STUDIES ON THE INTESTINAL ABSORPTION OF STEROLS IN THE RAT

by

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A THESIS

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TABLE OF CONTENTS

I. INTRODUCTION AND LITERATURE REVIEW 1

A. Physico-Chemical Aspects of Lipid Absorption 3

B. Physico-Chemical Aspects of Cholesterol Absorption 11

II. HYPOTHESIS AND PURPOSE OF THE STUDY 34

III. SECTION I: INTESTINAL ABSORPTION AND LYMPHATIC TRANSPORT OF CHOLESTEROL AND BETA SITOSTANOL IN THE RAT 41

INTRODUCTION 41

MATERIALS AND METHODS 43

A. Chemicals 43

B. Lymph Transport Study 44

C. Fecal Excretion and Blood Analysis 46

D. Calculation of Absorption 48

RESULTS 51

A. Lymphatic Recovery of ^{14}C Cholesterol and ^{3}H Beta Sitostanol 51

B. Effect of Cholestyramine 51

C. Absorption of Labeled Cholesterol and Beta Sitostanol 51

DISCUSSION 59

A. Fecal Recovery and Intestinal Absorption of Beta Sitostanol 59

B. Beta Sitostanol: A New Marker for Studying Cholesterol Absorption 62

C. Effect of Cholestyramine on the Excretion of Labeled Cholesterol 64

D. Lymphatic Recovery of ^{3}H Beta Sitostanol 65

TABLE OF CONTENTS (continued)

IV.	SECTION II: THE EFFECT OF BETA SITOSTANOL ON CHOLESTEROL ABSORPTION FROM MICELLAR SOLUTIONS IN JEJUNAL LOOPS *IN SITU*	67
	INTRODUCTION	67
	MATERIALS AND METHODS	69
	A. Chemicals	69
	B. Preparation of Micellar Solutions	69
	C. Animal Preparation	72
	D. Infusion	73
	E. Analysis of the Effluent and the Test Solution	76
	F. Calculations	77
	G. Statistical Methods	78
	RESULTS	79
	DISCUSSION	90
V.	GENERAL SUMMARY AND CONCLUSIONS	105
VI.	BIBLIOGRAPHY	107
VII.	APPENDICES	119
	Appendix A	119
	Appendix B	120
	Appendix C	121
	Appendix D	122

LIST OF FIGURES

NUMBER	TITLE	PAGE
1.	Structure of some of the common plant and animal sterols.	2
2.	Structure of commonly occurring bile acids.	5
3.	The general concepts of lipid absorption.	12
4.	Effect of added polar lipid on cholesterol solubility in micellar bile salt solution.	15
5.	Micellar/oil partition ratios versus bile salt concentrations for different lipids.	21
6.	Ratio between labeled sitosterol and cholesterol in the thoracic duct lymph of rats.	29
7.	Effect of sterol content on uptake from lipid emulsions.	35
8.	Cumulative percent recovery of cholesterol and beta sitostanol in the thoracic duct lymph as a function of time	52
9.	The effect of cholestyramine on fecal excretion of cholesterol and beta sitostanol	53
10.	Comparison of the Isotope Ratio Method and the two Total Fecal Recovery Methods.	56
11.	Schematic representation of the jejunal loop preparation.	74

LIST OF FIGURES (continued)

NUMBER	TITLE	PAGE
12.	Cholesterol absorption as a function of cholesterol concentration (control).	80
13A.	Effect of 0.20 mM beta sitostanol in 'solution-mix' on cholesterol absorption.	81
13B.	Effect of pre-flushing on cholesterol absorption from 'solution-mix'.	81
14A.	Effect of 0.30 mM beta sitostanol in 'solution-mix' on cholesterol absorption.	84
14B.	Effect of addition of a 'pre-flush' with 0.30 mM beta sitostanol micellar solution on cholesterol absorption from the 'solution-mix' used in Figure 14A above.	84
15.	Effect of 0.30 mM beta sitostanol in 'pre-mix' condition on cholesterol absorption.	86
16.	Cholesterol absorption as a function of cholesterol concentration in a cholesterol/cholesterol 'solution-mix'.	88
17	Diagrammatic representation of the cholesterol carrier hypothesis.	103

LIST OF TABLES

NUMBER	TABLE	PAGE
1.	Effect of stirring of the bulk buffer phase on the uptake of bile acid and fatty acid from micellar solutions.	8
2.	Distribution by centrifugation of total lipids in intestinal contents after a test meal.	14
3.	Gel filtration study showing the micellar distribution of sitosterol and/or cholesterol in emulsions.	19
4.	Effect of phosphatidylcholine on uptake of cholesterol from mixed micellar solutions by everted jejunal sacs.	24
5.	Absorption of fatty acids from segments of rat intestine _in vivo._	25
6.	Recovery of radioactive cholesterol from the gastrointestinal tract in rats studied 24 hours after receiving nondigestible phospholipid.	26
7.	Lack of effect of sitosterol on lymphatic transport of cholesterol.	28
8.	The absorption of cholesterol by rats fed diets containing cholesterol as the only sterol or cholesterol with 2% added beta sitosterol.	30

LIST OF TABLES (continued)

NUMBER	TABLE	PAGE
9A.	Accountability of ^{14}C in lymph of rats fed ^{14}C cholesterol or ^{14}C epicholesterol.	32
9B.	Paper chromatographic analysis of lymph from rats fed ^{14}C cholesterol or ^{14}C epicholesterol.	32
10.	Affect of soy sterols on cholesterol absorption in rats.	33
11.	Affect of cholesterol on sitosterol influx and the affect of sitosterol on cholesterol.	37
12.	Fecal recoveries of cholesterol and beta sitostanol and percent cholesterol absorbed.	54
13.	Group comparisons using the Mann-Whitney Test.	82

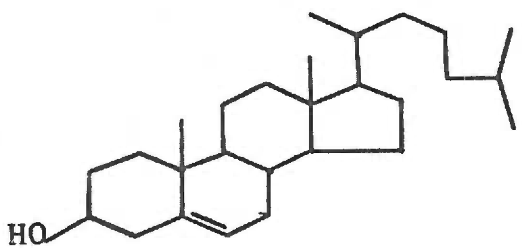
I. INTRODUCTION AND LITERATURE REVIEW

Cholesterol, an animal sterol, and phytosterols, a group of plant sterols which include beta sitosterol, campesterol and stigmasterol, are structurally related compounds (Figure 1). They all possess a cyclopentanophenanthrene ring, a 3 beta-OH group and a double bond between carbons 5 and 6. Structural differences between cholesterol and phytosterols (beta sitosterol will be used henceforth as the representative phytosterol because it is the most abundant phytosterol (1)) are present only in the highly mobile side-chains of the molecules. Despite the great structural similarity between cholesterol and beta sitosterol, these two substances have interesting biological differences which would not be expected a priori on structural grounds alone.

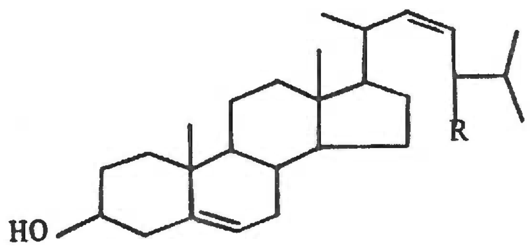
The average daily dietary intake of cholesterol in the human is about 500-700 mg (2). Most reports indicate that cholesterol absorption is incomplete and averages 40-60% of the dose fed (3-9). Cholesterol absorption in rats is similarly incomplete and averages about 50-70% (10-14).

The average daily dietary intake of plant sterols in the human is about 150-250 mg (15) and may be as high as 400 mg depending on the diet (16). Despite the significant intake of beta sitosterol, its absorption is quite limited (7,11-13, 17-19). Indeed, earlier studies by Schoenheimer on the absorbability of beta sitosterol (20), suggested that beta sitosterol was unabsorbable. However, Gould et al. using ^{3}H beta sitosterol for the first time (21), showed that beta sitosterol

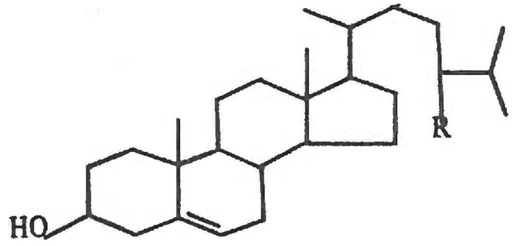
Figure 1. Structure of some of the common plant and animal sterols. The commonest plant sterols include beta sitosterol, campesterol and stigmasterol. Note the minor differences in structure among the different sterols. Epicholesterol has an alpha hydroxy group on C_3 instead of a beta hydroxy group like cholesterol and all the other sterols shown.



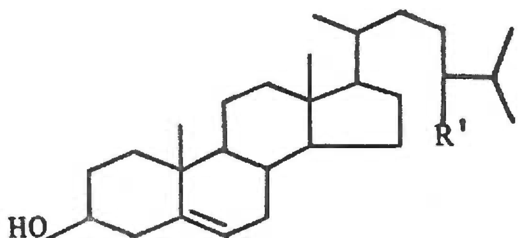
CHOLESTEROL



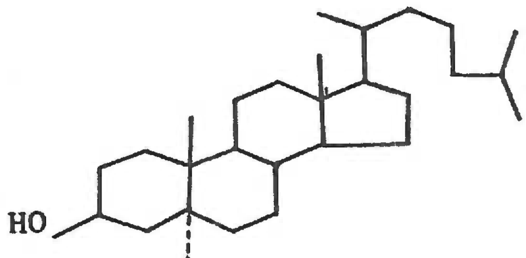
STIGMASTEROL



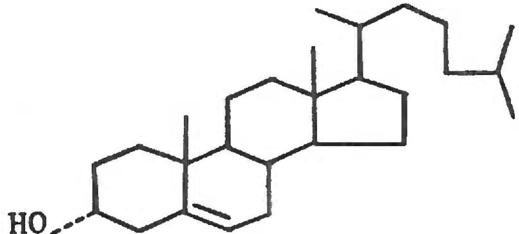
BETA SITOSTEROL



CAMPESTEROL



DIHYDROCHOLESTEROL



EPICHOLESTEROL

R= C_2H_5 ; R'= CH_3

could be absorbed to about 5% of the dose fed. Since then numerous reports have shown that beta sitosterol is absorbable, albeit to a limited extent.

Beta sitosterol has also been shown to reduce the intestinal absorption of cholesterol (5,17,22-24); humans and animals fed beta sitosterol absorb less cholesterol than controls not receiving beta sitosterol. In fact, a role for beta sitosterol as a hypocholesterolemic agent has also been suggested (25).

The reasons for the disparate absorption of two such compounds, beta sitosterol and cholesterol (structurally related), are not known. Furthermore, the mechanism of interference by beta sitosterol on cholesterol absorption is not well understood. It is pertinent, therefore, to examine the currently accepted views on the mechanism of absorption of lipids in general, and cholesterol in particular, to see if satisfactory answers to the problems posed above can be found.

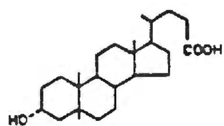
A. Physico-chemical aspects of lipid absorption: Dietary fat is mostly in the form of triglycerides; these are glycerol molecules esterified to three fatty acids (saturated or unsaturated) usually greater than 16 carbons in length. In the duodenum, these are dispersed into a stable emulsion by the presence of proteins, phospholipids, free fatty acids (FFA), bile salts and the churning of duodenal motility which serves to mix the intestinal contents. Under the action of pancreatic lipase, triglycerides are hydrolyzed chiefly into 2-monoglycerides and FFA's and some diglyceride (26). Because the

products of lipolysis are relatively insoluble in the aqueous phase, as are the triglycerides, absorption of the lipolytic products would be limited owing to their separation into an oil phase (27). Bile salts play a major role in the solubilization and consequent efficient absorption of monoglycerides and FFA's.

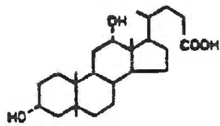
Derivatives of cholesterol (Figure 2), bile salts are a group of detergents with well-known properties (27-32). At a particular concentration, which depends on the type of bile salt (dihydroxy or trihydroxy), pH, concentration of other ions and temperature (31), bile salts spontaneously aggregate to form micelles. Bile salts also interact with FFA's and monoglycerides to form what are known as 'mixed micelles' (27). Formation of mixed micelles allows for solubilization of other water-insoluble lipids such as cholesterol. Such mixed micelles are believed to be macro-molecular aggregates (radius 30-60 Å (26)) with a hydrophobic lipid 'core' in which are dissolved FFA's, monoglycerides and other water-insoluble lipids (27). These aggregates, or mixed micelles, are believed to diffuse through the aqueous medium to the absorptive site where the lipids dissolved in the hydrophobic core of the micelles are absorbed. It is believed that a dynamic equilibrium exists between the lipids in the oil phase and the lipids in the micellar phase (33,34), so that, as the micellar lipids enter the mucosal cell, lipids from the oil phase enter the micellar phase.

The evidence for the existence of the oil and micellar

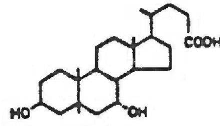
Figure 2. Structure of commonly occurring bile acids. The liver conjugates bile acids with either glycine or taurine. A glycine conjugate of cholic acid, for example, would be known as glycocholic acid whereas the corresponding taurine conjugate would be known as taurocholic acid. (From: Beher, W.T. Bile Acids. Chemistry and Physiology of Bile Acids and their Influence on Atherosclerosis. S. Karger, 1976.)



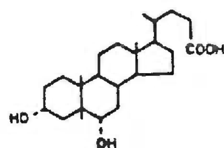
Lithocholic acid
3α-Hydroxy-5β-cholan-24-oic acid



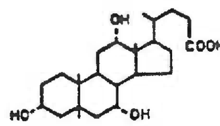
Deoxycholic acid
3α,12α-Dihydroxy-5β-cholan-24-oic acid



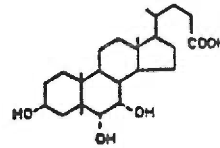
Chenodeoxycholic acid
3α,7α-Dihydroxy-5β-cholan-24-oic acid



Hyodeoxycholic acid
3α,6α-Dihydroxy-5β-cholan-24-oic acid



Cholic acid
3α,7α,12α-Trihydroxy-5β-cholan-24-oic acid



Hyocholic acid
3α,6α,7α-Trihydroxy-5β-cholan-24-oic acid

phases during fat digestion was first provided by Hofmann and Borgstrom (34). They showed that the luminal contents during fat digestion could be separated by ultracentrifugation into a micellar phase and an oil phase; the former phase contained bile salt, FFA's and monoglycerides but virtually no di- or triglycerides which were present in the latter phase. The concentration of other lipids in these two phases was said to be a function of the partition coefficients of those lipids between the oil and micellar phases.

Bile salts, then, are seen to possess two important physiological functions: 1) they are involved in the solubilization of relatively water-insoluble lipids, and 2) as mixed micelles, they allow for the 'transport' of lipids to the absorptive site.

The 'transport' role of bile salts has been given more credence by the studies of Wilson et al. (35), Dietschy (36, 37) and others (38-42). These studies have shown that the rate-limiting barrier in the mucosal uptake of lipids, particularly for those lipids that are water-insoluble, is the 'Unstirred Water Layer' (UWL) adjacent to the mucosal cell membrane. Bile salts, by forming mixed micelles, function as a shuttle transporting the solubilized lipid through the UWL to the absorptive site. As a result a high concentration of lipids is maintained in close proximity to the mucosal membrane. The lipids are believed to enter the cell by passive diffusion through the mucosal membrane which is not postulated to offer any significant resistance because the lipids are

thought to traverse the cell membrane by 'dissolving' in the lipid phase of the membrane. Wilson et al. (35) and others (39) have demonstrated that stirring of the incubation medium increased the uptake of lipids from micellar solutions by intestinal segments *in vitro* (Table 1). This phenomenon was attributed to the presumed decrement in the thickness of the UWL implying a reduction in the diffusion barrier leading to increased lipid uptake. It is evident that modifications of micelles (size and/or shape) diffusing through the UWL would also be expected to effect the uptake of lipids, at least in the *in vitro* system mentioned above.

The exact processes involved in the transfer of micellar lipids into the cell are not well understood. After crossing the UWL the micellar lipids could enter the mucosal cell in one of three ways:

1. The entire micelle could be taken up by the mucosal cell by a process similar to pinocytosis. However, Simmonds et al. (43) have shown that the various constituent molecules in the mixed micelles were absorbed at independent rates. They concluded, therefore, that the entire micelle was probably not taken up by the cell. Although there is some agreement on this point (26), there are also a few reports that suggest that the entire micelle is probably taken up by the mucosal cell (16,44,45).

2. On the other hand, a micelle could collide with and possibly bind to the mucosal cell membrane. Because of the hydrophobic nature of the membrane, the stability of the

Table 1. Effect of stirring of the bulk buffer phase on the uptake of bile acid and fatty acid from micellar solutions. (Data from: Wilson, F.A., Sallee, V.L. and Dietschy, J.M. Science, 174:1031, 1971.)

Test substance	J Unstirred	Stirred	Increase (%)
	From micellar solutions		
Fatty acid 16:0	21±6	45±8	114
Taurodeoxycholate	87±6	196±14	125

micelle would be decreased causing the micelle to disaggregate and liberate the lipid content within the membrane. The high concentration of such liberated lipid within the cell membrane would favor passive diffusion of those lipids into the cell down the concentration gradient (46).

3. It is believed that the micellar lipid is in dynamic equilibrium with the lipids dissolved in monomer form in the aqueous phase adjacent to the cell membrane. Equilibration between the lipids in the micelle and those in monomer form in the aqueous phase is believed to be very rapid so that a high concentration of lipids in the aqueous phase is always maintained. The high concentration of lipids in monomer form in the aqueous phase is believed to provide the favorable concentration gradient for entry of lipids into the mucosal cell by passive diffusion (42).

On the basis of theoretical derivations and *in vitro* experimental observations, Westergaard and Dietschy (47) concluded that lipids were taken up by the mucosal cell from the monomer form present in the aqueous phase adjacent to the mucosal membrane. They rejected the possibility of micelle-mucosal membrane interaction as the mechanism of entry of micellar lipid into the cell on the grounds that their experimental data did not fit their theoretical predictions of that model (47).

It is agreed that some lipid may be absorbed from the monomer form present in the aqueous phase (16); however, Westergaard and Dietschy's rejection of the micelle-mucosal

membrane interaction model should be viewed with some caution. There is evidence to suggest that observations with regard to lipid absorption *in vitro* may not correspond to that taking place *in vivo* (48). In addition there were a few peculiarities of the micellar solutions used by Westergaard and Dietschy (47) which may have had a bearing on their results and, therefore, their conclusions:

1. Taurodeoxycholic acid at 20 mM was used in most studies. It has been shown that taurodeoxycholate, even at physiological concentrations (10 mM), can cause histological damage to the small intestine mucosal membrane (49).

2. Taurodeoxycholic acid forms larger micelles with increasing concentration (30,32). This may lead to results which are opposite to expectations. That is, increased bile salt concentration would be expected to solubilize more lipid and therefore enhance absorption. However, if at the same time, the size of the micelle is increased, then the net result may well be a decrease in lipid absorption owing to the decreased rate of diffusion of the now enlarged micelle.

3. The micellar solutions used by Westergaard and Dietschy (47) were two component systems, i.e., they consisted of the bile salt and one lipid, fatty acid or cholesterol. It has been shown that the solubility of both fatty acid and cholesterol in pure bile salt micelles is minimal; inclusion of monoglyceride in the system greatly enhances their solubility (27). It is doubtful that mixed micelles are two-component systems *in vivo*; as such the use of pure two-component

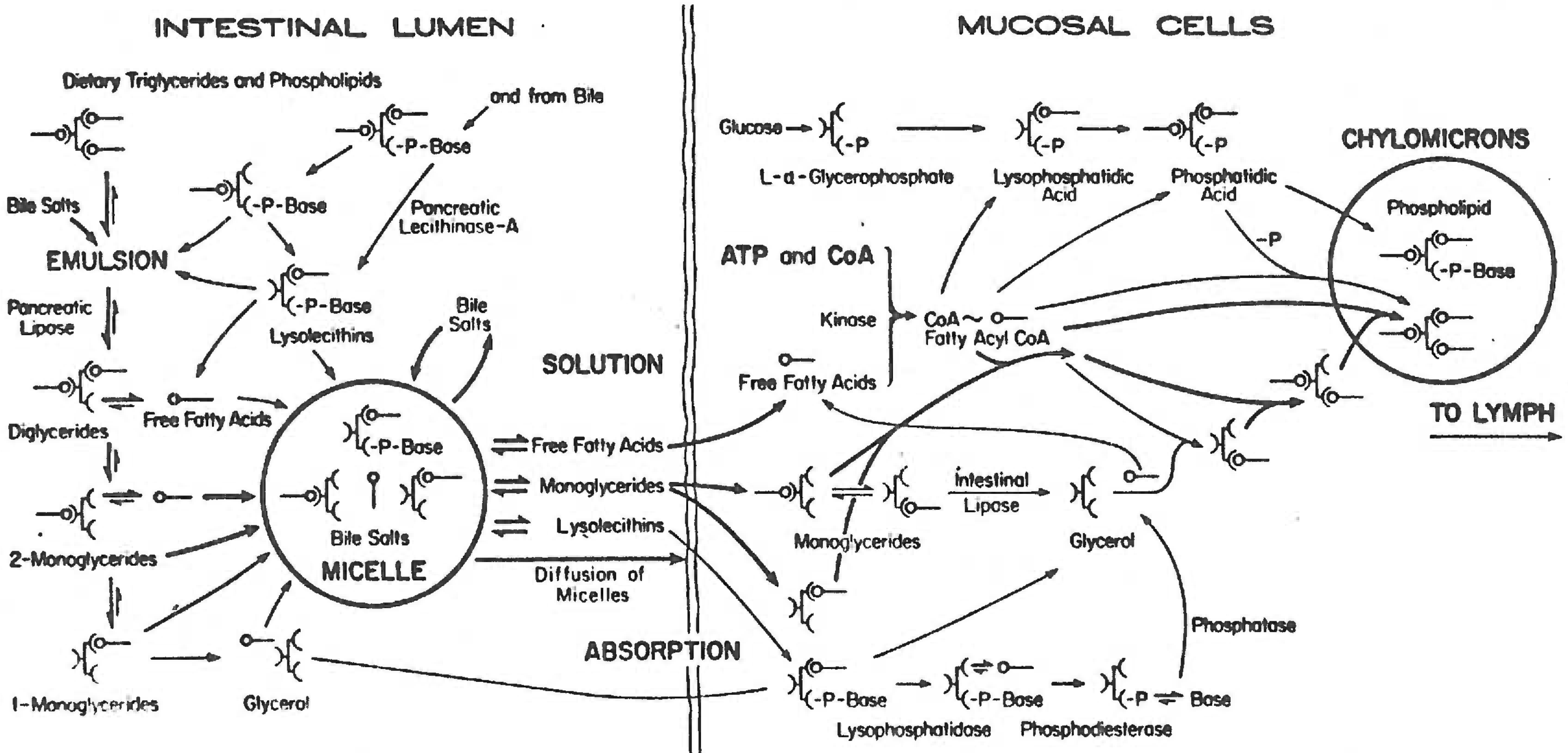
systems by Westergaard and Dietschy (47) in their *in vitro* studies seems questionable.

Based on this and the fact that there is some controversy regarding uptake of the intact micelle by the mucosal cell, selection of one mechanism of entry of micellar lipids into the cell over the others would be premature at best. More information is needed before a given mechanism can be selected as *the* mechanism whereby micellar lipids enter the mucosal cell. Until such information is obtained, all three alternatives must be entertained as possible.

The monoglycerides entering the mucosal cell undergo little or no further hydrolysis; they are resynthesized into triglycerides by esterification with fatty acids (26). FFA's are also combined with glycerophosphate to make phospholipids. Some FFA's may escape directly into portal blood; this is a function of the chain length (less than 10 carbons) (16). The newly synthesized triglycerides and phospholipids, together with other lipids and some proteins, are collected by the cell into droplets called chylomicrons. These are extruded from the lateral borders of the cell into the interstitial fluid by an unknown mechanism. The chylomicrons in the interstitial fluid enter the lymphatics and eventually enter the general circulation through the venous system (Figure 3 summarizes the general concepts of lipid absorption).

B. Physico-chemical aspects of cholesterol absorption: Cholesterol presented to the intestine for absorption comes from several sources: 1. diet, 2. bile, 3. desquamated cells,

Figure 3. The general concepts of lipid absorption. (From: Davenport, H.W. _Physiology of the Digestive Tract_. Year Book Medical Publishers, 4th edition, 1977.)


INTESTINAL LUMEN
MUCOSAL CELLS
Dietary Triglycerides and Phospholipids
and from Bile
-P-Base
Bile Salts
EMULSION
Pancreatic Lecithinase-A
Pancreatic Lipase
Lysolecithins
Bile Salts
Diglycerides
Free Fatty Acids
2-Monoglycerides
1-Monoglycerides
Glycerol
Bile Salts
MICELLE
SOLUTION
Free Fatty Acids
Monoglycerides
Lysolecithins
Diffusion of Micelles
ABSORPTION
Glucose
-P
L-α-Glycerophosphate
Lysophosphatidic Acid
Phosphatidic Acid
CHYLOMICRONS
Phospholipid
ATP and CoA
Kinase
CoA ~ Fatty Acyl CoA
Free Fatty Acids
TO LYMPH
Monoglycerides
Intestinal Lipase
Glycerol
Phosphatase
Lysophosphatidase
Phosphodiesterase
Base

and 4. intestinal secretion. Both free and esterified cholesterol are presented for absorption; however, only the free form is absorbed. The fatty acid ester is removed by pancreatic cholesterol esterase (2).

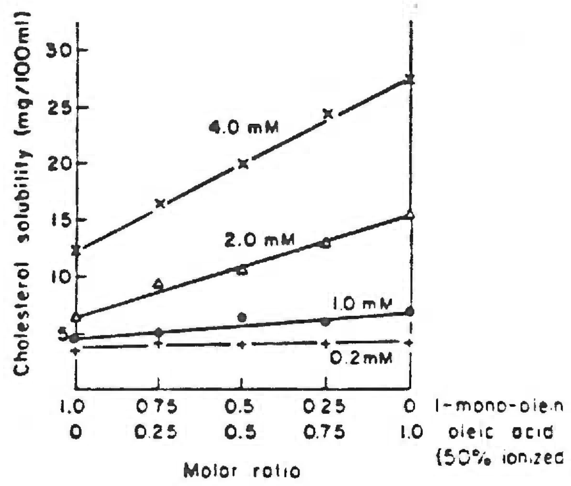
Cholesterol is classified as an 'Insoluble, Non-Swelling Amphiphile.' These lipids are insoluble in water and, depending on the temperature, form either oils or crystals in water (27,28). Because cholesterol is insoluble in water, the intestinal absorption of cholesterol would be quite limited (if at all) without the presence of solubilizing agents. Bile salts have an indispensible role in the solubilization and consequent absorption of cholesterol (50).

In the intestine, cholesterol is distributed between the two phases mentioned earlier: the oil and the micellar phases (33) (Table 2). The concentration of cholesterol in each phase is determined by the partition coefficient of cholesterol for the system. The extent of absorption of cholesterol depends on the concentration of cholesterol in the micellar phase (51), which in turn depends on the solubility of cholesterol in the micelle. It has been shown that the products of triglyceride hydrolysis, which form mixed micelles with bile salts (30), greatly increase the concentration of cholesterol in the micellar phase (Figure 4), as compared to the concentration of cholesterol in pure bile salt micelles, where because of its low solubility, cholesterol precipitates from solution and is unavailable for absorption (43). In the intestine, then, the extent of cholesterol absorbed depends

Table 2. Distribution by centrifugation of total lipids (TF), total cholesterol (TC), free cholesterol (FC) and ^{14}C activity between top layer (oil phase) and subnatant layer (micellar phase) in intestinal contents after a test meal. The test meal fed to the subjects (human) was composed of an emulsion of skim milk powder, glucose and corn oil. In addition the test meal contained 5 uCi of 4-^{14}C cholesterol with 100 mg of 'cold' cholesterol.

	TF mg/ml	FFA mg/ml	TC mg/ml	FC mg/ml	S.A. of TF cpm/mg	S.A. of TC cpm/mg
Top layer	3.5	1.2	0.19	0.15	662	3480
Subnatant layer	3.2	1.4	0.15	0.11	513	3420

Figure 4. Effect of added polar lipid on cholesterol solubility in micellar bile salt solution. Oleic acid or glyceryl-1-mono-oleate was added singly or in mixtures, in amounts and molar ratios indicated, to 10 mM sodium taurocholate-sodium taurodeoxycholate and phosphate buffer, pH 6.5. This figure is provided to simply illustrate that cholesterol solubility increases as the concentration of added polar lipids increases from 0.2 mM to 4.0 mM. No emphasis is placed on the relative effects of monoolein and oleic acid on cholesterol solubility. (From: Simmonds, W.J., Hofmann, A.F., & Theodor, E. J. Clin. Invest. 46:874, 1967.)



Cholesterol solubility (mg/100ml)
30
25
20
15
10
5
4.0 mM
2.0 mM
1.0 mM
0.2mM
1.0
0.75
0.5
0.25
0
1-mono-olein
0
0.25
0.5
0.75
1.0
oleic acid
(50% ionized
Molar ratio

not only on the presence of bile salts but also on the duration of the presence of monoglycerides and FFA's; cholesterol absorption will be decreased when the hydrolytic products of triglycerides have been completely absorbed (16).

The actual mechanism of solubilization of cholesterol in mixed micellar solutions is not known. It is believed that the interaction of monoglycerides with bile salt micelles 'swells' the micelles (27) allowing for incorporation of cholesterol and other lipids into the hydrophobic interior of the micelles. In this way cholesterol is 'transported' across the UWL to the absorptive site.

A few words concerning the plausibility of the Westergaard and Dietschy model (47) of uptake of micellar lipids as applied to cholesterol, are in order. It is generally accepted that cholesterol is insoluble in water (2,25,27). Based on this, the mucosal uptake of cholesterol, according to the hypothesis of Westergaard and Dietschy, should be zero or very near zero. Release of cholesterol from the mixed micelle to the water phase will result in the precipitation of cholesterol which will render it unabsorbable. Therefore, the hypothesis of Westergaard and Dietschy (47) appears to be untenable as far as the mucosal uptake of cholesterol is concerned.

After cholesterol enters the mucosal cell it is esterified. The cholesterol esters, along with some free cholesterol, are 'packaged' into the chylomicrons which enter the general circulation via the lymphatics (2).

It is evident from the above discussion, that the processes

involved in the intestinal absorption of cholesterol may be discussed under three broad categories:

1. Intraluminal events
2. Intramembranal events and
3. Intracellular events.

The difference in the absorbability of cholesterol and beta sitosterol, therefore, may be a function of the differences that may exist between the sterols at any one (or more) of the above steps in cholesterol absorption. In addition, interference in the absorption of cholesterol by beta sitosterol could also occur at any one (or more) of the above steps. Further discussion, then, will concentrate on the intraluminal and intracellular aspects of sterol absorption vis-a-vis cholesterol and beta sitosterol. It might be reiterated here, that the processes involved in the transfer of micellar sterol across the cell membrane into the mucosal cell (intramembranal events), are not well understood. As such this point will not be discussed any further.

1. Intraluminal events: It was mentioned earlier that the solubilization of cholesterol by mixed micelles is an essential pre-requisite to the absorption of cholesterol. Beta sitosterol, by interfering in the micellar solubilization of cholesterol, could possibly reduce the absorption of cholesterol. The lesser absorption of beta sitosterol could be accounted for if the partition coefficient of beta sitosterol were highly in favor of the oil phase.

In an attempt to elucidate the interactions between cho-

lesterol and beta sitosterol, Feldman and Borgstrom (52) studied the phase distribution of these sterols by gel filtration. They found that beta sitosterol was distributed more in favor of the oil phase than the micellar phase; for cholesterol the reciprocal was true. The presence of beta sitosterol in micellar solutions of cholesterol, increased the concentration of cholesterol in the micellar phase; cholesterol, however, reduced the concentration of beta sitosterol in the micellar phase (Table 3, lines 3 and 4). Beta sitosterol-containing micelles were found to be smaller than cholesterol-containing micelles as shown by the calculated value of K_{av} which is an indicator of the volume of gel available for the micelle containing the given sterol. The greater the value of K_{av}, the greater the volume of gel available to the micelle and the smaller the micellar size.

Carey and Small (27) have criticized the use of gel filtration for measurements of micellar size on the grounds that such techniques disrupt micelles by trapping the bile salts in the gel lattice thereby causing cholesterol to precipitate out as micro-liquid crystals.

Notwithstanding the criticism of Carey and Small (27) on the use of gel filtration for the measurements of micellar size, there are some interesting implications of the study. If the *in vivo* interaction of beta sitosterol and cholesterol were the same as that shown in the *in vitro* system, then it would appear that cholesterol would decrease the absorption of beta sitosterol by reducing the micellar solubility of

Table 3. Gel filtration study showing K_{av} and micellar distribution of sitosterol, and/or cholesterol emulsified in varying concentrations in 6 mM sodium taurodeoxycholate. (From: Feldman, E.B. and Borgstrom, B. Biochim. Biophys. Acta, 125:136, 1966.)

Sitosterol concn. (mM)	Cholesterol concn. (mM)	K_{av}	% micellar phase		umoles micelle	
			Sitosterol	Cholesterol	Sitosterol	Cholesterol
2.0	0	0.37	42		0.84	
0	1.0	0.28*		68		0.68
**2.0	1.0	0.30	5		0.10	
***2.0	1.0	0.28		84		0.84
6.0	1.0	0.31		83		0.83
14.0	10.0	0.30*		72		7.2
0	10.0	0.20		61		6.1

*Mean of two experiments

**1.0 mM cholesterol added to a 2.0 mM sitosterol-containing micellar solution

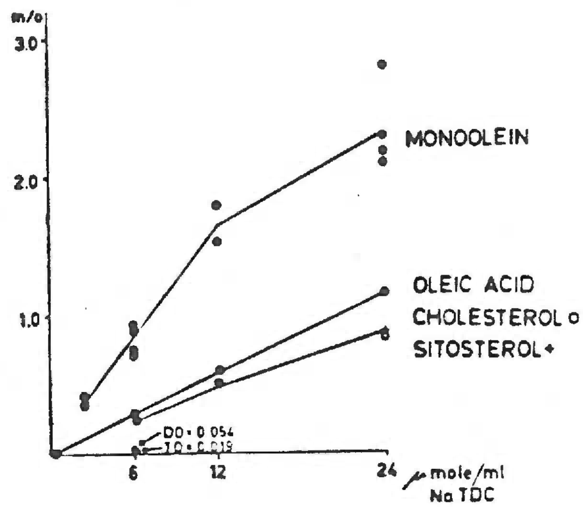
***2.0 mM sitosterol added to a 1.0 mM cholesterol-containing micellar solution

beta sitosterol; beta sitosterol, on the other hand, would enhance the absorption of cholesterol by increasing the concentration of cholesterol in the micellar phase. Whether the *in vivo* interaction of beta sitosterol and cholesterol is the same as that described *in vitro*, is not known. What has been observed *in vivo* is that beta sitosterol decreases the intestinal absorption of cholesterol both in animals (17,53) and man (23,24).

The observation (above) that the partition coefficient of beta sitosterol was in favor of the oil phase, could possibly account for the lesser absorption of beta sitosterol. However, in an *in vitro* study utilizing triolein-bile salt system and a more complicated system consisting of tri-, di- and monoolein and oleic acid in bile salt dispersions (these systems essentially give two phases: an oil phase and a micellar phase), Borgstrom (54) has shown that cholesterol and beta sitosterol had similar partition coefficients in similar systems (Figure 5). The partition coefficients were determined by separating the two phases with millipore filters and determining the concentration of the specific sterol in each phase.

If Borgstrom's conclusion (54), that the oil/micellar partition coefficients of cholesterol and beta sitosterol are similar, is acceptable, then, given the currently accepted mechanism of absorption of cholesterol, there is no reason to expect large differences in the intestinal absorption of cholesterol and beta sitosterol. Certainly if cholesterol

Figure 5. Micellar/oil (M/O) partition ratios versus bile salt concentration for different glycerides, oleic acid and sterols in dispersions of 2.5 umoles each triolein (TO), diolein (DO), and monoolein (MO), 7.5 umoles of oleic acid, and 1.0 umole of sterol per ml in 6,12 and 24 mM sodium taurodeoxycholate, pH 6.3. The values for cholesterol and sitosterol at 6 and 12 mM sodium taurodeoxycholate were identical and the symbols therefore blend. (From: Borgstrom, B., J. Lipid Res. 8:598, 1967.)


m/o
3.0
2.0
1.0
MONOOLEIN
OLEIC ACID
CHOLESTEROL o
SITOSTEROL +
DO = 0.054
6
12
24
μ mole/ml
Na TDC

(and beta sitosterol) is insoluble in water, then the mixed micelle containing cholesterol (or beta sitosterol) must come in contact with the membrane if cholesterol absorption is to occur. From that point, if cholesterol enters the cell by simply 'dissolving' in the lipid phase of the membrane, then there is no reason to suppose that the same would not hold true for beta sitosterol.

On the question of reduction of cholesterol absorption by beta sitosterol, it has been suggested that the large amounts of beta sitosterol usually required for the effect expands the sterol pool in the intestine thereby reducing the availability of bile salts and other lipids for solubilization of cholesterol (16). However, this argument is difficult to reconcile with the fact that while large amounts of beta sitosterol could possibly reduce the availability of solubilizing agents for cholesterol, beta sitosterol itself would be present in potentially absorbable form in large amounts, which would result in increased absorption of beta sitosterol. This is not observed.

Again, if the mixed micelle is the transport vehicle for cholesterol and beta sitosterol and if the observations on the UWL are valid, then it is evident that any factor that retards the diffusion of the micelles through the UWL will effect the rate of absorption of sterols. Obviously, there exist at least two possibilities here, either one of which could lead to decreased sterol absorption.

a. Increase in the thickness of the UWL would simply

increase the extent of the diffusion barrier, so that a longer time would be required for the mixed micelles to diffuse to the mucosal membrane. This would lead to a decreased rate and amount of uptake of sterols from mixed micelles. Although beta sitosterol has been reported to decrease the intestinal absorption of cholesterol, it is difficult to see how it does so if the UWL is the only parameter that is altered. Evidently, the next question would be concerned with the mechanism whereby beta sitosterol altered the thickness of the UWL, if it did so at all.

b. Alterations in the shape or size of mixed micelles would be expected to influence the diffusion parameters of the mixed micelles. This in turn would be expected to affect the rate of absorption of micellar sterols.

A number of factors are known to affect the size of simple bile salt micelles (31). In addition, Rampone (55,56) and Rodgers and O'Conner (57) in _in vitro_ studies have shown that the intestinal uptake of cholesterol from micellar solutions containing phosphatidylcholine or its ether analog, was reduced compared to controls (Table 4). Recently, a similar phenomenon has been demonstrated _in vivo_ both with fatty acid (58) and cholesterol (59). In both instances the absorption of the test substance under study was reduced compared to controls when phosphatidylcholine or its ether analog was included in the test solutions (Table 5 & 6).

The mechanism of action of phosphatidylcholine and its ether analog in reducing cholesterol absorption, is not known.

Table 4. Effect of phosphatidylcholine (diester and diether) on uptake of cholesterol from mixed micellar solutions by everted jejunal sacs. (From: Rodgers, J.B. and O'Connor, P. Biochim. Biophys. Acta, 409:192, 1975.)

Group	^3H Cholesterol uptake (nmol/min per g)	P
Control	49±3	-
Diester	29±3	<0.001
Diether	20±2	<0.0001

Table 5. Absorption of fatty acids from segments of rat intestine *in vivo*. (From: Saunders, D.R. and Sillery, J. *Lipids*, 11:832, 1976.)

Segment	Addition	Linoleate absorbed (nmoles/min/g)
Jejunum	None	471±24
	Lecithin, 3mM	290±53
	Lysolecithin, 3mM	552±60
Ileum	None	494±45
	Lecithin, 3mM	321±47
	Lysolecithin, 3mM	572±58

Table 6. Recovery of radioactive cholesterol from the gastrointestinal tract in rats studied 24 hours after receiving nondigestible phospholipid (% dose ± S.E.). (From: Rodgers, J.B., Fondacaro, J.D. and Kot, J.J. Lab. Clin Med. 89:147, 1977.)

	Stomach		Small bowel		Colon and feces		Total recovery	
	Per cent	*p**	*Per cent*	*p*	*Per cent*	*p*	*Per cent*	*p*
Control (8)†	1.0 ± 0.4	—	9.3 ± 1.3	—	44.2 ± 3.7	—	54.4 ± 2.6	—
Experimental 1 (8)‡	2.5 ± 0.8	NS	10.4 ± 1.7	NS	57.9 ± 2.9	<0.02	70.8 ± 2.2	<0.001
Experimental 2 (8)‡	0.2 ± 0.1	<0.02	4.7 ± 0.7	<0.02	69.0 ± 4.7	NS	73.9 ± 4.6	NS

*The p values given for Experimental 1 compare the means for this group with the respective means for the controls. The p values given for Experimental 2 compare the means for this group with the respective means for Experimental 1.

†Numbers in parentheses refer to the number of animals studied.

‡Experimental 1 received 3 mM diether phosphatidylcholine and Experimental 2 received 6 mM diether phosphatidylcholine in the test dose of labeled emulsion.

Neither is the size nor the shape of the cholesterol and sitosterol-containing mixed micelle known. Clearly, more definitive studies are needed to understand the interactions of beta sitosterol with cholesterol-containing micelles.

2. Intracellular events: Observations on the lymphatic transport of cholesterol and beta sitosterol in the rat (11) showed that only 3-6% of the administered beta sitosterol was transferred to the lymph in 24 hours independent of the dose fed. The total amount of beta sitosterol transferred to the lymph, therefore, was directly proportional to the amount administered. About 40-50% of the administered cholesterol was transferred to the lymph in the same time (Table 7) and the ratio of beta sitosterol to cholesterol in the lymph was constant regardless of the ratio of the two in the diet (Figure 6). Sylven and Borgstrom (11) concluded that cholesterol and beta sitosterol did not exhibit any mutual interference in their absorption and that the site of specificity for sterol absorption was located within the mucosal cell.

The results of Sylven and Borgstrom (11) showing no interference of cholesterol absorption by beta sitosterol are at variance with the results of Mattson et al. (60). The lymphatic transport of cholesterol in rats fed a sterol mixture containing cholesterol and beta sitosterol, was significantly lower than control (Table 8), suggesting that beta sitosterol had interfered with the absorption of cholesterol. At present there is no known reason for the different results obtained in the two studies, although it is clear that the

Table 7. Lack of effect of sitosterol on lymphatic transport of cholesterol. (From: Sylven, C. and Borgstrom, B. J. Lipid Res. 10:179, 1969.)

Cholesterol Fed (umoles)	Cholesterol Transported (umoles)
12.5	5.7
25.0	9.7
37.5	14.0
Cholesterol/Sitosterol Fed (Proportions) (umoles)	
12.5/37.5 (1:3)	6.3
25/25 (1:1)	9.8
37.5/12.5 (3:1)	14.7

Figure 6. Ratio between labeled sitosterol (^{3}H) and cholesterol (^{14}C) in the thoracic duct lymph of rats fed 50 umoles of total sterol in 800 umoles of triolein. The ratios of cholesterol to sitosterol were 1:3 (o), 1:1 (+), and 3:1 (•). (From: Sylven, C. and Borgstrom, B. J. Lipid Res. 10:179, 1969.)

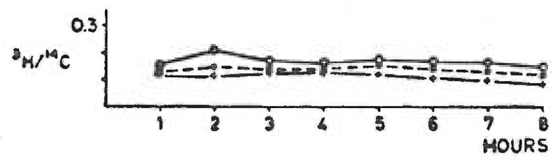

0.3
$^3H/^{14}C$
1
2
3
4
5
6
7
8
HOURS

Table 8. The lymphatic recovery of cholesterol (cholesterol absorption) in rats fed diets containing cholesterol as the only sterol or cholesterol with 2% added beta sitosterol. Each rat was administered an emulsion type diet that contained 15 mg of (4-^{14}C) cholesterol as a 60-40 mixture of cholesterol and cholesterol oleate. The total cholesterol equivalent was 1% of the dietary fat. The cholesterol-sitosterol ratio was 1:2. The values shown are the mean $\pm$ S.E.M. of 10 rats. (Data from: Mattson, F.H., Volpenhein, R.A., & Erickson, B.A. J. Nutr. 107:1139, 1977.)

Plant sterol	Level in dietary fat %	Cholesterol absorption %	Decrease in absorption %
None	-	56.8±2.8	-
Beta sitosterol	2.0	44.4±2.8	21.8

results of Mattson et al. (60) are consistent with those normally observed with beta sitosterol feeding (22,23,53).

Difference between cholesterol and beta sitosterol in esterification after entry into the mucosal cell has been suggested as a possible reason for the difference in the absorption of the two sterols (61). However, Salen et al. (7) have shown that the percent beta sitosterol found esterified in the human plasma was the same as that of cholesterol. Borgstrom (13) has shown that once cholesterol and beta sitosterol had been taken up by the mucosal cell, they did not differ in their subsequent rate of transit out of the cell. In addition, it has been shown that epicholesterol (an alpha-hydroxy analog of cholesterol) (Figure 1), for example, is absorbed and transported without esterification (Table 9) (62) suggesting that esterification may not be an obligatory process in the transfer of sterols from the mucosal cell to the lymph.

Finally, it has also been suggested that phytosterols interfere with the esterification of cholesterol and so lower plasma cholesterol (63). However, it has been shown that regardless of the amount of cholesterol absorbed, the range in percent of cholesterol found esterified in the lymph was the same whether or not the rats received the phytosterols (soy sterols) (Table 10) (64).

Table 9A. Accountability of ^{14}C in lymph of rats fed 4-^{14}C cholesterol or 4-^{14}C epicholesterol. Lymph collected from the animals was extracted with acetone-alcohol to extract the lipids. In order to remove extraneous material, the acetone-alcohol extract was subjected to alumina column chromatography. Note that essentially all (97%) of the radioactivity present in the original acetone-alcohol extract was recovered in the alumina column eluate.

Table 9B. Paper chromatographic analysis of lymph from rats fed 4-^{14}C cholesterol or 4-^{14}C epicholesterol. Note that before saponification, the radioactivity in the lymph recovered from rats fed ^{14}C cholesterol is found in two regions on the paper chromatogram. One region has an R_f equivalent to cholesterol palmitate, while the other has an R_f equivalent to free cholesterol. After saponification ^{14}C activity is only found in one region corresponding to free cholesterol. This shows that cholesterol found in the lymph is, in part, esterified. In contrast, paper chromatogram of lymph recovered from rats fed ^{14}C epicholesterol showed that radioactivity in lymph from these animals was found in the region corresponding to free epicholesterol whether or not the lymph was saponified. This shows that epicholesterol found in the lymph is free epicholesterol. (From: Hernandez, H.H., Chaikoff, I.L., Dauben, W.G. and Abraham, S. J. Biol. Chem. 206:757, 1954.)

A

C^{14} recovered in	Rat 3 fed cholesterol-4-C^{14}	Rat 18 fed epicholesterol-4-C^{14}
	total c.p.m.	*total c.p.m.*
Lymph sample	26,810	13,900
Acetone-alcohol extractions	27,210	14,000
Alumina column eluate	26,310	13,400

B

Rat No.	Material chromatographed	R_F* of radioactive spots 4 to 8 hr. sample	
		Before saponification	After saponification
	Cholesterol	0.56 ± 0.05	0.56 ± 0.05
	Cholesterol-palmitate†	0	0.56 ± 0.05
	Epicholesterol-4-C^{14}	0.91 ± 0.05	0.91 ± 0.05
1	Lymph extract from rat fed cholesterol-4-C^{14}	0 and 0.56	0.55
3	" " " " " "	0 " 0.51	0.56
5	" " " " " "	0 " 0.54	0.52
6	" " " " " "	0 " 0.58	0.59
19	" " " " " "	0 " 0.60	0.56
56	" " " " " "	0 " 0.59	0.53
2	" " " " " epicholesterol-4-C^{14}	0.91	0.90
4	" " " " " "	0.88	0.88
7	" " " " " "	0.92	0.94
18	" " " " " "	0.91	0.90
58	" " " " " "	0.93	0.87
59	" " " " " "	0.89	0.93

* R_F is measured as the distance of the front of the compound from the origin, divided by the distance of the front of the solvent from the origin.

† Prepared according to Page and Rudy (10).

Table 10. Effect of Soy Sterols on Cholesterol Absorption in Rats. (From: Hernandez, H.H. and Chaikoff, I.L. Proc. Soc. Exp. Bio. Med. 87:541, 1954.)

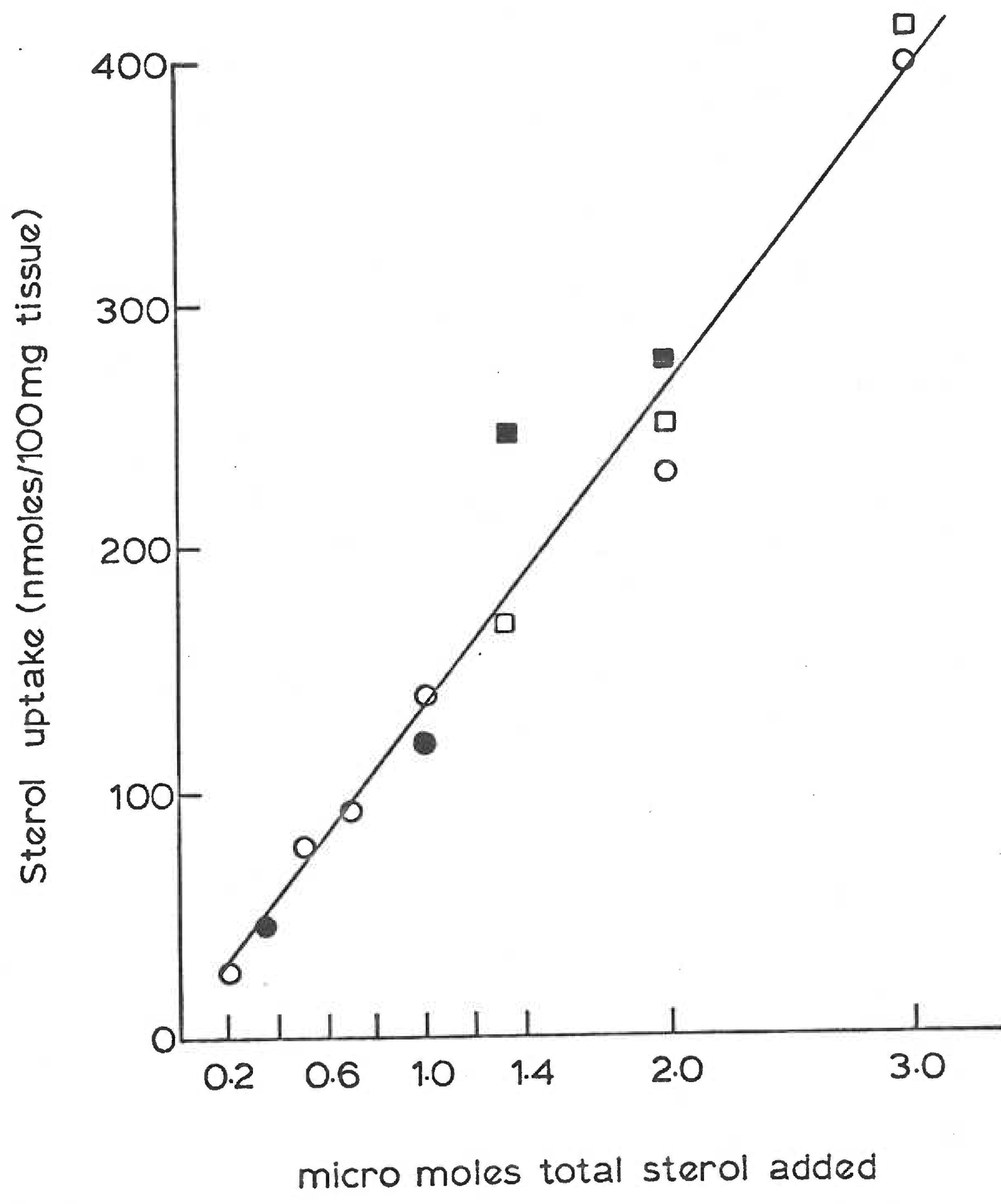
Lymph flow, cc	Test meal: Olive oil, cc	Test meal: Cholesterol (mg) * 4-C^{14}-labeled † Unlabeled	Test meal: Soy sterols, mg	24-hr lymph sample: Digitonin-precipitable, L. B. positive materials, mg	24-hr lymph sample: % of fed C^{14} recovered in digitonin-precipitable fraction	24-hr lymph sample: % of sterol esterified, Total	24-hr lymph sample: % of sterol esterified, C^{14}
45	3	100†	None	27	—	42	—
64	3	"	"	29	—	56	—
34	3	100*	"	23	12.2	65	40
60	3	"	"	29	14.4	62	60
51	3	"	"	27	14.6	53	57
57	3	"	"	31	13.8	63	52
45	3	"	100	20	8.0	48	60
33	3	"	"	20	8.0	56	79
63	3	100	"	18	9.2	62	67
67	.25	4*	None	12	24.6	56	74
94	"	"	"	17	27.0	58	75
93	"	4	"	14	29.8	59	68
90	"	4*	4	10	12.4	60	61
67	"	"	"	10	13.3	51	56
88	"	"	"	20	13.4	60	54

II. HYPOTHESIS

From the foregoing discussion on the intraluminal and intracellular aspects of sterol absorption, the role of intracellular events in the specificity of sterol absorption and the reduction of cholesterol absorption by beta sitosterol, does not appear to be very important. On the other hand, the inhibitory effects of beta sitosterol on cholesterol absorption may be explained by intraluminal events if beta sitosterol interferes with the formation of or alters the structure of cholesterol-containing micelles. Since, as mentioned earlier, the amount of cholesterol absorbed is dependent on the concentration of cholesterol in the micellar phase, reduction of cholesterol concentration in this phase could lead to reduced cholesterol absorption. Several hypotheses explaining how beta sitosterol interferes with the formation of cholesterol-containing micelles, have been suggested (60,65). Intraluminal events alone, however, do not explain the specificity in sterol absorption. Indeed, from the evidence to be presented, it appears that the mucosal cell itself in some way exerts the specificity in sterol absorption.

Feldman and Borgstrom studied the uptake of sterols by rat intestinal slices _in vitro_ (66). They found that the uptake of beta sitosterol was quantitatively similar to the uptake of cholesterol (Figure 7) and that beta sitosterol did not reduce the uptake of cholesterol. Schultz and Strecker (46) reported similar results with rabbit intestinal sli-

Figure 7. The effect of sterol content on uptake from lipid emulsions. To each incubation flask was added 1 ml emulsion containing 6 umoles of sodium taurodeoxycholate, 10.8 umoles of triolein, 5.4 umoles of diolein, 5.4 umoles of monoolein and 10.8 umoles of oleic acid in Krebs-Ringer buffer (pH 6.3). ^3H cholesterol or ^3H beta sitosterol were included at the indicated levels. In mixtures of two sterols, one labeled and one not, only the uptake of the labeled sterol is plotted. The ratio of sitosterol to cholesterol was 2:1 (molar). o, cholesterol; ●, labeled cholesterol plus sitosterol; □, sitosterol; ■, labeled sitosterol plus cholesterol. (From: Feldman, E.B. and Borgstrom, B. Biochim. Biophys. Acta, 125:148, 1966.)



400
300
200
100
0
Sterol uptake (nmoles/100mg tissue)
0.2
0.6
1.0
1.4
2.0
3.0
micro moles total sterol added

ces (Table 11). The results are contrary to what is observed in the intact animal and suggest that manipulations which might have an effect on the integrity of the mucosal cells, may result in the loss of the cell's ability to discriminate between sterols.

Support for the above thesis has been provided by Sylven (67). In studies of sterol uptake by rat intestinal loops *in situ*, Sylven showed that interruption of blood supply to the loops led to uptakes of cholesterol and beta sitosterol that were quantitatively similar; restoration of blood supply to the loops restored the normally observed pattern of sterol uptake, i.e., a high uptake of cholesterol and a low uptake of beta sitosterol. To explain his observations, Sylven invoked the mucosal membrane as the locus for specificity of sterol absorption. He suggested that the normal membrane shows 'vibration' which is dependent on an energy supply; this 'vibration' permits discrimination between sterols.

It is not known if the mechanism of specificity of sterol absorption, as suggested by Sylven, is actually operative. There is some evidence, however, suggesting that some component of the cell membrane might be responsible for exerting the specificity in sterol absorption. Thus, kinetic studies of cholesterol uptake by everted intestinal sacs *in vitro*, yielded a high free energy of activation (20 Kcal/mol) (68). This, coupled with the observation that an Arrhenius plot of cholesterol uptake vs temperature was linear over the range of temperature studied (26-38 ^{o}C), led the authors to suggest

Table 11. The effect of cholesterol on sitosterol influx and the effect of sitosterol on cholesterol influx. For determination of influx, the mucosal surface of segments of rabbit jejunum were exposed for 2 minutes to a radioactive test solution containing ^{3}H cholesterol or ^{14}C sitosterol and 13.6 mM monoolein and 10 mM taurodeoxycholate. In A, total sterol concentration varied while the cholesterol and beta sitosterol concentrations were constant. In B, the total sterol concentration was constant, while the concentrations of cholesterol and sitosterol varied. (From: Schultz, S.G. and Strecker, C.K. Am. J. Physiol. 220:59, 1971.)

A. *Cholesterol and sitosterol influxes*

	0.25 mM Cholesterol	0.25 mM Cholesterol + 0.25 mM Sitosterol	0.25 mM Sitosterol	0.25 mM Sitosterol + 0.25 mM Cholesterol
Cholesterol influx, n = 16	0.007 ± 0.001	0.006 ± 0.0003		
Sitosterol influx, n = 15			0.006 ± 0.002	0.006 ± 0.001

All solutions contained 10 mM taurodeoxycholate and 13.6 mM monoolien. All values expressed as μmoles/hr per cm² ± SE.

B. *Cholesterol and sitosterol influxes*

	0.5 mM Cholesterol	0.25 mM Cholesterol + 0.25 mM Sitosterol	0.5 mM Sitosterol	0.25 mM Sitosterol + 0.25 mM Cholesterol
Cholesterol influx, n = 6	0.013 ± 0.001	0.006 ± 0.001		
Sitosterol influx, n = 6			0.010 ± 0.002	0.006 ± 0.002

that, "an energy barrier for cholesterol uptake exists at the enterocyte luminal cell membrane and may be an important limiting step in cholesterol uptake." Further, they proposed "that a transient association between cholesterol and a component of the enterocyte luminal cell membrane is formed during initial uptake of cholesterol. The transient association may be an activated complex formed with proteins present at or within the luminal enterocyte membrane."

Based on the above studies, the following hypothesis may be suggested: the intestinal absorption of cholesterol occurs via a membrane-bound (protein?) molecule specific for cholesterol. Intact blood supply and cell metabolism are necessary for the proper function of this molecule. In essence, a receptor/carrier specific for cholesterol and dependent on metabolism for proper function, is postulated. The presence of such a receptor/carrier molecule would explain the specificity in sterol absorption; the inhibitory effects of beta sitosterol on cholesterol absorption may be explained by hypothesizing that beta sitosterol, being similar in structure to cholesterol, blocks the binding of cholesterol to the putative receptor/carrier by occupying the binding site. A corollary to this hypothesis is that a defective receptor/carrier may lead to a condition where beta sitosterol absorption is increased without any effect on cholesterol absorption as has been reported in the clinical literature. Thus, Bhattacharyya and Connor (15) described a disease entity in two sisters that appears to support the cholesterol receptor/

carrier hypothesis. The sisters had extremely high plasma levels of beta sitosterol (27.1 mg/100 ml and 17.7 mg/100 ml) compared to normal levels ($<$1.0 mg/100 ml). Plasma cholesterol was normal. Bhattacharyya and Connor (15) showed that the high plasma levels of beta sitosterol were, at least in part, due to the increased intestinal absorption of beta sitosterol. Although cholesterol absorption in these individuals was not directly evaluated, normal plasma levels suggested that cholesterol absorption was not impaired. Because neither parent of the girls was similarly affected, the authors proposed that the disease was an inherited recessive trait. More recently another individual with a similar disorder has been described (69).

The hypothesis of a receptor/carrier molecule specific for cholesterol was first proposed by Glover and Green (70, 71). Although no direct confirmation of the hypothesis has yet been obtained, studies like those of Bhattacharyya and Connor (15) certainly provide support for it. The hypothesis is further supported by the observations that there appears to be a pattern to the specificity in sterol absorption. For example, campesterol (Figure 1) is more absorbable than beta sitosterol (24,72); cholestanol, a reduced derivative of cholesterol, is less absorbable than cholesterol (73). This suggests that the receptor/carrier is probably specific for the double bond in the B ring and the substituent group on C_{24} in the side chain.

The present studies were undertaken in the hopes of

providing further insight into the intriguing interrelationships I have alluded to. First, I wanted to confirm the finding that cholesterol and one of its structurally related plant sterols, do, in fact, have dissimilar absorption patterns under the conditions employed, and then to show whether or not and in what manner the sterols might interact with one another. Two possible sites of interaction were examined, one which I have called "intraluminal", meaning interaction at the level of the micelles, and one which I have called "membranal", meaning interaction at possible absorption sites on the epithelial cell membranes.

The thesis is presented in two sections. The first section dealing with the relative absorption rates of cholesterol and a structurally similar plant sterol is entitled "Intestinal Absorption and Lymphatic Transport of Cholesterol and Beta Sitostanol in the Rat". For reasons which I hope will be made clear in the Introduction to Section I, beta sitostanol rather than beta sitosterol was selected as the representative plant sterol. The second section dealing with the mutual interactions of these compounds is entitled "The Effect of Beta Sitostanol on Cholesterol Absorption from Micellar Solutions in Jejunal Loops *in Situ*".

SECTION I

INTESTINAL ABSORPTION AND LYMPHATIC TRANSPORT OF CHOLESTEROL AND BETA SITOSTANOL IN THE RAT

I. INTRODUCTION

The reader may wish to refer to Figure 1 for a reminder of the structural similarities and differences between cholesterol and some of the phytosterols. The structural similarity between cholesterol and beta sitosterol, for example, makes it easy to accept the view that these compounds have similar physico-chemical properties (54). Their widely discrepant absorbabilities which I alluded to earlier then becomes especially interesting particularly in the light of the fact that most investigators hold to the view that all lipids, including the sterols, are absorbed passively based on their solubility in the lipoidal membranes of the epithelial cells. In addition, it appears that modification of the sterol nucleus profoundly affects its absorbability. For example, cholestanol, a derivative of cholesterol formed by the hydrogenation of the double bond in the B ring, is absorbed less than cholesterol (73). This suggests that sterol absorption might be accomplished through a process that is somewhat more complex than simple passive diffusion through the lipoidal membrane.

In order to investigate the interactions of cholesterol and plant sterols and to gain some insight into the mechanism of sterol absorption, I selected beta sitostanol as the representative phytosterol. The reasons for this choice were as follows:

A. Beta sitostanol is a saturated derivative of beta sitosterol and, in analogy to cholestanol vis-a-vis cholesterol (73), it might even be less well absorbed than its unsaturated counterpart (sitosterol).

B. The absorbability of beta sitosterol and its interaction with cholesterol have been widely examined while little is known about the absorbability of beta sitostanol and its interaction with cholesterol. In one laboratory where the absorbability of beta sitostanol has been examined, the expected result concerning its non-absorbability mentioned above, was obtained (17).

It was hoped, therefore, that this study would provide more information regarding the absorbability of beta sitostanol and its interaction with cholesterol.

In this section, experiments were designed to study the relative absorbabilities of cholesterol and beta sitostanol. In one experiment rats were fed a test meal containing ^{14}C-beta sitostanol and ^{3}H-cholesterol with or without varying amounts of cholestyramine. The purpose of varying amounts of cholestyramine was to introduce varying degrees of malabsorption of sterols so that the absorbabilities of the two compounds could be tested under normal and abnormal conditions. In a second experiment, rats were prepared with lymphatic fistulae and the lymphatic transport of the two compounds was studied after feeding them a test meal containing either ^{3}H-beta sitostanol or ^{14}C-cholesterol.

II. MATERIALS AND METHODS

A. Chemicals: (1α,2α(n)-^{3}H) Cholesterol was obtained from New England Nuclear (Boston, Massachusetts) and found to be 94% radiopure by thin layer chromatography (TLC) according to the method of Miettinen, Ahrens and Grundy (74) and used as such. Beta-(4-^{14}C) sitosterol was obtained from Amersham/Searle (Arlington Heights, Illinois); thin layer chromatography in chloroform:methanol:water, 65:38:10 (System XV (75)) showed that greater than 98% of the plated activity migrated with the band corresponding to beta sitosterol. Beta-(4-^{14}C) sitostanol was prepared by Dr. T.D. Lee[1] by the catalytic hydrogenation of beta-(4-^{14}C) sitosterol. Nuclear magnetic resonance (NMR) spectroscopy of the product showed that reduction was complete. Beta-(22,23(n)-^{3}H) sitostanol was prepared by Dr. Doyle Daves[2] by the catalytic hydrogenation of beta-(22,23(n)-^{3}H) sitosterol obtained from Amersham/Searle. Although NMR spectral analysis of the product showed that reduction was complete, the product was purified before use by TLC on 5% silver nitrate-impregnated silica gel plates developed in chloroform (76) (Appendix A). This was necessary owing to a mixing of the product with the catalyst used in the reduction process. (4-^{14}C) Cholesterol was obtained from Amersham/Searle and found to be better than 97% radiopure by TLC as described (74).

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[1]Oregon Graduate Center for Studies and Research, 19600 N.W. Walker Road, Beaverton, Oregon 97005.

[2]Ibid.

Cholesterol (99% pure) was obtained from Sigma Chemical Company (St. Louis, Missouri) and used as supplied. Beta sitosterol was obtained from Nutritional Biochemical Company (Irving, California); gas chromatographic analysis revealed that it was 91.67% beta sitosterol (Appendix B). Beta sitostanol was prepared by Dr. Doyle Daves by the catalytic hydrogenation of beta sitosterol. NMR spectral analysis of the product showed that reduction was complete. Gas chromatographic analysis of the product showed that it was 92.23% beta sitostanol (Appendix B). TLC of the reduced product (Appendix A) showed that it migrated with an R_f greater than that of beta sitosterol. The reduced product was recrystallized from ethyl acetate (17) before use. Beta sitosterol (chromatographic standard) was obtained from Applied Science Laboratories (Philadelphia, Pennsylvania). Cholestyramine (Cuemid^R) was obtained from Merck, Sharpe and Dhome[3] (West Point, Pennsylvania). All other chemicals were of reagent grade and used as supplied.

B. Lymph Transport Study: In order to evaluate the absorbability of beta sitostanol and cholesterol, lymph transport studies were carried out in male Sprague-Dawley rats in the following way. After anesthetizing the animals with sodium pentobarbital given intra-peritoneally (35 mg/kg body weight), the thoracic duct in the abdomen was cannulated with a P.E. 50 cannula as described (77). To avoid anesthetizing

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[3]From this point on, the concentration of Cuemid^R (90% cholestyramine resin) will be referred to as the concentration of cholestyramine for the sake of simplicity.

the animals again in order to feed the test meal, the stomach was also cannulated with a P.E. 50 cannula sutured in place. The stomach cannula was exteriorized through the original incision in the abdominal wall, while the lymphatic cannula was carefully exteriorized by threading through a tunnel on the right side of the abdominal wall created by a 15 gauge hypodermic needle. The animals were kept in restraining cages and lymph was allowed to flow freely into a collection tube containing heparin. During the period of study, no food was available to the animals; they had free access to 0.9% saline throughout the experimental period.

Only animals that had satisfactory overnight lymph flows (1-2 ml/hr) were considered for the study. Animals with satisfactory lymph flows were given either (4-^{14}C) cholesterol or (22,23-^{3}H) beta sitostanol in an ethanol solution through the gastric cannula. Injection of the test solution was followed by one ml of normal saline to flush out the cannula. Lymph was collected in hourly samples for eight hours following injection of the test meal and as single sample for the next sixteen hours. At the end of 24 hours the rats were sacrificed with an overdose of sodium pentobarbital. The abdomen was then opened to check for the placement of the cannulae and for any overt signs of dilatation of the mesenteric lymphatics.

The lymph samples were analyzed as follows: an aliquot of lymph was saponified for 90 minutes with 2 ml of 33% KOH in a hot water bath maintained at 90 ^{o}C. After cooling,

2 ml of absolute ethanol were added to the saponified mixture and the non-saponifiable neutral sterols were extracted into 20 ml of petroleum ether (boiling range 30-60 ^{o}C). The solvent was washed with 5 ml of water. An aliquot of the petroleum ether extract was pipetted directly into a scintillation vial and the solvent was evaporated under nitrogen. To the dried lipid extract, 10 ml of scintillation solution (4 gm 2,5 diphenyloxazole and 0.05 gm p-bis (2-(5-phenyloxazolyl))-benzene in one liter of toluene) was added and the radioactivity was assayed in a Packard Liquid Scintillation Spectrometer (Downers Grove, Illinois). The radioactivity recovered was expressed as a percent of the amount fed. This method of extraction recovered greater than 98% of both ^{14}C cholesterol and ^{3}H sitostanol added to lymph samples.

C. Fecal excretion and blood analysis study: Twenty male Sprague-Dawley rats weighing between 240 and 270 grams were divided into four groups with five rats in each group. Assignment of a rat to a group was based on the amount of cholestyramine (0,100,200 or 300 mg) the animal was to receive, which was determined on a random order. The purpose of cholestyramine was to induce varying degrees of malabsorption of sterols so that the absorbabilities of the two compounds could be tested under normal and abnormal condition. The rats were fasted overnight. On the day of the experiment, the animals were lightly anesthetized with ether; a gastric tube was introduced into the stomach and the following were injected in the order listed:

1. One ml of water as a check for placement of the tube.
2. Two ml of a fat free milk solution (13) containing no cholestyramine or with added cholestyramine in one of three concentrations (25,50,75 mg/ml).
3. Approximately 0.5 ml of an ethanolic solution of cholesterol (2 mg/ml), beta sitostanol (1 mg/ml), (4-^{14}C) beta sitostanol (tracer) and (1,2-^{3}H) cholesterol (tracer) from a pre-weighed syringe which was weighed after injection to determine the exact volume injected.
4. Two ml of the same milk solution as in 2 above.
5. One ml of water to flush the tube.

After injection of the test meal the animals were returned to separate cages with wire mesh floors to allow for collection of feces. No food was available to the animals for eight hours after receiving the test meal. They then had free access to rat chow for the duration of the experiment. Water was freely available to the animals at all times. Feces were collected daily for four days following the injection and frozen until analyzed. The experiment was terminated after the fourth fecal collection by sacrificing the rats with an overdose of sodium pentobarbital. Feces were analyzed according to Malinow et al. (14). The daily fecal samples were mixed with methanol, weighed and homogenized in a blender (Osterizer, Oster Corporation, Milwaukee, Wisconsin). An aliquot by weight was transferred to a centrifuge tube and 2 ml of 33% KOH were added to the homogenate in the tube.

Mini-condensers were fitted onto each tube and the feces were saponified under reflux for 90 minutes at 90 ^{o}C. After cooling, two ml of absolute ethanol were added to the saponified mixture and the non-saponifiable neutral sterols were extracted into 20 ml of petroleum ether (boiling range: 30-60 ^{o}C). The solvent was washed with 5 ml of water.

An aliquot of the petroleum ether extract was pipetted directly into a scintillation vial and the solvent was evaporated under nitrogen. The residue was dissolved in 2 ml of chloroform and the solution was ozonated to remove the color (13). The chloroform was evaporated under nitrogen and 12 ml of 10% Bio-Solv^{R} Solubilizer (Beckman Instrument Company, Fullerton, California) in toluene scintillation solution were added to the vial. Radioactivity was assayed in a Packard Liquid Scintillation Spectrometer with automatic external standard for quench correction. This method recovered 95.2 $\pm$ 0.6% (Mean $\pm$ S.D., N=6) of the cholesterol and 97.9 $\pm$ 1.6% (N=6) of the beta sitostanol added to fecal homogenates. The recoveries were in fairly good agreement with those obtained by Malinow et al. (14) for cholesterol and beta sitosterol under similar conditions.

Forty-eight hours after injection of the test meal a blood sample, with heparin as the anticoagulant, was obtained from the tail of each rat under light ether anesthesia. The plasma was saponified and extracted with ethanol and petroleum ether and analyzed in a manner similar to the lymph.

D. Calculation of Absorption: The absorption of labeled cholesterol from fecal excretion data was calculated

in three different ways:

1. Isotope Ratio Method:

   Based on certain assumptions concerning the non-absorbability of beta sitostanol to be described, the absorption of labeled cholesterol was calculated using the ratio of the two isotopes in the feces and test meal, according to the following formula:

$$\text{\% Cholesterol Absorbed} = \left[1 - \frac{{}^{14}C/{}^{3}H \text{ in test meal}}{{}^{14}C/{}^{3}H \text{ in first day fecal collection}}\right] \times 100$$

2. Total Fecal Recovery:

   The percent labeled cholesterol absorbed was calculated by the following formula:

$$\text{\% Cholesterol Absorbed} = \left[1 - \frac{\Sigma\ {}^{3}H \text{ Cholesterol Recovered}}{{}^{3}H \text{ Cholesterol Injected}}\right] \times 100$$

3. Total Fecal Recovery corrected for non-absorptive losses:

   This method corrects for incomplete recovery of cholesterol in feces due to non-absorptive losses which may include possible bacterial degradation of cholesterol, incomplete fecal collection, variations in fecal flow, sequestration of cholesterol, etc. Based again on the assumption about beta sitostanol mentioned earlier, the fecal recovery of labeled beta sitostanol was used to make correction for nonabsorp-

tive loss of labeled cholesterol according to the following formula:

$$\left[1 - \left(\frac{\Sigma\ ^{3}H\ \text{Cholesterol Recovered}}{^{3}H\ \text{Cholesterol Injected}} \times \frac{^{14}C\ \beta\text{-sitostanol Injected}}{\Sigma\ ^{14}C\ \beta\text{-sitostanol Recovered}}\right)\right] \times 100$$

III. RESULTS

A. Lymphatic Recovery of ^{14}C cholesterol and 22,23-^{3}H beta sitostanol: The cumulative percent recoveries of 4-^{14}C cholesterol and 22,23-^{3}H beta sitostanol, over a period of eight hours, in the thoracic duct lymph of rats are given in Figure 8, showing an average recovery of 6.5% of the administered cholesterol and 0.8% of the administered beta sitostanol. The 24 hour recoveries averaged 36.7% and 2.2% for labeled cholesterol and beta sitostanol respectively. The cholesterol recoveries are in agreement with the results of Sylven and Borgstrom (11). The lymphatic transport of beta sitostanol has not been studied previously.

B. Effect of cholestyramine: The effect of cholestyramine on the fecal excretion of labeled cholesterol and beta sitostanol is shown in Figure 9. Rats receiving cholestyramine, as expected, excreted significantly greater amounts of labeled cholesterol than rats receiving no cholestyramine ($p < 0.001$ by analysis of variance). A near maximum effect of the drug was achieved at a dose of 100 mg. The fecal excretion of labeled beta sitostanol was unaffected by cholestyramine at any dose.

C. Absorption of labeled cholesterol and beta sitostanol: The fecal recoveries of labeled cholesterol and beta sitostanol in the presence and absence of cholestyramine are shown in Table 12 together with computed values of cholesterol absorption using the three different methods. Note that the fecal

Figure 8. Cumulative percent recovery in the thoracic duct lymph as a function of time. Circles ---^{14}C cholesterol; triangles---^{3}H beta sitostanol. Each line represents an individual animal.

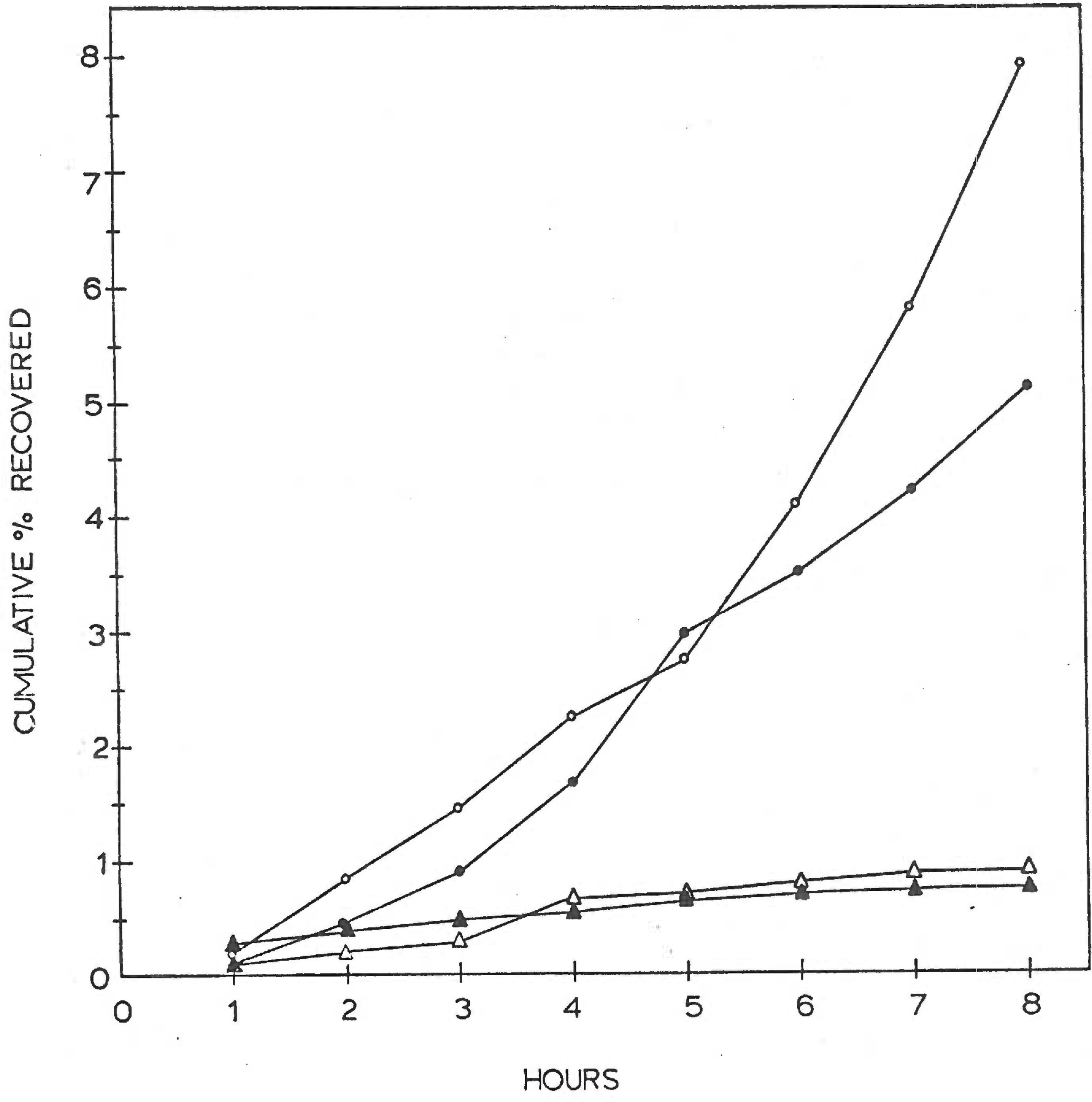

CUMULATIVE % RECOVERED
HOURS
0
1
2
3
4
5
6
7
8

Figure 9. The effects of cholestyramine on fecal excretion of: A. ^{14}C beta sitostanol and B. ^{3}H cholesterol. Values are percent fed amounts as a function of cholestyramine dose. The fecal ^{3}H cholesterol recoveries were corrected for non-absorptive losses. Each point is the mean of five rats. The vertical bars represent standard errors of the mean, offset in B to avoid overlapping.

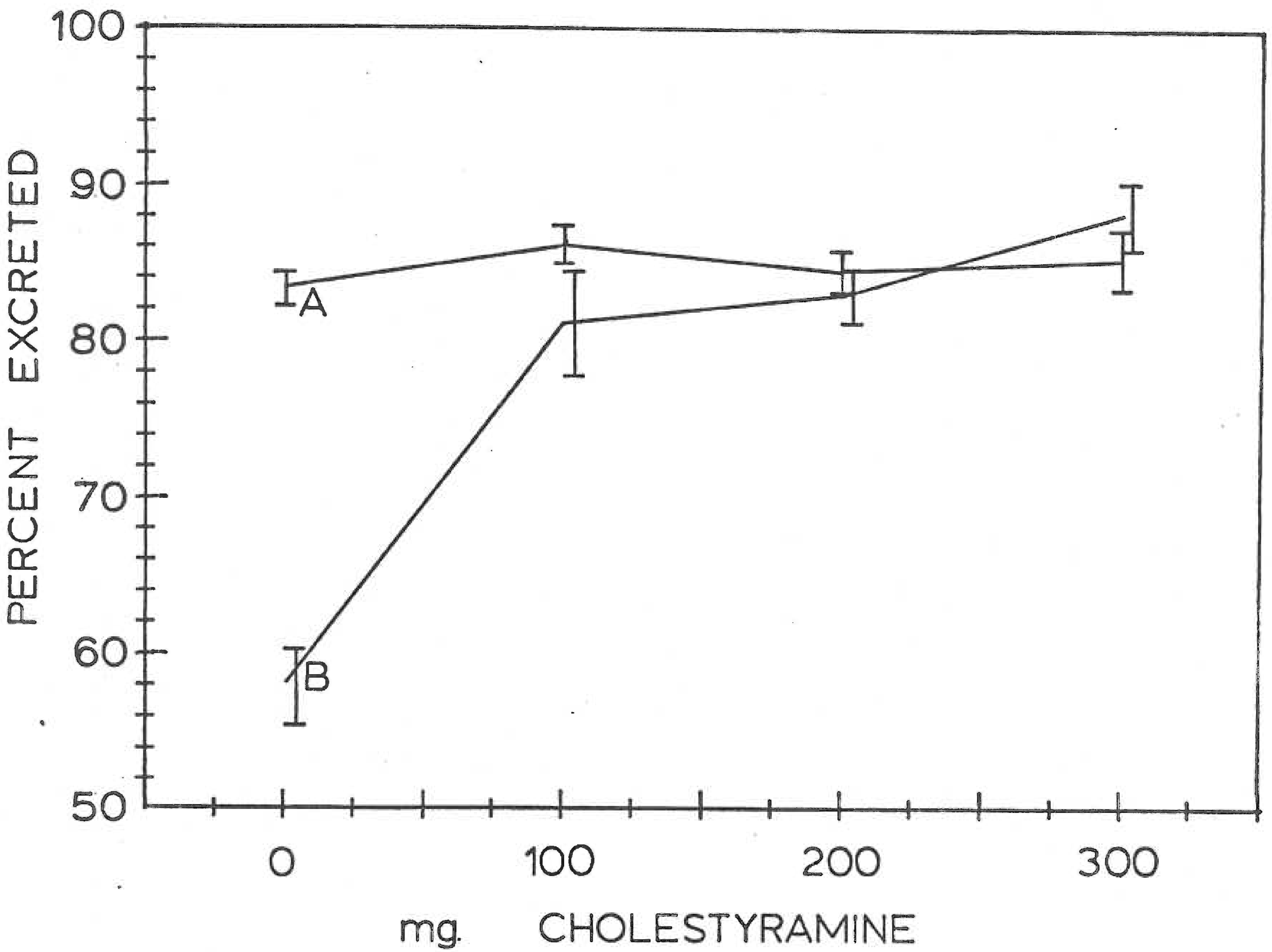

PERCENT EXCRETED
100
90
80
70
60
50
0
100
200
300
mg. CHOLESTYRAMINE
A
B

Table 12. Fecal recoveries of ^{3}H cholesterol and ^{14}C beta sitostanol and percent cholesterol absorbed as calculated by the Isotope Ratio Method (Method A) and two Fecal Recovery Methods. (Method B: Total Fecal Recovery and Method C: Total Fecal Recovery corrected for non-absorptive losses.)

Rat #	Cholestyramine fed (mg)	Cumulative % Recovery		Percent Cholesterol Absorbed		
				Isotope Ratio Method	Total Fecal Recovery Method	
		^{3}H Cholesterol	^{14}C Beta sitostanol	Method A	Method B	Method C
3		45.11	83.38	52.14	54.89	45.90
4		55.06	83.40	38.75	44.94	33.98
10	0	55.23	84.87	39.32	44.77	34.92
12		49.53	85.22	47.22	50.47	41.88
17		48.90	79.80	44.32	51.10	38.72
Mean ± S.E.M.		50.77 ± 1.94	83.33 ± 0.96	44.35 ± 2.51	49.23 ± 1.94	39.08 ± 2.21
5		71.70	85.70	16.91	28.30	16.34
11		75.64	82.23	10.97	24.36	8.01
13	100	64.40	86.27	27.19	35.60	25.35
16		73.24	86.20	16.33	26.76	15.03
19		69.58	90.19	24.73	30.42	22.85
Mean ± S.E.M.		70.91 ± 1.90	86.12 ± 1.26	19.23 ± 2.96	29.09 ± 1.90	17.52 ± 3.06
1		73.99	84.97	14.17	26.01	12.92
6		74.18	87.64	15.63	25.82	15.36
8	200	75.25	86.28	14.19	24.75	12.78
9		68.98	82.63	19.71	31.02	16.52
15		63.27	80.83	23.21	36.73	21.72
Mean ± S.E.M.		71.13 ± 2.25	84.47 ± 1.23	17.38 ± 1.77	28.87 ± 2.25	15.86 ± 1.63
2		73.75	79.50	7.93	26.25	7.23
7		74.11	82.68	11.22	25.89	10.37
14	300	79.12	85.19	7.32	20.88	7.13
18		73.75	89.38	18.39	26.25	17.49
20		77.75	89.36	14.21	22.25	12.99
Mean ± S.E.M.		75.70 ± 1.14	85.22 ± 1.92	11.81 ± 2.06	24.30 ± 1.14	11.04 ± 1.94

excretion of beta sitostanol averaged 85% independent of the presence or absence of cholestyramine. Since only 2% of the labeled beta sitostanol was recovered in the lymph, this means that 87% of the labeled beta sitostanol was accounted for. The remaining 13% must have been either degraded during passage through the intestine, sequestered in the gut or absorbed through non-lymphatic channels (all of which were collectively referred to earlier as non-absorptive losses). Since labeled beta sitostanol was not detected in the blood, we assume that none was absorbed through non-lymphatic channels.

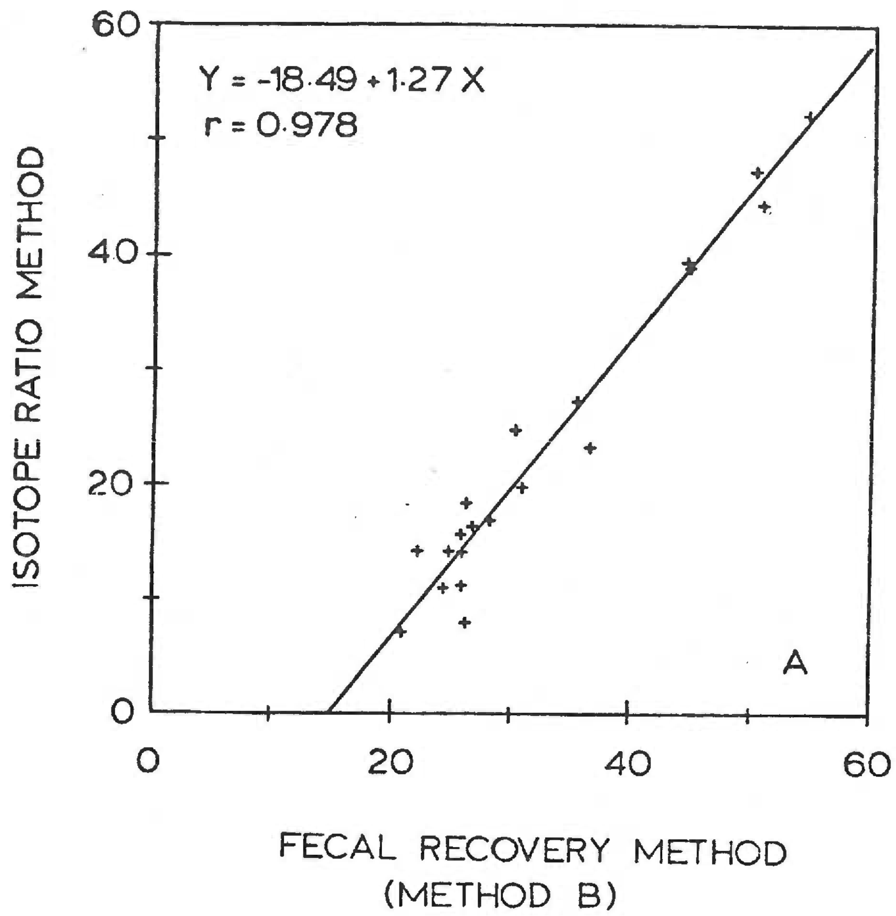
The computation of cholesterol absorption by the three methods in the last three columns of Table 12 are based on the following assumptions:

1. Labeled beta sitostanol was absorbed to the extent of only approximately 2% and was regarded as negligible.

2. The non-absorptive losses of labeled beta sitostanol by bacterial or chemical degradation or by sequestration were matched by similar non-absorptive losses of labeled cholesterol. There is no a priori reason to doubt the validity of this assumption since the two compounds are similar to one another in structure.

In Figure 10, the values for absorption of labeled cholesterol from the isotope ratio method (method A) are plotted against each of the values from the two total fecal recovery methods -- method B (uncorrected) and method C (corrected for non-absorptive losses).

Figure 10. Comparison of the Isotope Ratio Method and the two Total Fecal Recovery Methods (Figure 10A, Total Fecal Recovery, uncorrected and Figure 10B, Total Fecal Recovery corrected for non-absorptive losses).


Y = -18.49 + 1.27 X
r = 0.978
ISOTOPE RATIO METHOD
FECAL RECOVERY METHOD
(METHOD B)
A
0
20
40
60
0
20
40
60

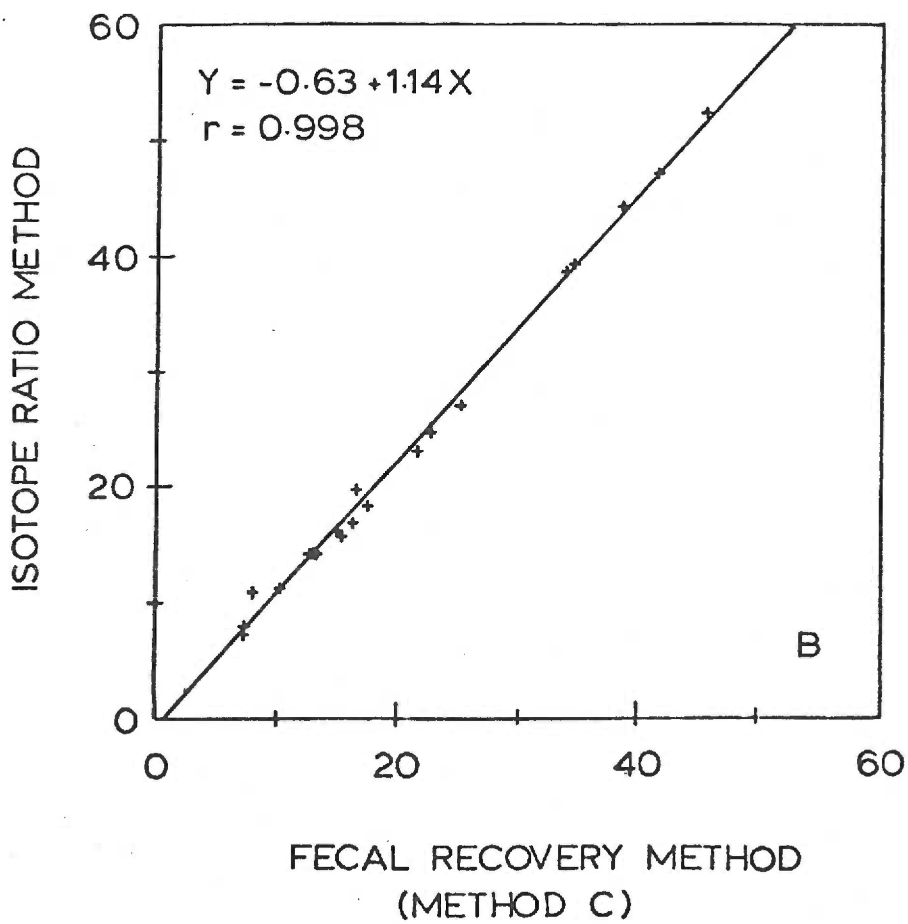

Y = -0.63 + 1.14 X
r = 0.998
ISOTOPE RATIO METHOD
FECAL RECOVERY METHOD
(METHOD C)
B
0
20
40
60
0
20
40
60

Figure 10A, comparing methods A and B, shows a correlation coefficient of 0.978, but the values from these (uncorrected) fecal recoveries consistently exceeded those from the isotope ratios as evidenced by the positive x-axis intercept of the regression line. This would be expected if there were equivalent non-absorptive losses of labeled cholesterol and beta sitostanol since equivalent non-absorptive losses would yield reduced fecal recoveries of labeled cholesterol (and hence greater apparent absorption) while the ratio of the two compounds in the feces would be unaffected. That the non-absorptive losses of both labeled cholesterol and beta sitostanol were similar was evidenced by the fact that when the fecal recoveries of labeled cholesterol and beta sitostanol, in the group not receiving cholestyramine, were summed with the recoveries of the two isotopes in the thoracic duct lymph, the sums were nearly identical (51 + 36 = 87% for labeled cholesterol and 83 + 2 = 85% for labeled beta sitostanol). If 100% minus these values were taken to represent non-absorptive losses, then the non-absorptive losses of labeled cholesterol and beta sitostanol were similar.

In Figure 10B, the isotope ratio method is compared in a similar fashion with method C (fecal recoveries after correcting for non-absorptive losses). The correlation coefficient was increased to 0.998. In addition, the slope of the regression line was closer (1.14) to the slope of the line of identity and the intercept was not significantly different from the origin. Thus, one can see that the isotope ratio

method for estimating cholesterol absorption compares favorably with estimates based on the total fecal recovery of labeled cholesterol, particularly when account is taken of a possible source of error in the fecal recovery method, which is the non-absorptive losses of labeled cholesterol during intestinal transit.

IV. DISCUSSION

A. Fecal recovery and intestinal absorption of beta sitostanol. The mean recovery of labeled beta sitostanol in this study was 85%. This could mean that 15% of the compound was absorbed. However, the fecal recovery of plant sterols (beta sitosterol) is variable (18) and, despite the claim that plant sterols (beta sitosterol) are minimally absorbed, there are several reports that show low fecal recoveries of beta sitosterol both in rats (14) and in humans (6,78). The fecal recoveries of sterols in general is believed to be a function of:

1. Bacterial degradation of sterols (79)
2. Intestinal transit rates (80)
3. Diet (81)
4. Intestinal absorption

Recently, Newton and Mansbach (82) found that 11-12% of an administered dose of beta sitosterol was not recovered in ileostomized subjects. On the basis of previous studies (79), they postulated that surface adsorption of beta sitosterol probably accounted for the low recoveries of beta sitosterol in these patients, and reiterated a previous conclusion (79) that the fecal recovery of beta sitosterol after an oral dose may be an unreliable way of determining its absorption.

In the present study, there are three lines of evidence to suggest that beta sitostanol was not absorbed or it was absorbed in minimal amounts only.

a. In contrast to cholesterol, cholestyramine

failed to cause an increase in the fecal excretion of labeled beta sitostanol. Cholestyramine binds bile salts (83) which have been shown to be essential for the absorption of cholesterol (50). The failure of cholestyramine to alter the fecal excretion of labeled beta sitostanol may mean either that: beta sitostanol does not aggregate with bile salts to form mixed micelles as does cholesterol or beta sitostanol does enter into the formation of mixed micelles with bile salts, but is still unable to be absorbed. In either case, cholestyramine would not be expected to have any effect on the fecal excretion of beta sitostanol.

It seems most unlikely that the lack of micellization of beta sitostanol was responsible for the lack of effect of cholestyramine on the fecal excretion of the compound because micellar solutions of beta sitostanol have been prepared in connection with this study (see Section II). Therefore, the complete lack of effect of cholestyramine on the fecal excretion of beta sitostanol may be taken as indirect evidence that beta sitostanol is unabsorbable and it was not absorbed to any appreciable extent in this study.

b. No ^{14}C (beta sitostanol) activity was detectable in the non-saponifiable fraction of the plasma taken from any of the animals 48 hours after feeding the test meal, although significant ^{3}H (cholesterol) activity was detectable in the plasma of all but one rat (rat #14 in the group receiving 300 mg of cholestyramine). It is still possible, however, that absorption of beta sitostanol did not follow the

same time course as cholesterol. Thus, labeled beta sitostanol absorbed after 48 hours may have escaped detection since later plasma samples were not taken. Considering the relatively short intestinal transit time of the rat (approximately 24 hours) and the fact that sterols are not absorbed from the colon (84), it seems unlikely that any beta sitostanol was absorbed after 48 hours. In any case, lack of ^{14}C activity in the plasma 48 hours after feeding the compound supports the conclusion that beta sitostanol was not absorbed to any appreciable extent.

c. An average of only 2.2% of the administered beta sitostanol was found in the thoracic duct lymph of two rats in 24 hours. Even though a small percentage of administered beta sitostanol was recovered in the lymph, it is interesting to note that the compound could not be detected in the plasma as mentioned above. This may have been due to the dilution of the small amount of isotope (^{14}C beta sitostanol) entering the circulation. On the other hand, it is possible that the small amount of beta sitostanol absorbed was rapidly excreted. Salen et.al. (7) have shown that in the human the small amounts of beta sitosterol absorbed are excreted more rapidly than cholesterol. In the rat, intravenously injected beta sitostanol has been shown to be excreted more rapidly than cholesterol (85). Whether the same rate of excretion exists with the small amounts of beta sitostanol that are absorbed, is not known. In the present study then, lack of detection of labeled beta sitostanol in the plasma after an

oral dose, could have been possibly due to the rapid excretion of the small amounts absorbed.

Taken together, the incomplete recovery of beta sitostanol may be ascribed to non-absorptive losses as a result of any of the factors, or a combination thereof, listed above. It is also evident that beta sitostanol was either not absorbed or absorbed in minimal amounts only. Further support for this conclusion has been provided by the studies of Sugano et.al. (17) and Ikeda and Sugano (85).

B. Beta sitostanol - a new marker for studying cholesterol absorption. An ideal marker or reference compound for studying cholesterol absorption should at least be: 1) non-absorbable, 2) similar to cholesterol in physico-chemical properties, and 3) subject to the same non-absorptive losses as cholesterol during transit through the intestine. The last criterion has come to be recognized as important because of the possibility of degradation of neutral sterols in transit through the intestine (79,86). The extent of degradation (or lack of it) is believed to vary depending on species (87), intestinal transit rates (80) and diet (81). It is important, therefore, that an ideal marker for cholesterol be treated in the same way as cholesterol by the intestinal bacterial flora.

Over the years, a number of substances such as yttrium chloride (88,89), inulin (38,39), dextran (38,39), and beta sitosterol (3,6,79,89) have been used as markers to study the absorption of cholesterol. Among these, only beta sito-

sterol is lipid soluble and has been shown to have physico-chemical properties similar to cholesterol (54). Evidence also indicates that it is treated by the intestinal bacteria in the same way as cholesterol (86). However, it absorbed to varying degrees (18) and to the extent that it is absorbed, it is less than ideal as a marker.

Results from the present study indicate that beta sitostanol possesses properties required of a valid marker for studying cholesterol absorption. These include:

1. The non-absorptive losses (due to degradation by intestinal bacteria, etc.) of cholesterol and beta sitostanol were similar. The sum of the fecal and lymphatic recoveries of the two isotopes were nearly identical.

2. The absorption of labeled cholesterol calculated by the isotope ratio method agreed closely with the corresponding values obtained by the total fecal recovery method, particularly when corrections for non-absorptive losses of cholesterol were made. As pointed out earlier, this correction should be made before comparing values of cholesterol absorption obtained by the two methods.

3. The absorption of beta sitostanol was minimal and may be regarded as insignificant.

Further, because beta sitostanol is prepared by the catalytic hydrogenation of beta sitosterol, it may be reasonably assumed that the physico-chemical properties of beta sitostanol are similar to beta sitosterol and hence to cholesterol.

Thus, beta sitostanol may be considered to be a <u>valid</u>

marker for studying cholesterol absorption in normal rats and in rats with impairment in cholesterol absorption induced by the administration of cholestyramine.

The isotope ratio method used for calculating cholesterol absorption used in the present study was validated by Sodhi et al. in humans and in rats (8,9). These authors reported the "necessity of obtaining the very first sample of feces containing the unabsorbed radioactivity" (9). This caution was mentioned in order to obtain fecal samples uncontaminated with absorbed labeled cholesterol secreted in the bile. In the rats, it was not clear how they adhered to their recommendation. As such, considering the relatively short intestinal time of the rat, the first day's fecal sample was used to determine the isotope ratios. The excellent agreement between the values of cholesterol absorption obtained by the isotope ratio method and by the total fecal recovery method (corrected for sterol losses) further validates the isotope ratio method for calculating cholesterol absorption based on the ratio of the two isotopes obtained in the first day's fecal collection (in the rat).

C. Effect of cholestyramine on the excretion of labeled cholesterol: In the present study, varying amounts of cholestyramine were fed to rats to achieve varying degrees of maladsorption of cholesterol. Cholestyramine, by binding bile salts (83), reduces the availability of bile salts in the intestinal lumen and hence reduces the absorption of cholesterol. The binding of bile salts to cholestyramine, however,

shows saturation (83). Binding of bile salts to cholestyramine is linear at low concentrations of cholestyramine. A further increase in the concentration of cholestyramine does not result in a directly proportional increase in the binding of bile salts. Since the presence of bile salts is essential for the absorption of cholesterol (50), the pattern of absorption of cholesterol in the presence of cholestyramine would be the reciprocal of the pattern of binding of bile salts to cholestyramine. In other words, the fecal excretory pattern of cholesterol when cholestyramine has been administered, would be expected to be similar to the pattern of binding of bile salts to cholestyramine. This expectation was realized in the present study in which the fecal excretion of cholesterol (Figure 9) achieved a near maximum at a dose of 100 mg of cholestyramine. Further increase in the amount of cholestyramine administered led only to small additional increases in the fecal excretion of cholesterol.

D. Lymphatic recovery of ^{3}H beta sitostanol: In order to directly evaluate the absorbability of beta sitostanol, the recovery of ^{3}H beta sitostanol in the lymph was studied. Only 2.2% of the administered label was recovered in the thoracic duct lymph in 24 hours. Interestingly, both the rate of recovery and the total recovery of sitostanol in the lymph shown in the present study differed from that of sitosterol shown in a previous study (11). Why two substances so similar to one another should exhibit these differences is not clear. What is also not well understood is why these compounds, beta

sitosterol and beta sitostanol, are absorbed so minimally when structurally they are quite similar to cholesterol. It is apparent that more work at a molecular level is needed to clarify the mechanisms involved in the intestinal absorption of sterols.

SECTION II

THE EFFECT OF BETA SITOSTANOL ON CHOLESTEROL ABSORPTION FROM MICELLAR SOLUTIONS IN JEJUNAL LOOPS *IN SITU*

I. INTRODUCTION

As has been mentioned, plant sterols are less well absorbed than cholesterol (7,11-13,17-19) despite their similarity in structure (Figure 1). Plant sterols have also been shown to reduce the intestinal absorption of cholesterol (5, 17,22-24) and although several hypotheses for this effect have been proposed (60,65), none of these hypotheses can account for the specificity of sterol absorption mentioned above.

Glover and Green (70,71) proposed that the absorption of cholesterol was mediated by a membrane-bound receptor/carrier lipoprotein specific for cholesterol. Such a carrier could account for both the specificity of sterol absorption and the inhibitory effects of plant sterols on cholesterol absorption. To date, no direct proof of this hypothesis has been obtained.

Recently, Sugano et al. (17) showed that the reduced derivative of beta sitosterol, beta sitostanol, was more potent than beta sitosterol in reducing intestinal absorption of cholesterol in rats. No mechanism for the increased potency of the reduced derivative was suggested.

The experiments described in this section were undertaken to study the effects of beta sitostanol on the intestinal absorption of cholesterol in micellar solutions, in an attempt

to see if beta sitostanol would affect cholesterol absorption under the present conditions and, if possible, to elucidate the mechanism(s) of the inhibitory effect. It was hoped that the results would also provide further insights into the mechanism of absorption of cholesterol.

II. MATERIALS AND METHODS

A. Chemicals: Labeled compounds, 4-^{14}C cholesterol and 22,23-^{3}H beta sitostanol, used in these experiments were obtained from the batch used in the previous experiments. Sodium taurocholate (98%) was obtained from Sigma Chemical Company (St. Louis, Missouri). Thin layer chromatography of the bile acid in butanol:glacial acetic acid:water (100:10:10) (90), revealed only one band. Monoolein (99%), oleic acid (99%) and cholesterol (99%) were all obtained from Sigma Chemical Company and used as supplied. Beta sitostanol was from the batch used in the previous study. All other chemicals were of reagent grade and used as supplied.

B. Preparation of micellar solutions: The concentrations of the various components of the micellar solutions are shown below:

1.	Monoolein	0.60 mM
2.	Oleic Acid	1.20 mM
3.	Cholesterol	0.05-0.3 mM
4.	Beta sitostanol	'0',0.20 mM or 0.30 mM
5.	Sodium taurocholate	10 mM

4-^{14}C cholesterol and 22,23-^{3}H beta sitostanol were used as tracers.

To prepare a micellar solution, the appropriate amounts of stock solution of the lipids in benzene were added together in a tube; the appropriate tracer, ^{14}C cholesterol and/or ^{3}H beta sitostanol was also added to the tube. The solvent was evaporated under nitrogen and the appropriate volume of 10 mM

sodium taurocholate in Ca^{++}-free Krebs-Ringer 0.10 M phosphate buffer (KRB) (pH 6.25) (91) was added to the dried lipids. The mixture was stirred vigorously with a vortex stirrer to yield an optically clear solution. All solutions so obtained were optically clear unless stated otherwise.

The following test solutions were used:

a. Control: This solution contained no beta sitostanol except that present as a tracer. Cholesterol concentration ranged from 0.05 to 0.30 mM.

b. Solution-mix: Two separate micellar solutions were made, one containing cholesterol and the other containing beta sitostanol. The concentrations of cholesterol and beta sitostanol in these 'parent' solutions were twice those in the final test solution to be infused. Under these conditions, the 'parent' beta sitostanol solution at a concentration of 0.60 mM was cloudy. To prepare the test solution, equal aliquots of the two micellar solutions were added in a test tube and mixed thoroughly with a vortex stirrer. Test solutions obtained by the mixing of the cloudy beta sitostanol solution with an equal volume of the clear cholesterol micellar solution were also cloudy. Beta sitostanol concentration in the final test solution was 0.20 mM or 0.30 mM.

In one set of experiments, the beta sitostanol solution was replaced by a cholesterol solution so that a cholesterol/cholesterol mixture was obtained instead of the usual beta sitostanol/cholesterol mixture.

c. Pre-mix: All the lipids including cholesterol and beta sitostanol were added together in a tube; after evaporation of the solvent, the appropriate volume of the bile salt solution was added to make the final test solution. The concentration of beta sitostanol was constant at 0.30 mM while the concentration of cholesterol ranged between 0.05 to 0.20 mM.

d. Pre-flush/solution-mix: The final test solutions were prepared as in the solution mix ((b) above) except that the loops were pre-flushed with a beta sitostanol-containing micellar solution (without radioisotopic tracer). The concentration of beta sitostanol in the flushing solution was equal to its concentration in the test solution to be infused.

The following assumptions served as the basis for using micellar solutions prepared in the two different ways, that is, either by mixing the sterol together before dispersing them with bile salts (pre-mix) or by dispersing them separately with bile salts before mixing (solution-mix):

i. Cholesterol and beta sitostanol are insoluble in water. There is no information available on the solubility of beta sitostanol in water. The solubility of cholesterol in water has been reported to be 4×10^{-3} mM (92); however, there was sufficient evidence for uncertainty in obtaining this value which is 10^{5} times greater than the most conservative estimate for the solubility of cholesterol in water (65).

ii. The intermicellar concentration of cholesterol

and beta sitostanol was zero.

If the assumptions are valid, then cholesterol and beta sitostanol would not exchange across micelles. By adopting the approach just described, it was hoped to be able to see whether or not beta sitostanol interfered with cholesterol absorption and, if so, to see if the interference occurred by virtue of some type of interaction at the micellar level (or intraluminal phase) in contradistinction to the membrane level (membranal or cell uptake phase).

C. Animal preparation: The decision to use an *in vivo* approach was based on reports that *in vitro* studies of sterol absorption failed to show the specificity of the process as is normally observed *in vivo* (46,66) and that metabolism requiring an intact blood supply might have an important role on the question of specificity (67).

The animals in the present study were male Sprague-Dawley rats weighing between 200-220 grams purchased from King Animal Supply (Oregon, Wisconsin). They received standard laboratory rat chow and water ad libitum to the time of the experiments.

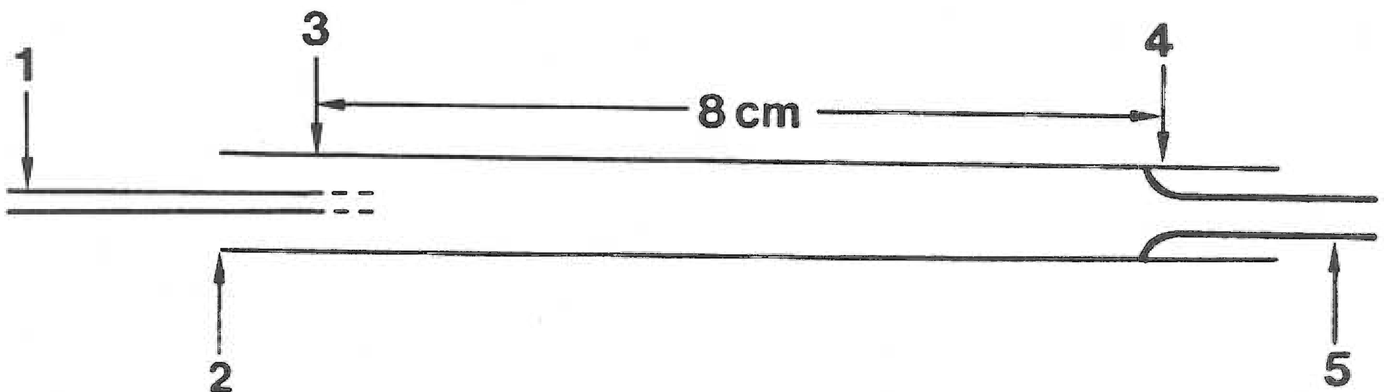
On the day of the experiment, the rats were anesthetized with sodium pentobarbital (35 mg/kg body weight) administered intraperitoneally. The abdomen was opened along the midline, the ligament of Trietz was located and a proximal ligature was placed around the jejunum immediately distal to the ligament taking care not to ligate the blood supply to the segment of interest. Eight centimeters distal to the proximal liga-

ture, a polyethylene cannula (P.E. 280) was introduced into the intestinal lumen and tied securely in place. This was the effluent collection cannula. Immediately distal to the proximal ligature another polyethylene cannula (P.E. 10) was introduced into the lumen and tied in place. This was the infusion cannula (See Figure 11).

Selecting an eight centimeter segment of jejunum was based on preliminary experiments (Appendix C). These studies showed that cholesterol absorption normalized to unit tissue weight actually decreased as the loop length increased because they failed to fill completely when the small volumes of test solutions were infused. On the other hand, loops shorter than eight cm were at risk from possible overdistension. Therefore, a loop length of eight cm and a test solution volume of 0.5 ml were used in all the experiments.

D. Infusion: Before starting the infusion the effluent collection cannula was clamped. The test solution was then infused into the loop from a pre-weighed syringe at a rate of 1.35 ml/min using a Harvard continuous infusion pump. At the completion of the infusion, the syringe was again weighed to determine the exact volume of the test solution injected. After 20 minutes, the effluent collection cannula was unclamped and the loop contents were flushed into a graduated tube with 10 ml of KRB at a rate of 0.67 ml/min. The segment was then carefully removed, placed in a tared container and dried at 90 ^{o}C for 24 hours to obtain the dry weight. The protocol was the same in all experiments except in the pre-flush con-

Figure 11. A highly schematic representation of the loop preparation. The figure is not drawn to scale and is given only to illustrate the general features of the loop preparation and the relative positions of ligatures, ligament, etc.



1. Infusion cannula (P.E.10)
2. Ligament of Trietz
3. Proximal ligature
4. Distal ligature
5. Effluent collection cannula (P.E. 280)

dition in which the effluent cannula was clamped after the loop had been pre-flushed. The pre-flush was a micellar solution of beta sitostanol in the same concentration as the test solution, and was infused at a rate of 1.35 ml/min.

The rate of infusion of the test solution used, was based on preliminary experiments. In these experiments it was found that no cholesterol was absorbed during the infusion period when the test solution was infused into the loops at a rate of 1.35 ml/min. Therefore, an infusion rate of 1.35 ml/min was selected as the infusion rate for the present study.

Two points were considered in selecting the duration of the absorption period:

1. It should be short enough to avoid any possible saturation.

2. It should be long enough to allow for equilibration of the test solution with the absorptive surface and for absorption to occur.

In preliminary experiments, (Appendix D), a time of ten minutes was found to satisfy the above requirements. Absorption of cholesterol over this time period was modest and averaged 34.71%/100 mg tissue dry weight. However, in these experiments the unabsorbable reference compound was ^{3}H inulin, a water-soluble substance. Initial studies on cholesterol absorption by jejunal loops with ^{3}H beta sitostanol as the reference compound, showed that the amount of cholesterol absorbed over 10 minutes was about 25% of the value obtained with inulin as the reference compound. This difference was ascribed to the

difference in the physical properties of the two reference compounds vis-a-vis cholesterol. It was pointed out earlier that an ideal reference compound for cholesterol is one that has physico-chemical properties similar to cholesterol in addition to being non-absorbable. Inulin, although non-absorbable, is a water-soluble oligosaccharide. The difference in the physico-chemical properties of inulin, the reference compound, and cholesterol, the test substance, could lead to overestimation of the absorption of cholesterol. Because of this observation, it was decided that the absorption period, in all the studies with ^{3}H beta sitostanol as the reference compound,would be increased to 20 minutes.

E. Analysis of the effluent and the test solution: 0.50 ml. of the effluent was added to a screw-capped centrifuge tube containing 0.50 ml of methanol. The tube was stirred and 10 ml of benzene were added following which the mixture was shaken in an Eberbach shaker for 30 minutes. It was then centrifuged at 1500 rpm for 10 minutes. An aliquot of the benzene extract was pipetted into a scintillation vial, the benzene was evaporated under nitrogen and 10 ml of Bioflour^{R} (New England Nuclear, Boston, Massachusetts) were added. Radioactivity was assayed in a Packard Liquid Scintillation Spectrometer with an external standard for quench correction.

The original micellar solutions (diluted 20 times with KRB) were similarly extracted and counted. Recovery of both ^{14}C cholesterol and ^{3}H beta sitostanol was greater than 99%

by this method.

F. Calculations: In the previous section beta sitostanol was shown to be a valid marker for studying cholesterol absorption. In this study, therefore, ^{3}H beta sitostanol was used as the reference compound. Since beta sitostanol is unabsorbable, cholesterol absorption could be easily calculated knowing the ratio of the two isotopes (^{3}H beta sitostanol and ^{14}cholesterol) in the test solution and the effluent because the change in the ratio of the two isotopes would be a direct reflection of the absorption of cholesterol. It might be pointed out here that exchange of isotopic cholesterol for cholesterol in the mucosal membrane would tend to increase the calculated value of cholesterol absorption. Grundy and Mok (5) found that the extent of exchange was variable and averaged 23%/hour of the net absorption/hour (in the human). In the present study no attempt was made to determine the extent of isotopic exchange; however, since absorption was studied over a period of only 20 minutes, isotopic exchange was assumed to negligible.

Cholesterol absorption was calculated according to the following formula:

$$\left[1 - \frac{{}^{3}H/{}^{14}C \text{ (in test solution)}}{{}^{3}H/{}^{14}C \text{ (in effluent)}}\right] \times \left[\text{Cholesterol infused (n moles)}\right]$$

where: ^{3}H refers to ^{3}H beta sitostanol and ^{14}C refers to ^{14}C cholesterol. The uptakes were normalized and expressed as nmoles $\cdot$ 100 mg^{-1} tissue dry weight $\cdot$ 20 min^{-1}.

G. Statistical Methods: Given the small number of N's in the present study, which in some instances are unequal, and the degree of variability , it was decided to use non-parametric statistics to evaluate the data. Thus the following tests were used:

1. Kruskal-Wallis H Test: This statistic tests whether three or more independent samples have been drawn from the same population (93). In essence, this is a non-parametric one-way analysis of variance and was used to test if the absorption of cholesterol was a function of cholesterol concentration in the two micellar solution.

2. Mann-Whitney Test (94): This test was used to make the following comparisons:

a. Data obtained between experimental groups.

b. Data obtained to assess the effects of cholesterol concentration within an experimental group.

c. In certain instances when the comparisons between groups showed no overall differences, the groups were compared at a particular cholesterol concentration.

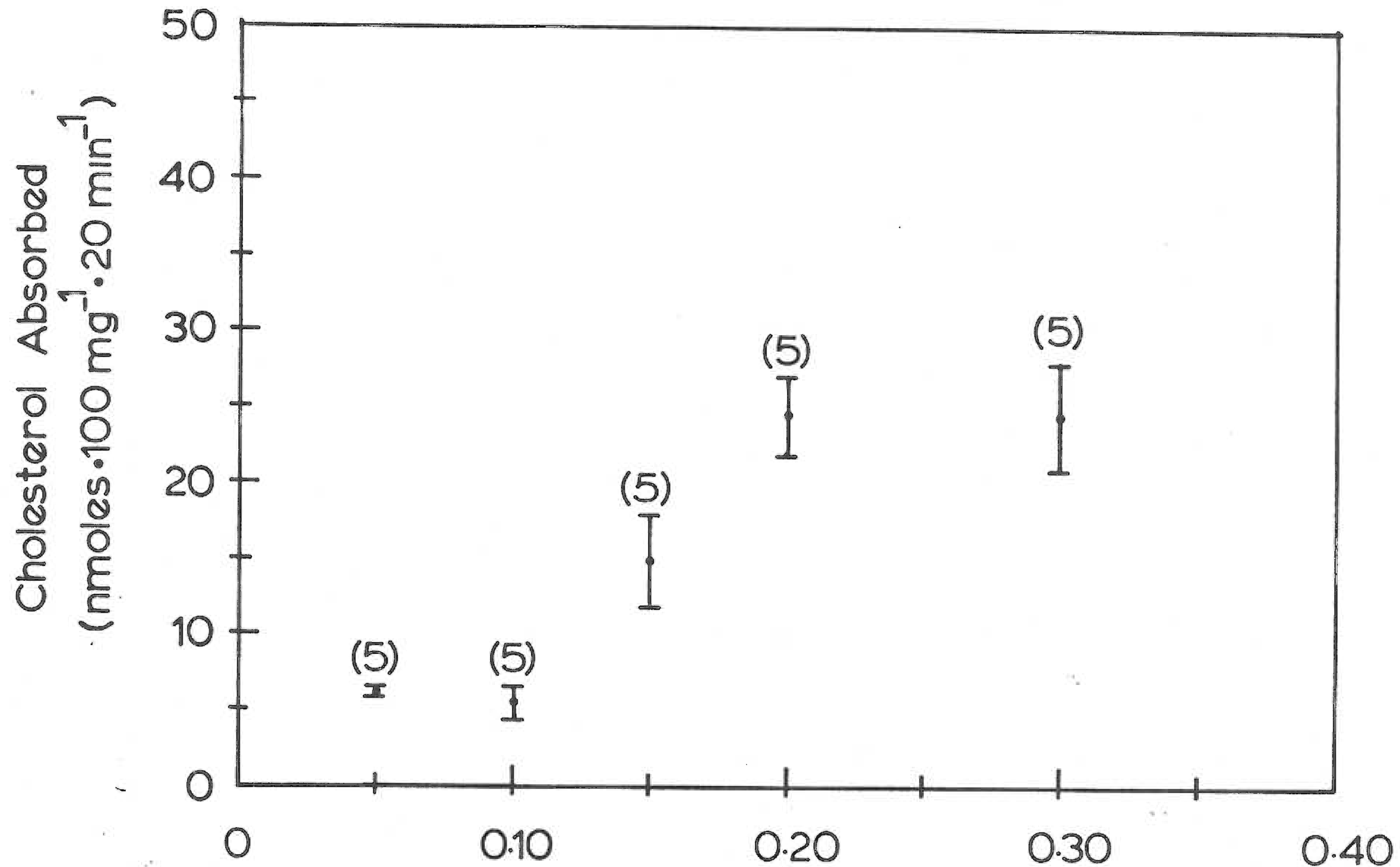
III. RESULTS

Figure 12 shows the absorption of cholesterol (nmoles · 100 mg^{-1} · 20 min^{-1}) as a function of cholesterol concentration. Beta sitostanol was present in the solution only as a non-absorbable tracer. As expected, cholesterol absorption increased with increasing concentration ($p < 0.01$). It appeared to achieve a maximum at a concentration of 0.20 mM since increasing cholesterol concentration to 0.30 mM did not result in a further increase (absorption at 0.20 mM was not significantly different from that at 0.30 mM). At the plateau, absorption averaged about 25 nmoles · 100 mg^{-1} · 20 min^{-1}. At concentrations of 0.05 mM and 0.10 mM, cholesterol absorption was also found not to be different statistically. No significance has been attached to it, however, because, as will be seen, it was not observed in any of the other experiments.

The effect of beta sitostanol in a concentration of 0.20 mM (given as a 'solution-mix') is shown in Figure 13A. Cholesterol absorption in this condition was not significantly different from control (See Table 13 for group comparisons). As before, cholesterol absorption at a concentration of 0.20 mM was not significantly different from that at 0.30 mM. Because cholesterol absorption achieved an apparent saturation at a concentraion of 0.20 mM, it was decided that in the experiments to follow, observations would be confined to the linear portion of the curve.

Since a 'solution-mix' experiment with beta sitostanol

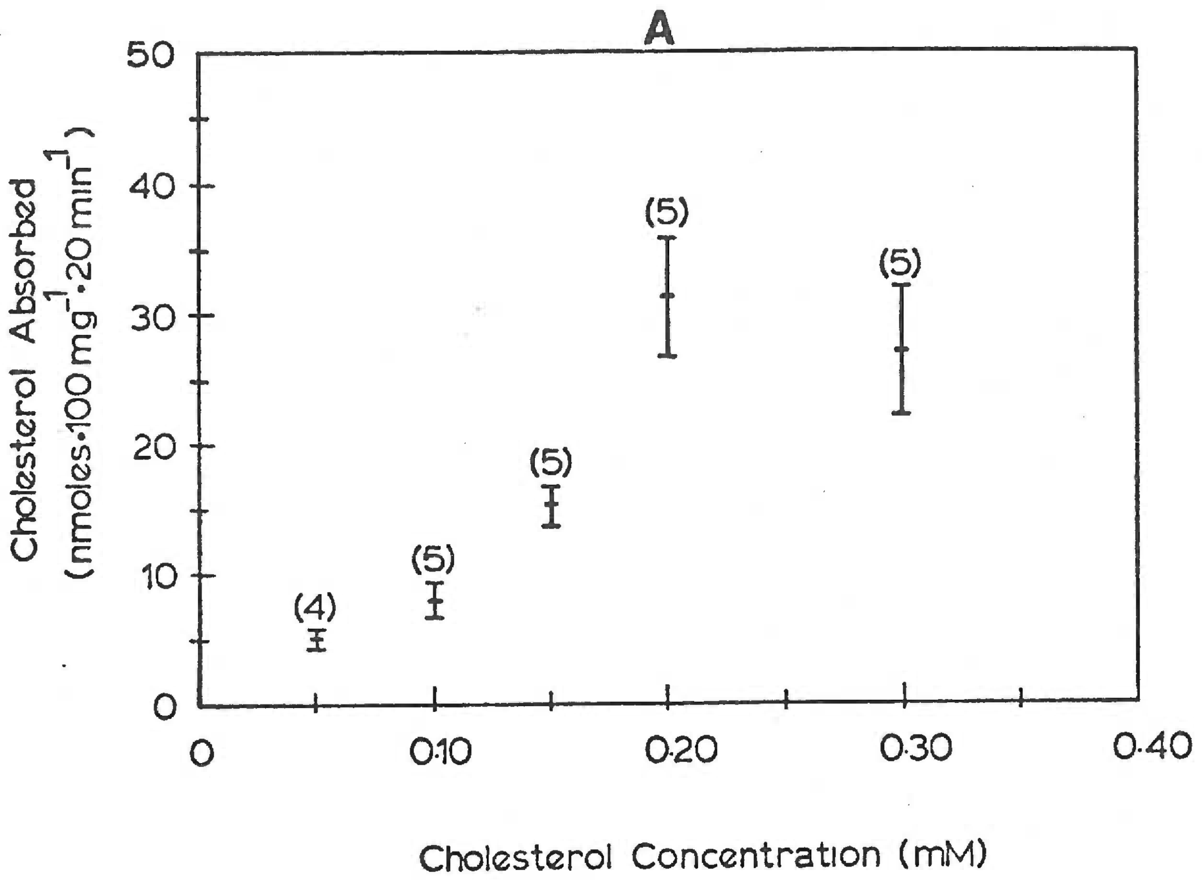
Figure 12. Cholesterol absorption as a function of cholesterol concentration. Beta sitostanol was present only as a non-absorbable marker. The numbers in parentheses refer to the number of animals studied at that concentration and each point is the mean of the values obtained from those animals. The vertical bars represent ± one standard error of the mean (S.E.M.).



Cholesterol Absorbed
(nmoles·100 mg^{-1}·20 min^{-1})
50
40
30
20
10
0
0
0·10
0·20
0·30
0·40
(5)
(5)
(5)
(5)
(5)
Cholesterol Concentration (mM)

Figure 13A. Effect of 0.20 mM beta sitostanol in 'solution-mix' on cholesterol absorption. The numbers in parentheses have the same significance as before and the values plotted are the mean $\pm$ S.E.M. of the values obtained at each concentration.

Figure 13B. The effect of pre-flushing on cholesterol absorption from 'solution-mix'. The concentration of beta sitostanol in both the 'pre-flush' and the 'solution-mix' was 0.20 mM. As described in Methods, the loop was pre-flushed with the beta sitostanol micellar solution followed by infusion of the 'solution-mix' test solution. Data are plotted as before. Cholesterol absorption at 0.20 mM in this experiment is significantly different ($p < 0.06$) from cholesterol absorbed at the same concentration in Figure 13A above, or Figure 12 (control).


A
50
40
30
20
10
0
Cholesterol Absorbed
(nmoles•100 mg^{-1}•20 min^{-1})
(4)
(5)
(5)
(5)
(5)
0
0·10
0·20
0·30
0·40
Cholesterol Concentration (mM)

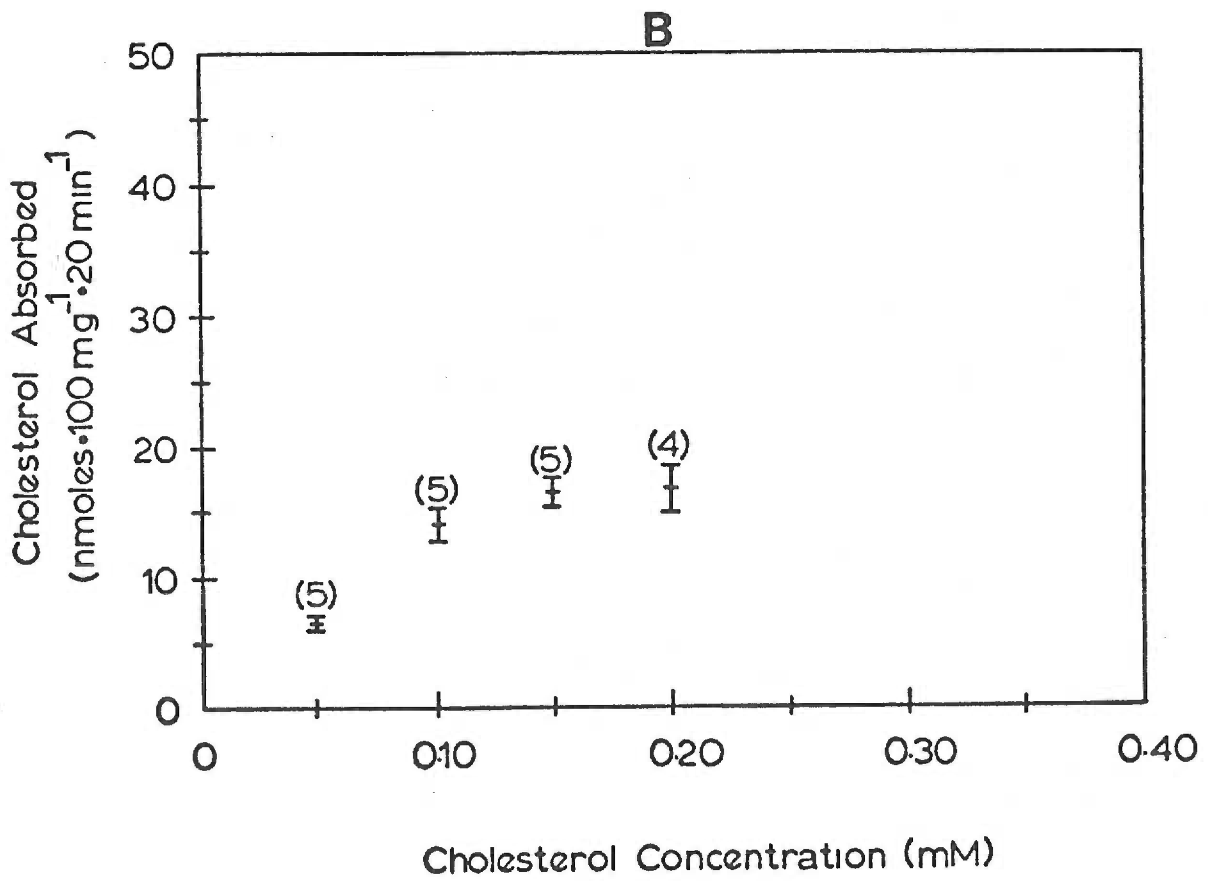

B
50
40
30
20
10
0
Cholesterol Absorbed
(nmoles•100 mg^{-1}•20 min^{-1})
(5)
(5)
(5)
(4)
0
0·10
0·20
0·30
0·40
Cholesterol Concentration (mM)

Table 13. Group comparisons using the Mann-Whitney Test. The numbers reading horizontally are p values for group comparisons. For example, control group (C, second line) vs 'Pre-flush/solution-mix' group with a beta sitostanol concentration of 0.30 mM ($P.F._{0.30}$, third column), is significantly different at $p<0.01$; or 'Pre-flush/solution-mix' at 0.30 mM ($P.F._{0.30}$, fifth line, third column) vs 'Pre-flush/solution-mix' at 0.20 mM (column 5) is significantly different at $p<0.0005$.

C	$S.M._{0.30}$	$P.M._{0.30}$	$P.F._{0.30}$	$S.M._{0.20}$	$P.F._{0.20}$	$S.M.^{*}_{0.30}$
C	<0.01	N.S.	<0.01	N.S.	N.S.	N.S.
	$S.M._{0.30}$	<0.0005	N.S.	<0.001	<0.0005	<0.0005
		$P.M._{0.30}$	<0.0005	N.S.	N.S.	N.S.
			$P.F._{0.30}$	<0.001	<0.0005	<0.0005
				$S.M._{0.20}$	N.S.	N.S.
					$P.F._{0.20}$	N.S.

C: Control

S.M.: Solution-mix

P.M.: Pre-mix

P.F.: Pre-flush/solution-mix

Numbers in subscript refer to the concentration of beta sitostanol in the solution.

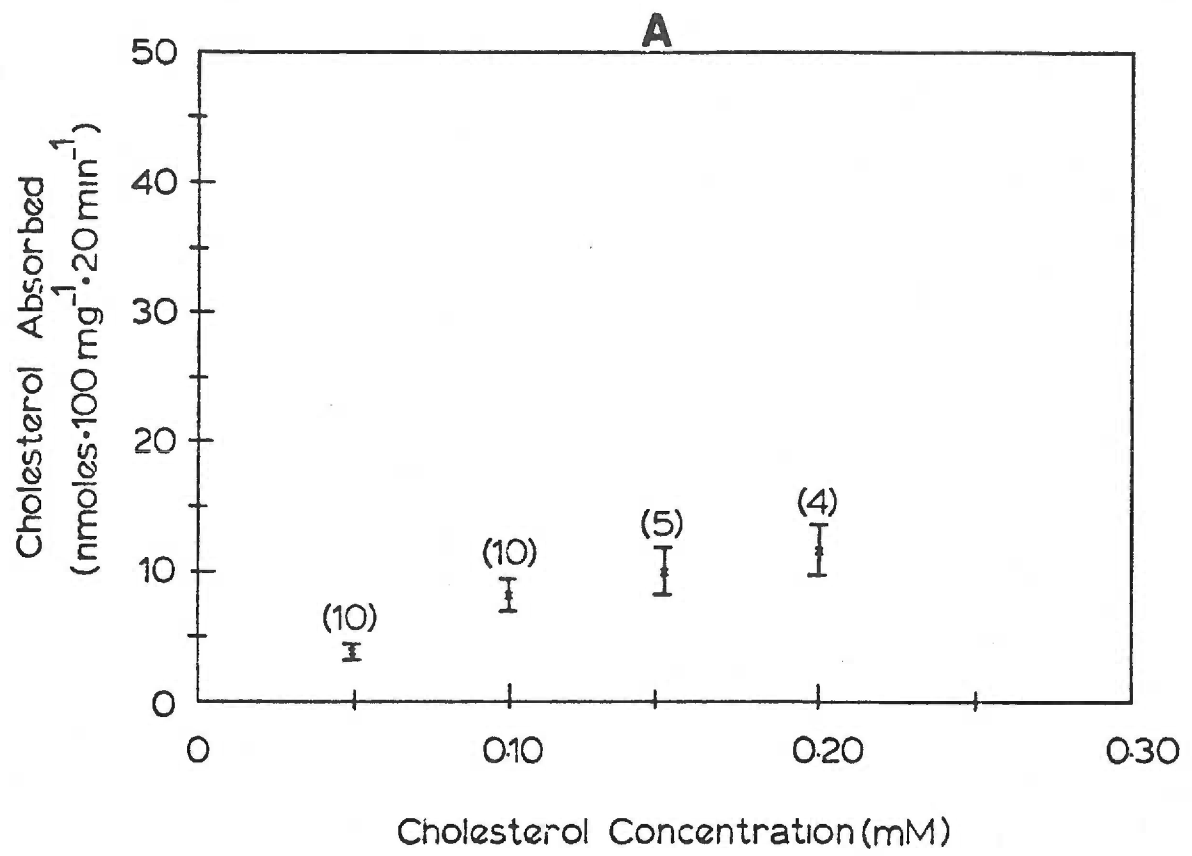
*This was the cholesterol/cholesterol solution mixture and the number in the subscript refers to the concentration of cold cholesterol.

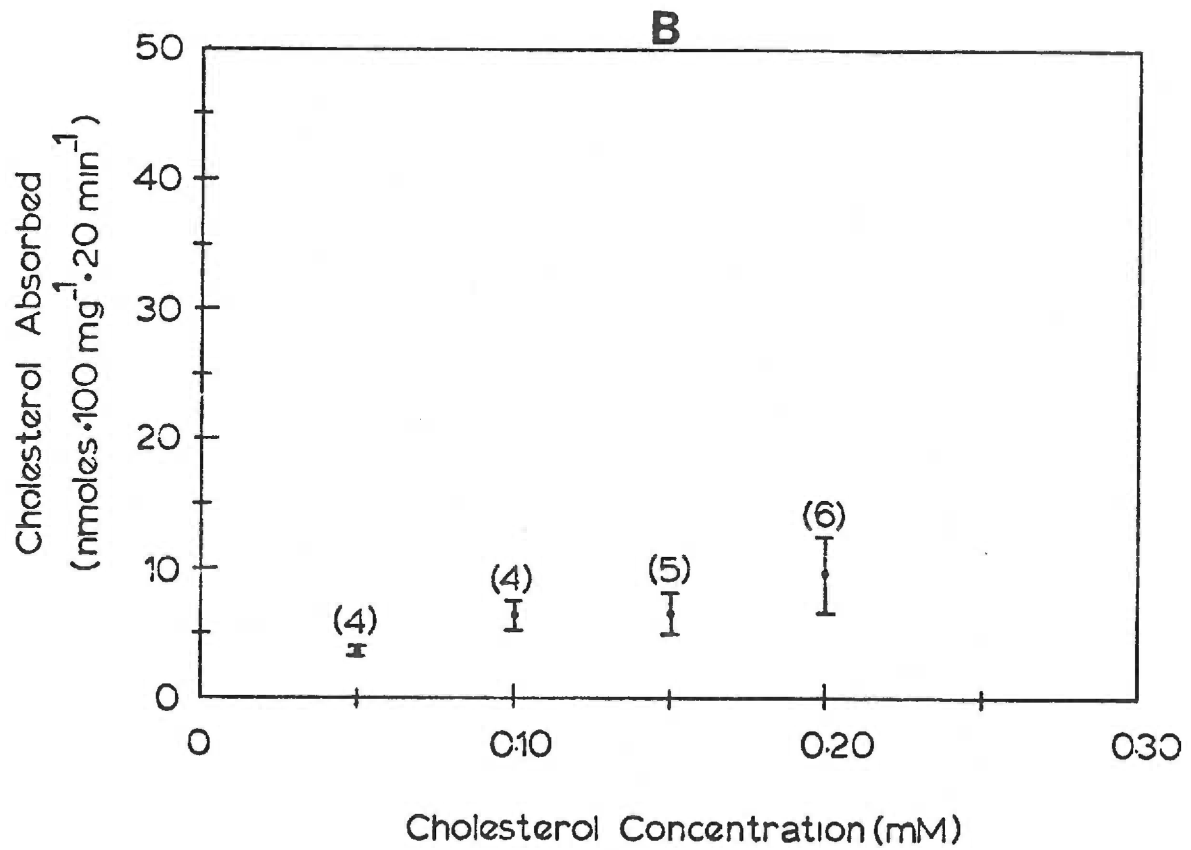
at a concentration of 0.20 mM had no effect on cholesterol absorption, it was decided to carry out a 'pre-flush/solution mix experiment with a beta sitostanol concentration of 0.20 mM to see if the addition of the pre-flush would affect cholesterol absorption. The results of this experiment are shown in Figure 13B. Absorption of cholesterol in this group was not significantly different from the control (Figure 12; see Table 13 for group comparisons). However, the cholesterol absorbed at a concentration of 0.20 mM in this experiment was significantly less than cholesterol absorbed at the same concentration in the control group ($p < 0.06$). This suggests that the effect of pre-flushing the loops with beta sitostanol at 0.20 mM on the absorption of cholesterol from a following 'solution'mix', was only manifest at a cholesterol concentration of 0.20 mM. In addition, cholesterol absorption appeared to achieve a near maximum at a concentraion of 0.15 mM and this plateau corresponded to a mean cholesterol absorption of about 18 nmoles $\cdot$ 100 mg^{-1} $\cdot$ 20 min^{-1} compared to about 30 nmoles $\cdot$ 100 mg^{-1} $\cdot$ 20 min^{-1} in the 'solution mix' alone (Figure 13A).

Increasing the concentration of beta sitostanol to 0.30 mM in the 'solution-mix' reduced cholesterol absorption dramatically compared to control ($p < 0.01$, Table 13). This is shown in Figure 14A. Cholesterol absorption was a function of cholesterol concentration ($p < 0.01$), although it achieved a near maximum at a concentration of only 0.10 mM. The plateau corresponded to a mean cholesterol absorption of

Figure 14A. The effect of 0.30 mM beta sitostanol in 'solution-mix' on cholesterol absorption. Data are plotted as before.

Figure 14B. The effect of the addition of a pre-flush' with a 0.30 mM beta sitostanol micellar solution on cholesterol absorption from the 'solution-mix' used in Figure 14A above.


A
Cholesterol Absorbed
(nmoles·100 mg⁻¹·20 min⁻¹)
50
40
30
20
10
0
(10)
(10)
(5)
(4)
0
0·10
0·20
0·30
Cholesterol Concentration (mM)

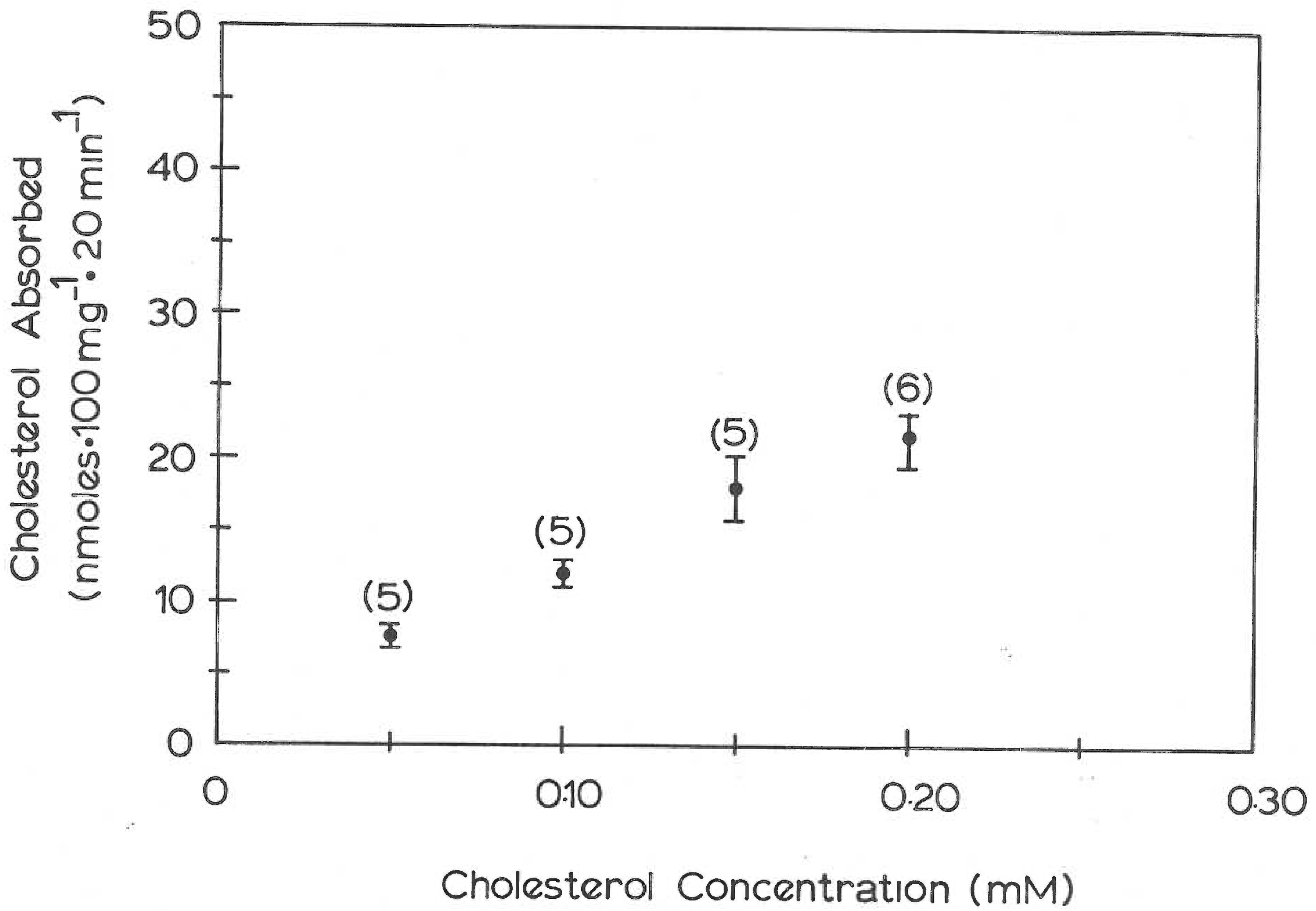

B
Cholesterol Absorbed
(nmoles·100 mg⁻¹·20 min⁻¹)
50
40
30
20
10
0
(4)
(4)
(5)
(6)
0
0·10
0·20
0·30
Cholesterol Concentration (mM)

about 10 nmoles $\cdot$ 100 mg^{-1} $\cdot$ 20 min^{-1} compared to about 25 nmoles $\cdot$ 100 mg^{-1} $\cdot$ 20 min^{-1} in the control (Figure 12).

Figure 14B shows the results of a 'pre-flush/solution-mix experiment. The loop was first flushed with a micellar solution of beta sitostanol at a concentration of 0.30 mM. This was immediately followed by infusion of the test solution which was the same 'solution-mix' used above. The pre-flush, it was thought, might reduce cholesterol absorption further than that observed with the 'solution-mix' experiment above. Although cholesterol absorption was again reduced compared to control ($p < 0.01$), there was no significant difference between this group, and the group given the 'solution-mix' alone. One-way analysis of variance (the Kruskal-Wallis Test) showed that in the present group (Figure 14B) cholesterol absorption was independent of cholesterol concentration. This was in contrast to the 'solution-mix' group (Figure 14A). The differences between Figures 14A and 14B were small, however, and there is some doubt whether or not any significance could be attached to them.

Since 0.30 mM beta sitostanol reduced cholesterol absorption previously, without pre-flushing, it was of interest to see if beta sitostanol, at the same concentration, in the 'pre-mix' condition would have the same effect. As can be seen in Figure 15, pre-mixing beta sitostanol with cholesterol, to give what was called 'pre-mix' solution, failed to suppress cholesterol absorption in contradistinction to marked suppression achieved by beta sitostanol when given in the 'solu-

Figure 15. Effect of 0.30 mM beta sitostanol in 'pre-mix' condition on cholesterol absorption.



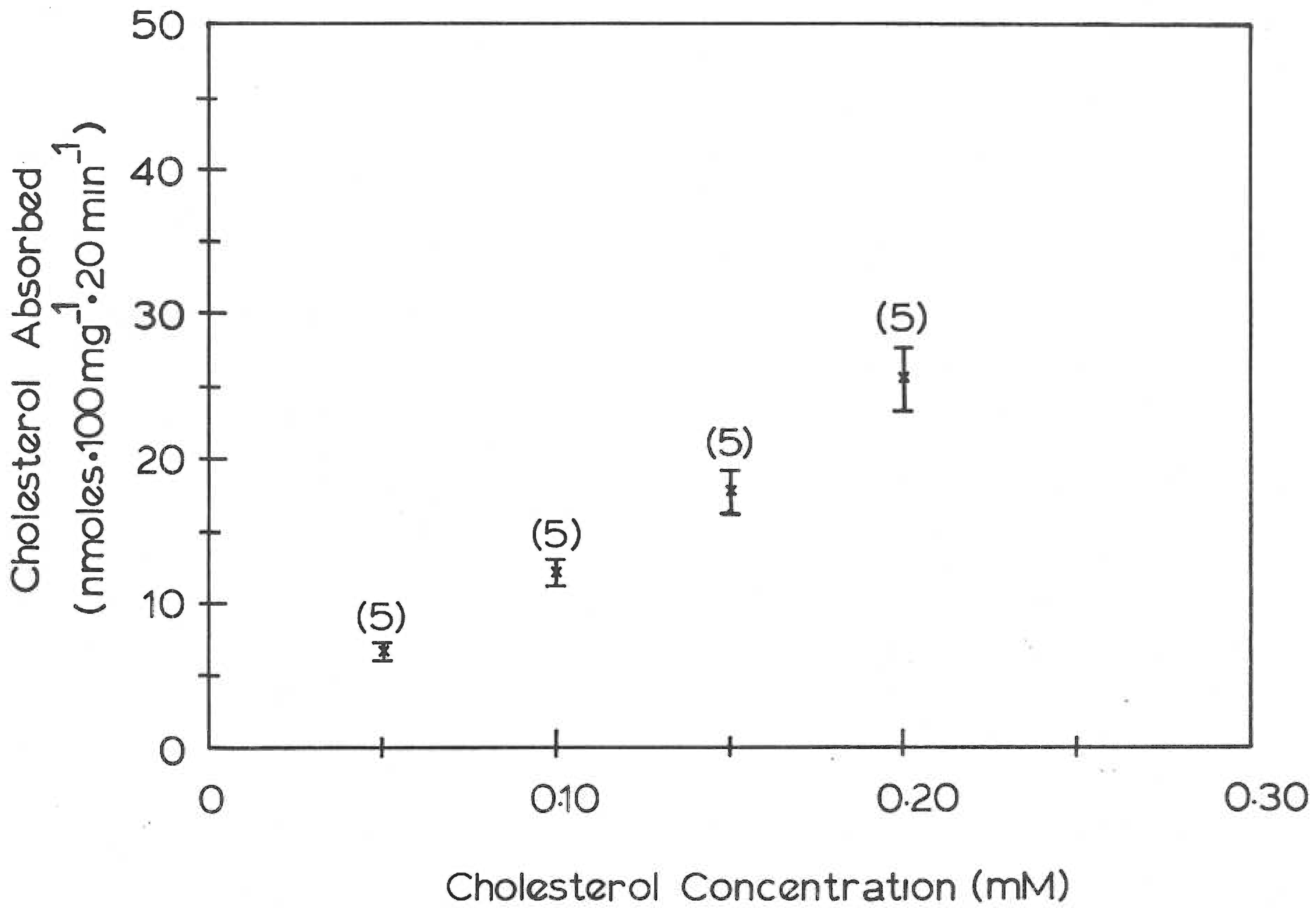
Cholesterol Absorbed
(nmoles·100 mg^{-1}· 20 min^{-1})
50
40
30
20
10
0
(5)
(5)
(5)
(6)
0
0·10
0·20
0·30
Cholesterol Concentration (mM)

tion-mix' (See Table 13 for group comparison). Absorption of cholesterol was a function of cholesterol concentration ($p < 0.01$) and it appeared to be linear over the entire range. A best fit line (not shown) to the data by the method of least squares yielded a coefficient of determination (r^2) of 0.95.

Finally, in order to see if the reduction in cholesterol absorption achieved by 0.30 mM beta sitostanol in 'solution-mix' was due specifically to beta sitostanol and not to some non-specific effect related to total sterol concentration, a 'solution-mix' experiment was carried out using a cholesterol/cholesterol mix. The solution was prepared by mixing an aliquot of a 0.60 mM cholesterol micellar solution without any radioactive labels, with equal aliquots of other micellar solutions containing cholesterol in concentrations varying from 0.10 mM to 0.40 mM and containing the radioactive labels. The resulting solutions, therefore, were 0.30 mM in unlabeled cholesterol and 0.05 to 0.20 mM in labeled cholesterol. The conditions were analogous to the previous 'solution-mix' experiments except that cholesterol was substituted for beta sitostanol.

The results are given in Figure 16 showing that at a concentration of 0.30 mM, cholesterol had no effect on its own absorption. As with most experiments, cholesterol absorption was a function of cholesterol concentration ($p < 0.01$) and the relationship was linear. A best fit straight line (not shown) according to the method of least squares yielded

Figure 16. Cholesterol absorption as a function of cholesterol concentration in a cholesterol/cholesterol 'solution-mix'. This 'solution-mix' was analogous to the beta sitostanol/cholesterol 'solution-mix' used in Figure 14A, except that beta sitostanol was replaced with cholesterol.



Cholesterol Absorbed
(nmoles·100mg⁻¹·20 min⁻¹)
50
40
30
20
10
0
(5)
(5)
(5)
(5)
0
0·10
0·20
0·30
Cholesterol Concentration (mM)

a coefficient of determination of 0.97. This suggests that the reduction in cholesterol absorption seen with beta sitostanol was probably due to beta sitostanol and not a nonspecific effect of total sterol concentration.

IV. DISCUSSION

It was mentioned earlier that plant sterols could suppress cholesterol absorption in one of two ways.

A. By altering the micellar distribution of cholesterol and/or changing the shape or size of the micelles, plant sterols could suppress cholesterol absorption. In the latter case it would be due to the possible decreased rate of diffusion of the micelle through the unstirred water layer, while in the former case the suppression would be due to the decreased amount of solubilized cholesterol available for absorption.

B. Plant sterol could interfere with cholesterol absorption at the level of the cell membrane either directly or by competing with cholesterol for specific receptors or carrier molecules.

An attempt to discriminate between the two possibilities was a major part of this investigation. Since I was using a new plant sterol, it was necessary first to verify that beta sitostanol could suppress cholesterol absorption and to identify the conditions under which it did so. These data are given in Table 13, showing that beta sitostanol inhibited cholesterol absorption under the 'solution-mix' condition but not under the 'pre-mix' condition. The significance of this observation will be brought out in the ensuing paragraphs.

If cholesterol and beta sitostanol are assumed to be insoluble in water (there are no data indicating otherwise) then, under the conditions of the experiments, the two compounds could only exist in solution as micelles. Provided that the solubil-

izer (bile salt, monoolein and oleic acid) is present in sufficient concentration, a micellar solution of either compound would be optically clear, that is, all the solubilizate (cholesterol or beta sitostanol) would be present in micellar form and the intermicellar concentration of these sterols would be essentially zero. On the other hand, insufficient solubilizer would result in a cloudy solution in which part of the sterol would not be truly solubilized. In addition, because micelles can be, and have been, subjected to separation procedures such as gel filtration (52) without apparent disruption, it is not unreasonable to assume that the micelle is a fairly stable structure requiring special forces to be disrupted.

Given this, if two separate micellar solutions were made, one of cholesterol and the other of beta sitostanol, and then mixed together, it would be reasonable to suppose that the micelles would retain their identity. That is to say beta sitostanol would not enter into a cholesterol-containing micelle and vice versa.

In the present study, no direct attempt was made to determine if the micelles did indeed retain their identity. However, there is some evidence that such was the case. It may be recalled that a 0.60 mM beta sitostanol solution was cloudy, suggesting that some beta sitostanol was not in micellar solution. Furthermore, mixing an aliquot of this solution with an equal aliquot of a clear micellar solution of cholesterol resulted in a solution that was still cloudy, a situation

which would be unexpected if there were intermicellar exchange of sterols. A suppressor effect of beta sitostanol on cholesterol absorption in this 'solution-mix' condition would be due to an interaction between beta sitostanol and the mucosal cell membrane, although, some type of a micelle-micelle interaction could not be ruled out.

In contradistinction to the 'solution-mix', when both beta sitostanol and cholesterol were mixed together before being dispersed with the solubilizer, a given micelle would be expected to contain both the sterols. Such solutions were optically clear even though the concentrations of cholesterol and beta-sitostanol were the same as in the (cloudy) 'solution-mix' conditions. Under these 'pre-mix' conditions any suppressor effect of beta sitostanol would be due to some type of intramicellar interaction leading to possible enlargement of the micelles (as has been suggested for lecithin (55-59)) and/or in some other way reducing the diffusivity of the micelles in the aqueous environment of the intestinal lumen.

Thus by using these two basic solutions and by the addition of the pre-flush experiments with beta sitostanol, it should be possible to distinguish between intraluminal interactions vs membrane interactions as the basis for the inhibition of cholesterol absorption by the plant sterols. These two possibilities will now be discussed in some detail under their respective headings.

1. The Intraluminal Hypothesis: Although it has been argued that the addition of plant sterols in the diet

expands the intestinal sterol load thereby reducing the availability of solubilizers for cholesterol (16), this argument is not a factor in the present experiments because cholesterol was administered always in micellar form. Therefore, other intraluminal events have to be sought to explain the effects of beta sitostanol.

When plant sterols are administered _in vivo_, one would expect to see several different micellar species. Some micelles would contain cholesterol as the only sterol, others would contain only plant sterols, and some (probably most) would contain both.

It is interesting to note that in the 'pre-mix' experiments in which the micelles contain both cholesterol and beta sitostanol, there was no demonstrable effect of the beta sitostanol on cholesterol absorption (Figure 15). On the other hand, in the 'solution-mix' experiment (beta sitostanol concentration of 0.30 mM) in which two separate micellar species were assumed to be present, beta sitostanol markedly suppressed cholesterol absorption (Figure 14A and 14B). This suggests that beta sitostanol reduces cholesterol absorption by some means other than by altering the shape or size of the micelles.

It is worth discussing the question of cloudiness in some of the micellar solutions. Beta sitostanol suppressed cholesterol absorption only in the 'solution-mix' conditions and only when the solutions infused were cloudy (Figure 14A and 14B). Cloudiness occurred only when the beta sitostanol

concentration was greater than 0.20 mM. Solutions containing beta sitostanol in concentrations of 0.20 mM or less were optically clear and did not suppress cholesterol absorption (Figure 13A). One could argue that the unsolubilized beta sitostanol in the cloudy solution reduced the micellar distribution of cholesterol thereby decreasing cholesterol absorption. However, this seems unlikely in light of Borgstrom's experiments showing that addition of sitosterol had no effect on the micellar distribution of cholesterol in an emulsion even when sitosterol was added in ten times the concentration of cholesterol present in the emulsion (54). Thus, the argument is not a strong one in support of the intraluminal hypothesis.

A final argument in support of the intraluminal hypothesis is that the suppressor effect of beta sitostanol was related to the total sterol concentration of the infusate. Evidence against this argument is provided by the experiment where cholesterol, in a solution analogous to the beta sitostanol 'solution-mix,' failed to suppress its own absorption (Figure 16).

2. The Membranal Hypothesis:

a. Recently there has been a growing interest in the effect of cholesterol on membrane permeability. It is thought that cholesterol affects the distribution of the phospholipids in the membrane leading to changes in membrane permeability (95). This effect is apparently not limited to cholesterol; any substance with a 3 beta-OH group, a planar

ring and a hydrophobic side chain at C_{17} is capable of inducing permeability changes in the membrane (94). Because beta sitostanol fulfils the criteria listed above, it is reasonable to suppose that beta sitostanol inhibits cholesterol absorption by altering the mucosal membrane permeability characteristics. The argument is strengthened by the observation that large quantities of plant sterols are usually administered *in vivo* to achieve suppression of cholesterol absorption (24). Although the observation does not itself point to a membrane effect, it at least suggests that the effect is non-specific.

However, the situation *in vivo* is not as simple as it may seem. While it is true that large amounts of plant sterols have been used to achieve suppression of cholesterol absorption, the plant sterols consisted of a mixture of beta sitosterol and campesterol (60% and 40% respectively). Because campesterol is relatively more absorbable than sitosterol (24), its effectiveness in suppressing cholesterol absorption would, a priori, be expected to be less than sitosterol. In essence, the large doses of plant sterols were administered to achieve an adequate dose of beta sitosterol. Arguing against the large dose idea is the fact that as little as 3 grams of a plant sterol preparation consisting of greater than 90% beta sitosterol, can bring about a marked suppression of cholesterol absorption in man (23). A daily dose of 3 grams of plant sterols is probably not an overwhelming dose considering that the small intestine (in

man) is probably faced with a daily load of 3 grams of cholesterol.

Even if plant sterols inhibited cholesterol absorption by altering membrane permeability *in vivo*, it does not stand to reason that they themselves should not be absorbed. Increasing the amount of cholesterol fed, for example, leads to an increased absorption to a point after which the percent cholesterol absorbed decreases. Why the same pattern should not be observed for plant sterols is not answered by the alteration in membrane permeability hypothesis.

In the present study, if the suppressor effect of beta sitostanol is attributed to alteration in membrane permeability, then a similar effect should have been observed in the analogous cholesterol/cholesterol 'solution-mix' since the essential requirements for the membranal effect are possessed by both sitostanol and cholesterol. Since cholesterol had no demonstrable effect on its own absorption under the present conditions (Figure 16), it does not seem likely that the mechanism of suppression of cholesterol absorption by beta sitostanol is due to a non-specific effect on the membrane.

b. Beta sitostanol could interfere with the absorption of cholesterol by binding to cholesterol-specific binding sites on the membrane. This is simply a restatement of the Glover and Green hypothesis of cholesterol absorption (70,71). They proposed that cholesterol absorption was mediated through a membrane-bound receptor or carrier specific for cholesterol. If there were such a carrier, beta sito-

stanol, being similar in structure, could possibly occupy the binding site, blocking the binding of cholesterol and thereby reducing its absorption. On the other hand, because of the small structural differences between beta sitostanol and cholesterol, transport of beta sitostanol into the cell might be limited.

Whether or not such a carrier actually exists is, at present, unknown. Therefore, for the moment, it would be well to consider the characteristics of carrier-mediated (passive-facilitated) transport and whether the results in this study support the presence of such a process.

Three criteria characterize carrier-mediated transport not requiring energy. These include:

i. Specificity

ii. Saturability

iii. Inhibition

i. Specificity: A carrier for a certain substance is usually specific for that substance and to very closely related analogs. For example, the carrier for glucose in the small intestinal mucosal membrane also transports other sugars but with differing efficiencies (96). That certain specific requirements are necessary for efficient absorption of a given sterol is suggested by the following: cholesterol is absorbed more than cholestanol and campesterol is absorbed more than sitosterol under similar conditions. A glance at Figure 1 will reveal that cholesterol and cholestanol differ by a double bond in the B ring, while campesterol and sitosterol differ

only by the substituent group on C_{24} of the side chain. This suggests that if there is a carrier for cholesterol, it is specific for substituents around C_5 and C_6 in the B ring, and on the C_{24} position. A compound like beta sitostanol which has a reduced B ring like cholestanol and an ethyl group on the C_{24} position, would be expected to quite unabsorbable as was shown in the previous section. Thus, there is evidence that sterol absorption is a specific process.

ii. Saturability: With many substances of physiological importance (e.g. glucose and amino acids), the rate of entry of the substance into the cell initially increases with increasing concentration of the substance on the outside of the cell. Ultimately, however, an external concentration is reached at which the entry of the substance is maximal and not increased in the presence of higher external substrate concentrations. This is referred to as saturation kinetics denoting the saturability of the carrier.

In the present study, absorption of cholesterol as a function of concentration shows saturation in five out of the seven sets of experiments. This was true whether or not beta sitostanol was present. In Figures 15 and 16, there is no evidence for saturation over the range of concentrations studied. However, that might conceivably be due to the fact that absorption in these two cases was not studied at cholesterol concentrations greater than 0.20 mM where saturation might well be seen. Thus, there is evidence that the cholesterol absorption process is saturable. If cholesterol absorp-

tion was a simple passive diffusion process, one would expect to see a linear relationship between cholesterol absorption and concentration.

iii. Inhibition: This characteristic is, in effect, a consequence of specificity. Substances closely related to the transported substrate may inhibit the transport of the substrate. This is believed to be due to the binding of the analog to the carrier, preventing the binding of the transported substrate and thereby reducing the absorption of the substrate.

In the present study, beta sitostanol at 0.30 mM in the 'solution-mix' clearly suppressed cholesterol absorption. In addition, in the presence of beta sitostanol, saturation of cholesterol absorption was achieved at a lower cholesterol concentration compared to control (Figure 14A vs Figure 12). This suggests that in the presence of beta sitostanol, fewer carriers are available to bind and transport cholesterol. Once again, if sterol absorption were a simple passive diffusion process, one would not expect to see any mutual interference in the absorption of different sterols.

Therefore, in the present study, there is evidence for a carrier mechanism operative for cholesterol absorption. There are, however, a few interesting points to be made about this putative carrier.

It may be recalled that beta sitostanol at 0.20 mM in the 'solution-mix' had no effect on cholesterol absorption (Figure 13A), whereas at 0.30 mM it suppressed cholesterol

absorption markedly (Figure 14A). This suggests that a beta sitostanol concentration of 0.20 mM is not sufficient to occupy enough carriers to produce an effect, while at 0.30 mM, the effect of beta sitostanol is near maximal. This conclusion is strengthened by the results of the pre-flush experiments. These experiments show that pre-flushing with a beta sitostanol solution at a concentration of 0.30 mM had little additional effect on cholesterol absorption from the subsequent test solution (Figure 14A vs 14B). On the other hand, pre-flushing with a beta sitostanol solution at a concentration of 0.20 mM, had a significant additional effect on cholesterol absorption from the subsequent test solution (Figure 13A vs 13B). Note that saturation of cholesterol absorption now occurred at a lower cholesterol concentration and that the plateau corresponded to a mean cholesterol absorption of about 18 nmoles $\cdot$ 100 mg^{-1} $\cdot$ 20 min^{-1} (Figure 13B) compared to nearly 30 nmoles $\cdot$ 100 mg^{-1} . 20 min^{-1} without the pre-flush (Figure 13A) or 25 nmoles $\cdot$ 100 mg^{-1} $\cdot$ 20 min^{-1} in the controls (Figure 12). This does not necessarily imply that the effect of beta sitostanol is an 'all-or-none' phenomenon; clearly there must be a concentration between 0.20 mM and 0.30 mM at which there is the first evidence of inhibition which then progresses rapidly with increasing beta sitostanol concentration. Because it was not the purpose of this study to determine the characteristics of the putative carrier, no attempt was made to determine the minimum concentration of beta sitostanol required for an effect.

Finally, it is interesting to note that beta sitostanol at a concentration of 0.30 mM given as a 'pre-mix' had no effect on cholesterol absorption (Figure 15). The lack of effect here may be understood by considering the differences between the 'pre-mix' and the 'solution-mix' solutions. In the 'solution-mix' two independent micellar species were assumed and, if so, there may have been intermicellar competition for a carrier. However, once a micelle was at the carrier site, either cholesterol _or_ beta sitostanol would bind to the carrier depending on their relative affinities and which micelle arrived at the carrier first. On the other hand, since a micelle in the 'pre-mix' solution was assumed to contain both beta sitostanol and cholesterol together, the two sterols would arrive at the site at the same time and inhibition could mean competition for binding based on relative affinities.

If it may be assumed that the affinity of the carrier for cholesterol is greater than that for sitostanol, then it is likely that more cholesterol than beta sitostanol would bind to the carrier. In this situation, sitostanol may have no effect on cholesterol absorption.

To extend this argument further, one might predict that cholesterol absorption _in vivo_ would be more effectively suppressed by beta sitostanol or beta sitosterol if the plant sterols were administered in micellar form instead of as a component of the diet. The latter situation would be akin to the 'pre-mix' solution, while the former would be analogous

to the 'solution-mix' condition. Indeed, Grundy and Mok (5) have shown that in the human, intraduodenal infusion of even small amounts of beta sitosterol in micellar form (30-101 mg/hour) markedly suppressed cholesterol absorption. This observation supports the findings in the 'solution-mix' experiments and suggests that the important factor in plant sterol suppression of cholesterol absorption might be the amount of plant sterol in micellar form.

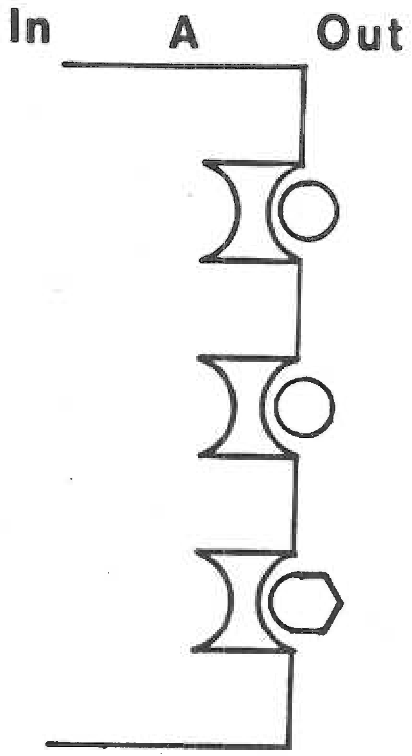
The lack of suppression of cholesterol absorption in the cholesterol/cholesterol 'solution-mix' (Figure 16) was unexpected. A priori, one would predict that in this condition 'cold' cholesterol would effectively compete with radioactively labeled cholesterol for the carrier. This would be the case if there was one binding site per carrier. However, if the carrier has two (or more) binding sites then the situation might be different. Therefore the following hypothesis is suggested: There is a membrane-bound carrier specific for cholesterol. This carrier has at least two binding sites; binding of cholesterol at one site allows the carrier to undergo a rotational movement such that the bound cholesterol is internalized while another binding site is exposed to bind another molecule of cholesterol. The binding of beta sitostanol to the carrier not only prevents cholesterol from binding, but also prevents the rotational movement of the carrier so that the second binding site is not exposed (See Figure 17). Thus, beta sitostanol would suppress cholesterol absorption without being absorbed.

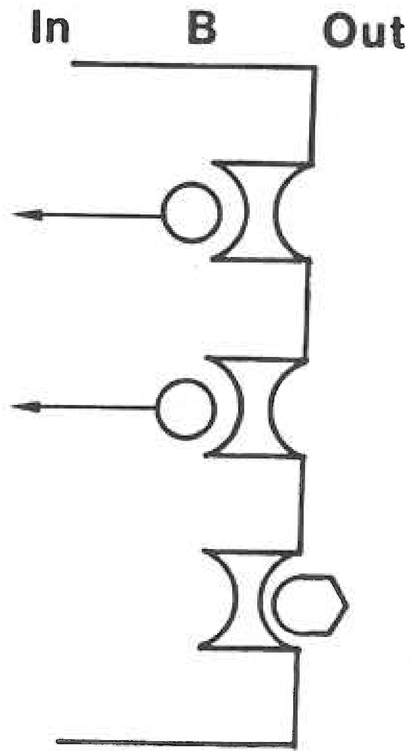
Figure 17. A diagrammatic representation of the cholesterol carrier hypothesis. The figure depicts part of a cell membrane with carriers for cholesterol. Each carrier is shown as having two binding sites. 'In' refers to inside the cell, while 'Out' refers to outside the cell.

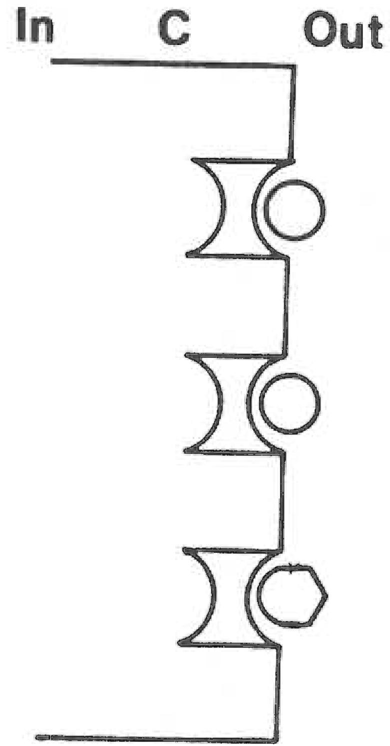
In A, two carriers are shown with bound cholesterol while one is shown with beta sitostanol bound to it.

In B, the carriers with cholesterol have undergone a rotational movement, thereby internalizing the bound cholesterol (shown by the arrows), while exposing the second binding site. Note, that the carrier with beta sitostanol bound to it, has not undergone the rotational movement.

In C, more cholesterol is shown binding to the carrier and the situation is analogous to A.







Cholesterol
Beta sitostanol
Carrier

No attempt was made to determine the kinetics of inhibition (competitive or non-competitive) of cholesterol absorption by beta sitostanol. Such characterization will have to await future work under more controlled conditions.

In the end, it must be stressed that the cholesterol-carrier hypothesis in not intended to represent the truth necessarily; the carrier hypothesis is consistent with the observations in this study and observations in other studies discussed elsewhere in this thesis. Clearly the processes involved in the absorption of cholesterol are complex and more work at a more fundamental level is necessary to achieve better understanding of the process. It is sincerely hoped that this study has provided some insights into the investigation of a complex question which probably originated from Schoenheimer in 1931 when he stated that plant sterols were unabsorbable (20).

SUMMARY AND CONCLUSIONS

Studies on the intestinal absorption of sterols in the rat were carried out in two sections. In the first section, the relative absorbabilities of cholesterol, and beta sitostanol, the reduced derivative of beta sitosterol, were examined *in vivo*. It was found that:

1. Beta sitostanol was relatively non-absorbable compared to cholesterol. In fact evidence was presented to show that beta sitostanol was absorbed to the extent of only 2% compared to 36% for cholesterol under the same conditions.

2. Cholestyramine, a bile salt binding resin, had no effect on the fecal excretion of beta sitostanol while it markedly increased cholesterol excretion.

3. Cholesterol absorption calculated by two separate methods, an isotope ratio method and a total fecal recovery method, were in excellent agreement.

It was concluded that beta sitostanol was a non-absorbable substance and could be used as a valid marker for studying cholesterol absorption in the rat, perhaps the best one yet used.

In the second section, the effect of beta sitostanol on cholesterol absorption was studied in jejunal loops *in situ*. Micellar solutions of varying composition were infused into the loops and the absorption of cholesterol was followed. It was found that:

1. Cholesterol absorption was a function of cholesterol concentration with evidence for saturation at higher concen-

trations.

2. Beta sitostanol given as mixed micelles with cholesterol ('pre-mix') had no effect on cholesterol absorption. On the other hand, cholesterol absorption was markedly reduced when the solution contained beta sitostanol in separate micelles ('solution-mix').

3. Cholesterol did not suppress its own absorption within the concentration range studied.

The evidence supported the notion that beta sitostanol suppressed cholesterol absorption probably by binding to a membrane-bound, cholesterol-specific carrier. The carrier was postulated to have at least two binding sites and the ability to undergo rotational movement after the binding of cholesterol. This would allow for internalization of the bound cholesterol and, at the same time, expose another binding site to bind more cholesterol. Binding of beta sitostanol was postulated to prevent: 1. Cholesterol from binding and, 2. the rotational movement of the carrier, thereby suppressing cholesterol absorption without beta sitostanol itself being absorbed.

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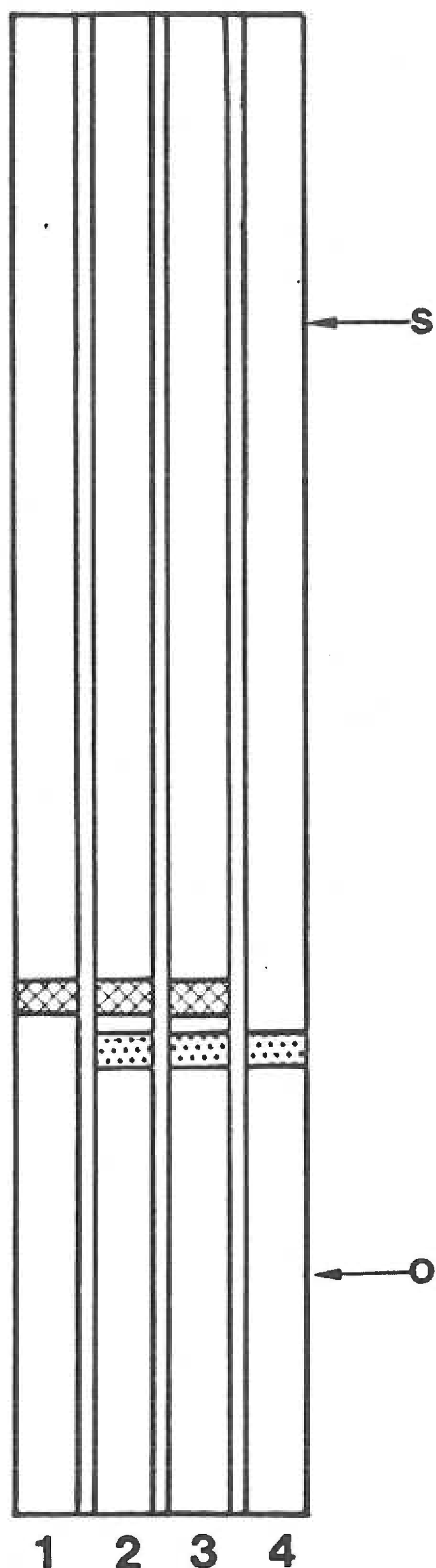
APPENDIX A

Argentation Thin Layer Chromatography of Beta Sitosterol and Beta Sitostanol

Procedure:

1. Plate: Quantagram®, LQDF (Kontes of California); thickness: 250 microns.
2. Solution: 5% silver nitrate in methanol:water, 70:30.
3. Impregnation: The silver nitrate solution was poured into a developing tank and the thin-layer plate to be impregnated was placed upright in the tank. The silver nitrate solution was allowed to ascend on the plate for 4-6 hours, depending on the extent of the plate to be impregnated. The plate was then removed and allowed to dry overnight. The plate was activated in an oven for an hour at 110 °C before use.
4. Sample separation: 20 μg of the sample was spotted on each lane and the plate was developed in chloroform. The bands were visualized under U.V. light after spraying the plate with a saturated solution of Rhodamine 6G in methanol.
5. Even though the two compounds, beta sitosterol and beta sitostanol, were clearly separated after the plate had been developed only once, the plate was developed a second time to assure complete separation. The figure on the opposite page is a sketch of an actual run; the plate was only developed once in this case.



O ---Origin

S ---Solvent front

Lane #	Material Plated
1	Beta sitostanol
2	Beta sitosterol and beta sitostanol
3	Beta sitosterol and beta sitostanol
4	Beta sitosterol

Compound	R_f (Mean $\pm$ S.D.; n=3)
Beta sitosterol	0.2533 $\pm$ 0.009
Beta sitostanol	0.3120 $\pm$ 0.008

APPENDIX B

A. Gas chromatographic analysis of beta sitosterol purchased from Nutritional Biochemical Company (Irving, California) using cholesterol (Peak #1) as reference:

Peak #		
2	Campesterol	7.36%
3	Stigmasterol (+Campestanol?)	0.97%
4	Beta sitosterol	91.67%

B. Gas chromatographic analysis of reduced beta sitosterol purchased from Nutritional Biochemical Company (Irving, California)

Peak #		
1	Campestanol	7.77%
2	Beta sitostanol	92.23%

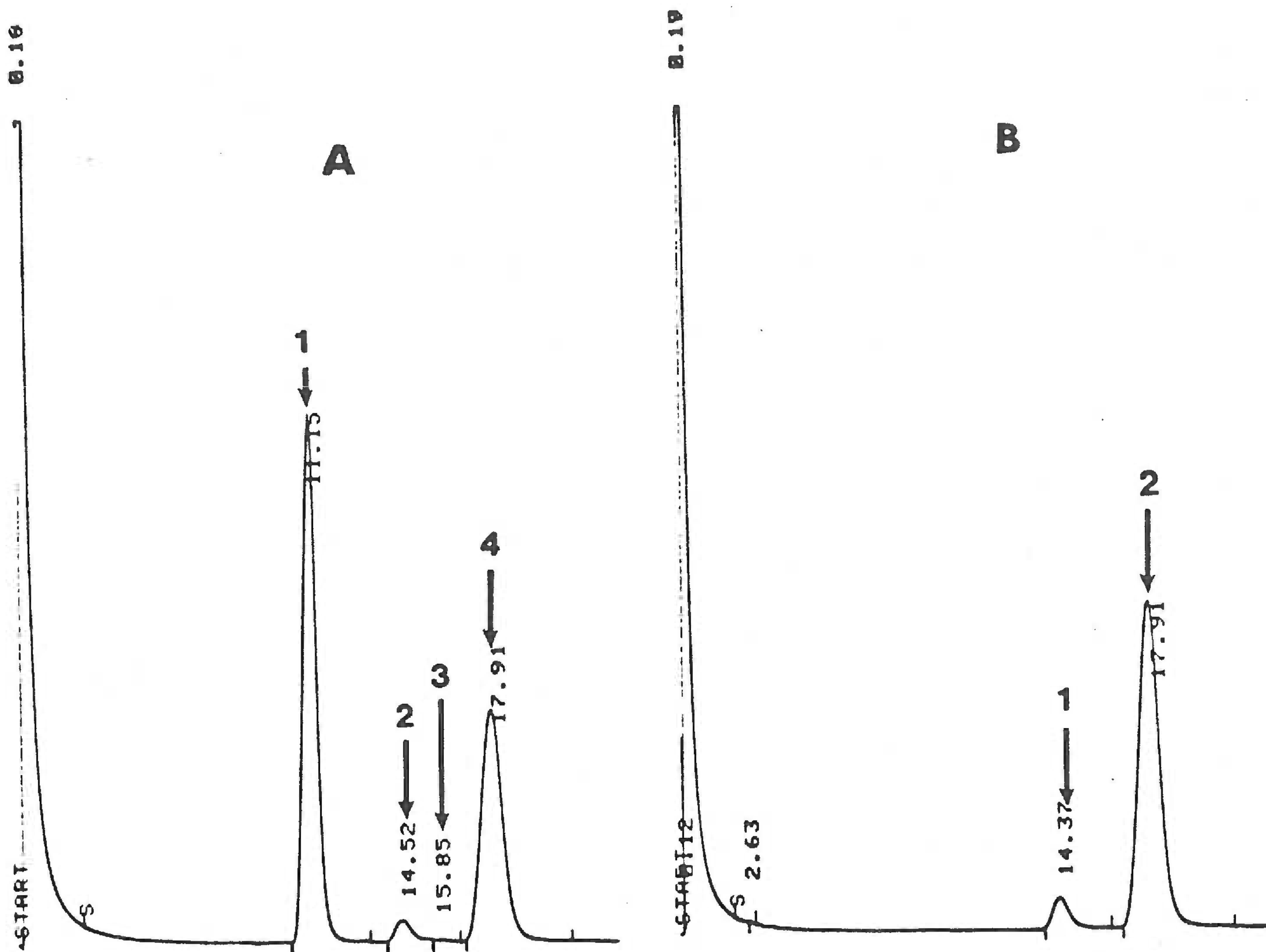
Hewlett-Packard Gas Chromatograph Model #5830A Operating Conditions:

Column: 3% OV-17

Carrier Gas: Nitrogen;31ml/min.

Temperature

1. Column: 260 ^{o}C
2. Injection: 275 ^{o}C
3. Detector: 310 ^{o}C



A

Peak #	Compound	Relative Retention Time* Parodi**	Present Study
1.	Cholesterol	1.00	1.00
2.	Campesterol	1.34	1.30
3.	Stigmasterol	1.47	1.42
4.	Beta sitosterol	1.68	1.61

B

Peak #	Compound	Parodi**	Present Study
1.	Campestanol	1.33	1.29
2.	Beta sitostanol	1.68	1.61

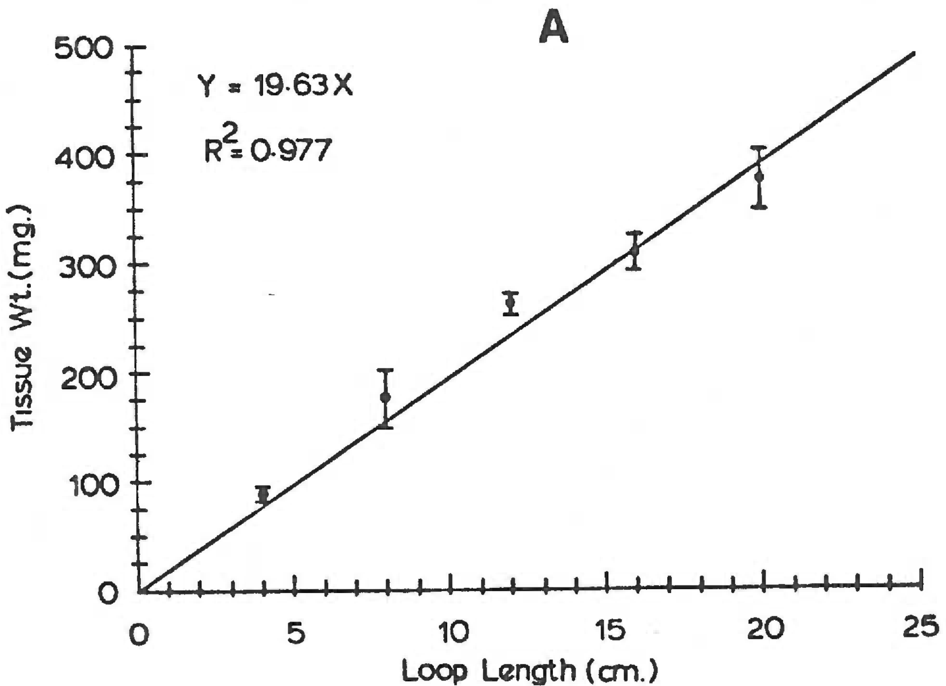
* Retention time relative to cholesterol

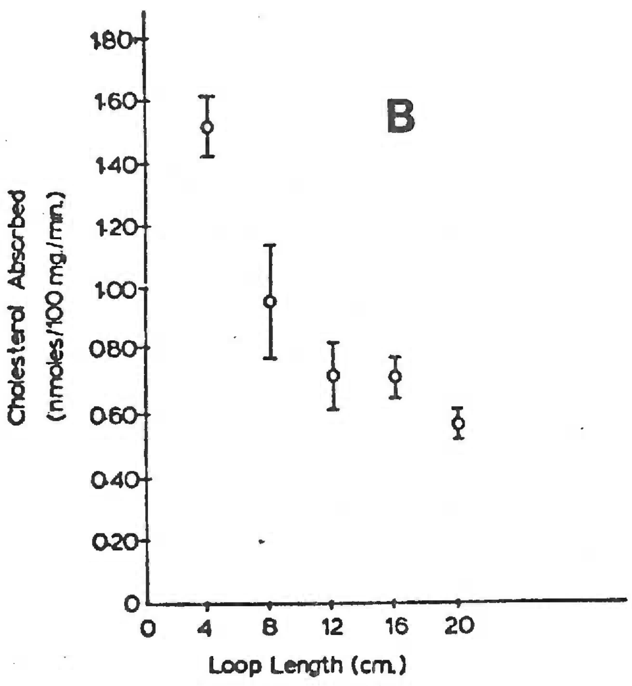
** Parodi, P.W. J.Amer.Oil Chem.Soc. 52:346, 1975.

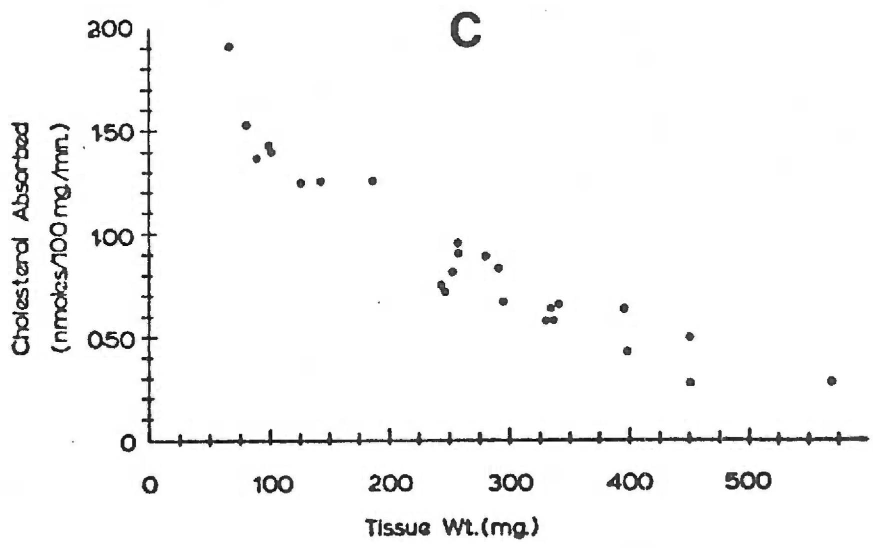
APPENDIX C

A. Tissue dry weight (mg) as a function of loop length (cm). Each point is the mean of five determinations; the vertical bars represent ($\pm$) one standard error of the mean. The line is a best fit straight line of the form, Y = bX, calculated by the method of least squares.

B. Cholesterol absorption (nmoles/100mg/min) as a function of loop length (cm). Each point is the mean of values obtained from five animals. The vertical bars have the same significance as above. In C, the same data is plotted but as a function of the loop dry weight. Note that cholesterol absorption decreases as a function of either loop length or weight. This is to be expected because loop length and loop weight are directly related as shown in A. Cholesterol concentration in the infused solution was 0.20 mM and absorption was studied for 40 miuntes after infusion of the solution.

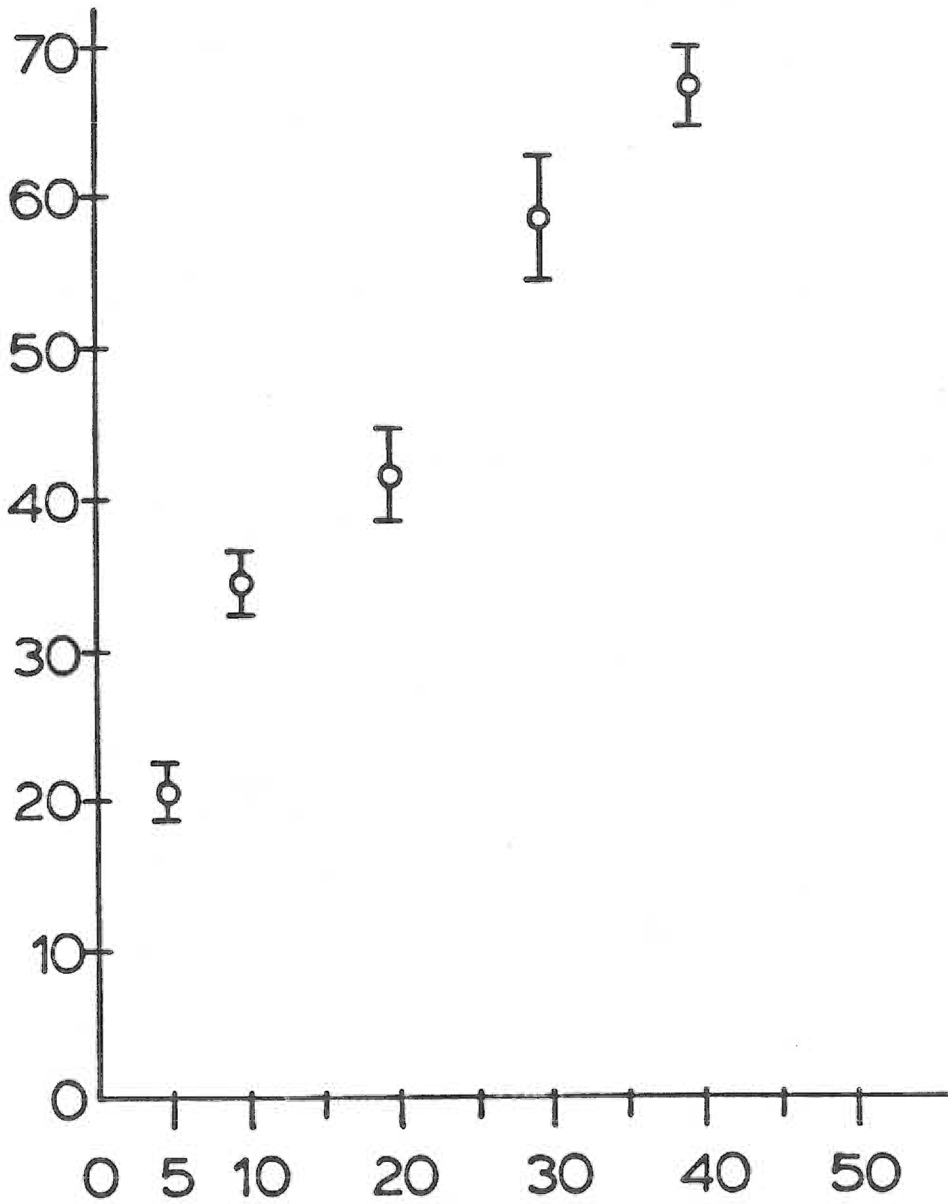

A
Y = 19.63 X
R^2 = 0.977
Tissue Wt. (mg.)
Loop Length (cm.)


B
Cholesterol Absorbed (nmoles/100 mg./min.)
Loop Length (cm.)


C
Cholesterol Absorbed (nmoles/100 mg./min.)
Tissue Wt. (mg.)

APPENDIX D

Cholesterol absorbed by *in situ* jejunal loops as a function of time. Each point is the mean of values obtained from five animals. The vertical bars represent the standard error of the mean. Cholesterol concentration was 0.20 mM and the non-absorbable reference compound was ^{3}H inulin.



Cholesterol Absorbed
(%/100 mg.)
70
60
50
40
30
20
10
0
0 5 10 20 30 40 50
Time (min.)