in the proximal half. Uptake in third-quarter intestinal segments was by both passive and

active transport processes, with a slight tendency toward the latter, whereas uptake in the distal quarter (ileum) was almost entirely due to active transport. These findings have been confirmed by other investigators (74, 75, 77).

Passive Bile Acid Uptake

Passive absorption of bile acids may be ionic or non-ionic depending on the pK_a of the particular bile acid, which in turn depends on the nature of the moiety with which the bile acid is conjugated. In passive ionic diffusion, the absorbed bile acids are completely ionized. An example of this type of diffusion exists in taurine conjugated bile salts. These are completely ionized at every level of the small intestine because of their relatively low pK_a values (of the order of 2.0) compared to the pH range of 6-8 that exists in the small intestine. For this reason, taurine conjugated bile acids may be absorbed by passive ionic diffusion. Glycine conjugates, on the other, have a pK_a of the order of 4.0 and in situations where the small intestinal pH is abnormally low, significant amounts of the protonated (non-ionic) species may exist. In such a situation, glycine conjugated bile acids may be absorbed by both passive ionic and non-ionic diffusion. Similarly, since the pK_a of free bile acids is about 6.0, appreciable amounts of both protonated and unprotonated species exist for both passive ionic and passive non-ionic uptake to occur.

It may be inferred, from the lipoid nature of epithelial membranes, that passive non-ionic diffusion is a much more rapid process than passive ionic diffusion. Dietschy et al (76) have shown this to be the case. The nature of the barriers that determine the rate of passive bile acid uptake

from the small intestine has been studied by Wilson and Dietschy (77). With highly ionized bile salts, the lipiod cell membrane was found to constitute the greatest resistance to penetration of the brush border. With unionized bile acids, the lipoid cell membrane was more penetrable but the fluid layer in immediate contact with the brush border offered a considerable amount of resistance on account of the inherently poor mixing of the fluid lamella.

It must be reiterated that in most species, including man, the quantitative contribution of passive bile acid absorption to the total bile acid pool in the enterohepatic circulation is minor. In certain pathological situations, however, passive bile acid uptake may be quantitatively significant or may even predominate. Thus, in syndromes in which there is gastric hypersecretion, the pH of the small intestine may be quite low and, as noted above, unionized glycine conjugates may be absorbed in significant amounts. Similarly, in the blind loop syndrome, bacterial overgrowth of the small bowel results in rapid deconjugation of bile acids. The pK_a of the latter is high enough for significant quantities of free bile acids to be absorbed.

Active Transport of Bile Acids

Active bile acid transport is an energy-dependent process and as such is inhibited by metabolic inhibitors (73, 74) and by oxygen deprivation (73). Active uptake of bile acids is also dependent on sodium. Thus, the substitution of K^+ for Na^+ in everted gut sac preparations results in an almost instantaneous cessation of bile salt transport (74, 75). However, when bile salts are incubated with Na^+ under anerobic conditions, uptake into the intestinal wall occurs but concomitant concentration across the

serosa requires Na^+ ions in addition to some energy-yielding process that is oxygen dependent. A sodium ion-activated ATPase is thought to mediate this process (75). Indeed, the cardiac glycoside ouabain, an ATPase inhibitor, is known also to inhibit bile acid transport (78). These facts imply that uptake of bile acids is coupled to the sodium pump and is thus an example of a secondary active transport process.

Conjugation of a bile acid is not necessary for its active transport. However, the nature of the conjugating group may affect the rate of transport. There is some indirect evidence that only one carrier system is involved in shuttling the different kinds of bile acid across the ileal wall. Holt (74) and Playoust and Isselbacher (75) have shown that the uptake of sodium taurocholate by the ileum may be competitively inhibited by the prior presence of other bile acids, suggesting that a single carrier is involved. Infact, the kinetics of active bile acid transport has been shown to follow the saturation kinetics of Michaelis-Menten (73, 75, 76). For a particular bile acid, the maximum velocity V_{max} for its transport across the ileal wall increases rapidly from the proximal to the distal end (76), consistent with the rapid increase in receptor sites form the proximal to the distal end of the ileum.

CHAPTER 2

MATERIALS AND METHODS

2.1 Solutions and Reagents

1. Buffers for dissociation of enterocytes: "Buffer I" had the following composition: 1.5 mM in KCl, 96 mM in NaCl, 27 mM in sodium citrate, 8 mM in KH_2PO_4 and 5.6 mM in Na_2HPO_4. The pH was 7.4. "Buffer II" contained 2.7 mM KCl, 137 mM NaCl, 1.5 mM KH_2PO_4, 8 mM Na_2HPO_4, 1.5 mM Na_2EDTA, and 0.5 mM in dithiothreitol. The pH was 7.4.

2. Hanks buffer was purchased as such from Grand Island Biological Company, Grand Island, New York.

3. Krebs-Ringers-Bicarbonate solution had a pH of 7.4.

4. Cell culture medium consisted of 1.5 gm% BSA in Cbl-free NCTC-135. NCTC-135 was obtained from GIBCO, Grand Island, New York.

5. Intrinsic Factor: The source of intrinsic factor was human gastic juice, obtained during gastric secretion studies after petagastrin stimulation. Only the first 30 minute post pentagastrin aspirates were used. In order to prevent the degradation of IF, the gastric juice was depepsinized by elevating the pH to 10 by the addition of 1 N NaOH. After 30 minutes, the pH was returned to 7 using HCl.

   We used human intrinsic factor because previous work has shown that the receptors on guinea pig ileal mucosa bind human IF.

6. IF-^{57}CoCbl: Human gastric juice was reacted with an excess of ^{57}CoCbl and the free ^{57}CoCbl was removed using hemoglobin-coated charcoal (84). ^{57}CoCbl, 10 µCi/0.667 ug/ml, was obtained from Radiochemical Center, Amersham, England.

7. A 5 mM solution of non-labeled sodium taurocholate (NaTC) was prepared by dissolving 13.4 nm NaTC in 5 ml of NCTC/BSA medium. A 5 µM solution of taurocholate, containing 1 µCi/ml of ^{3}H-labeled taurocholate was prepared by mixing the appropriate quantity of ^{3}H-taurocholate with enough non-labeled taurocholate to make the final solution 5 µM in taurocholate. Taurocholic acid-^{3}H(G) was obtained from New England Nuclear, Boston, Mass.; the activity was 1 µCi/0.15 mg/ml.

2.2 Animals

Adult guinea pigs of either sex weighing between 400 and 800 gm were used. The animals lived under constant artificial illumination and had unrestricted access to food and water. Animals were selected at random and starved overnight before use.

2.3 Preparation of Villous and Crypt Cell Suspensions

Under ether anesthesia, the small intestine of the animal was removed and divided into three equal segments. The proximal third (jejunal segment) and distal third (ileal segment) were used in these studies. The middle third was discarded. The mucosal surface of the intestinal segments were washed thoroughly with ice-cold saline to remove mucus and food residues. One end of each intestinal segment was clamped with a Spencer Wells artery

forceps and the lumen was filled with "Buffer I", previously equilibriated to 37°C and oxygenated. The open end of the intestinal segment was clamped and the entire loop of intestine placed in a 250 ml plastic Erlenmeyer flask containing 50 ml of "Buffer I". The flask was then incubated in a water bater bath at 37°C with continuous agitation. The contents of the flask were maintained under constant oxygenation with a mixture of 95% O_2 and 5% CO_2. After incubating for 15 minutes in "Buffer I", the contents of the intestinal loop were discarded, the lumen was rinsed throughly with "Buffer II" and filled with this latter buffer in the manner described above for "Buffer I". The cells obtained at the end of this incubation were kept (this represented the "villous cell fraction"), and the series of incubations and washings were continued, as shown schematically in Fig. 2.1.

Enzyme analyses of the cell fractions obtained at each of the above steps established the existence of a gradient of enzyme activity that was consistent with the release of predominantly villous cells at the end of the first Buffer II incubation and the release of predominantly crypt cells at the end of the last Buffer II incubation (see "Results").

The cells were centrifuged at 150 g for 5 minutes. A small aliquot was removed, washed in KRB, resuspended in water, homogenized, and used for enzyme assays. The remaining cells were rinsed twice with Hanks Buffer and finally suspended in NCTC/BSA.

2.4 Enzyme Assays

After appropriate dilution of the harvested cells, aliquots were taken and homogenized. The protein content of the cell homogenate was determined by the method of Lowry (85) using BSA as standard. Sucrase

FIGURE 2.1

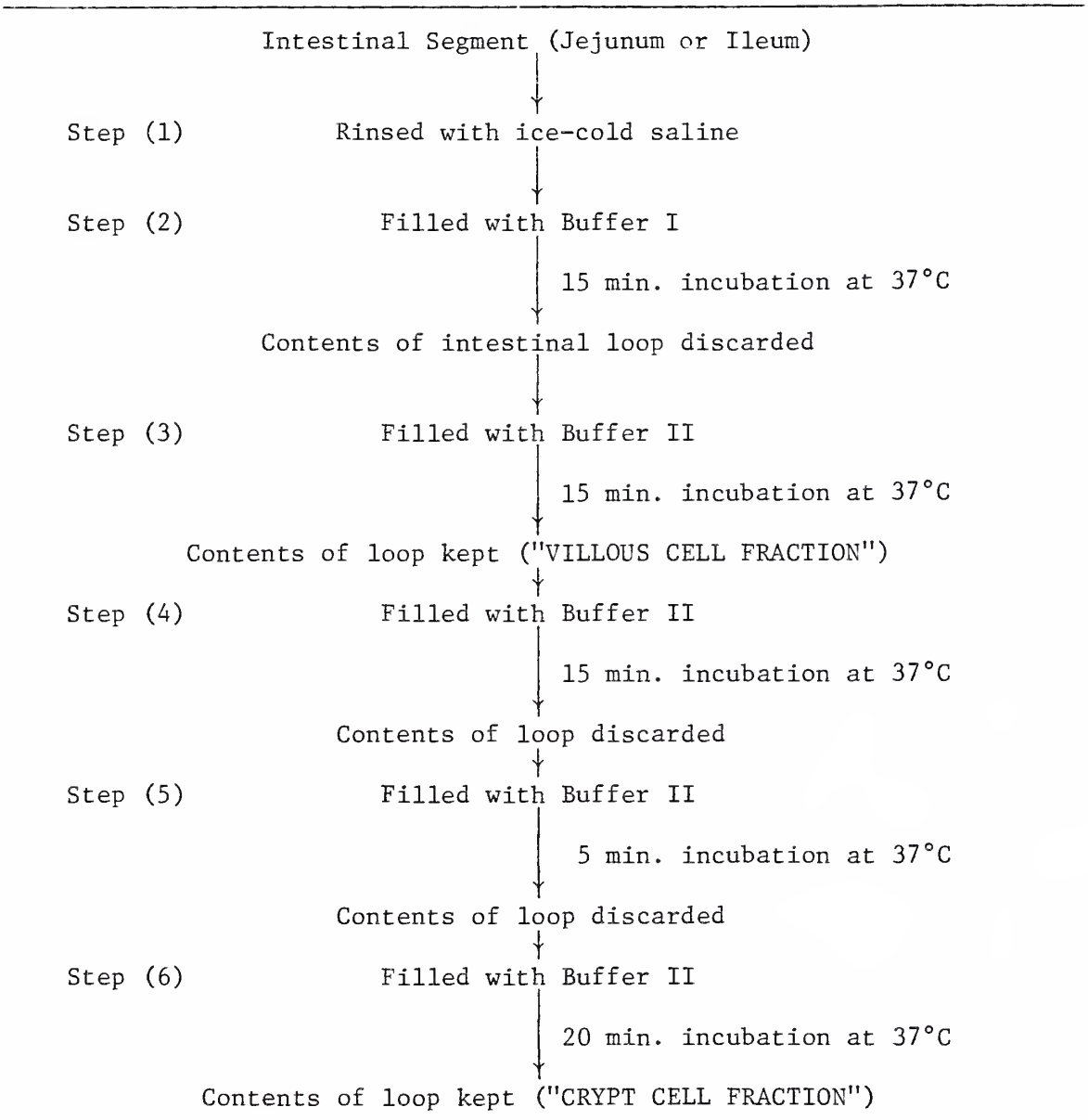


Figure 2.1: Flow diagram for obtaining villous and crypt cell preparations.

activity was determined by the method of Dahlqvist (81); using the results of the protein assay, the sucrase activity was expressed as "enzyme units per minute incubation per mg. protein", where a unit of sucrase activity is defined as that quantity of enzyme which hydrolyzes 1.0 µmole of disaccharide (sucrose) per minute (81).

Alkaline phosphatase activity was determined by the method of as modified by Weiser (82). The assay was carried out in 10 mM Tris-HCl buffer (pH 9.5) using disodium p-nitrophenyl phosphate as substrate.

Acid phosphatase was assayed using disodium p-nitrophenyl phosphate as substrate in an 0.1 M acetate buffer (pH 5.2). Incubation time was 10 minutes and enzyme activity was expressed as µmole p-nitrophenol per min. per mg. protein.

Thymidine kinase was assayed according to the method of Chen et al (83), based on the conversion of (2-^{14}C) thymidine to (2-^{14}C) thymidylate. Because the total final volume of the assay mixture was only 55µl, the assay was carried out in small conical plastic tubes which permitted good vortexing. At the end of the 8-minute incubation period, a 25 µl aliquot was transferred onto 23 mm discs of Whatman filter paper impreganted with DE 81. The latter were placed in a beaker containing 25 ml of a 1 mM solution of ammonium formate at room temperature. The ammonium formate wash was necessary to remove unreacted (2-^{14}C) thymidine. The beaker was swirled for 15 minutes by means of a gyratory water bath and the ammonium formate was decanted off. After a further rinsing with 25 ml of ammonium formate and two water rinses, the disc was oven dried and counted in a toluene-based scintillation fluid. Thymidine kinase activity was expressed as counts per min. per min. incubation per mg. protein.

2.5 Cell Viability Studies

To verify the viability of the cell preparations, 1.0 ml aliquots of crypt and villous cells in NCTC/BSA were incubated with 2 μCi each of ^{14}C-labeled leucine. Incubation was done in duplicate for six time points (5, 10, 15, 20, 30, and 45 minutes) and was terminated with 10 ml of ice-cold KRB containing a large excess (20 mM) of unlabeled leucine. The products were centrifuged at 2,000 g, and the resulting pellets washed with KRB and taken up and homogenized in 4.0 ml of 20% Trichloroacetic acid (TCA) containing 20 mM unlabeled leucine. The homogenized cells were centrifuged at 27,000 g for 10 minutes and washed thoroughly by successively suspending and centrifuging in 10% TCA, 5% TCA, and 50:50 v/v methanol/chloroform. The methanol/chloroform was evaporated in air and the pellet solubilized using a quaternary ammonium base in toluene (NCS, Amersham/Searle). The solubilized pellet was transferred quantitatively with a toluene-based scintillation fluid into counting vials and the radioactivity determined against a toluene blank. The results were expressed as disintegrations per minute (dpm) of leucine incorporated into 1 mg of cell protein.

2.6 IF-Cbl Binding Studies

Freshly prepared intestinal cells were washed twice with Hanks buffer and resuspended in NCTC/BSA medium. The volume of NCTC/BSA medium used to resuspend the cells depended on the yield of the latter. The "crypt" cell fraction was suspended in about 5 ml of medium. Because of the usually larger yield of "villous" cells, they were made up in about 12 ml of medium in order to achieve cell concentrations comparable to the "crypt"

cell fraction. 3.75 ml of NCTC/BSA medium was pipeted into four of eight 25 ml palstic Erlenmeyer flasks and equilibriated to 37°C in a gyratory water bath. 3.96 ml of medium was pipeted into each of the remaining four Erlenmeyer flasks (see table 2.1). The flasks were continuously gassed with 95% O_2, 5% CO_2. Four of the flasks represented duplicate incubations of villous cells with free Cbl and with IF-Cbl; the other four represented similar incubations with "crypt" cells.

1.0 ml aliquots of the appropriate cell suspension were then pipeted into the flasks. This was followed by the addition of the appropriate quantities (see table 2.1) of either free Cbl or IF-Cbl containing 2.0 ng of ^{57}Co. The flasks were incubated at 37°C for 30 minutes. At the end of this time the reaction was terminated by adding 10 ml of ice-cold KRB to each flask. The contents of each flask were transferred quantitatively to plastic tubes, which were then centrifuged at 2000 g. The resulting cell pellet was washed twice with 10 ml of KRB and the radioactivity determined. Uptake of free $^{57}CoCbl$ or of IF-$^{57}CoCbl$ was calculated as picogram $^{57}CoCbl$ bound per mg. cell protein. The experimental protocol for the cobalamin binding experiment is summarized in Table 2.1.

2.7 Bile Acid Uptake Studies

Freshly prepared intestinal cells (jejunal and ileal) were washed twice with Hanks buffer and resuspended in NCTC medium containing 0.5% BSA. Because of its larger yield, the "villous" cell preparation was resuspended in a larger (about 12 ml) volume of NCTC medium compared to the "crypt" cell fraction which was suspended in about 5 ml of medium.

TABLE 2.1

	Sample No.	NCTC medium (ml)	Cells in NCTC medium (ml)	IF-^{57}CoCbl 8.0 ng/ml (ml)	Free ^{57}CoCbl 5.34 ng/ml (ml)
"VILLOUS"	1	3.75**	1.0	0.25*	-
CELL	2	3.75	1.0	0.25	-
FRACTION	3	3.96	1.0	-	0.44
	4	3.96	1.0	-	0.44
"CRYPT"	5	3.75	1.0	0.25	-
CELL	6	3.75	1.0	0.25	-
FRACTION	7	3.96	1.0	-	0.44
	8	3.96	1.0	-	0.44

*These volumes contain 2.0 ng of ^{57}Co; IF-Cbl preparations were always checked against a ^{57}Co standard at the start of each experiment.

**These volumes are necessary to make the final volume 5.0 ml in each flask.

Table 2.1: Experimental protocol for the binding of free ^{57}CoCbl or IF-^{57}CoCbl to "villous" and "crypt" cells.

3.90 ml of NCTC/BSA medium was pipeted into each of six plastic 25 ml Erlenmeyer flasks and incubated at 37°C. These six flasks represented triplicate incubations of "villous" and "crypt" cells at a total taurocholate concentration of 1 μM (see table 2.2). 2.90 ml of NCTC/BSA medium and 1.0 ml of 5 mM unlabeled taurocholate were pipeted into each of four plastic Erlenmeyer flasks which were similarly equilibriated to 37°C. These represented duplicate incubations of "villous" and "crypt" cells at a total taurocholate concentration of 1 mM. (Since the total taurocholate concentration in the latter set of flasks was 1000 fold greater than that in the first set, it was possible to correct for the uptake of radioactive taurocholate that was due to "nonspecific" binding; see "Results").

1.0 ml aliquots of the appropriate cell preparation were then pipeted into each flask. This was followed by the the addition of 0.1 ml of the labeled taurocholate solution to each flask. Labeled taurocholate solution was added at half-minute intervals so that each flask was incubated for exactly 5 minutes at 37°C.

The incubations were stopped by the addition of 10 ml of ice-cold KRB, with quantitative transfer of the contents of the flasks to plastic tubes which were then centrifuged at 2000 g. The resulting pellet was washed with 15 ml of KRB and solubilized using 1 ml of NCS. The solubilized pellet was taken up quantitatively in a toluene-based scintillation fluid and its radioactivity determined. Bile acid uptake was calculated as the amount of ^{3}H uptake (in cpm) in 5 minutes per mg. protein. Table 2.2 summarizes the protocol for the bile acid uptake studies.

TABLE 2.2

		Sample No.	Medium (ml)	5 mM NaTC (ml)	Cells (ml)	^{3}H-TC (ml)
"VILLOUS" CELL FRACTION	1.0 µMTC	1	3.9	-	1.0	0.1*
		2	3.9	-	1.0	0.1
		3	3.9	-	1.0	0.1
	1.0 mMTC	4	2.9	1.0	1.0	0.1
		5	2.9	1.0	1.0	0.1
"CRYPT" CELL FRACTION	1.0 µMTC	6	3.9	-	1.0	0.1
		7	3.9	-	1.0	0.1
		8	3.9	-	1.0	0.1
	1.0 mMTC	9	2.9	1.0	1.0	0.1
		10	2.9	1.0	1.0	0.1

*contains 1.0 µCi of ^{3}H-TC

Table 2.2: Experimental protocol for the uptake of ^{3}H-Taurocholate by guinea pig ileal cells.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Enzyme Assays

Tables 3.1 and 3.2 show the results of the enzyme assays on the villous and crypt cell fractions respectively in five different experiments. Most of the enzyme activities shown represent the mean of two or three determinations depending on the number of serial dilutions used. The only exceptions to this are the sucrase data in Fig. 3.2; these represent single determinations for each experiment because only the smallest dilutions showed any measurable sucrase activity.

In Fig. 3.1, the mean enzyme activities for the five experiments have been calculated from Tables 3.1 and 3.2 and the results are shown graphically for easy comparison. The highest specific activity for each enzyme has been arbitrarily set at 100% to obtain relative activity. It can be seen that most of the sucrase and alkaline phosphatase was located in the villous cell fraction, with the crypt cell fraction showing only 9-10% of the activity of the villous fraction. Acid phosphatase, on the other hand, was about equally distributed between the two cell fractions whilst thymidine kinase predominated in the crypt cell fraction.

An alternative way of examining the data that allows easy comparison with previous work done in this field is shown in Table 3.3. The ratios of enzymatic activity in crypt cells to villous cells were calculated from the mean enzymatic activities of Tables 3.1 and 3.2. Comparison of Table 3.3

TABLE 3.1

Expt. No.	Sucrase (enzyme units/min/ mg protein)	Alkaline Phos. (μmoles pnp/min/ mg protein)	Acid Phos. (μmoles pnp/min/ mg protein)	Thymidine Kinase (cpm/min/ mg protein)
1	0.092	0.10	0.09	351
2	0.065	0.59	0.085	496
3	0.069	0.65	0.087	663
4	0.063	0.123	0.069	250
5	0.076	0.232	0.119	245
Mean ± S.D.	0.0730	0.3390	0.090	401
	± 0.0117	± 0.2622	± 0.0181	± 178

Table 3.1: Results of enzyme assays on the first 15 minute ("villous") fraction of guinea pig ileal cells. The method of preparation of cells is described in Sec. 2.3; the various enzyme assays are described in Sec. 2.4.

TABLE 3.2

Exp. No.	Sucrase (enzyme units/min/ mg protein)	Alkaline Phosph (µmoles pnp/min/ mg protein)	Acid Phosph. (µmoles pnp/min/ mg protein)	Thymidine Kinase (cpm/min/ mg protein)
1	0.0195	0.02	0.07	4096
2	0.006	0.046	0.079	4797
3	0.003	0.020	0.073	6124
4	0.0075	0.025	0.082	8499
5	< 0.001	0.038	0.099	4462
Mean ± S.D.	0.0074	0.0298	0.0806	5596
	± 0.0072	± 0.0117	± 0.0113	± 1794

Table 3.2: Results of enzyme assays on the last 20 minute ("crypt") fraction of guinea pig ileal cells. The method of preparation of cells is described in Sec. 2.3. The various enzyme assays are described in Sec. 2.4.

FIGURE 3.1

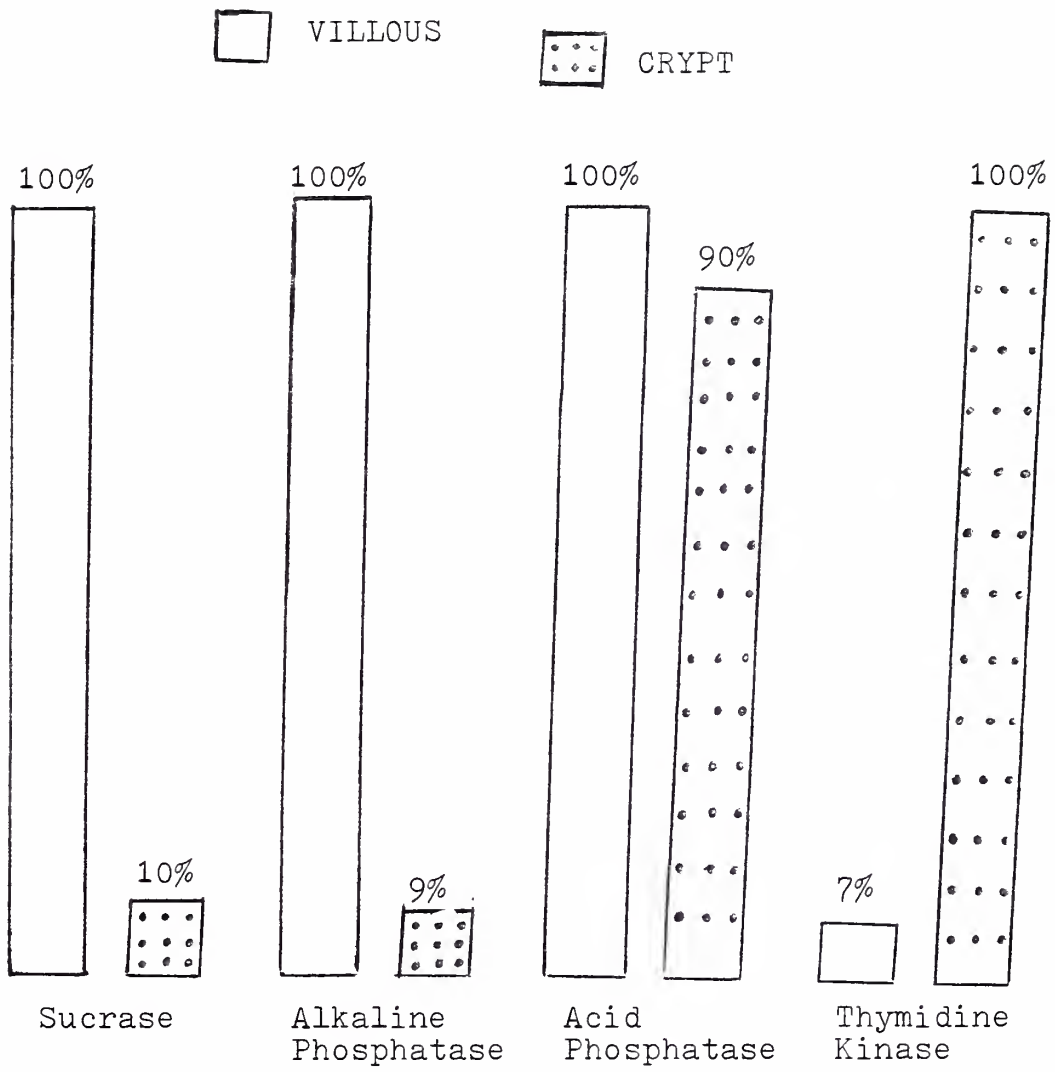


Figure 3.1: Distribution of enzymes in the two cell fractions of the guinea pig ileum. The highest specific activity for each enzyme was arbitrarily set at 100% to obtain relative activity. Each bar represents the mean of five different cell preparations.

with Table 1.1 shows excellent agreement, indicating good separation of crypt from villous cells by the method we used.

3.2 Cell Viability Studies

Fig. 3.2 shows a plot of the uptake of radioactive leucine by ileal villous and crypt cells. The ordinate represents the amount of ^{14}C-leucine incorporated up to the time shown on the absicca. Excluding the outlier at the 45 minute point for the crypt cells, the rate of incorporation is seen to be linear initially and to gradually level off with time, suggesting that both crypt and villous cell preparations were viable, at least with respect to protein synthesis.

3.3 Cobalamin Binding Studies

The first set of experiments (Nos. 1-5 in Table 3.4) compared the uptake of free and IF-bound ^{57}CoCbl in ileal villous cells. IF-mediated uptake was found to be 40-fold greater than uptake of free Cbl by the same cells under identical conditions.

In the second set of experiments (Nos. 6-10, Table 3.5), uptake of free and IF-bound ^{57}CoCbl by ileal crypt cells was measured. IF-mediated uptake was absent with ileal crypt cells. In fact, the uptake of IF-^{57}CoCbl by these cells was not signicantly different from their non-specific binding to free Cbl.

Having established the lack of uptake of IF-Cbl by ileal crypt cells, it was of interest to compare jejunal and ileal crypt cells with respect to their capabilities for IF-Cbl uptake. To do this, crypt cells from the proximal third (jejunum) and the distal third (ileum) were harvested

TABLE 3.3

	Ratio of enzymatic activity in crypt cells to villous cells	Literature Values	Reference
Sucrase	0.10	0.11	(29)
Alkaline Phosphatase	0.09	0.49	(34)
Acid Phosphatase	0.90	0.83	(31)
Thymidine Kinase	14.0	50.00	(26)

Table 3.3: Crypt/villous ratios of enzymatic activity calculated from the mean enzymatic activities of Tables 3.1 and 3.3. Comparison is made with literature values. Numbers in parentheses refer to the sources of the literature values. Qualitative agreement the measured ratios and the literature values is excellent.

FIGURE 3.2a

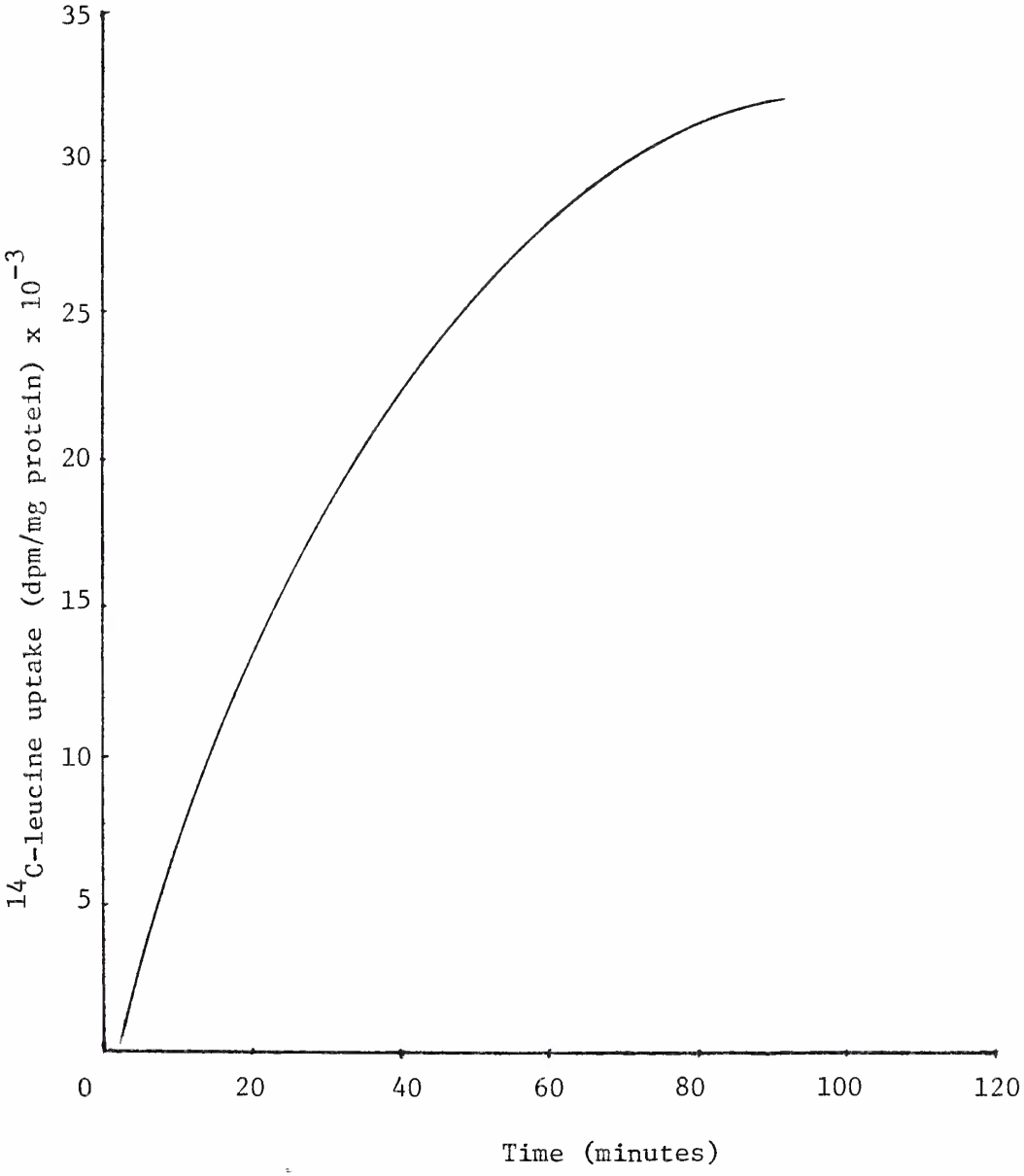


Figure 3.2a: Incorporation of ^{14}C-labeled leucine into protein by ileal villous cells. The graph is linear initially but tends to level off with time, thus verifying cell viability.

FIGURE 3.2b

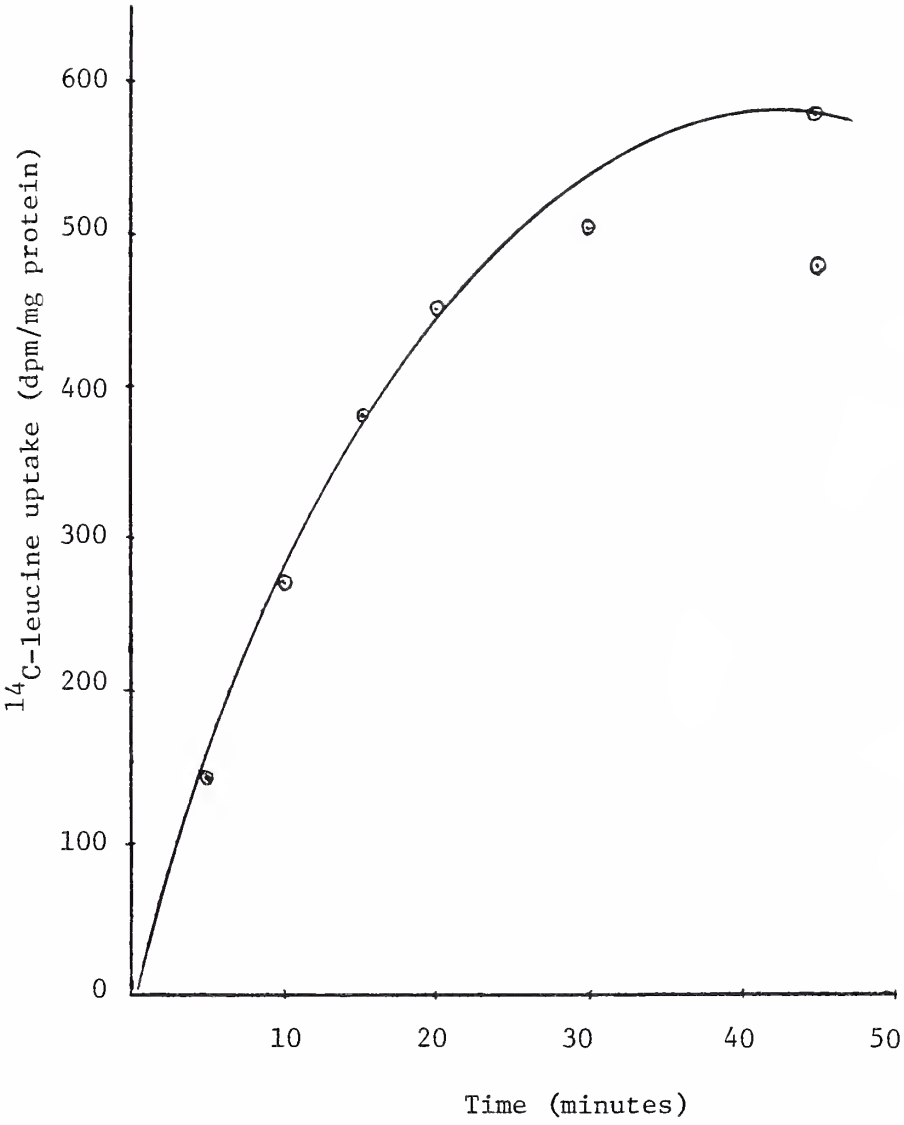


Figure 3.2b: Incorporation of ^{14}C-labeled leucine into protein by ileal crypt cells. The graph is linear initially but tends to level off with time, thus verifying cell viability.

TABLE 3.4

Expt. No.	Ileal villous cell uptake of Free ^{57}CoCbl (pg/mg protein)	IF-^{57}CoCbl (pg/mg protein)	Ratio of uptake of IF-^{57}CoCbl to Free^{57}CoCbl
1	4.0	180	45.0
2	2.0	110	55.0
3	6.0	193	32.2
4	7.0	281	40.0
5	4.0	140	35.0
Mean $\pm$ S.D.	4.60 $\pm$ 1.9	181 $\pm$ 65	41.4 $\pm$ 9.0

Table 3.4: Results of the uptake of Free and IF-bound ^{57}CoCbl by ileal villous cells.

TABLE 3.5

Expt. No.	Ileal crypt cell uptake of Free ^{57}CoCbl (pg/mg protein)	IF-^{57}CoCbl (pg/mg protein)	Ratio of uptake of IF-^{57}CoCbl to Free ^{57}CoCbl
6	Not done	4.25	-
7	4.0	6.0	1.5
8	3.0	6.0	2.0
9	3.4	5.6	1.6
10	2.2	1.8	0.8
Mean $\pm$ S.D.	3.2 $\pm$ 0.8	4.73 $\pm$ 1.8	1.5 $\pm$ 0.5

Table 3.5: Results of the uptake of free and IF-bound ^{57}CoCbl by ileal crypt cells.

TABLE 3.6

	IF-^{57}CoCbl uptake (pg/mg protein)	Free ^{57}CoCbl uptake (pg/mg protein)
Jejunal Crypts	0.68	2.1
Ileal Crypts	3.0	3.2

Table 3.6: Comparison of the uptake of free and IF-bound ^{57}CoCbl by jejunal and ileal crypt cells.

and used for binding experiments in the manner already described. The results of this experiment are shown in Table 3.6. Again, IF-^{57}CoCbl uptake was absent or insignificant with either ileal crypt or jejunal crypt cells. IF-mediated uptake of ^{57}CoCbl by ileal crypt cells exceeded slightly the corresponding uptake by jejunal crypt cells. Because of the low levels of uptake in both ileal crypt and jejunal crypt cells, this may just represent experimental error. More likely, the difference was the result of the inevitable slight contamination of crypt cells with villous cells. Contamination of ileal crypt cells with ileal villous cells would be expected to lead to a higher than expected level of uptake by the former since ileal villous cells bound IF-Cbl avidly. On the other hand, contamination of jejunal crypt cells with jejunal villous cells would not be expected to result in any higher levels of Cbl uptake since jejunal villous cells themselves lack the capacity for such. Fig. 3.3 shows graphically the results of this section.

3.4 Bile Acid Uptake Studies

Tables 3.7 and 3.8 summarize the data for the uptake of ^{3}H-Taurocholate by jejunal and ileal villous and crypt cells. It can be seen that ileal villous cells showed a level of uptake 35-40 fold greater than uptake by ileal crypt or jejunal crypt or villous cells incubated under identical conditions. This is shown graphically in Fig. 3.4. It must be remembered that in these data, the term "uptake" refers to the net uptake after correction has been made for the so-called "non-specific binding". The latter refers to the binding of bile acids to sites other than the surface receptors specific for the transport of bile acids. To correct for this,

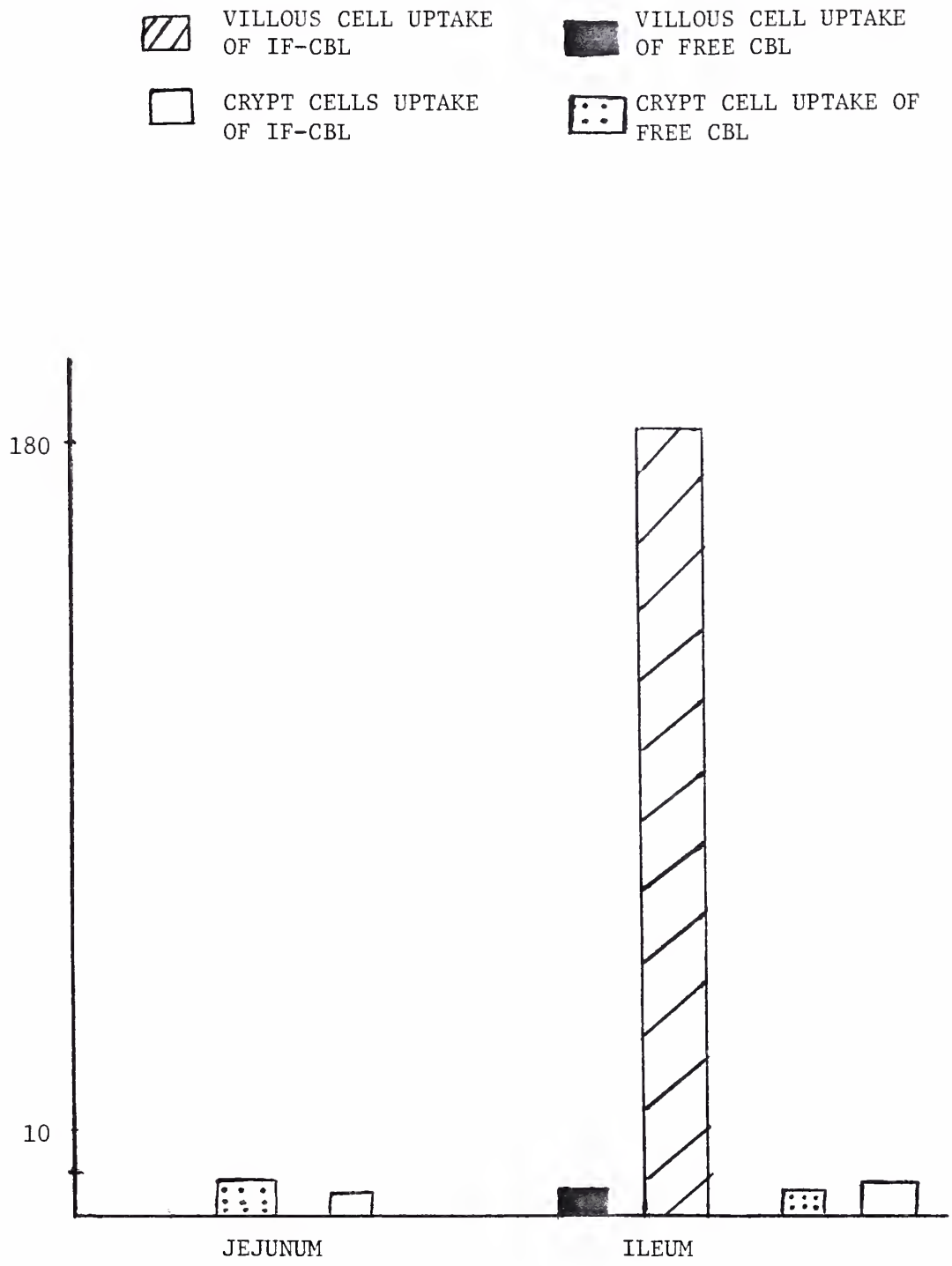


Figure 3.3: Uptake of Free and IF-bound ^{57}CoCbl by guinea pig jejunal and ileal crypt and villous cells. Drawn from Tables 3.4 - 3.6.

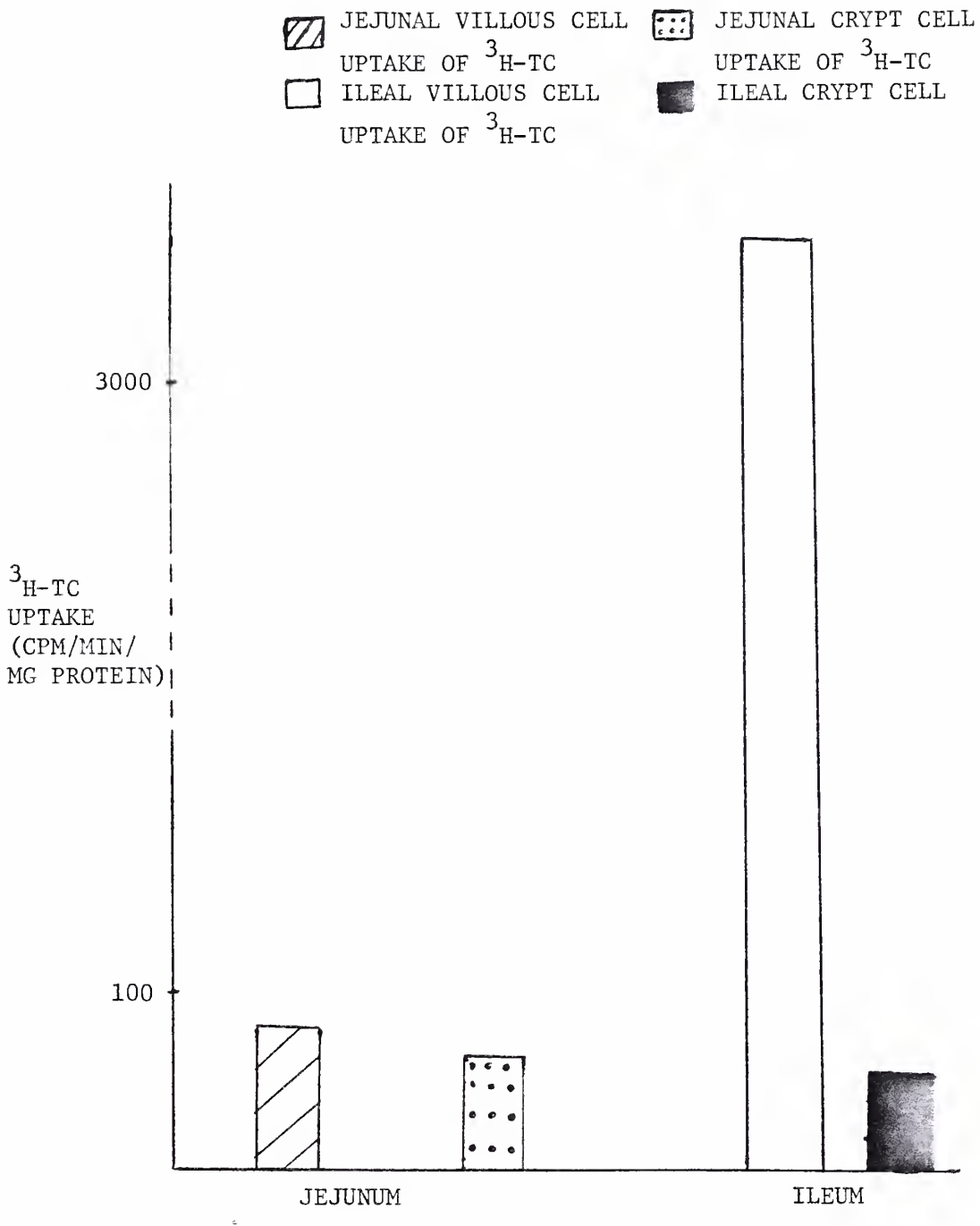


Figure 3.4: Uptake of ^{3}H-Taurocholate by guinea pig jejunal and ileal crypt and villous cells. Drawn from Tables 3.7 and 3.8.

TABLE 3.7

	Uptake of ^{3}H-Taurocholate by villous cells of	
Expt. No.	Jejunum	Ileum
1	87	3463
2	112	3521
3	72	2485
Mean ± S.D.	90 ± 20	3156 ± 582

Table 3.7: Results of the uptake of ^{3}H-Taurocholate by jejunal and ileal villous cells.

TABLE 3.8

	Uptake of ^{3}H-Taurocholate by crypt cells of	
Expt. No.	Jejunum	Ileum
4	93	115
5	61	99
6	74	44
7	110	90
Mean ± S.D.	84 ± 21	87 ± 30

Table 3.8: Results of the uptake of ^{3}H-Taurocholate by jejunal and ileal crypt cells.

incubations were done in solutions containing 1 μM of total taurocholate (labeled and unlabeled) as well as in 1 mM solutions. Since the total amount of labeled taurocholate was constant in each flask, the ratio of labeled to unlabeled taurocholate was 1000-fold greater in the 1 μM solutions than in the 1 mM solutions. Subtraction of the "uptake" of the 1 mM incubations from that in the 1 μM incubations gave the desired corrected uptake that is shown in Tables 3.7 and 3.8.

It may be concluded from the results of this section that only ileal villous cells prossess the mechanism for the active uptake of bile salts.

3.5 Conclusion

The process by which epithelial cells of the crypts of Lieberkuhn divide, differentiate, migrate to the tip of the villus, and eventually desquamate has intrigued cell biologists because of its suitability for the biochemical study of cell maturation in general. It has also been of keen interest to investigators of intestinal absorption and secretion inasmuch as the enzymatic potential of crypt cells may be of importance in the overall absorptive and digestive capacity in those disease states in which either the intestinal surface is continually denuded, or the normal crypt to villous maturation is impaired.

Several different approaches to studing this phenomenon have been reported, including autoradiographic (1), biochemical (12, 13, 14, 89) and histochemical (90). In earlier studies, the histochemical approach was favored bacause it was capable of providing information on the distribution of enzymatic activity as a function of the location of the cell along the

crypt-villous axis. With the advent of biochemical methods of cell fractionation, it has been possible to study not only crypt-villous differences but also differences in subpopulations of villous cells according to their age and/or location on the villous axis (12, 89, 25).

Our studies have established the existence of biochemical and functional differences between the undifferentiated crypt cells and the well differentiated villous cells. Whereas the villous cells were rich in alkaline phosphatase and sucrase, the crypt cell fraction contained nearly all of the thymidine kinase, consistent with the propensity of the crypt cell for cell division. It may be inferred from this distribution of enzymatic activity that as the crypt cell matures, certain gene regulators are activated whilst other genes are repressed; this results in the cessation of the synthesis of the enzymes necessary for DNA synthesis and the initiation of the production of brush border enzymes. By a similar mechanism, maturation of the crypt cell is accompanied by the synthesis of cell surface receptor proteins that are necessary for the binding and transport of dietary and other nutrients such as bile salts and cobalamin.

In previous work, the absorption of glucose, amino acids and electrolytes have been shown to be a function of the villous cell (91), as is the absorption of triglycerides (92). On the other hand, the secretion of electrolytes is thought to be a function of the crypt cell. On the basis of this rather limited information, the generalization has been made that the villous cell serves a primarily absorptive function whereas the crypt cell, in addition to its role in replenishing the villous cell population, has a secretory function. Our study has shown that two very different molecules - conjugated bile salts and cobalamin - both of which

share a common feature in the sense that their active transport occurs only in the ileum, are taken up by villous cells and not by crypt cells. This further supports the concept that the absorptive functions of the intestine are performed by the villous cells.

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