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Lipid binding from aqueous solution by lipid conjugated hydroxypropyl methylcellulose (HPMC)

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LIPID BINDING FROM AQUEOUS SOLUTION BY LIPID CONJUGATED HYDROXYPROPYL METHYLCELLULOSE (HPMC)

A Novel Food Additive for Reducing Cholesterol and Fat Intestinal Absorption

by

James A.S. Nightingale

A dissertation submitted in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

University of Washington

1988

Approved by: ______________________
(Chairman of Supervisory Committee)

Program Authorized
to Offer Degree: Center for Bioengineering

Date: May 25, 1988

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Abstract

LIPID BINDING FROM AQUEOUS SOLUTION BY LIPID CONJUGATED HYDROXYPROPYL METHYLCELLULOSE (HPMC)

A Novel Food Additive for Reducing Cholesterol and Fat Intestinal Absorption.

by James A.S. Nightingale

Chairman of the Supervisory Committee: Professor Allan S. Hoffman
Center for Bioengineering

This research project focuses on the development and testing of a novel, medically useful lipid or steroid sequesterant that can be administered orally. Specifically, this compound is designed to absorb cholesterol and fats from the bulk phase of the intestinal pool by sequestering these constituents and then pass out of the body with the feces. A summary of food additives as they are used to accomplish the same goal is reviewed as are the interactions of polymers with amphiphilic compounds.

To accomplish the goal we have examined the physico-chemical nature of our hydrophobic affinity polymer which is created by the attachment of lipophilic moieties to a hydrophilic polymeric backbone. These compounds are specifically designed to be non-biodegradable and non-absorbable in the intestinal tract. The effectiveness of different conjugated lipids is evaluated using *in vitro* experiments. The lipids are octadecyl, oleyl and cholesteryl attached through glycidyl ether chemistry to hydroxyls on HPMC using stannic chloride as a catalyst.

It has been found (using equilibrium dialysis experiments) that sodium taurocholate (NaTC) binds HPMC and that binding is enhanced by the attachment of glycidyl ethers to this polymer. A maximum binding of physiologically relevant NaTC concentration is reached when approximately 2-3 wt% oleyl is attached to the polymer. The binding of mixed micelles containing NaTC, sodium

oleate (NaOl), lysolecithin, and cholesterol were also studied at concentrations similar to those found in the human gastrointestinal tract. It was found that all three lipids conjugated to HPMC (octadecyl, oleyl, and cholesteryl) bound NaOl and cholesterol from mixed micelles better then NaTC and this binding was enhanced as more lipid was attached to HPMC. Lipid conjugated HPMC was also shown to have *in vivo* activity in rats. Mechanisms governing these interactions are proposed.

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Glossary

Ag#	aggregation number
CAP	3-carboxyaldehyde pyrene
CGE	cholesteryl glycidyl ether
CMC	critical micelle concentration
CPT	cloud point
DS	degree of substitution
DEAE	diethyl aminoethyl
ECH	epichlorohydrin
EDTA	ethyl diamine
FDA	Food and Drug Administration
FH	familial hypercholesterolemia
G.I.	gastrointestinal
GE	glycidyl ether
GPC	gel permeation chromatography
HDL	high density lipoprotein
HM	hydrophobically modified
HM-HEC	hydrophobically modified hydroxyethyl cellulose
HM-HEC	hydrophobically modified hydroxyethyl cellulose
HMG-CoA	hydroxy methylglutaryl-CoA
HPMC	hydroxypropyl methyl cellulose
IDL	intermediate density lipoprotein
IP	intraperitoneal
IPT	incipient precipitation temperature
LCAT	lecithin: cholesterol acetyltransferase
LCST	lower critical solution temperature
LDL	low density lipoprotein
LP_4GE	lauryl PEG_4 glycidyl ether
MS	molar substitution
MeC	methyl cellulose
N.M.R.	nuclear magnetic resonance
NHLBI	National Heart, Lung, and Blood Institute
NSL	nonsaponifiable lipids
NaDC	sodium deoxycholate
NaTC	sodium taurocholate
ODGE	octadecyl glycidyl ether
OGE	oleyl glycidyl ether
PEG	polyethylene glycol
PEO	polyethylene oxide
PGE	phenoxyl glycidyl ether
PPO	polypropylene oxide
PVA	polyvinyl alcohol
PVAc	polyvinyl acetate

SAD	special atherogenic diet
SANS	small angle neutron scattering
SDS	sodium dodecyl sulfate
SPE	sucrose polyester
TGE	Triton-X glycidyl ether
TLC	thin layer chromatography
UPI	United Press International
UWL	unstirred water layer
VLDL	very low density lipoprotein
XX%OGE/HPMC	OGE attached to HPMC at XX wt%
XX%ODGE/HPMC	ODGE attached to HPMC at XX wt%
XX%CGE/HPMC	CGE attached to HPMC at XX wt%
XX%LP_4GE/HPMC	LP_4GE attached to HPMC at XX wt%
η_r	relative viscosity
η_{sp}	specific viscosity (η/η_o - 1)
$\eta_{sp/C}$	reduced viscosity
$[\eta]$	intrinsic viscosity $\lim_{C \to 0} \frac{\eta_{sp}}{C}$

Acknowledgements

An engineering Dissertation can be construed as a complete story that recounts the trials and triumphs of understanding a complex problem. As with any tale there are many people involved. The author brings to bear the sum of his experience in creating and presenting it. This is certainly the case with this work. It would be difficult to name all of the people that participated to one degree or another in this process however, a few deserve special note.

The author wishes to thank his mentor and good friend Professor Allan S. Hoffman for his patience, insight and support while I attempted to reconcile an applied science in the framework of a doctoral degree. His teachings on education and research will be treasured and shared throughout my career. I would also like to thank my supervisory committee including: Professors Niels Andersen, John Berg, John Glomset, David Saunders and especially the members of the reading committee, who waded through this document, Pedro Verdugo, and Paul Yager. They have all contributed to my education.

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Finally, to the people that made all this possible and without whose support I wouldn't have come this far; I thank my parents - Harvey and Helen, wife - Jennifer and family who have endured this educational experience.

Dedication

This Dissertation is dedicated to Jennifer, who had to do without her husband, and Alexandra, and Mackenzie who haven't seen much of their father for the past few years.

CHAPTER 1

Introduction and Significance

The founding goal of this research was to design and reduce to practice a non-absorbable/non-biodegradable polymeric material that could be ingested and then adsorb cholesterol and/or fats in the intestinal tract of humans using hydrophobic interactions. During the course of this study we developed a material which showed *in vivo* activity in rats. To study the physical chemical nature of the polymer activity of operation we have utilized *in vitro* methods to examine the interaction of this material with amphiphilic compounds coincident with the goal of binding fats in the gastrointestinal tract. To appreciate the products potential we need to examine the problem of excessive cholesterol in the body.

It is generally accepted that dietary avoidance of cholesterol can reduce the risk of death from the number one killer: coronary heart disease [1]. Results from a major NHLBI study, examining the effect of reduced intestinal absorption of bile salts and cholesterol, were reported in 1984 [2-5]. The study involved 3,806 men with high plasma cholesterol levels (>265 mg/dl), and examined the effect of reduced dietary cholesterol and a non-absorbable food additive - a synthetic anionic exchange resin (cholestyramine) - over a 7 to 10 year period. It was determined that a diet alone reduced blood plasma cholesterol by 3.5% while, men who consumed 83 to 100% of the prescribed food additive showed 19% reductions in plasma cholesterol. The significance of these reductions is realized when the incidence of heart attack, bypass surgery and symptomatic heart disease are considered.

It was reported that a 1% reductions in plasma cholesterol resulted in about a 2% reduction in heart disease risk. Unfortunately, the medication is not without side effects including: constipation, nausea and high cost. In addition, this material is unpalatable and long-term compliance can be a

significant problem particularly when treating children [6]. It should be noted that 27% of the men in the NHLBI study were not consuming any of the prescribed medication (placebo or cholestyramine). This may be due to the physical nature of the placebo (a silica mixture [7]) and cholestyramine both of which have a gritty texture.

Heterozygous familial hypercholesterolemia (FH) is a primary genetic cause of high plasma cholesterol, occurring in about one of five hundred births. This disease is the result of a mutant allele at the LDL (low density lipoprotein) receptor locus that results in production of only about one-half the normal number of LDL receptors per cell. This results in poor uptake of blood plasma LDL's which are related to the formation of arteriosclerosis [8]. Polygenic hypercholesterolemia affects many more people than the monogenic cause. Durrington and Miller suggest that these people have "inherited a kindred of genes that interact with diet or some other aspect of life in industrialized communities to produce hypercholesterolaemia" [9]. Homozygous FH is rare (one in a million) and few effective therapies exist.

The number of Americans' suffering from high serum cholesterol represents a large market for a potential dietary additive that would either sequester cholesterol and/or bile acids and/or fats in the intestinal tract. Furthermore, Americans' preference for diets high in cholesterol and fats suggests that a market for a food additive may exist. A food additive that would sequester lipids or bile acids in the intestine - preventing their absorption - might be welcomed by the medical community and perhaps the general public. It seems to be the general consensus of experts in this field that Americans' need to adopt diets lower in fats and cholesterol [10]. In addition, the need for "more effective, better tolerated, safer, and more economical drugs for lowering blood cholesterol levels" is recognized [11].

The interactions of polymers with amphiphilic compounds is currently a highly published research area. The primary reason for this has to do with tertiary oil recovery. In one scheme, a detergent is pumped into a well to "wet" the rock which has small fissures containing previously

non-retrievable oil. A viscous polymeric solution is then pumped into the well which hydrodynamically drives the oil/surfactant complex to recovery wells. The goals of this research do not include this application, however knowledge gleaned from our work may be applicable since some of our products are both surface active and form viscous aqueous solutions.

Since this project involves the application of technology to a specific problem, the dissertation will begin with a description of cholesterol absorption which we have considered a method for attacking high blood plasma cholesterol levels. The same chapter contains a review of methodologies and theories on food additives which reduce cholesterol absorption. The aims of the project are presented in Chapter 3 with the rationale for the design of our food additive. The food additive is synthesized by conjugating lipids to hydroxypropyl methylcellulose (HPMC). Other researchers have referred to similar materials as hydrophobically modified (HM), we will use the same convention (HM-HPMC).

Chapter 4 details the methods and results for synthesis, and characterization of HM-HPMC. Chapter 5 presents the methods and results for the interaction of HPMC and HM-HPMC with amphiphiles including both *in vitro* and *in vivo* experiments. Finally, conclusions and recommendations can be found in Chapter 6.

CHAPTER 2

Review of Cholesterol Absorption in the Body

Circulation of cholesterol in the body is a complicated process involving many compartments and pathways. In addition to dietary sources it is synthesized in the body and used as a building block for many other compounds which are crucial for growth and survival. One class of these compounds are the bile salts which actively participate in the intestinal absorption of lipophilic dietary constituents. The role of cholesterol in the development of atherosclerosis is not completely understood, however a recent study indicates that even mildly elevated levels of cholesterol in the plasma will increase the risk of heart attack. In 1985, Stamler stated; "The claim is incorrect that excess risk does not start until cholesterol reaches a markedly elevated level" (as reported by UPI [12]). Critical review of the cholesterol absorption process and its circulation will not be reported here. Subjects that are important to understanding the research goals of this project will however, be briefly reviewed.

2.1. Cholesterol Circulation in the Body

Fig. 1 presents a simplified view of the absorption process for cholesterol and its derivatives in the intestinal tract. This figure is simplistic and does not illustrate many of the complex theories that have been published. In a review on cholesterol absorption McIntyre presented many conflicting descriptions of the processes involved [13]. My aim is to use this figure as a guide to possible methods for attacking the problem and as a rationale for our design of a synthetic fat adsorbing food additive.

It is possible to postulate several sites for disrupting the normal uptake of cholesterol prior to absorption into the intestinal mucosal cells. I have numbered many of these sites in Fig. 1 which is

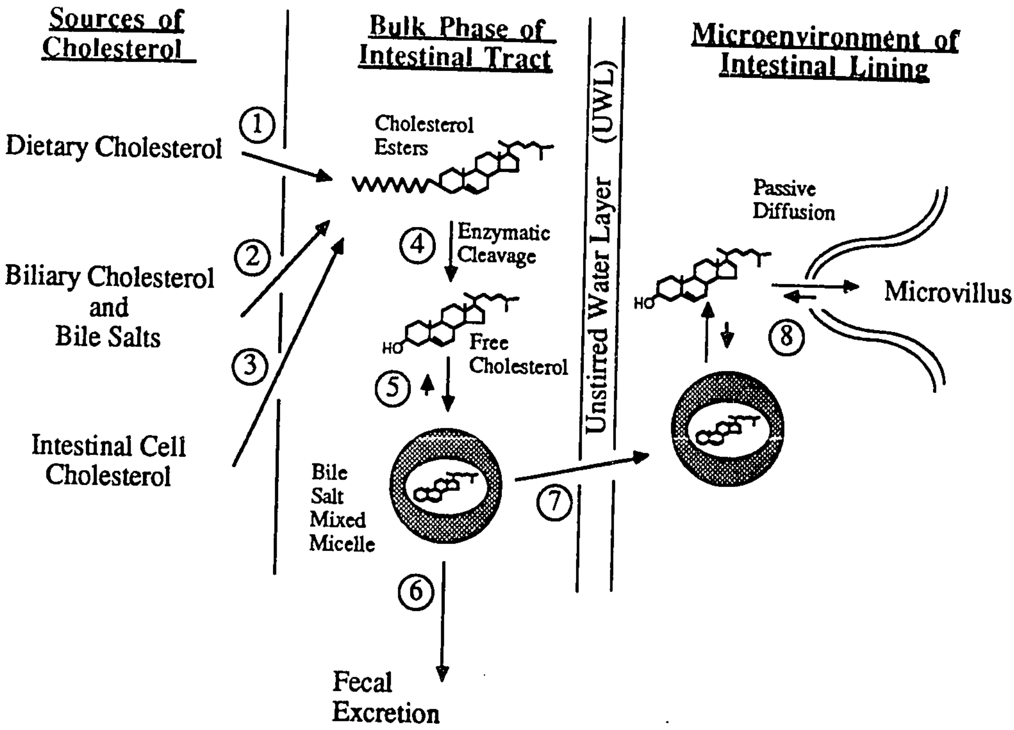


Fig. 1 - Simplified View of Cholesterol Absorption.
Including: a) primary sources of cholesterol,
b) the bulk phase of the gastrointestinal tract, where cholesterol partitions into mixed bile salt micelles,
c) the diffusion of these micelles across the unstirred water layer (UWL) which is a primary barrier to lipid absorption and
d) the microvillus where cholesterol is absorbed into the body.
Numbers indicate sites where cholesterol absorption can be altered.
These are reviewed in Sections 2.3.1-2.3.8.

based on Westergaard and Dietschys description of cholesterol uptake [14]. Section 2.3 will discuss each of these sites with reference to previous or possible methods for treating hypercholesterolemia. Much of this section was taken from general texts on cholesterol and a few articles dealing with LDL formation [8,15-17].

On the left side of the figure we see primary sources of cholesterol including: dietary (0.5-0.75 gm/day), biliary (1-4 gms/day), and intestinal cell (mostly the result of sloughing off of dead cells - which contain membrane cholesterol - but also includes cholesterol synthesized by the intestinal

cells). In the center panel the bulk phase of the intestines is depicted, it is in this phase that cholesterol esters are enzymatically cleaved to the free sterol and fatty acids, and where bulk phase incorporation of cholesterol into the bile salt mixed micelle occurs - the "average" composition of which can be found in Table 1 [18]. Note that for simplicity, I have shown all sources as esterified cholesterol. Actually, only some of the cholesterol is in this form, some of it is the alcohol.

The bile salt micelle (containing cholesterol) then diffuses through the unstirred water layer (UWL - shown separating the bulk from the microenvironment phases) into close proximity of the intestinal microvilli (as seen in Fig. 1 on the right) where cholesterol partitions out of the micelle (down a concentration gradient) and passively diffuses across the microvillus membrane from a "monomer phase" (non-micellar) [14]. 1-2 grams a day of cholesterol is eliminated from the body in the feces. Some of this excreted cholesterol is in the form of bile salts, which represents a pathway for removal of cholesterol from the body.

Following absorption of cholesterol by the cells lining the intestinal tract some of the cholesterol is made into esters and packaged into chylomicrons which are transported away by the lymph. It has been speculated that the assembly and exocytosis of chylomicrons represents the rate-limiting steps for fat absorption [19]. Removal of fats and cholesterol from the intestinal

Table 1 - Properties and Composition of Human Intestinal Lipid Micelles. (Mansbach, *et al.* [18] as summarized by Sabine [15])

Properties	
Average Density	1.25
Stokes Radius	2.3-3.5 nm
Estimated Molecular Weight	27,000-39,000
Composition	
(molar ratio relative to bile acid)	
Bile acid	1.00
Fatty Acid	1.40
Lysolecithin	0.15
Cholesterol	0.06

absorptive cells provides the driving force for diffusion of intestinal cholesterol into these cells. After entering the bloodstream, the chylomicrons deliver triglycerides to adipose and muscle tissue. Their remnants are then removed from circulation by specific receptors found on liver cells. The liver then secretes very low-density lipoproteins (VLDL). Through a process in which materials are taken out of these particles (going through an intermediate-density lipoprotein - IDL) some of these are converted into LDLs. The LDL serves as a circulating storehouse of cholesterol in the blood.

All cells in the body require cholesterol for incorporation into newly synthesized surface membranes. Specialized cells also use cholesterol as a precursor for steroids and in the liver - synthesis of bile acids. To acquire cholesterol from the bloodstream the cell uses a process called receptor mediated endocytosis. LDL receptor sites exist which bind a specific protein (apoprotein B-100) which is found on LDLs. As noted previously, the primary genetic origin of familial hypercholesterolemia result in the production of a lower number of these sites. LDLs are made up primarily of cholesterol esters (in the core), phospholipids, and free cholesterol (on the surface with the apo B). After endocytosis, the receptor bound LDL is delivered to lysosomes which break down the cholesterol esters to free cholesterol. The effect of the most popular treatment (for hypercholesterolemia - cholestyramine) on the number of LDL receptor sites will be discussed in the next section.

Each cell can then use the cholesterol for making new membranes or steroids. If the cell accumulates too much, a feedback mechanism results in the production of fewer LDL receptors. The majority of cells can also synthesize their own cholesterol using a pathway in which the enzyme - HMG-CoA reductase - catalyzes the formation of mevalonate from β-hydroxy-β-methylglutaryl-CoA. Cholesterol concentration determines the activity of this enzyme. This mediation is not completely understood, however many patented treatments rely on either natural or synthetic compounds that will inhibit this enzyme.

LDLs can also be cleared from the blood by "scavenger cells" (primarily macrophages). Recently, a specific sorbent for apo B has been developed and successfully tested using rabbits for plasmapheresis applications [20]. Cholesterol released from cells into the plasma are associated with HDL and ultimately return to the LDL form (in a circulating pathway) by the action of lecithin:cholesterol acetyltransferase (LCAT) in the plasma. Glomset has been active in discerning these pathways. Although there is still some argument concerning the process by which HDL influences cellular and serum cholesterol, most authorities seem to agree that HDL is responsible for picking up extra cholesterol from the tissues and transporting it back to the liver. High HDL levels are associated with decreased atherosclerotic problems. In a crude sense HDL formation is good since it picks up peripheral cholesterol, while high LDL levels are bad since they are associated with the deposition of atherosclerotic plaque.

2.2. Bile Salt Circulation in the Body

Since bile salts are metabolic products of cholesterol and are involved in the intestinal absorption of cholesterol a brief description of their circulation will be presented. Hofmann has documented much of the work reported in this section [21]. Bile salts for the most part are not seen in the peripheral blood. Most will be found in the gallbladder or the intestines. After eating a meal, the gallbladder (storage device for bile) excretes bile which enters the intestines. Here the bile salts are active in the transport of cholesterol and fats across the UWL. After transporting cholesterol across this layer they can recirculate back into the bulk phase of the intestines and pick up more cholesterol. Most of the bile salts (95%) are absorbed in the terminal ileum and enter the portal blood where they are picked up by the liver (a little spilling over into peripheral blood) and rapidly excreted back into the bile.

The liver takes unconjugated bile salts (either recently synthesized from cholesterol or picked up from portal blood) and conjugates them with taurine or glycine. The conjugated form is important since they resist precipitation by acids or calcium ions and are less slowly absorbed in the

proximal region of the small intestines. This increases their efficacy in lipid and cholesterol transport since they will be present (not absorbed) to make micelles later in the intestinal tract. Some of the conjugated bile acids in the intestines are de-conjugated by bacteria in the ileum before being absorbed.

Cholestyramine resin binds bile salts in the intestines and reduces the formation of mixed micelles (decreasing cholesterol absorption) and indirectly the number of LDL receptors. Fig. 2 shows the reasons for the latter and the effect of combined cholestyramine and HMG-CoA reductase inhibitor therapies [8].

On the left we see the "normal" synthesis from cholesterol and recycling of bile acids in a liver cell. In the middle panel, blockage of the recycling of bile acids (using a bile acid sequestrant) results in the increased catabolism of cholesterol to form more bile acids resulting in increased formation of LDL receptors and *de novo* synthesis of cholesterol. Finally in the right panel the synthesis of cholesterol can be blocked with other drugs which results in the formation of more LDL receptors thus clearing LDL from the plasma. A variety of bile salts exist (see a few in Fig. 3). They form small, highly charged micelles (this is in contrast to "normal" detergent micelles). In simple - single component solutions they can either form primary or secondary structures, depending on the bile salt concentration. Proposed structures can found in Fig. 4. By themselves they are unable to dissolve cholesterol, however in the presence of other detergents (such as sodium oleate or lecithin) they form mixed micelles which can absorb fat soluble vitamins and cholesterol [22]. The characteristics of a couple of these can be found in Table 2. We note that mixed micelles increase in size (over that of the single component bile salt micelles) with the bile salt occupying sites at the interface and the lecithin forming an oily core.

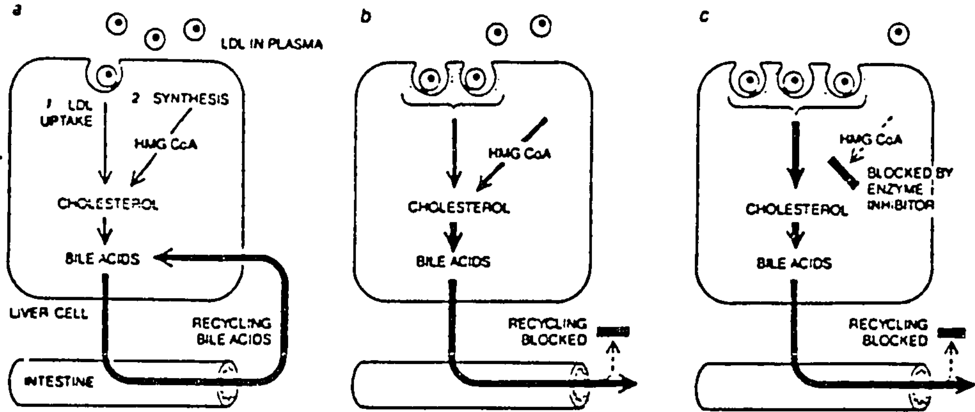


Fig. 2 - Rationale for the Use of Combined Therapies in Treating FH [8].

a) Liver gets cholesterol for conversion into bile acids from IDL and LDL taken up from the circulation (1) or by synthesizing it *de novo* (2). A key step in the long synthetic pathway is reduction of HMG-CoA to mevalonic acid, a reaction catalyzed by the enzyme HMG CoA reductase.

b) Intestinal bile acid absorption can be blocked by administering cholestyramine resin with the diet. This increases the demand for cholesterol which is necessary to produce bile acids. This results in the formation of more LDL receptor sites.

c) The enzyme can be inhibited by the drugs compactin or mevinolin, whose side chain is so similar to that of HMG CoA that it blocks the enzymes active site. This results in the formation of more LDL receptor sites which clears LDL from the plasma.

Sodium Cholate

Sodium Deoxycholate

Sodium Chenodeoxycholate

Sodium Glycocholate

Sodium Taurocholate (NaTC)

Fig. 3 - Chemical Structure of Bile Acids.
Sodium taurocholate has a sulfonate residue which resists precipitation at acidic pH.

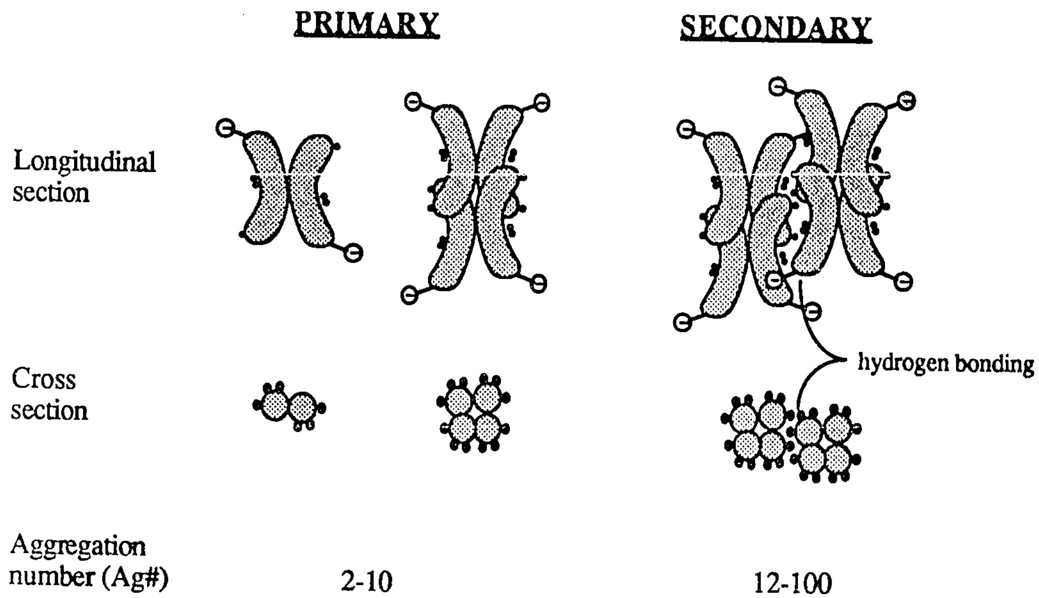


Fig. 4 - Proposed Structures for Bile Salts in Aqueous Solution [22].

Table 2 - Characteristics of Mixed Bile Salt Micelles [22].

Composition	Bile Salt (NaTC) & Cholesterol	Bile Salt (NaTC) & Lecithin	Bile Salt (NaDC) & Na Oleate
Molar saturation ratio: solubilizate per solubilizer	0.008	2.0	Miscible in all proportions
Micellar characteristics	"Saturated" with cholesterol	Saturated with lecithin	Data on 50/50 (w/w) mixture
CMC	3.2mM	0.2mM	Not known
Shape	Spherical	Almost spherical	spherical
Size-micellar-weight	2,450	125,000	11,200 (interpolated)
Ag# of bile salt molecules per micelle	5	62	10
No. of solubilizate molecules per micelle	none	125	17
Other	No changes in micellar characteristics from pure NaTC. Probably not true micellar solubilization.	True micellar solubilization. Such micelles can easily solubilize cholesterol and long chain fatty acids.	Mixed micellar aggregates form in all proportions.
Proposed Structure of Micelles			
Longitudinal Section	unknown		
Cross Section	unknown		

2.3. Methods for Altering Normal Cholesterol Absorption

The hypocholesterolemic effects of many food additives have been patented. These include; natural products, [23-32]; chemically modified natural products, [33-35]; and synthetic compounds [36-42]. Table 3 lists these materials, the year patented and the appropriate reference. The reasons for physiologic activity of many of these materials is not presented in the patent literature. Frequently, the materials were examined using only simple *in vivo* tests that, showed reduced plasma cholesterol but, reveal little concerning the mechanism responsible for activity. From the design standpoint, this hampers the search for better hypocholesterolemic agents.

Table 3 - Food Additive Hypocholesterolemic Agents Patented in the U.S.

Substance	Assignee	Date	Reference
Natural Products			
mucilaginous substances	American Cyanamid	1964	[23]
guar gums	American Cyanamid	1968	[24]
polysaccharides of D-glucopyranose	self	1970	[25]
sugars	USA-NASA	1974	[26]
konjac mannon	Kabushiki-Kaisha	1974	[27]
konjac mannon	Kabushiki-Kaisha	1975	[43]
maltitol and lacitol sugars	Kabushiki-Kaisha	1976	[28]
antibiotics (Levorin)	Schmid	1977	[29]
oat or barley gums	Quaker Oats	1979	[30]
psuedomonas polysaccharogens M-30	Mitsubishi Petrochemical	1980	[44]
extracts of yucca plants	Yushiro Chemical	1984	[45]
extracts of yucca plants	Yushiro Chemical	1984	[31]
antibiotics (endomycin)	Schmid Labs	1984	[32]
Modified Natural Products			
polyglycerol esters of fatty acids	Unimed, Inc.	1970	[46]
anion exchangers based on polysaccharides	Upjohn Co.	1971	[33]
diethylaminoethyl (DEAE) dextran	Kabushiki-Kaisha	1974	[34]
n-acetyl candicidin	Schmid Labs	1976	[47]
sucrose polyester (SPE)	Proctor & Gamble	1976	[35]
chitosan/fatty acid complexes	self	1980	[48]
hydrolyzed saponins	USA	1980	[49]
Synthetic Compounds			
polymeric amines	Merck & Co.	1967	[36]
polymeric amines	Merck & Co.	1968	[50]
alkylidenedithiobisphenols	Consolidation Coal Co.	1971	[51]
thienyl aliphatic hydrocarbon amides	Sumitono Chem. Co.	1972	[52]
copolymer of ethylenepolyamine and epichlorohydrin	self	1972	[53]
heterocyclic amides	Sumitomo Chem. Co.	1974	[54]
quaternary poly[(alkylimino)alkylene] polymers	Merck & Co.	1977	[55]
copolymers of carboxylic acids and unsaturated olefins	Monsanto Co.	1978	[37]
dialkylaminoalkyl imides of alkene/maleic anhydride copolymers	Monsanto Co.	1980	[38]
porous membranes containing a bile acid sequestrant	Evreka, Inc.	1982	[39]
anionic ion exchange resins	Etablissement Texcontor	1983	[40]
polymeric agent having vinylimidazole pendant groups	Mitsubishi Petrochemical	1983	[41]
benzodiazerpine derivatives	Hoffman-La Roche	1984	[42]

2.3.1. Removal of Cholesterol from the Diet (1)

An attractive method for reducing the available cholesterol would be eliminating the dietary source. This is represented by number 1 in Fig. 1. The American Heart Association recently recommended that all Americans adopt a diet that will maintain plasma cholesterol near a desirable range [56]. Furthermore, Nye and many others have recommended that drug therapies be used only after it has been proven that dietary methods do not work [10,11,57]. Unfortunately, it has been found that many peoples' plasma cholesterol does not respond favorably to reduced dietary intake of cholesterol and fats alone indicating that more aggresive therapy is needed.

2.3.2. Reducing the Absorption of Biliary Cholesterol (2)

Another method for the elimination of a source of cholesterol would be to reduce levels of cholesterol and/or bile acids in the bile or eliminate bile altogether. Surgical intervention in the form of an ileal exclusion operation has been proposed in the most extreme cases (such as homozygous FH).

2.3.3. Altering Cholesterol Release from Mucosal Cells (3)

As noted earlier, much of the cholesterol released from mucosal cells is due to the high turnover of cells in the intestines. Synthesis of cholesterol by these cells contributes the rest of this source. Depending on the diet, it should be possible to control the rate of cell turnover. It has been theorized that high fiber diets increase this source of cholesterol. Of course it depends on where the bulk of the cells are sloughed off. Cellular debris (including cholesterol) in the colon will not be effectively absorbed thereby increasing the quantity of cholesterol excreted in the feces [15]. The opposite holds for cells lost in the upper gastrointestinal tract. Cellular cholesterol joining the bulk phase here should be available for absorption. Overall, high fiber diets appear to cause a net loss of cholesterol from the body.

While it is generally accepted that the administration of cholestyramine to the diet stimulates cholesterolgenesis in the intestines, there is some controversy as to the effects of this treatment, dietary influence and species differentiation. It has been shown (for the rat) that addition of safflower oil to a fat free diet will increase the incorporation of ^{14}C-acetate into nonsaponifiable lipids (NSL, includes squalene and sterols) by the intestinal cells [58]. It is concluded that diet, serum cholesterol, food intake, and fat content of the diet all appear to play roles in the regulation of intestinal synthesis of cholesterol. The same authors suggest that increased intestinal synthesis of cholesterol may play a role in hypercholesterolemia. Even though no treatment explicitly attempts to exploit this method (for reducing cholesterol) it might play some role in current therapies.

2.3.4. Inhibiting the Enzymatic Formation of Free Cholesterol (4)

Only a portion of the dietary cholesterol is in the esterified form. A therapy relying solely on this method would probably not result in significant changes in plasma cholesterol. A description does serve to illustrate the effects of some polymers on enzymatic activity, however.

Several polymeric materials have been shown to decrease the activity of pancreatic lipase. These include: Triton WR-1339 [59], Tweens [60], and Pluronic L-101 [61]. Triton and Pluronics affect activity thru "hydrophobic" interactions with the enzyme, while Tween is believed to act as a less reactive substrate.

2.3.5. Reducing the Formation of Bile Salt Micelles (5)

The most popular treatment for patients afflicted with type II hypercholesterolemia works by disrupting micellization of cholesterol. Almost any drug that operates by reducing the formation of bile salt micelles will also increase elimination of cholesterol with the feces. For the most part this section will deal with sequestration of bile salts. Cholestyramine (a quaternary ammonium styrenevinylbenzene copolymer shown in Fig. 5 is sold as Questran® - Mead Johnson Pharm.) and colestipol (a high molecular weight copolymer of tetraethylenepentamine and epichlorohydrin -

where one fifth of the amines are protonated; brand name - Colestid® - Upjohn Manufacturing Co.) both act by sequestering bile acids [62,63], which are known to be crucial for intestinal absorption of cholesterol [14]. Cholestyramine and colestipol have also been used in conjunction with other food additives or drugs including; pectin [64], neomycin [65], nicotinic acid, probucol (Lorelco® - Merel Dow) [66], and mevolin (Merck Sharp & Dohme - compactin is the Japanese version of this drug [67,68] the later two are competitive inhibitors of HMG-CoA reductase) [66]. Though these last two drugs do not directly relate to reducing cholesterol absorption, they do provide interesting additions to potential therapies such as the food additive described here.

Other charged exchangers that have shown similar activity to bile acid sequestrants include: diethylaminoethyl (DEAE) Sephadex [69], and chitosan [70]. Neomycin and kanamycin are known to precipitate the bile salt sodium glycocholate and bilirubin [71,72]. Specific interactions are apparently responsible since only neomycin is able to precipitate sodium taurocholate [71]. Combined use of neomycin and niacin results in normalization of plasma lipoprotein concentrations for type II hyperlipoproteinemia [73,74]. The combined use of cholestyramine and neomycin resulted in small decreases of total plasma cholesterol [65]. Unfortunately, this reduction was due to a lower HDL cholesterol. The combined therapy did however reduce the constipation frequently

Fig. 5 - Chemical Structure of Cholestyramine
Dowex® 1-X2-Cl - Dow Chemical Company
Questran® - Mead Johnson Pharmaceutical

observed with cholestyramine administration. Because of the drop in HDL cholesterol the authors recommend against the administration of this combination [65].

Tetronic 701 (formed by the block copolymerization of ethylene oxide and propylene oxide onto ethylenediamine) has been shown to be effective at precipitating cholesterol from mixed micelles *in vitro* [75]. The tetrabenzoate ester of Tetronic 701 showed enhanced hypocholesterolemic effects (over the parent compound) and also showed less toxicity (as indicated by its effect on animal weight gain).

Using *in vitro* techniques Eastwood (reported in 1968) found that bile salts and acids are strongly absorbed by the lignin content of vegetable fibrous tissue [76]. He suspected that this bonding was of a "hydrophobic" nature. In addition, the hydrophilic colloid derived from blond psyllium seed (Metamucil® - G.D. Searle) can moderately reduce plasma cholesterol for normal males [77]. At first glance this would seem to indicate the use of cellulosic products as bile acid sequestrants.

In 1983 and 1984 several articles and a book [78] were published concerning the hypocholesterolemic effects of gums, and fibers. An overview of some of this work follows. The neutral detergent fiber and guar gums from tender cluster bean pods were shown to reduce plasma cholesterol in rats [79]. Daily administration of locust bean gum reduces LDL-cholesterol in FH patients [80]. It has also been shown that the hypocholesterolemic effects of guar gum decreases for long-term administration [81]. The defatted portion of fenugreek seeds ($\cong$ 54% fiber) have been shown to have hypocholesterolemic effects on diabetic and normal dogs [82]. Unfortunately, experimental design did not allow determination of the reasons for activity.

Schweizer and coworkers study on the effects of oral administration of dietary fiber, concluded that, dietary fiber from soybeans did not contribute to the hypocholesterolemic effects of soya [83] however, they witnessed an increase in bile acid excretion for purified soybean fiber. Lindgarde however found that concentrated wheat fiber reduced VLDL and LDL cholesterol while

increasing HDL cholesterol in HC men [84].

The activity of some of these plant fibers might be explained by Cummings 1981 report. He found that certain soluble plant fibers (such as pectin and oat bran) are fermented by colonic bacteria to produce short chain fatty acids such as acetate, proprionate, and butyrate [85]. In light of this, the addition of sodium proprionate to the diet of rats was found to significantly reduce serum and liver cholesterol levels. It is believed that this action is due to altered hepatic cholesterol synthesis [86].

The demonstrated hypocholesterolemic effects of saponins might also explain differences in some studies examining different fiber sources [87]. Depending on the source, saponins have been shown to have different activities. This will be discussed briefly in the next section.

2.3.6. Increasing Fecal Elimination of Cholesterol (6)

A variety of substances increase fecal elimination of cholesterol. Many of these are noted in the section above. A few others will be presented here. Their action is to either sequester cholesterol or alter the bile acid micelle.

Natural food products commonly are made up of many substituents. The hypocholesterolemic effects of one of the constituents - dietary oils - may be due to the presence of triterpene alcohols (cycloartenol and 24 methylenecycloartanol) [88]. Enhanced fecal elimination of cholesterol was observed. Mattson *et al.* have noted that β-sitosterol or β-sitosteryloleate increases the cholesterol content of the feces of humans [89]. They theorized that this activity was due to a co-precipitation process. It was noted that these materials did not prevent the elevation of plasma cholesterol which is commonly seen with the ingestion of saturated fatty acids. Recently, the standard clinical use of β-sitosterol (Cytellin®) for treating hypercholesterolemia has been recommended [90]. It is noted that β-sitosterol is poorly absorbed by the intestinal tract.

The administration of chemically modified natural products can also cause a decrease in cholesterol absorbed. Rogers *et al.* have discovered that non-digestible (diether) phosphotidyl choline inhibits the absorption of cholesterol in rats [91]. They postulated that this action may be due to alterations in the physical properties of mixed micelles. Aryl sulfonate esters of long chain fatty alcohols lower the body cholesterol levels of rats by inhibiting absorption from the bulk phase of the gastrointestinal tract [92-94]. Nondigestible synthetic diether phospholipids are also known to inhibit the absorption of cholesterol [91]. The reasons for this activity are however, unknown.

It has been shown that block copolymers of polyoxyethylene and polyoxypropylene decrease lipid absorption [95]. Though their effect on plasma cholesterol is minimal, use as anti-obesity agents have received some attention [61]. The hydrophobic content of the polymer must be high (at least 80%) and decreasing the molecular weight results in a decrease in activity. The hypocholesterolemic effects of guar gum and konjac mannon on chicks did not correlate with molecular weight however (as measured by viscosity) [96].

Only one material, sucrose polyester (SPE), is designed specifically to sequester cholesterol and fats. The approximate structure of this compound can be found in Fig. 6. It is synthesized by the transesterification of hexa-, hepta- and octa- methylesters (total of 12 moles) with sucrose (1 mole) using sodium hydroxide as a catalyst [97]. It was found that when at least five of the hydroxyls are esterified then the material became hydrophobic enough to resist mixing with water and possible enzymatic attack. SPE is an artificial non-absorbable fat, developed by Proctor and Gamble, which forms an oil phase in the gastrointestinal tract. This hydrophobic phase attracts many lipids including cholesterol and fat soluble vitamins. This unspecificity to attracting fatty compounds results in the need to administer excess dietary fat soluble vitamins (including vitamins A, D, E, and K [98]). A recent study indicates that SPE interferes with the intestinal absorption of vitamin A in rats by 8.7% in acute studies [99]. In addition the use of this food additive results in an increase in the frequency of stools [100] which can be treated by administering at least 10%

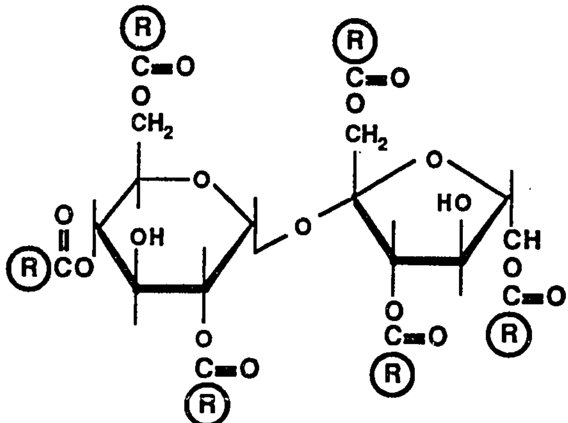


Fig. 6 - Approximate Chemical Structure of Sucrose Polyester (SPE).
R- is either hexyl, heptyl, or octyl

anti-anal leakage agents by weight of SPE [101]. The NIH has advanced the use of SPE in weight reduction therapy [102]. The use of this material by obese HC patients indicates that a reduction of vitamin E, glucose, LDL-cholesterol, and weight occurs [103-105]. Furthermore, reductions in LDL-cholesterol were greater than would be expected due to the weight loss alone. Recently, a study was performed examining the effect of SPE on turnover of plasma cholesterol [106]. Rats were injected with ^{14}C-cholesterol and it was found that SPE administration (8% of the diet) resulted in more rapid removal of cholesterol from the body. SPE did not affect the quantity of bile salts in the feces while it did significantly increase the level of neutral steroids.

As noted above, SPE is made by the formation of ester bonds between fatty acids and sucrose. Since these bonds are hydrolyzable in acidic media, the potential for chemical breakdown and possible absorption of synthetically modified sucrose is real - though their research results have suggested that this doesn't occur. Use of ester linkages in their material should be considered a major drawback.

Nondigestible synthetic diether phospholipids are also known to inhibit the absorption of cholesterol [91]. Again the reason for this action is, unknown. Recently, the ability of saponins to

reduce cholesterol absorption has been noted [107]. Saponins are naturally occurring compounds that contain a steroid or triterpene moiety and a sugar (glucose, galactose, pentose). There are many known (hundreds), and their chemical structure may be important to physiologic activity [87]. Story *et al.* have shown that alfalfa saponins bound significant quantities of cholesterol from *in vitro* micellar solutions of cholesterol, monoolein, oleic acid and taurocholate [107]. They concluded after *in vivo* studies using cholesterol-fed rats that saponin-cholesterol interactions are a "important part of the hypocholesterolemic action of alfalfa but interaction of bile acids with other components of alfalfa may be of equal importance".

2.3.7. Decreasing the Diffusion of Micelles Across the UWL (7)

The principle responsibility of the bile salt micelle is to transport lipids across the UWL. If the permeability of this layer was modified it might be possible to decrease absorption of components from the bulk phase into the intestinal absorptive cells. Perfluorooctyl bromide has been suggested as an anti-obesity agent [108] for this reason. The authors suggested that by coating the intestinal tract with this lipid impermeable material the uptake of fatty acids would be reduced.

2.3.8. Decreasing Permeability of the Microvillus Membrane to Cholesterol (8)

Though no treatment has been shown to function by reducing the permeability of the microvillus membrane to cholesterol - it remains as a possible method. Using *in vitro* techniques it has been shown that acute ethanol exposure results in a selective decline in passive permeability towards acetic, lauric, myristic, and palmitic acids while cholesterol uptake is increased [109].

A number of hypocholesterolemic agents have been studied that act by decreasing *de novo* cholesterolgenesis [110-113]. Though most of our interest lies in reducing the level of cholesterol absorbed across the gastrointestinal tract it should be noted that changes in the level of exogenous cholesterol can also affect the efficiency of endogenous cholesterolgenesis and utilization. Klein and Rudel have found that increasing the exogenous cholesterol can reduce the endogenous cholesterol

absorption [114,115] in nonhuman primates. This may be related to the ability of the intestinal cells to take free cholesterol and "package" it as cholesterol esters. Both cholesterol esters and free cholesterol are transported in the lymph.

2.4. Conclusions to be Drawn from Previous Work

The number of published studies dealing with compounds that reduce plasma cholesterol is immense. Obviously it would be difficult to comment on all of them. More articles come out every month dealing with this subject. We can however draw some conclusions from those studies reviewed here.

First, the "cure all" prophylactic treatment for arteriosclerosis does not exist. While the reduction of LDL cholesterol in the bloodstream is recognized as beneficial, there are no materials available today that will accomplish this for everyone. Cutting down on dietary cholesterol would seem to ward off the disease for many people. Since this will probably not occur soon, the need for a mild treatment seems to be in order. For those people suffering from type II FH, even this treatment will not produce the desired results (though it might decrease the risk).

Second, it becomes clear that very little is known concerning the exact mechanism by which many compounds reduce plasma cholesterol. A primary reason for this void is probably a reflection of how little is known about the complex and numerous processes occurring in the gastrointestinal tract. In many cases it is only possible to speculate on mechanisms for activity. Possible reasons for activity of non-absorbed polymeric materials include: 1) Stabilization of the emulsion phase. 2) Binding of anionic constituents. 3) Binding of neutral lipids. 4) Decreased activity of pancreatic lipase. 5) Increase in the viscosity, effectively increasing the UWL.

Gums and other polymers may act by stablizing the emulsion phase, in a manner analogous to the stabilization of latex by polymers. We might hypothesize that as the oily phase of a meal is broken into small droplets by gastric mechanical action the small lipid droplets become stabilized by surface active polymers. This would probably restrict the access of pancreatic lipase to the bulk

lipid, thereby slowing down the metabolism of fats to free fatty acids. It would also retard the exit of fats, cholesterol and fat soluble vitamins into micelles. We can regard the polymer acting as a diffusional barrier. While this explanation has not been considered by previous workers it seems reasonable when the stabilization of latexes considered. Water soluble polymers would also increase the viscosity of the G.I. tract contents. This would effectively increase the UWL.

We have seen that Cholestyramine and other charged exchangers bind anionic bile salts. SPE acts as a non-absorbable fat in the G.I. tract and binds many lipids. We might expect that any nonabsorbable fat would accomplish the same result. Several polymers have been shown to decrease enzymatic activity as well.

Finally, the lack of conformity in testing materials becomes evident. Each group has their favorite *in vivo* test. It's difficult to compare results between different studies because of this. A simple screening method for compounds seems to be indicated. Understanding the physical chemical interactions of materials may aid the design of future compositions since educated guesses could then be made.

CHAPTER 3

Rationale for Design and Aims of Research

As noted in the previous chapter, many materials (both natural and man-made) have been tested (and frequently patented) as hypocholesterolemic food additives. With few exceptions, materials which interfere with the normal intestinal uptake of fats and cholesterol have been used *off the shelf.* Only a few have been designed specifically for this purpose (e.g. colestipol and SPE). We elected to design a material which would bind lipophilic constituents of the gastrointestinal tract using hydrophobic interactions. Dr. Christian Braud had worked on attaching lipids to cellulose particles for the same application in Dr. Hoffman's laboratory and found that only low quantities of cholic acid could be sequestered from solution. This was believed to be due to the low surface area of the cellulose particles. To overcome this problem, it was decided that the material should be soluble or at least swellable in aqueous solution; individual polymer molecules providing sequestering sites. So we decided to use a water soluble polymer as a base for lipid attachment.

3.1. Rationale for Hydrophobic Affinity Polymer Design

In 1976, Hofmann (University of San Diego) suggested that intestinal cholesterol absorption might be effectively reduced by the synthesis of a "cage-like molecule" that could trap cholesterol in the lumen of the intestine and prevent intestinal absorption [116]. Conceptually, several possible methods exist for designing such a "trap". Specific binding might be achieved by mimicing the active sites present on enzymes which use cholesterol or cholesterol esters as substrates. Recently, it has been hypothesized that a fatty acid binding protein from bovine liver cytosol uses hydrophobic interactions to bind the lipophilic substrate [117]. Perhaps an understanding of the binding site would allow the custom synthesis of a site for cholesterol adsorption.

We have taken a more general approach which also relies on hydrophobic interactions to bind lipids. Fig. 7 shows the cross-section of a hypothesized fat sequestering polymer. The similarity between this hypothesized system and models for micelles should be evident. Lipid groups (solid) are covalently attached to a hydrophilic polymer. Hydrophobic cholesterol or other lipids (cross-hatched) might then be sequestered in the interior of this "micelle" in a manner analogous to the action of detergents. This type of material is frequently referred to as a "poly-soap".

We elected to make this hydrophobically modified polymer by the reaction of the glycidyl ethers of selected alcohols with a polymer which has pendant hydroxyl groups. This reaction forms non-biodegradable ether bonds between a non-ionic hydrophilic polymeric backbone and the alcohol. Fig. 8 shows the chemistry of this reaction. A similar method was used by Hjerten *et al.* for the attachment of pentanol, dodecanol, hexanol, and octanol to Sepharose® [118] to yield a reverse phase chromatography packing. Using the attachment chemistry as a starting point we began

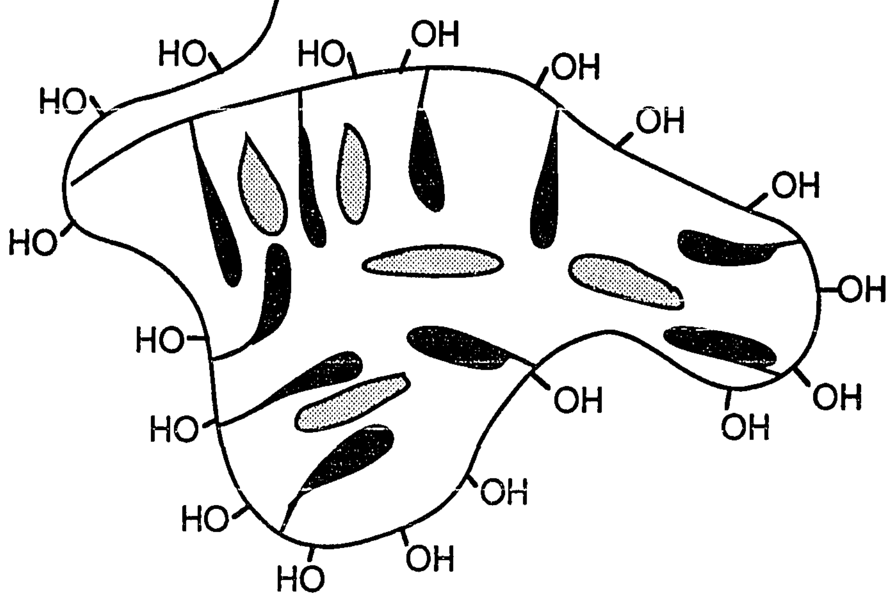


Fig. 7 - Cross Section of a Cholesterol Sequestering Polymer. Showing, neutrophilic backbone, attached lipids (solid), and sequestered cholesterol (cross-hatched).

Alcoholic Glycidyl Ether

$SnCl_4$

Dioxane/CH_2Cl_2 (3:1), room temp. overnight

Hydroxypropyl methylcellulose (HPMC)

HM-HPMC (poly soap)

Fig. 8 - Method of HM-HPMC Synthesis.
Reaction between a glycidyl ether and polymer having hydroxyl groups using a Lewis acid catalyst.

looking for a polymer and lipids which could be used. It is noted that SPE (described in Chapter 2) is formed by the formation of ester bonds between fatty acids and sucrose. While esters are acid labile (an expected condition in the stomach), ether bonds are quite resistant to chemical degradation (except in the presence of active oxygen or strong electrophiles) and thus represent an important advantage of our material over that developed at Proctor and Gamble. By selecting the correct combination of polymeric backbone and lipid we will be creating an "affinity polymer" which may sequester lipids. We expect that by attaching a sufficient number of lipids to a hydrophilic backbone through biologically stable linkages we should be able to accomplish solubilization (or sequestration) of cholesterol and/or bile acids in the digestive tract while reducing possible enzymatic or chemical breakdown of the modified polymer.

"Hydrophobic interactions" (HI) play a major role in precipitation of bile salts and reducing enzymatic activity (as reported in sections 2.3.4 and 2.3.5). A good definition of HI is not universally accepted. I will use one summarized by Ben-Naim [119]. HI is not a force of

interaction between two species such as Van der Waals, covalent, ionic etc., (i.e. a property possessed by the associating particle) rather it is governed by the properties of the solvent (water in biological fluids). We can think of water *pushing* a hydrophobic solute out of solution into close proximity of other hydrophobic solutes. The driving force for the aggregation of hydrophobes is a thermodynamic property of water not an attractive force between the hydrophobes. This definition is important in several aspects of this research, including: precipitation behavior of HM-HPMC and sequesteration of lipids.

Many of the successful bile acid precipitators are cross-linked solids. It is unknown how important the phase behavior is. As we shall see our polymer can be made either soluble or non-soluble in water, depending on the degree of lipid conjugation.

Hypothetical arguments on what form the material should be in the G.I. tract can be presented though this research does not attempt to determine what the optimum phase would be. If the polymer dissolves in aqueous media it would probably increase the viscosity of the bulk phase. This would effectively increase the thickness of the UWL (UWL presented in 2.1) and decrease fat absorption. It is unknown whether this would have any effect on overall fat absorption since our bodies are designed to take full advantage of fats in the meal. Most of the fats are absorbed within the first meter of the intestine. The remaining "reserve" (3 meters) represents a formidable opponent to lipid sequesterants. Balancing the viscosity effect would be a possible transport increase across the UWL by a polysoap. If the polymer dissolves and forms a true intramolecular micelle it may be able to pass through the UWL (like a bile salt mixed micelle) which represents a principle barrier to fat adsorption in the intestinal tract. A solid material could bind the lipid and due to it's size would probably not transport across the UWL. For this reason we have felt that a solid material with hydrophobic affinity sites would be the best phase since it could bind fats and not pass through the UWL. A solid which does not swell (or is porous) though would not be able to absorb much lipid because only the surface area would be effective.

As an aside, the material could also be used to remove lipids or cholephilic materials from the blood in the form of a packed column. The selective removal of bile acids [120,121] lipoproteins [122] and bilirubin [123] have been reported in the literature using packed columns. Realization of this application might require some chemical modification through extensive cross-linking. Another application might be the absorptive immobilization of proteins (similar to methods using palmitoyl-sepharose [124,125]). It may also be possible to use these compounds for drug delivery in a manner analogous to the proposed use of liposomes for insulin administration [126]. These materials may find industrial applications as well. We have found that our polymer undergoes a phase transition at elevated temperatures; its use as a lipid sorber through this route may find such applications.

3.1.1. Selection of Hydrophilic Polymer Backbone

A large number of non-biodegradable synthetic hydrophilic polymers are commercially available. Characteristics of the polymeric backbone which might affect *in vivo* performance of a poly-soap include: toxicity, molecular weight, solubility in aqueous media, existence (or absence) of ionic character, and degradability by gastrointestinal enzymes or conditions. From a manufacturing view the cost, availability, reactivity, and solubility in organic media are important considerations. Table 4 shows the advantages of cellulose ethers in this context. Dow Chemical Co. literature provided most of the information for this table [127]. For these reasons we selected HPMC as the polymeric backbone for this study.

Table 4 - Advantages of Cellulose Ethers as a Polymeric Backbone.

1) Resistant to enzymatic degradation.
2) Stable from pH 3 to 12.
3) Water soluble.
4) FDA approved for food usage.
5) Low cost.
6) Nonionic polymer (it can be made ionic however).
7) Readily available (Dow Chemical Co. Methocel®)
8) Hydroxyls can be reacted to form stable ethers.
9) Naturally surface active (44-56 dynes/cm).

3.1.2. Rationale for the Selection of the Attached Lipid

The choice of a lipid that can be conjugated to a hydrophilic polymer is limited by the availability, cost and reactivity. These criteria are summarized in Table 5. We elected to examine several lipids; octadecanol, oleyl alcohol, and cholesterol. These three lipids represent major components of interest in the intestinal tract. The primary constituents of mixed micelles in the intestinal tract are fatty acids and bile salts (Table 1). With this in mind we chose C_{18} as the ligand most likely to succeed as a hydrophobic affinity moiety. Octadecanol was selected since it is the simplest C_{18} lipid. It is saturated and we would expect it to form highly order - almost solid - structures at 37°C. Oleyl alcohol was chosen because it forms a liquid phase (this alcohol is a liquid at room temperature) which might encapsulate a lipid. Being a liquid we might expect that a hydrophobic domain made of oleyl would be easily penetrated by cholesterol. Cholesterol derivatives are known to form liquid crystal structures as well [128]. It is this ability of cholesterol to associate with itself to form organized layered structures that we hoped to capitalize on. We had hoped that these structures would provide an environment for the selective sequestration of cholesterol.

We have found in this study that these alcohols can be reacted with epichlorohydrin (ECH) then sodium hydroxide to yield glycidyl ethers which can then be reacted with hydroxyls on the HPMC to yield ether bonds (detailed in section 4.2.1). We also attached a nonionic surfactant to HPMC using the same procedure. We conjugated Brij® 30 (dodecane-PEG_4), this represents a novel non-ionic surfactant attached to a polysaccharide.

Table 5 - Criteria for Lipid Ligand.

1) Reactable to form ether or other biologically stable bonds (alcoholic).
2) Readily available in pure forms.
3) Low cost.
4) Similarity to cholesterol or intestinal mixed micelle components.

3.2. Aims of Present Work

Our approach to reducing plasma cholesterol involves the systematic molecular design, synthesis, physico-chemical characterization, *in vitro* modeling and testing of a new class of synthetic lipid sequestering agents. The aims can be found in Table 6.

To answer these questions we used the methods outlined in Fig. 9. We have already addressed the specific reasons for selecting the starting materials (shown on the left side of this figure). The rationale for selecting the methods of analysis are presented in Chapter 5. The model compounds to be tested have been divided into two general classes, water soluble and water insoluble. We have found that for low levels of lipid attachment, the polymers produced become water soluble. At higher lipid attachment the polymers are water insoluble. The phase of the

Table 6 - Aims and Questions Addressed by Project

Aims of Project

1) Design and synthesize a series of hydrophobic affinity polymers by attaching a variety of lipophilic side chains to a water soluble polymer.

2) Develop models to evaluate the ability of such polymers to bind amphiphiles and hydrophobic components of physiologically relevant mixed micelles, especially cholesterol.

3) Propose mechanisms of interactions of such polymers with lipids and mixed micelles as they relate to lipid sequesteration in biological fluids.

Questions Addressed

1) Is it possible to design a nonionic polymeric material that will bind cholesterol or fats from mixed bile salt micelles.

2) Can selectivity be enhanced by using cholesteryl as a lipid conjugated to a water soluble polymer?

3) Does the nonionic polymeric backbone bind bile salts and other surfactants?

4) What effect does the amount of lipid conjugated to the polymer have on binding and physical character?

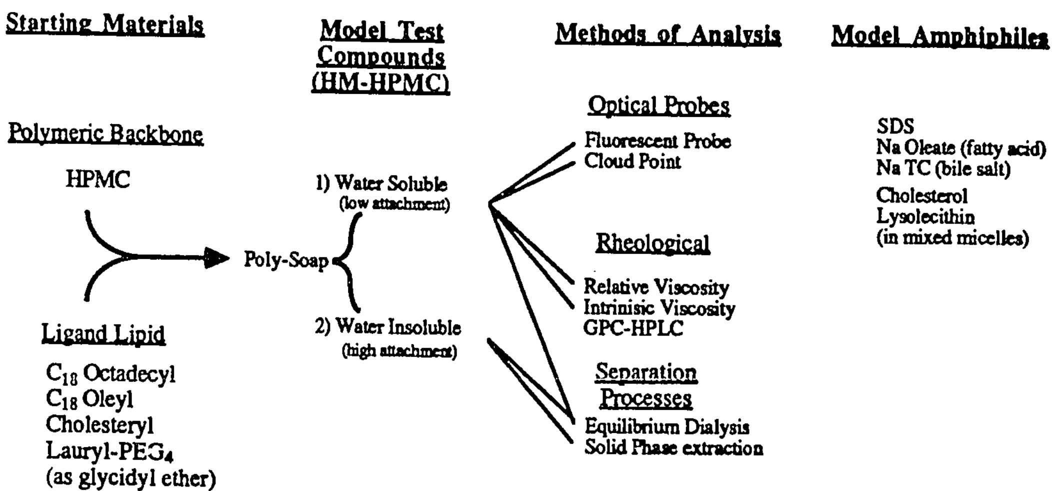


Fig. 9 - Methods to Examine Amphiphilic Interactions with HM-HPMC. Showing: starting materials, model compounds tested, methods of analysis, and amphiphiles.

materials (either solid or water soluble) somewhat determines what method of analysis are possible. This is reflected in the figure as well.

CHAPTER 4

Synthesis and Characterization of HM-HPMC

This Chapter presents the synthesis of the polymeric hydrophobic affinity polymer and methods of analysis. This includes a history of HPMC and other hydrophobically modified saccharides.

As outlined in Chapter 3 we wish to design an affinity polymer which will bind hydrophobic components of the intestinal tract. We have selected a hydrophilic polymer which has hydrophobic moieties attached thru ether linkages. Using a single hydrophilic polymer we attached three different ligands which range from a simple straight chain through an unsaturated fat to a complex heterocyclic lipid. By varying lipid attachment we have examined what effect the ligand has on the products ability to sequester amphiphiles and cholesterol from mixed micelles. A non-biodegradable hydrophilic polymer with attached cholesterol (with or without other lipids), which can be dispersed and bind cholesterol *in situ*, then pass out of the intestines with the feces, might represent a new and significant addition to the current treatment of high plasma cholesterol.

4.1. Background on HM-polymers

The use of cross-linked celluloses or other poly-saccharides as supports for the liquid chromatographic separation of compounds has existed for many years. The availability and excellent properties of cellulose have made it one of the most utilized polymers for many applications. Only recently have these supports been actively used in hydrophobic interaction chromatography where hydrophobic, lipid groups have been attached through covalent bonds to the support. In addition, water-soluble ether derivatives of cellulose have been used in many applications. It has been estimated that 5.5% of the total world-wide use of dissolving pulp is used

in cellulose ethers [129]. This amounts to about 320 thousand metric tons annually. Primary uses include applications as thickeners and glues. Modified celluloses have also found widespread use as protective colloids for emulsion polymers. Most of this work has focused on their use in latex paints [130,131]. The polymer forms a protective coating of polymer and bound water around the emulsion particle thus improving stability and rheological character. The flocculation behavior of latexes by the addition of water soluble cellulosics is believed to be due to volume restriction forces [132]. This may be important in some of the work we are presenting, since the upper gastrointestinal tract has a bulk emulsion phase which may be coated by the polymer.

4.1.1. Methods of Cellulose Modification

Cellulose is a polysaccharide with monomer (saccharide) units linked through their β-1,4-hydroxyl groups (see Fig. 10). The importance of this linkage is realized when one considers conformation and stability of this material. The 1,4-linkage allows the polymer to form an almost fully extended polysaccharide chain by flipping successive saccharide units over 180°. In contrast, the α-1,4-linkage does not allow this to occur (e.g. amylose adopts a coiled helical structure in water). The β-linkage results in a polymer that has a large end to end distance in solution and can be considered a rigid straight chain for low molecular weights. This linkage is not particularly susceptible to degradation by enzymes produced by mammals. Herbivores that consume cellulose

Fig. 10 - Repeating Structure of Cellulose.

require the presence of microbes (who have the necessary enzymes) in the intestinal tract which degrade the polymer so it can be utilized by the animal as a foodstuff. Following sufficient etherification of this polymer it is highly resistant to attack by microorganisms [127].

HPMC is produced by the reaction of methylchloride and propylene oxide with cellulose in an alkaline slurry. This produces substituted cellulose ether, sodium chloride and water. The structure of this material is shown in Fig. 11. Additionally, a host of side reactions are possible. Primarily, the hydroxyl on the propyl group is accessible for additional reaction, thus the necessity of reporting the molar degree of substitution (MS) for the ligands.

Cellulose ethers are characterized by both MS and the degree of substitution (DS) of hydroxyl groups on the glucose ring. Since there are only three hydroxyls available the DS must be less than or equal to three. If subsequent reaction of ligands with other groups is likely then it is possible to have a MS greater than three. This is illustrated in Fig. 12 [133].

Patents assigned to Hercules Inc. in 1980 and 1981 describe the formation of "surfactant-soluble cellulose derivatives" [134,135]. Landoll (at Hercules) produced hydrophobically modified (HM) cellulose ethers of a similar structure to the ones that we are now reporting [136]. Primarily, they have used hydroxyethyl cellulose as a starting material and react this with epoxy-alkyl ligands. Their polymers appear to have similar structures and properties as our polymer. We have found

Fig. 11 - Approximate Chemical Structure of Hydroxypropyl Methylcellulose (HPMC) [127].

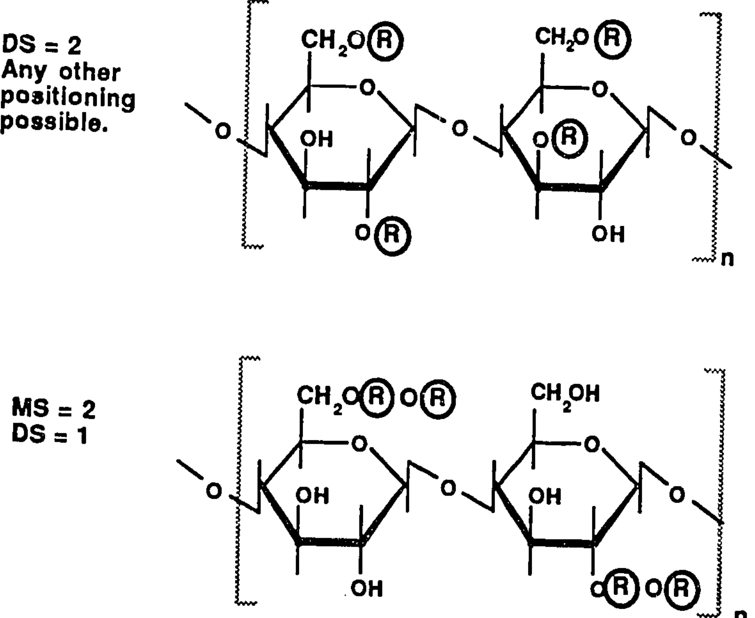


Fig. 12 - Example of Degree of Substitution (DS) and Molar Substitution (MS) for Cellulose Ethers [133].
The ligand R can be a methoxyl in the first case where 2 of the three hydroxyl's on the saccharide monomer are capped. In the second case, R could be a hydroxy propoxyl or hydroxy ethoxyl residue where the hydroxy terminal group has reacted with a second propoxyl or ethoxyl residue.

their work to provide an interesting comparison to our's. After reading the literature on these materials it appears that they have a product looking for an application. Apparently, they have not considered it's use as a food additive. It seems that primary uses would be as thickeners in detergent solutions. An example might be as an additive in hair shampoo. One study has been published discussing the interaction of HM-HEC with amphoteric surfactants [137].

4.1.2. Functionalization and Analysis of Polysaccharides

The reaction of epichlorohydrin (ECH) with cellulosics is a standard method of activating the polymer. It is frequently used to prepare a polysaccharide support for subsequent reaction with specific groups [138]. One difficulty in these reactions is the formation of crosslinks in alkaline

systems. After initial activation by the ECH the epoxy ring reforms (with the loss of chloride), this can then react with other hydroxyls forming crosslinking bridges between polymer chains [139]. This has been used intentionally as a crosslinking method [140]. There have been numerous papers describing the binding of enzymes to cellulose derivatives. These include the use of ECH as an activating agent [141].

The attachment of lipids to Sepharose is known to produce chromatographic supports which can separate proteins [142] and many other compounds [143,144]. The founding fathers of this chemistry and it's application appear to be Ellingboe, Nystrom and Sjovall [145-147]. The glycidyl ether of Triton-X (a nonionic surfactant) has also been investigated. Pitha, Kociolek, and Caron have attached Triton-X glycidyl ether (TGE) to cellophane, dextran, amylose, and inulin [148]. Their experiments did not however, result in very good attachment of the detergent. 15 gms of polysaccharide were dissolved in aqueous NaOH and 100 gms TGE added. This resulted in from 5 to 30% attachment of the TGE. TGE has also been attached to Sepharose® using dioxane as solvent and boron trifluoride etherate as catalyst [149]. This system resulted in attachment of only 3mmol TGE per mol galactose. Similarly, palmityl has also been attached by reacting the alcohol with ECH [124,150].

An ASTM test for the determination of alkoxyl (methyl, ethyl and propyl) substitution for cellulose ethers has been issued [151]. The method involves Zeisel cleavage of ethers using hydroiodic acid in an organic media contained in sealed containers at high temperatures [152]. Most industrial groups have used this method to determine the MS of these compounds.

For the analysis of larger alkyl ethers conjugated to hydroxy polymers several methods have been utilized. Landoll used exhaustive cleavage of the ethers using HBr in acetic acid followed by GC analysis on his HM-cellulose ethers [136]. Another method has been used, primarily by the groups hydrophobically modifying Sepharose [149,153]. They degrade the HM-agarose derivatives using hot acetic or formic acid. This is followed by removal of the acid and examination of the

polymer remnants using NMR.

The use of enzymatic degradation of cellulose ethers has been used to determine the homogeneity of substitution [154-157]. Experimental results using carboxy methylcellulose indicate that the derivatization pattern is not homogeneous [158] (i.e. regularly spaced along the cellulose polymer). The relative accessibility of the enzyme to the β-1,4 linkage is assumed to be a function of substitution patterns [159]. Sarkar has suggested that the heterogeneity of substitution (block vs. uniform) has a direct influence on the surface tension of cellulose ethers (methyl and hydroxypropyl methyl) [160]. He suggests that large block substitution results in very low surface tension, intermediate substitution (not very large blocks) leads to higher surface tension, and very small blocks distributed along the cellulose polymer results in low surface tension. Note that a low surface tension indicates high surface activity, the polymer reduces the surface tension of water to a high degree. This indicates that the polymer will orient hydrophobic groups towards air and hydrophilic moieties to water. The maximal surface tension for intermediate substitution being due to the inability of the polymer to orient itself to minimize surface free energy.

These observations may be important with regard to two aspects of our research. First, the heterogeneity of methoxyl and hydroxypropoxyl substitution on the polymer used in our study was not determined so to what degree this influences the HPMC binding of amphiphiles is unknown. We can speculate that the more surface active the polymer the greater its ability to stabilize emulsions (such as those found in the digestive tract). As we shall see it was discovered that the backbone polymer (HPMC) does bind a bile salt (NaTC) and interacts with a detergent (SDS). It would have been interesting to investigate what heterogeneous substitution has on this. Second, the heterogeneity of methoxyl and hydroxypropoxyl substitution has a direct influence on that of our conjugated lipids since the glycidyl ether chemistry used for this reaction utilizes free hydroxyls. A high degree of methoxyl heterogeneity would cause the availability of these groups to be heterogeneous as well which would lead to non-uniform substitution patterns of the conjugated

lipids.

4.1.3. LCST Behavior of Modified Celluloses

Lower critical solution temperature (LCST) behavior of polymers is well known [161]. This phenomena is characterized by the precipitation of polymers as the temperature is raised. Such behavior is contrary to the "normal" behavior of solutes where they go into solution better at elevated temperatures. The precipitation phenomena can be of two general types. In one case the polymer precipitates as an amorphous solid which forms a suspension of small solid particles in solution. I will refer to this as sol formation. For other polymers, heating leads to gelation. This behavior is the reverse of the more familiar gelation on cooling seen for agar, carrageenans and gelatin.

Both the sol and gelation processes are reversible. In the first situation, the sol rapidly dissipates, the solution turning from milky white to clear within a very short time after cooling to the critical temperature. An example is the sharp LCST seen for poly(N-isopropyl acrylamide) [162]. The gels dissipate more slowly. In the three-dimensional structure, chain mobility is restricted and reconstitution of the aqueous solution is retarded. It was found that we could control both the form of the precipitate and the LCST for the lipid conjugated HM-HPMC by altering the degree of attachment.

Taylor and Biasotti have found that the cloud point is a sensitive indication for the DS of aldehydes (C_2-C_4) to PVA (using acetal linkages) [163]. They observed a decreasing cloud point as more ligand was attached to the polymer. After a master curve is generated it was possible to use the cloud point as an indication of ligand attachment.

Cellulose ethers also undergo a phase transition at elevated temperatures. This behavior is used as a quick method (Pharmacopeia) to determine whether an unknown sample is HPMC [164]. For cellulose ethers with heterogeneous substituent patterns (i.e. methyl groups attached non-

uniformly on cellulose) it is believed that the gel forms because regions with high substitution aggregate at some temperature. This has been described as "cross-links by micelles" [165]. This description was found to be appropriate for HM-HPMC.

The precipitation process is driven by an entropy gain. In water, a poorly solvated molecule will cause a high degree of water structure. This "ice-like" ordering greatly reduces the entropy of the water molecules. As the temperature is increased the water molecules are released and hydrophobic interactions form between polymer segments. This hydrophobic interaction theory is applied generally to nonionic polymers in solution [166]. The thermodynamics of this process are complex and many theories have been proposed to explain it. The classical is the Flory Huggins theory. In 1983 Franks pointed out the short comings of this theory when applied to aqueous systems [167]. He states that attempting to rationalize LCST behavior within the confines of FH theory are hardly realistic.

Thermal gelation properties of HPMC have been reported by Sarkar (from Dow Chemical Co.) [168]. In this study he examined both gelation and the precipitation of HPMC as measured by light transmission. This method employs the gradual heating of a sample in a spectrophotometer. The light transmission is measured as a function of temperature. These are known as cloud point (CPT) curves. He defined the CPT as the temperature at which the light transmission falls to 50%. The incipient precipitation temperature (IPT) is defined similarly, as the temperature where light transmission is 97.5%. We have utilized these definitions in our studies.

4.1.4. Poly-soaps

Numerous papers have described poly-soaps. Poly-soaps (usually poly-electrolytes with hydrophobic side chains) have been known to form intramolecular micelles for some time (for a short review see [169]). Poly-4-vinylpyridine quaternized with n-dodecyl bromide (28-38%) has been shown to solubilize n-decane, benzene and 1-heptanol [170]. Furthermore, a critical dodecyl group content (similar to a critical micelle concentration - CMC) for the polymers ability to

sequester lipids has been observed [171]. Other poly-soaps that have shown similar behavior include; poly-2-vinylpyridine quaternized with n-dodecyl bromide [172], and 1-1 copolymers of alkyl vinyl ether and maleic anhydride [173,174] (where the anhydride group is hydrolyzed to the salt). Strauss suggests that the chemical nature of the groups that lend water solubility to polysoaps (cationic, anionic, or nonionic) should not be critical [169]. This concept would support the structure drawn in Fig. 7 which shows the cross-section of our fat sequestering polymer.

4.2. Synthetic Methods and Materials

4.2.1. Glycidyl Ether Synthesis Method

The glycidyl ethers of oleyl alcohol, octadecanol and cholesterol were synthesized by the reaction shown in Fig. 13. The alcohol is reacted with epichlorohydrin in the presence of stannic chloride (a Lewis acid catalyst). The chlorohydroxy intermediate of this reaction is then dehydro-dehalogenated with sodium hydroxide to form an alcoholic glycidyl ether.

Ⓡ-OH + CH_2(-O-)CH-CH_2Cl —($SnCl_4$)→ Ⓡ-O-CH_2-CH(OH)-CH_2Cl

Alcohol Epichlorohydrin (ECH)

↓ NaOH

Ⓡ-O-CH_2-CH(-O-)CH_2 + H_2O + NaCl

Alcoholic Glycidyl Ether

Fig. 13 - Two Step Preparation of an Alcoholic Glycidyl Ether. **R-OH** can be a primary or secondary alcohol. First reaction is run in methylene chloride using Lewis acid catalyst. Second reaction is run in dioxane at 80°C.

The structure of the glycidyl ethers used in this study can be found in Fig. 14. Phenoxyl glycidyl ether (PGE) was purchased from Aldrich Chem Co. When we started working on this project, octadecyl glycidyl ether (ODGE) could be purchased from Polysciences. It is no longer available commercially, however the synthesis is almost identical to the synthesis of oleyl and cholesteryl glycidyl ethers (OGE and CGE). We have also found that the hydroxyl of the Brij® nonionic detergents can be made into a glycidyl ether using almost the same protocol.

The reaction of cholesterol with ECH had been attempted unsuccessfully in Dr. Hoffman's laboratory [175] using perchloric acid as a catalyst. This catalyst had been used by others for the synthesis of alkyl glycidyl ethers [176]. Since one of our goals was to attach cholesteryl to a polymeric backbone using this chemistry we had to synthesize CGE. Initially, we tried boron trifluoride etherate as a catalyst for this reaction with limited success. A large number of products (determined by TLC) were formed indicating that polymerization of the ECH was occurring. Dr. Niels Andersen (Chemistry Department, University of Washington) suggested that we try stannic chloride as a catalyst. This worked well and the following paragraphs summarize the method we found to work best for the formation of CGE from cholesterol.

10 gms (26 mmoles) of cholesterol (Baker >98%) was added to a 500 ml 3-neck round bottom flask which was fitted with a short path distillation tower, a rubber septum and a thermometer. It was dried by introducing approximately 50 ml of benzene which is azeotropically distilled. This was performed since cholesterol is known to frequently be in the form of a mono-hydrate. Following distillation, trapped vacumn was used to remove most of the remaining benzene. Dry nitrogen was then used to fill the reactor before the addition of other reagents.

200 mls methylene chloride was added to the cholesterol and 0.65 mls of 1.0 M stannic chloride in methylene chloride (Aldrich) admitted through the septum utilizing standard methods of handling air-sensitive reagents [177] (a dry glass syringe and needle). This resulted in a 1:40 molar ratio of catalyst to cholesterol. While stirring, the reactor was heated to 40°C (reflux) and a 2:1

$\overset{CH_2CHCH_2O}{\setminus O /} - CH_2(CH_2)_{16}CH_3$ **Octadecyl Glycidyl Ether (ODGE)**

$\overset{CH_2CHCH_2O}{\setminus O /} - (CH_2)_8CH{=}CH(CH_2)_7CH_3$ **Oleyl Glycidyl Ether (OGE)**

$\overset{CH_2CHCH_2O}{\setminus O /} - (CH_2CH_2O)_4(CH_2)_{11}CH_3$ **Lauryl-PEG Glycidyl Ether (LP_4GE)**

CH_3 CH_3 $\overset{CH_2CHCH_2O}{\setminus O /}$ **Cholesteryl Glycidyl Ether (CGE)**

$\overset{CH_2CHCH_2O}{\setminus O /}$ **Phenoxyl Glycidyl Ether (PGE)**

Fig. 14 - Structure of Glycidyl Ethers Used in This Study.
PGE can be purchased from Aldrich Chem. Co., ((±)1,2-epoxy-3-phenoxypropane), the remainder were synthesized for this study.

molar ratio of ECH to cholesterol added using an addition funnel (2.4 gms). To follow the course of this reaction we used silica gel thin layer chromatography (TLC) (Eastman Kodak) which provided rapid feedback of the effect of conditions on extent of reaction. A 5:1 mixture of hexane and ethyl acetate was used to develop the plates and the spots visualized using iodine vapor.

Within two hours the reaction was complete and the product isolated by stripping off the methylene chloride (using a Buchi rotary evaporator) and washing the organic phase with water and ethyl ether. When reacting ECH with the Brij® surfactants it was necessary to modify the procedure by replacing the wash water with brine since stable emulsions were otherwise formed. Finally, the ether was stripped off and the intermediate product was ready for reaction with sodium

hydroxide to form the glycidyl ether. A rapid method to see if ECH has reacted with the alcohol is possible using a Beilstein flame test. A clean copper wire is loaded with some intermediate product and flamed. If chlorine is present then a bright green flame will be readily evident. After dehydro-dehalogenation the product should produce little green flame - depending on the purity of the sample.

For this reaction we used the protocol from Zielinska and Gasztych as a guide [176]. The reaction was carried out in dioxane (100ml) at 80°C by adding 1 gm of NaOH (50% in water - 1:2 molar ratio of cholesterol to NaOH) to the dioxane/cholesteryl solution. The reaction was again followed using TLC and was essentially complete in 4 hours. It was found essential that TLC be used for optimizing this reaction. A number of other investigators have used ice-water cooled NaOH with good results. We found these conditions to be unsuitable for the conversion of the alcoholic ECH intermediate to the glycidyl ether. After washing with water/ethyl ether, until the pH of the wash water is neutral, the ethyl ether was removed (rotary evaporator) resulting in an off white amorphous material which hardens at room temperature. It is interesting to note that the CGE formed colored liquid crystalline structures as it hardened. Again, if the glycidyl ether of a Brij® compound is being produced then brine must be used in place of the water wash.

It was found that CGE, ODGE, and OGE could be purified using liquid chromatography. A Rainen 100X2.5 cm column packed with Silicar CC-7 and a gravity feed of hexane/chloroform was used. 15 ml fractions were collected using a LKB Superac fraction collector. TLC was used to identify purity of the fractions.

4.2.2. HM-HPMC Synthesis Method

HPMC was used as received with the exception that it was dried in a vacumn oven at 60°C before reaction with epoxy ligands. Initially we tried boron trifluoride etherate or stannic chloride as a catalyst in several aprotic but polar solvents including dimethyl formamide and dimethyl sulfoxide. Both of these solvents dissolved the HPMC (forming a viscous solution) but the reaction

proceeded with little or no conversion. We suspect that the Friedel Crafts catalysts were reacting with the electron donor solvents. Subsequently, we found that a 3:1 mixture of dioxane to methylene chloride dispersed HPMC (almost dissolved it at higher temperatures) sufficiently to allow reaction while also solvating the glycidyl ether and the stannic chloride catalyst.

We have used phenoxyl glycidyl ether (PGE - Aldrich, structure also shown in Fig. 14) as a model compound (λ_{max} 268nm) with stannic chloride as catalyst. The reaction was carried out at room temperature in dioxane/methylene chloride (3:1) and aliquots were withdrawn over the course of the reaction. By dissolving these samples in 0.01 M SDS/water it was possible to quantitate the degree of substitution using U.V. absorbance.

The reaction of HPMC with ODGE, OGE, and CGE has also been accomplished. Weight ratios of 0.1 to 75% relative to HPMC have been reacted. We also allowed the native polymer to interact with stannic chloride without glycidyl ether present. This is referred to as control HPMC in subsequent discussions. The following paragraph describes the preparation of 25% CGE-HPMC.

10 gms of HPMC was introduced into a 500ml 3-neck round bottom boiling flask containing a magnetic stirring bar. A vacumn was applied (with a coarse filter at the top of the reactor) and the HPMC gently heated with a flame to pull off any remaining water. The polymer was dispersed in methylene chloride (40ml) and dioxane (150ml, anhydrous, Aldrich) was added while stirring. The solution was heated to about 40°C and the HPMC allowed to disperse and dissolve as much as possible. Stannic chloride (4ml of 1M methylene chloride solution, Aldrich) was added to the reaction mix and allowed to dissolve. This produced some fuming of the stannic chloride (decomposition into HCl) even when all materials were carefully dried. The glycidyl ether (3.3 gms) was dissolved in methylene chloride (10 ml) and slowly added over a period of about 15 minutes. The reaction was allowed to proceed over 24-48 hours at room temperature and the product isolated by precipitation using hexane. Initially, the modified polymer was washed over a 12-24 hour period using a Soxhlet extractor running a 50:50 mix of acetone/hexane which forms a

59/41 azeotrope. It was found that stannic chloride and unreacted lipid dissolved in this solvent. Unfortunately, we discovered that the resulting polymer sometimes had residual acid remaining. In addition, the very high substitution polymers began to dissolve in this solvent system. To overcome this problem we used dialysis of the products with water-methanol and acetone. Using a dialysis bag (12-14,000 MWCO) and water the polymer swells. Over several days the dialysis solution was changed from water to methanol to acetone and the polymer usually precipitated as the water was replaced. At high acetone we obtained a dense gel which was dispersed in a small quantity of acetone using a mortar and pestle and vacumn dried.

4.3. HM-HPMC Characterization Methods

The HPMC used in the study was purchased from the Aldrich Chemical Co. They reported the characteristics shown in Table 7. They considered the source of their chemicals to be proprietary so we were unable to determine what company manufactured the material and what (if any) quality assurance was provided. Examination of the Dow Corning literature on Methocel® products reveals that these specifications are quite close to those for Methocel E, however. We wished to confirm that the degree of attachment of methoxyl and hydroxypropoxyl was consistent with the values reported by the supplier. The following section outlines this method.

Table 7 - Reported Specifications of HPMC Used in Study.

Property	Value
Hydroxypropyl Content	10%
Methoxyl Content	30%
Number Average Molecular Weight (M_n)	86,000
Viscosity (2% solution)	4,000 cp
Manufacturer	Unknown
Sold by Aldrich Chemical Co.	

4.3.1. Methoxyl and Hydroxypropyl Substitution Determination Method

ASTM D3876-79 was used as a guide for the determination of methoxyl and hydroxypropyl substitution [151]. We found a description by Hodges *et al.* essential to understanding the method [152]. When HPMC is reacted with hydroiodic acid in the presence of adipic acid (a catalyst), quantitative liberation of methyl iodide and isopropyl iodide is achieved for each mole of methoxyl or hydroxypropoxyl substituted on the cellulose. The method utilizes *in situ* extraction of the alkyliodides into xylene all in a sealed vial. These iodide components can be detected quantitatively using gas chromatography (GC). With slight modification, this method was also utilized for the determination of glycidyl ether substitution (see the next section). The following paragraphs accurately describe the method used.

Apparatus:

A Hewlett-Packard model 7620A gas chromatograph equipped with a 7670A auto-sampler and a flame ionization detector (FID) was used to analyze the iodides. The column was a 10m X 0.53mm NON-PAKD® capillary column from Alltech Associates, Inc. Deerfield, IL. The stationary phase was 2.65μm thick and composed of RSL®-160 polydimethylsiloxane. This type of coating is considered non-polar so constituents are separated by boiling point, generally with lower boiling point materials coming off the column before higher boiling components. Helium (~ 3.5ml/min) was used as the carrier gas (it was run through an OXY-TRAP® to remove oxygen). Approximately 1μl injections were split 1:33 to prevent overloading the column. It was found that no make-up gas was necessary for good flame ionization with hydrogen (30cc/min) and "zero air" (300cc/min) used for the flame. The signal from the FID electrometer was captured using a Spectra Physics model SP4290 integrator. This unit was controlled by the auto-sampler so multiple samples could be easily analyzed. The syringe was washed using a full stroke volume 10 times between each sample using ultra-pure *o*-xylene. The injector and detector temperature were 200°C while the oven was maintained isothermally at 50°C. Since the reaction is carried out at 150°C in a sealed

container the type of seal for the vial is important. As recommended in the ASTM protocol we used Reacti-vials® from Pierce Chemical Co.. Each vial was closed with a Mininert® top which seals using a Teflon® valve. A small stirring vane was placed in each vial before weighing out samples.

Reagents:

Hydroiodic acid (HI), 99.99%, distilled and stabilized with hypophosphorous acid, Aldrich analyzed (~56.7% I) was stored under nitrogen in the refrigerator between uses. Adipic acid m.p. 151-153°C Mallincrodt, and *o*-xylene, ultrapure, Alfa Products, Thiokol were used. Iodomethane (99%), and 2-iodopropane (97%) from Aldrich were analyzed on the GC to confirm their purities. This allowed the accurate preparation of standards. Nominally, 25mg/ml of toluene in *o*-xylene was used as an internal standard. This was prepared by weighing 25±0.001 gms of toluene into a 100ml volumetric flask which was filled to volume using *o*-xylene.

Procedure:

Approximately 60 ±0.1 mg of HPMC was weighed into each Reacti-Vial. The sample was then dried for 1 hour at 105°C, cooled in a dessicator (P_2O_5) and reweighed to accurately determine the actual weight of sample. We found this procedure preferable to that given in the ASTM method since the accurate weighing of pre-dried samples was difficult owing to rapid pick-up of water from the atmosphere. Another departure from the ASTM method was the use of the magnetic stirring vanes. In the ASTM test the operator is required to manually agitate the vial at 5 and 30 minutes. On several occasions when using this method the hot pressurized contents breached the seal. To avoid compromising the safety of the operator we stirred the vials continuously without diminishment of quantitation.

An amount equal to or slightly greater than the HPMC of adipic acid was added to each vial followed by 2 ml of internal standard stock solution using a glass pipet. 2 ml of HI was then added and the vial quickly capped with the Mininert valve. The vial was weighed before and after

reaction to determine any loss of contents due to leakage (if more than 0.01 gms of material was lost then the sample was discarded, an ASTM recommendation). The vial was placed in the heating block which had been pre-heated to 150°C. The heating stirring module was kept in the fume hood during reaction in case of accidental release of hot/pressurized contents. After 1 hour the vial was carefully removed and placed in an aluminum block at room temperature until cool. The upper (xylene) phase was then removed using a glass syringe and placed in vials suitable for the autosampler (Wheaton 1ml). These were capped with crimp top Telfon backed rubber seals.

A calibration solution was prepared by the addition of 40μl of iodomethane and 10μl of 2-iodopropane (weighed to ±0.01 mg) to a vial containing approximately 65 mgs of adipic acid, 2 ml of HI, and 2ml of *o*-xylene internal standard solution. The equivalent weight of alkoxyl was then determined by using the following equations:

$$\text{mg } OCH_3 = \text{gm } CH_3I \left[\frac{\text{mol wt } OCH_3 \times 1000}{\text{mol wt } CH_3I}\right] \times \text{purity}$$

$$\text{mg } OC_3H_7O = \text{gm } C_3H_7I \left[\frac{\text{mol wt } OC_3H_7O \times 1000}{\text{mol wt } C_3H_7I}\right] \times \text{purity}$$

The detector response factor for each component of interest was then determined by the following equation

$$RF = \frac{\text{mg alkoxyl in std}}{\text{alkyliodide peak area}} \times \frac{\text{toluene peak area}}{\text{mg toluene in std}}$$

This response factor was then used to determine the amount of methoxyl and isopropoxyl attached to the cellulose using the following equation:

$$\text{Alkoxyl Bound (\%)} = RF \times \frac{\text{alkyliodide peak area}}{\text{sample mass}} \times \frac{\text{mg toluene in std}}{\text{toluene peak area}} \times 100$$

In all cases the analysis was performed in triplicate with each sample run on the GC three times (this results in nine data points).

4.3.2. Quantitation of Glycidyl Ether Attachment Methods

The same procedure as that described above was used for the determination of CGE, ODGE, OGE, and LP_4GE attached to HPMC. The reactions were carried out using the same protocol while the standards were prepared using a different method since the iodides of oleyl alcohol and cholesterol are not commercially available. 1-iodo standards for octadecane and dodecane are available and were used to compare the effectiveness of the method used.

Several mgs (~5 ± 0.01mgs) of the alcohol (oleyl and cholesterol) were added to control vials which were reacted with the samples. This resulted in substitution of the alcohol by iodine and for oleyl alcohol and cholesterol presumed hydroiodination of the double bonds. The corresponding quantity of glycidyl ether was then calculated using the equations given above. It was found that the alcohols reacted quantitatively only when dissolved in low quantities of xylene (0.2ml). When a full 2 ml of xylene was placed in the vial containing the alcohol little conversion was witnessed. This was probably due to poor solubility of the alcohol in the HI phase. Even with continuous stirring little or no conversion occurred. 1-iodohexadecane was used as the internal standard (~1 mg/cc). The column was changed to a high temperature version of the same used above (10m X 0.53mm NON-PAKD® capillary column, Alltech Associates) The stationary phase was 0.25μm thick and composed of RSL®-150 polydimethylsiloxane. The temperature of the injector and detector were maintained at 300°C and the oven temperature ramped according to the conditions shown in Table 8. The integrated results were calculated using the method described above.

Table 8 - GC Conditions for Analysis of Glycidyl Ethers.

Glycidyl Ether	Initial Temp.	Heating Rate	Final Temp.
ODGE, OGE	180°C for 6 min.	4°C/min	215°C
LP_4GE	120°C for 4 min.	6°C/min	180°C
CGE	180°C for 2 min.	6°C/min	280°C

To confirm that the Zeisel cleavage method of analysis yielded correct results, we attached ^{14}C labeled OGE to HPMC. This glycidyl ether was prepared from 1-^{14}C oleic acid (10μCi/gm) which was prepared from an NEN product diluted with cold oleic acid (99% Sigma). The acid was reduced to the alcohol by $LiAlH_4$ [178]. 466 mgs (1.65mmoles) of oleic acid was placed in 5cc ice-water cooled ethyl ether (anhydrous) which contained 82 mgs (2.1mmoles) $LiAlH_4$. Water was added after 15 minutes to decompose excess hydride and re-acidified by the addition of 5cc of 10% H_2SO_4 in water. The product was then purified using water/ethyl ether wash in a separatory funnel. The yield was very good as evidenced by TLC. The product was then reacted with ECH and NaOH using the procedure outlined previously. The product was purified using 10:1 hexane:ethyl acetate on a silica gel column using flash chromatography [179].

The resulting glycidyl ether was reacted with sufficient HPMC to yield a 20% reaction ratio. Following dialysis of the HM-HPMC, the sample was examined using the Ziesel cleavage method. After analysis on the GC the remaining (xylene) upper phase was analyzed using liquid scintillation counting (LSC). Due to the high quench observed by the xylene phase (which contained some iodine) it was necessary to use an internal standardization technique to obtain the quantity of cleaved ^{14}C oleyl iodide. After correction for quenching the quantity of OGE attachment was determined.

In addition, a small quantity of the product was dispersed in either 5cc water or 4% SDS in water and analyzed in a gel of Aquasol II (in triplicate). A 25% water 75% Aquasol II mixture produces a gel which suspended the polymer particulates. It was found that the 4% SDS dissolved the polymer however, some precipitation was observed when the scintillation fluid was added. Following counting, a small amount of ^{14}C oleic acid was added to each sample to provide an internal standard. The samples were recounted and the wt% attachment determined.

4.3.3. Characterization of HM-HPMC in Aqueous Solution

Several methods were employed to characterize the water soluble HM-HPMC products in aqueous solution. The pH was determined using an ASTM method. Both relative and intrinsic viscosity measurements were obtained for HPMC and one case of HM-HPMC (LP_4GE-HPMC). Aqueous phase GPC was also carried out. Finally, cloud points were also determined.

4.3.3.1. Surface Tension Determination by Wilhelmy Plate Method

The surface tension of HPMC and other solutions was determined using the Wilhelmy plate method. Numerous papers have discussed the effect of temperature, plate geometry and other aspects of using this method [180-185]. Andrade *et al.* provides the simplest discussion [186]. In this method a clean platinum blade is lowered into a test solution and the force determined at zero immersion depth. By knowing the surface area of the blade in contact with the liquid it is possible to determine the surface tension (dynes/cm) using the following equation.

$$\gamma_{LV} = \frac{F - mg + F_b}{P \times \cos\theta}$$

where:

γ_{LV} = surface tension (liquid/vapor, dynes/cm)

F = total force recorded on balance (dynes)

mg = mass of plate × acceleration due to gravity (dynes)

F_b = buoyancy force (dynes)

P = perimeter of the plate (cm)

θ = contact angle

By balancing the recorder to the weight of the blade, determining the force at zero immersion depth and assuming that the contact angle is zero we get the following equation.

$$\gamma_{LV} = \frac{F_m}{P}$$

where:

F_m = force measured by balance (dynes)

Our system consisted of a Cahn RG electrobalance which was calibrated using class S weights. The blade had a perimeter of 1.506 cm and was cleaned between each experiment by liberal washing with deionized/distilled water and flaming to a red-orange color. The sample was placed in a scintillation vial which was elevated using a specially designed and manufactured fluid drive. This system allowed for smooth and slow movement of the sample into contact with the blade. After immersing the blade into the sample it was pulled out at the same rate and the force at zero immersion determined using an x-y recorder (Western Graphtec WX1000). One axis recording the force while the second records the vertical displacement of the sample (using a LVDT - linear variable displacement transducer). A leveled cathetometer (Gaertner) was used to calibrate the LVDT and determine the translational speed of the movement. This was found to be 2.7mm/min. The surface tension of the water used in the study was 72.2 dynes/cm in good agreement with the results of others.

4.3.3.2. pH Determination Method

The pH of the raw material was determined due to problems during reaction in which the stannic chloride (a Lewis acid) was apparently being deactivated. Since HPMC is frequently prepared by reaction in alkaline slurry, it was believed that incomplete removal of alkali resulted in the deactivation. pH was also determined after reaction to check on complete removal of the stannic chloride during work-up of the HM-HPMC. ASTM D 2363-79 was used as a guide for the determination of pH [187] in HPMC. A 2% solution of HPMC in water was used for this determination. Enough sample was weighed out so a corrected weight (dried) would be 0.5 ±0.01 gms. This sample was placed in a 25 ml Erlenmeyer flask (with a ground glass stopper) and 24.5 gms of hot water (deionized/distilled, >1MΩ resistance) (85-90°C) added. The sample was then agitated on an orbiting shaker until fully dissolved (overnight). The hot water disperses the polymer which dissolves at a lower temperature. As noted, HPMC has a lower critical solution temperature

(LCST) in water. This means that the polymer will precipitate when the temperature is raised above the LCST, which is approximately 65°C. If cold water is added to the dry HPMC sample then it will form a gel which will take some time to dissolve due to chain entanglement. The pH was determined using an Orion Digital Ionalyzer (model 601A) calibrated according to the manufacturer's instructions.

4.3.3.3. Viscosity Measurement Methods

In all viscometric determinations Ubbelohde viscometers were used. For intrinsic viscosity the flow time for water was about 90 seconds. Intrinsic viscosities were determined for native HPMC, HPMC control (no glycidyl ether in reaction pot), and HPMC reacted with LP_4GE. Low concentrations of these polymers in water were prepared by dissolving known amounts of polymer in 25 ml volumetric flasks. A small sample of the polymer used to make each solution was also weighed into tared weighing bottles and dried in a vacumn oven for several days at 80°C. The true weight of polymer in each solution was then calculated. We preferred this to drying the polymer and then weighing it (as recommended in ASTM protocols) since the polymer absorbs water from the atmosphere rapidly making determination of actual weight difficult.

The viscometer was acid cleaned using Nochromix solution followed by liberal washing with deionized/filtered water. The temperature of the viscometer was controlled to 25±0.02°C and each sample was allowed to equilibrate to the viscometer temperature for at least 30 minutes. The efflux time was determined 5 to 10 times (depending on variability). Samples were kept in the refrigerator overnight (3°C) to promote deaggregation. On the day of the experiment the samples were each allowed to remain in the bath for 2 hours, then filtered using 1.2μm glass filters in a syringe filter housing. Care was taken not to entrain air into the samples when charging the viscometer.

Relative viscosity measurements were determined using a similar technique with the exception that the samples were not filtered (some were very viscous). The Ubbelohde viscometer was changed to a larger bore to reduce efflux times.

4.3.3.4. GPC Measurement Methods

Aqueous phase GPC measurements were taken using a Water's 6000A pump, a Water's U6K sample injector, and a Water's R401 differential refractometer. We used a Water's Ultrahydrogel 1000 column in series with filter and guard columns. For low temperature studies we used an Alltech column jacket hooked up to a Precision Scientific refrigerated bath.

PEO molecular weight standards used, were a product of Toyo Soda Manuf. Co., Ltd. They were provided with calibration data comparing light scattering, GPC and intrinsic viscosity measurements. The water (deionized/distilled) was vacumn filtered using 0.45μm filters to remove particulates and de-gas the solvent. In one case we used isotonic Sorenson's buffer to see what effect this had on HPMC elution. All samples were filtered using 1.0μm Teflon filters in a syringe holder. For the low temperature work the samples were stored overnight at 3°C to promote deaggregation. The plate count of the column was determined at the conditions that the column was operated at (the manufacturer recommended a different setting). This was performed each day using ethylene glycol.

4.3.3.5. Cloud Point Curves for HPMC and HM-HPMC Methods

Cloud points were determined using a Beckman DB Spectrophotometer (set to 600 nm) which had water circulated through it from a Precision Scientific water bath. The water bath temperature was ramped by heating at constant wattage. This arrangement provided excellent temperature linearity up to about 60°C. Above this temperature the rate decreased a little. Polystyrene cuvettes were used for the sample and the reference. Both were sealed using Parafilm®. A copper-constantan thermocouple was placed in the reference side to provide temperature rise data. The analog output from the spectrophotometer and the temperature was displayed on a x-y recorder (Western Graphtec WX1000). The effect of polymer concentration and attachment of hydrophobic ligand were examined. In addition, the effect of sodium chloride on the cloud point was also determined.

4.4. Results of HM-HPMC Synthesis

4.4.1. Glycidyl Ether Synthesis Results

The primary product from the cholesterol reaction with ECH was shown to be correct using NMR in deuterated chloroform. The appearance of peaks at $\delta \sim 3.5$ when compared to cholesterol indicate that protons are adjacent to chlorines on this intermediate. The final yield was about 60% (estimated from TLC, and HPLC) after reaction with NaOH. This value is about the same as that for production of polyglycidyl ethers using a similar method [188], and about three times that for the synthesis of palmityl glycidyl ether [124]. Similar yields were obtained for OGE, ODGE, and LP_4GE. Since purification is the most time consuming step in the synthesis of the glycidyl ether of alcohols, we prepared our own ODGE as well (this Polysciences product was less than about 70% pure).

4.4.2. Hydrophobic Affinity Polymer Synthesis Results

Analysis of the methoxyl and hydroxypropoxyl content of the native polymer worked well. MeI chromatographed as a single peak at 1.05 minutes. Isopropyl iodide was incompletely separated as a large peak with a small leading shoulder at 2.44 minutes. This was seen for both the standard and the cleaved HPMC samples and may be due to the iodine occupying either the 1 or 2 position. When the integrator separated these into two peaks they were summed for analysis. Finally, toluene and xylene were chromatographed at 4.95 and 12.5 minutes respectively. It was found the *ultrapure* xylene was only 92% with impurities at 10.18, 14.7 and 15.65 min. These peaks did not interfere with the analysis.

For the two lots used in the study it was found that the methoxyl content was the same (within the precision of the experiment). The attachment was 28.1±1.9 wt%. This is very close to that specified by Aldrich (30%). The hydroxypropoxyl content was significantly different between the two lots however. Aldrich had specified 10% attachment. We found that only one lot was close

with 9.55±0.27 wt% while the lot used to prepare most of our samples was only 7.08±0.11 wt%. No attempt was made to see if these differences had a significant effect on amphiphile interaction.

According to Dow literature these values result in a DS for methoxyl of 1.9. This means that almost two of the three available hydroxyls on the cellulose saccharide are capped by methoxyls. A 10% hydroxypropoxyl content results in a MS of 0.25. The HPMC used in our study is somewhat less then this. Since methoxyl substitution patterns on the cellulose chain have been shown to be heterogeneous by other we would expect that glycidyl ether attachment would reflect this. Each hydroxyl capped by a methyl being unavailable for reaction with glycidyl ether. We did not attempt to examine this however heterogeneity is probably important in the aggregation and surface active behavior of the HM-HPMC.

Recall that Landoll had used HEC for the production of HM-HEC. This has a distinct advantage over HPMC in terms of reactivity since it will contain primary hydroxyls where the hydroxyethyl has attached. HPMC will probably only have secondary hydroxyls which are known to be less reactive with glycidyl ethers. The PGE reaction with HPMC provided surprising results with this in mind.

The DS of PGE attached to HPMC was determined for two ratios of stannic chloride to PGE. For the amounts used, 100% reaction would result in 1 out of 8 saccharides on the polymer chain conjugated with phenoxyl. A plot of percent reaction against time is presented in Fig. 15. We note a strong dependence on the quantity of tin catalyst. The apparent decrease in percent reaction for reaction I is probably an artifact since at long times of reaction this product was not completely soluble in the SDS solvent system used to measure the extent of reaction (by U.V. absorbance). A possible error to this method might be polymerization of PGE which has been reported in sealed tube reactions to produce polymers which are insoluble in common solvents [189]. Noshay et al. reported that this polymer was only soluble in *hot* monomer, cyclohexanone, dimethyl formamide and o-dichlorobenzene [190]. This would make it difficult to remove the PGE which polymerized

from that conjugated to HPMC using Soxhlet extraction. This dependence on stannic chloride concentration may be a result of either incomplete drying or residual alkali (from the manufacturer). The pH of a 2% solution of HPMC was found to be 8.52 indicating that the latter is at least partially responsible.

The reaction of ODGE, OGE and LP_4GE with HPMC were completed as well. Fig. 16 shows the attachment of these glycidyl ethers with HPMC plotted against the quantity of the glycidyl ether in the reaction pot. We note that the attachments were quite low (about a tenth). This result is not too surprising when compared to the results of others. Landoll obtained the same attachment when using an alkaline slurry of HEC in isopropyl alcohol; reacting 1,2 straight chain epoxides (rather than glycidyl ethers). As noted in section 4.1.2 the reaction of glycidyl ethers with saccharides is generally found to be low. The difference between the PGE reaction (nearly 100%) and these glycidyl ethers may reflect steric hindrance by the long chain ligands to penetrate into the area of hydroxyls on the HPMC especially when stannic chloride must be present to catalyze the reaction

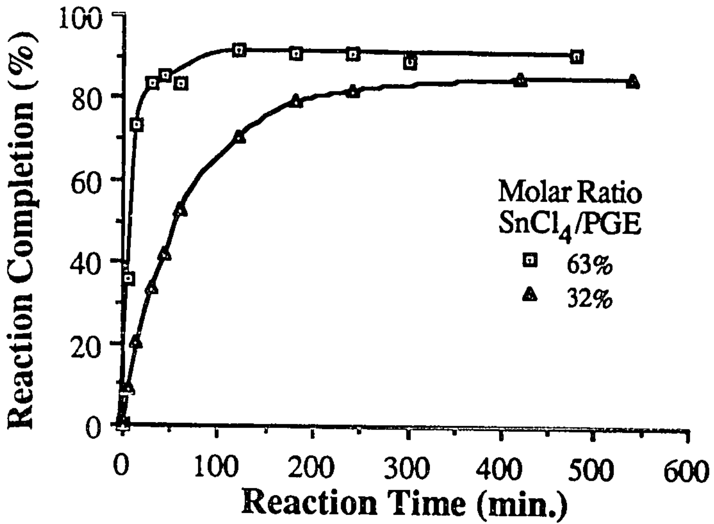


Fig. 15 - Percent Reaction of PGE with HPMC vs Time.
100% reaction equals 1/8 PGE/saccharides (10%). Note dependence on tin catalyst, with catalyst being used at high levels.

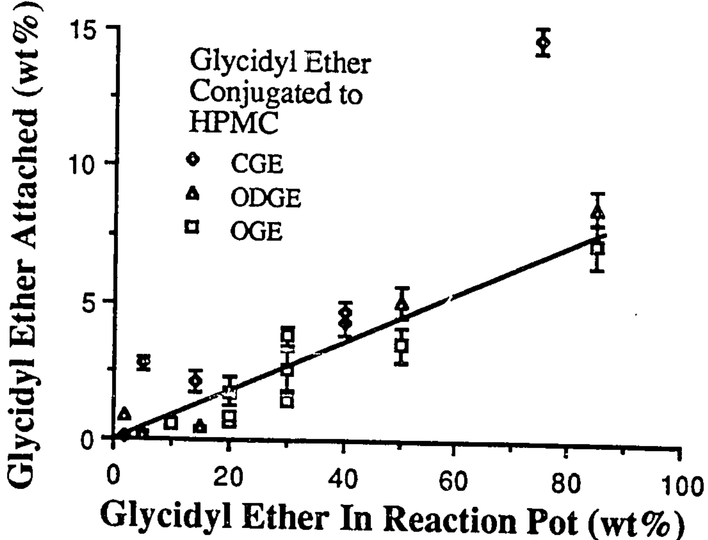


Fig. 16 - Attachment of OGE, ODGE, and CGE to HPMC.
With the exception of one outlier the points are all very close to the same attachment. A least squares fit to the data (neglecting the CGE outlier) is: *wt% attached) = (0.088±0.008)(reacted wt%) - 0.03± 0.3, r^2=0.87

(this is a large molecule). The Zeisel cleavage method of determining the attachment of the glycidyl ethers was quite sensitive when one considers the low attachment levels. As an aside, we found that the reaction of the glycidyl ethers of cholesterol, octadecyl, and oleyl alcohol reacted with Sepharose® beads at higher attachments. The low number and secondary nature of hydroxyls on HPMC apparently resulting in lower conjugation levels.

The method of creating the iodo substituted alcohols of cholesteryl and oleyl and using them as standards, was validated by comparing the chromatograms of 1-iodododecane and 1-iodooctadecane with dodecanol and octadecanol. It was found the the alcohols were quantitatively (1:1) converted when molecular weight and purity corrections were applied. Cholesterol was apparently cleaved into several products which came off the column well before cholesteryl iodide (Sigma Chemical Co.) and cholesterol. No attempts were made to determine what these products were since the chromatograms revealed quantitative and reproducible conversion. We added all of

these peaks together when calculating the attachment of CGE. In addition, it was found that hydroiodination of the oleyl alcohol (and OGE attached to HPMC) produced about 5% 1-iodooctadecane and several other unidentified products. These were not considered in evaluating the weight percent attachment of OGE. Table 9 shows the RF values for the samples and comments on their calculation.

The GC analysis of the ^{14}C-OGE conjugated HPMC was validated by analysis of the Zeisel cleavage products using GC and scintillation counting of the xylene phase of cleaved products and OGE-HPMC. LSC of the xylene phase revealed that attachment was 2.87 wt%, while the finely divided SDS suspended OGE-HPMC (not cleaved off) was 2.75 wt%. These values indicate that GC analysis of cleaved ethers does provide reproducible and quantitative evaluation of alcoholic glycidyl ether conjugation levels. The water suspended OGE-HPMC produced results which were about 5% less than the SDS suspended OGE-HPMC. This was probably due to their large aggregate size in the scintillation cocktail gel quenching the emission. By assuming that the molecular weight of the HPMC is 86,000 and that the molecular weight of a saccharide unit is 192 (Dow Methocel) it is possible to generate theoretical plots of the number of glycidyl ethers attached to the polymer (Figures 17 and 18). Note that the number of saccharide units per polymer is 450 for these assumptions. These calculations assume an ideal mono-disperse molecular weight of the polymer. As we shall see the polymer molecular weight is quite broad.

Table 9 - RF Values for Ziesel Cleavage of Alcohols
(relative to 1-iodohexadecane ~1mg/cc)

Alcohol	RF	Comments
LP_4	1.4	(corrected from I-dodecane peak for LP_4GE)
Octadecanol	2.11	(corrected for ODGE attachment)
Oleyl Alcohol	4.25	(only 1,9 diodooctadecane peak for OGE)†
Cholesterol	2.58	(all peaks added together for CGE)

† - calculated from ^{14}C-OGE/HPMC results

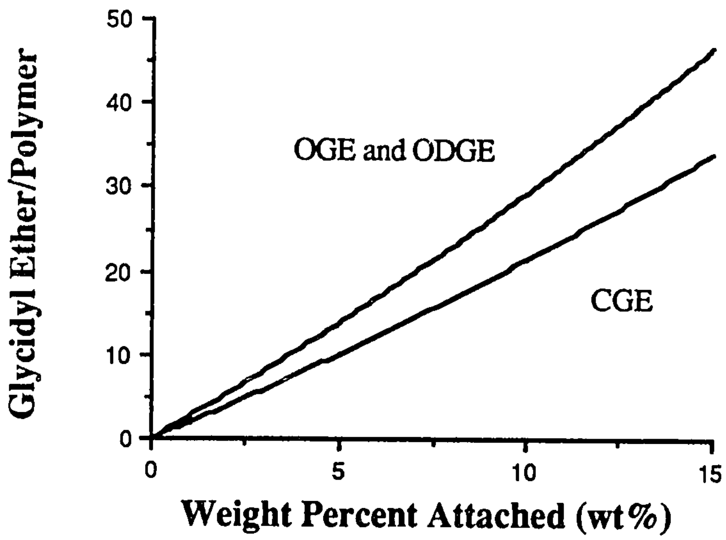


Fig. 17 - Theoretical Glycidyl Ether's per Polymer Chain. (86,000 molecular weight assumed)

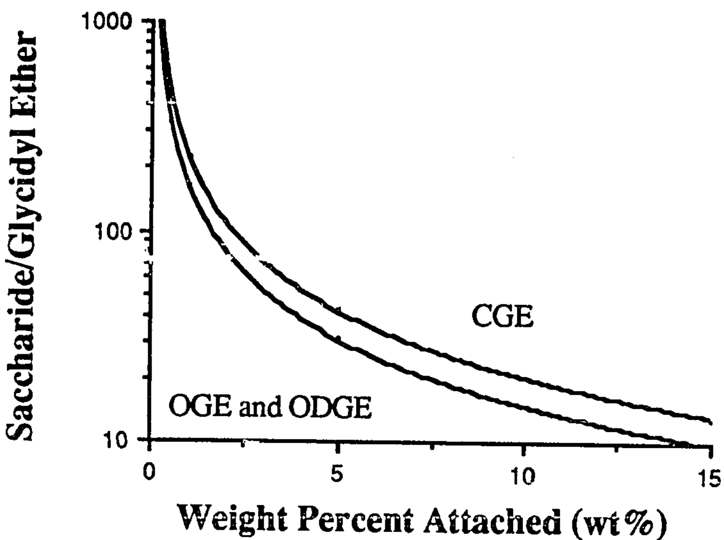


Fig. 18 - Theoretical Saccharide Units per Glycidyl Ether. (molecular weight of saccharide unit assumed to be 192)

4.4.3. Characterization of Water Soluble Polymers Results

As the weight percent of ODGE (we will consider this to be an example for all glycidyl ethers attached) is increased the modified polymer exhibits increased viscosity in water, with a clear solution. At 0.22 wt% attachment the modified polymer forms a very viscous thixotropic 1% solution. Finally, when ODGE was attached in proportions greater than about 0.44 wt% the resulting polymer wets well in water but does not appear to go into solution. We have found that it makes an opaque gel which can be easily broken-up with agitation. These results are quite similar to those from Landoll's work at Hercules [136] on HM-HEC. As an added note: if the attachment of hydrophobe is not too high then surfactant solutions will dissolve the polymer. Steiner (again of Hercules) has found this be true for HM-HEC [137].

The identity of the ligand has an effect on the dissolution of HM-HPMC in detergent solutions. We found that when octadecyl and oleyl were attached to the polymer in quantities less then about 5 wt% then 100mM NaTC (a bile salt) would dissolve them. For cholesteryl conjugated to the polymer attachment had to be less then about 2% for this to occur.

The surface tension of a 0.1% aqueous solution of HPMC was found to be 53±1 dynes/cm (water was 72.2 dynes/cm) indicating that the native polymer is somewhat surface active (it reduces the surface tension of water moderately). This quantity is near the upper end of values reported in the literature for HPMC (44-56 dynes/cm) and may suggest that the heterogeneity of substitution is of an intermediate nature. Recall that Sarkar attributed high surface activity to both small and large blocks of substitution while lower surface activity was ascribed to blocks of alkoxyl substitution being in between the two extremes. High surface tension indicates that the polymer does not alter the air/water interface much and is not very surface active. It may also indicate that the polymer forms stable aggregates in solution with hydrophobic moieties preferring this environment to the air/water interface. The heterogeneity of methoxyl substitution directly affects that for glycidyl ether substitution. As noted, the effect of this on amphiphile interaction and aggregation in aqueous

solution was not investigated.

4.4.3.1. Viscometric Analysis Results

The effect of LP_4GE attachment on relative viscosity (η_{water} = 1.0) is presented in Fig. 19. We note that between 0.5 and 0.7% attachment this quantity take a considerable jump. Landoll observed a similar change for HM-HEC. For C_{12} HEC this was found to be ~1.8% using 2% solution's in a Brookfield spinning viscometer. We observed a value considerably less then this (~0.6 wt% attachment) using Ubbelohde viscometry of 0.5% solutions. The higher hydrophobic character of HPMC, when compared to HEC, may result in this difference. This viscosity jump probably indicates enhanced polymer-polymer interactions. Beyond 0.85% ligand the polymer forms a gel and does not go into solution. This is represented in the figure as a dashed line. It appears that the ligand is acting like a crosslinker between polymer chains. The LCST phase transition for cellulose ethers (e.g. HPMC or MeC) has been described as cross-links by micelles (Section 4.1.3)

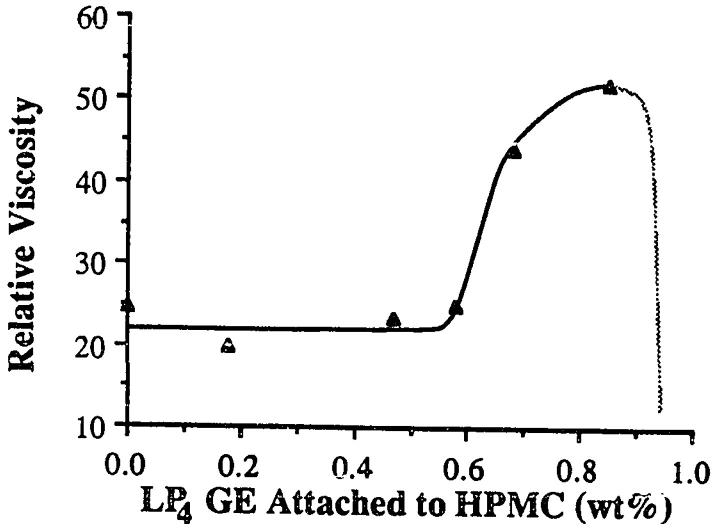


Fig. 19 - Effect of LP_4 Ligand Attachment on Relative Viscosity.
(η_o, Water = 1.0, 0.5% solution using Ubbelohde viscometry)
The sharp drop above 0.85% attachment indicates precipitation of the polymer.

[165]. By attaching long chain lipids we should be enhancing this property. If the 0.85% ligand polymer is placed in solution at a higher concentration then it forms a clear gel which is very thixotropic (it has a "yield value"). Since the "crosslinks" are not covalent in nature they can be easily disrupted by a shearing force (the gel is solid until sufficient shear is applied then it flows). This break point for viscosity (between 0.5 and 0.7% attachment) might represent a critical ligand concentration analogous to that observed by Strauss for HM-polyelectrolytes [171]. He observed from solubilization studies that a critical concentration of conjugated lipid produces a poly-soap which solvates oils. We have found that while HPMC interacts with amphiphiles (next chapter) it does not solubilize large amounts of a water insoluble dye (sudan black) until sufficient lipid is attached to precipitate the polymer.

Intrinsic viscometric examinations were used to study the effect of ligand on the molecular size. By definition, the intrinsic viscosity ($[\eta]$) assumes that polymer/polymer interactions are at a minimum (the viscosity is extrapolated to zero concentration). In an early paper, Landoll (at Hercules) stated that the reaction conditions used to produce the HM-HEC resulted in very little change of the molecular weight of the polymer as determined by the intrinsic viscosity [136]. We were surprised to see this since a reduced intrinsic viscosity would be expected by the addition of lipid. Our theory was that the added lipid would cause the polymer to curl-up on itself to "hide" the ligand from aqueous solution. This structure would support our proposed cholesterol binding polymer. We undertook a study to examine this using LP_4-HPMC. Using a range of ligand attachment (0 to 0.85%) we determined the intrinsic viscosity and found that there was a decrease followed by a rise at high lipid conjugation. The decrease would be described by our theory while the increase may indicate that even at very low concentrations there is aggregation of the polymer.

Fig. 20 shows the reduced viscosity ($\eta_{sp/c}$) plotted against concentration of polymer in solution. We see that for concentrations above about 0.1 gm/dl the linear relationship does not hold. There is apparently some inter-polymer interactions since the highest ligand attachment shows an

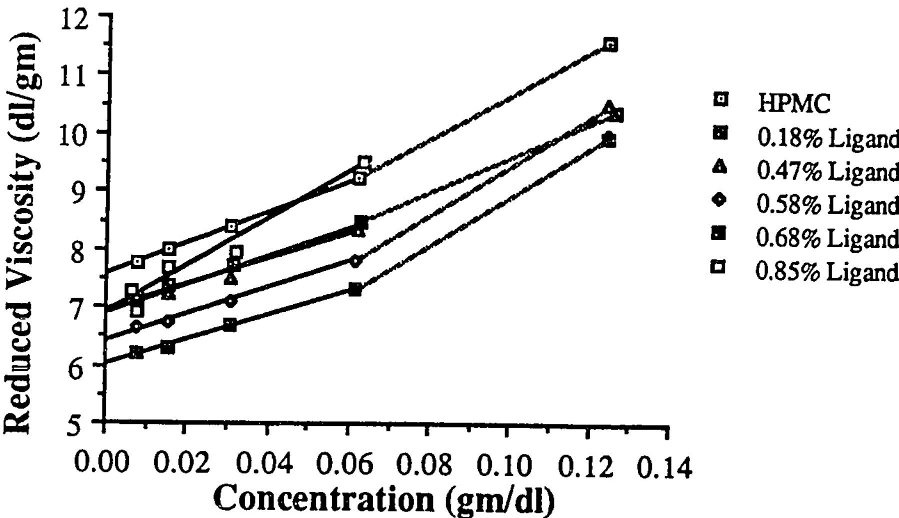


Fig. 20 - Effect of LP_4GE Conjugation on Reduced Viscosity

increasing reduced viscosity at 0.125 gm/dl. We note the 0.85% ligand attachment shows much different behavior than the other polymers. We had some difficulty in obtaining reproducible viscosities for this sample. After filtering, the sample showed a decrease in viscosity leading us to believe that the polymer was aggregating. We may have selectively removed some of the sample in the filter. It was also found that the viscosities had a high error. Over the course of the experiment they changed (irreproducibly). We feel that this indicates aggregation which formed while the experiment was taking place. These aggregates may have been broken-up to some irreproducible degree during the time when the sample is being sucked into the flow reservoir (by shearing forces). This point should be regarded with some scepticism for these reasons.

With the exception of the 0.85% ligand sample we found that the samples obeyed the Huggin's equation:

$$\frac{\eta_{sp}}{C} = [\eta] + k_1[\eta]^2C$$

η_{sp} = specific viscosity at C

C = Concentration (gm/dl)

$[\eta]$ = intrinsic viscosity

k_1 = Huggin's Constant

These values can be found in Table 10. For 0.85% ligand the sample showed high error for both $[\eta]$ and k_1. This may be due to aggregate formation as previously mentioned. It is well known that a Huggin's constant of about 0.3 indicates a polymer in a "good" solvent. A Huggin's constant of 0.7 indicates that the solvent is "poor" and represents the theta condition. The values indicate that the Huggin's constant rises slowly for the polymer samples until the 0.85% ligand sample is reached.

The intrinsic viscosities have been plotted against ligand attachment in Fig. 21. We see that the intrinsic viscosity of native polymer is near 7.5 dl/gm. This value is in excellent agreement with values in the literature for MeC with a 2% viscosity of 4,000 cP (the same as the HPMC used in our study). Studies on MeC indicate that this value corresponds to a MW of 86,000. Apparently, this relationship was used to yield the MW value reported by Aldrich. The control HPMC was a little higher (shown as Control in the figure). This may indicate that the dialysis step during purification has washed out lower molecular weights.

Table 10 - Intrinsic Viscosities and Huggin's Constants for LP_4GE-HPMC.

Ligand Concentration	$[\eta]$	Huggin's Constant
0.0%	7.55±0.01	0.39±0.06
0.18%	6.97±0.02	0.40±0.05
0.47%	6.91±0.09	0.43±0.16
0.58%	6.44±0.03	0.47±0.10
0.68%	6.08±0.02	0.48±0.12
0.85%	6.95±0.17	0.69±0.46

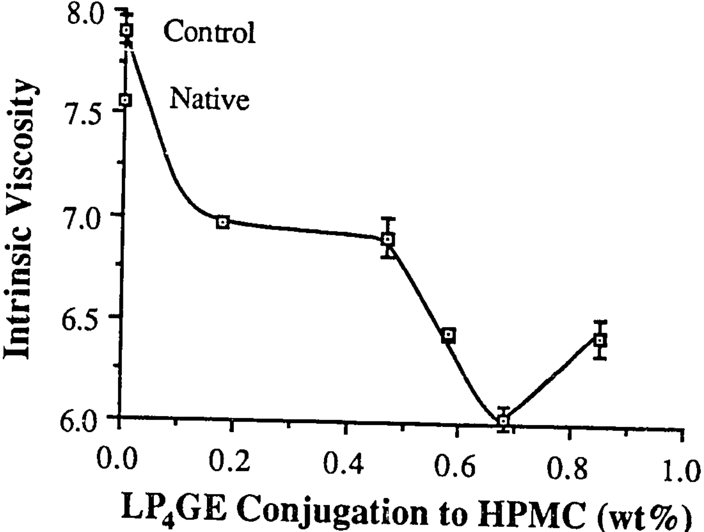


Fig. 21 - Effect of LP_4 Lipid Conjugation on Intrinsic Viscosity
Native: HPMC as purchased, Control: HPMC reacted as per other samples w/o ligand.

Two weeks after this study was completed a paper by Gelman and Barth (again of Hercules) appeared [191]. They examined HM-HEC and found similar results. These workers took the experiment one step further and added either sodium oleate or methanol to disrupt intra- and intermolecular associations. This "normalized" the data making all samples yield very similar Huggin's constants. This suggested that the amphiphile or methanol acts to alter the environment of the HM-HEC so it resides in a "good" solvent.

4.4.3.2. GPC Analysis Results

Initially, we wanted to confirm the intrinsic viscosity results using polymers that had ligand attached. Using a column (Ultrahydrogel 1000) which excludes PEO molecular weights greater than about one million it was found that our polymer was for the most part excluded. This indicates that the polymer molecule is not able to enter pores of a radius less than ~0.1 micron. Assuming that the end to end length of a saccharide "monomer" is about 0.42 nm then a polymer having 450 saccharide units (assuming that the Aldrich reported M.W. is correct) would have a straight end to

end length of about 0.19 μm. Since we wouldn't expect the HPMC chain to assume a perfectly linear orientation and be excluded from 0.1μm pores, we were surprised by the total exclusion of the polymer by the column. The column showed good linearity when the log of the PEO MW standards was plotted against elution volume for 10^6 to 10^4 MW.

It seemed that some type of polymer chain aggregation was occurring. Neely has found this to be true for MeC at room temperature [192]. He also reported that refrigerated samples deaggregate. It is believed that at lower temperature the polymer chains have lower mobility which decreases the probability of polymer/polymer contact. To examine this we refrigerated the polymer samples overnight and ran the column at 10°C (the lower limit as recommended by the manufacturer). We found that the polymer was separated by the column and that the apparent peak molecular weight (relative to PEO at the same temperature) was about a million. We also observed a very long tail extending into quite low molecular weights. This lower molecular weight constituent may be removed in dialysis and agrees with the rise of intrinsic viscosity seen for control HPMC in Fig. 21. We are not certain whether the polymer has been completely deaggregated at this temperature, however the knowledge that the polymer is probably aggregated has proven useful in the analysis of data from the experiments examining the interaction of SDS with HPMC. The efficiency of the column decreased at lower temperature from a little over 10,000 plates (room temperature) to about 9,000 plates (10°C). When the eluting phase was changed from deionized water to Sorenson's buffer pH 7.35 at room temperature the sample eluted in a very similar fashion as the low temperature study. The presence of the salt apparently either reducing the aggregate formation or causing the polymer to be somewhat hydrophobically adsorbed by the column.

Fig. 22 presents a mechanism consistent with the results obtained from viscometry and GPC experiments. The concentration of polymer in solution is schematically shown plotted against the attachment of LP_4GE to HPMC. For low polymer concentrations the polymer/polymer interactions

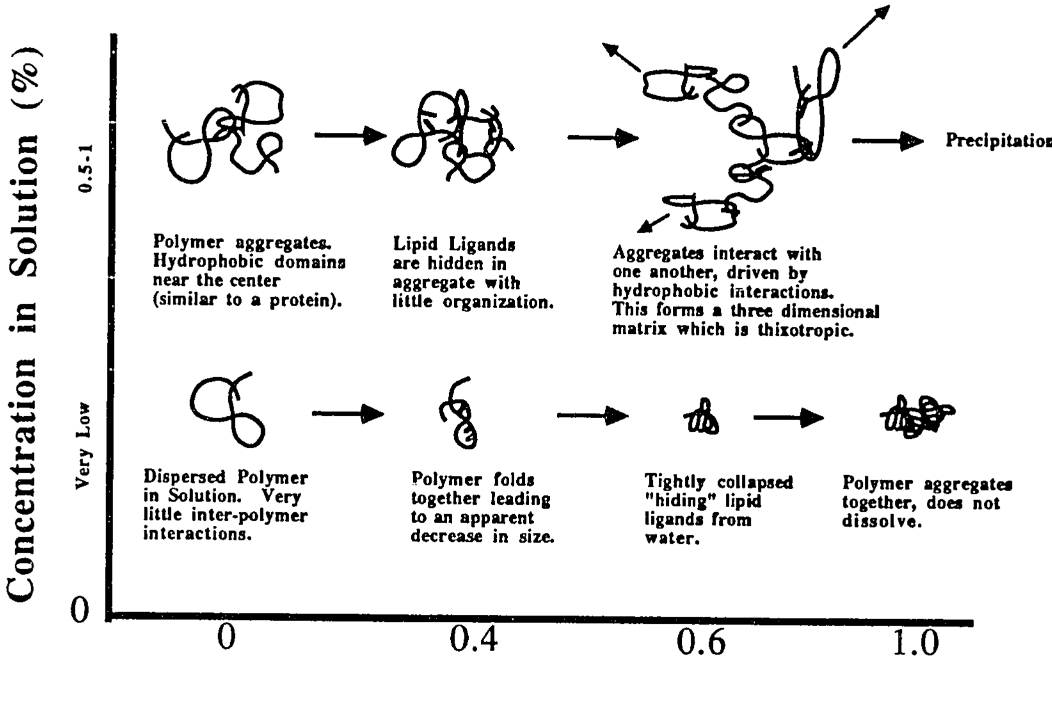


Fig. 22 - Effect of LP_4GE Attachment on HM-HPMC in Solution.

are assumed to be negligible (intrinsic viscosity) and the effects of intra-polymer interactions are observed. We see that as lipid/ligand is attached to HPMC the polymer collapses hiding the lipid from aqueous solution until enough ligand is attached to precipitate the polymer. When the concentration of polymer is higher, inter-polymer complexation occurs. This leads to aggregation of both the native polymer and HM-HPMC. These aggregates are stable enough that they are not separated using GPC until lower temperatures are used. The lower temperature deaggregates the polymer. As ligand is attached the aggregation process progresses, and the complex becomes more stable until a three-dimensional network is formed. The gel is easily broken due to the weak forces of HI. At lipid conjugation of 1% or above the gels collapse into a water wettable opaque mass which is easily dispersed in solution.

In conclusion, the effect of lipid conjugation to HPMC on aqueous solution behavior is dependent on the concentration of HM-HPMC. At very low concentration the polymer collapses onto itself as more lipid is attached. At a more physiologically relevant concentration the polymers ability to aggregate is enhanced until a three-dimensional network is formed. If still more lipid is conjugated to HPMC the polymer will not "dissolve" in solution - the hydrophobicity overcomes the hydrophilic component. The author is aware that a good definition of "dissolved" in solution would imply a lack of aggregation. I have used the operational definition of optically clear solution (aggregates of size less then the wavelength of white light) as indicating dissolution. Obviously, a true dissolved state is not present for HPMC in water at concentrations of 0.5-1% since the presence of aggregates was indicated by GPC data.

4.4.3.3. Cloud Point Measurement Results

The first experiment examined the cloud point change as LP_4GE was attached to HPMC. We found (as expected) that the addition of more hydrophobic groups to the polymer resulted in a material which precipitated at lower temperatures. Fig. 23 shows the raw data presented as percent light transmission plotted against temperature in the reference cell while Fig. 24 cross plots the IPT and CPT against lipid conjugation. Remarkably, as the attached lipid is increased the polymer precipitates from solution at first a decreasing temperature then at an elevated temperature. This indicates that the ligand at higher attachments may be interfering with the ability of the HPMC chains to aggregate and precipitate due to steric effects. Alternately, it is possible to argue that the polymer has assumed a different orientation in solution. We might hypothesize that it has formed a structure similar to the proposed "cholesterol sequestering molecule" (Fig. 7). The attached lipids forming a "micelle" which resists precipitation because they find this configuration favorable. For the polymer backbone to precipitate it would have to expose the lipids to aqueous solution as it unfolds to precipitate with neighboring polymers.

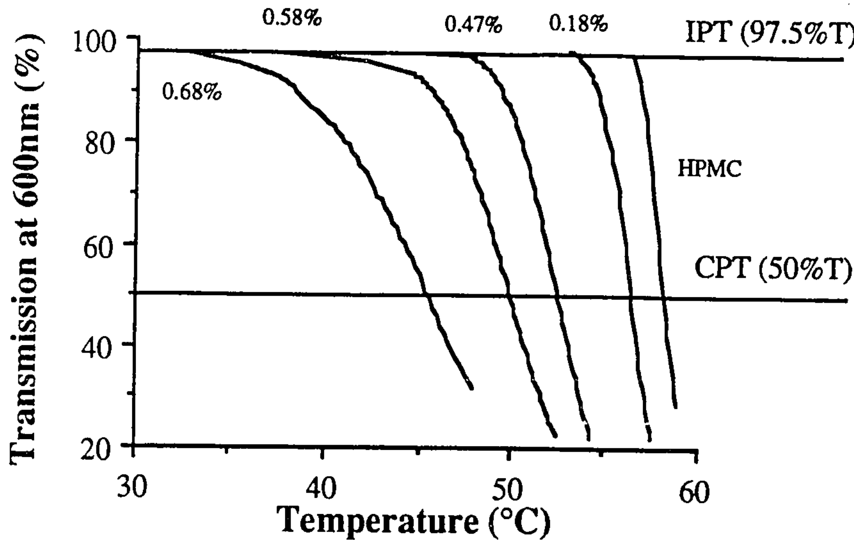


Fig. 23 - Effect of LP_4 Lipid Conjugation on Cloud Point Curves.
Showing the IPT and CPT definitions used for the following Fig. Note that HPMC precipitates very rapidly while 0.68% conjugated HPMC precipitates more slowly.

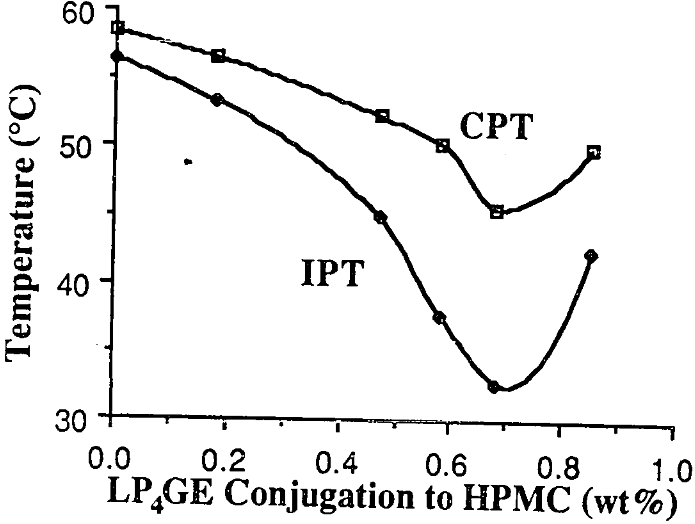


Fig. 24 - Effect of LP_4 Lipid Conjugation on Cloud Point (CPT) and Incipient Precipitation Temperature (IPT).

We find in the figure a broadening of the gap between the CPT and IPT until the minima is

observed. This indicates that the precipitation process is being influenced by the presence of attached lipids. Apparently, at intermediate lipid conjugation levels the temperature induced aggregation process is more heterogeneous. Some polymers coming out of solution at low temperatures while others precipitating at higher temperatures. This is probably due to heterogeneous lipid substitution to the backbone polymer. These results suggest an interesting experiment (that we did not perform). By using the cloud point precipitation to fractionate the polymer then analyzing the resulting fractions for molecular weight and ligand attachment we could determine to what extent actual ligand attachment influences the polymers LCST behavior. Obviously, this is a very complicated system where the hydrophobic regions aggregate at lower temperatures then the backbone polymer.

We found that the concentration of polymer in solution influences the cloud point as well. Fig. 25 shows the effect of concentration on CPT and IPT for HM-HPMC. We notice that polymer concentrations above 0.5% show little dependence of concentration on these values. Recall at low concentrations (<0.5%) we concluded that individual polymer molecules collapsed onto themselves resulting in decreased intrinsic viscosities. Individual polymer molecules folding onto themselves to "hide" the attached lipids from aqueous solution (as presented in Fig. 7). At higher concentrations (0.5 to 1%) the polymer interacts with neighboring molecules by forming cross-links by micelles. This results in a system that precipitates independent of concentration.

Fig. 26 shows the effect of sodium chloride on cloud point. We notice that at physiologic conditions (0.15M) the CPT and IPT have not decreased much. We also note that the spacing between the IPT and CPT does not vary much. In all cases the polymer precipitates rapidly (the light transmission falling quickly with respect to temperature).

Overall, we conclude that the attachment of lipid to HPMC results in the formation of hydrophobic domains that can either cause the polymer to collapse onto itself (intramolecular) at low concentrations or aggregate with neighboring polymer molecules (intermolecular association) at

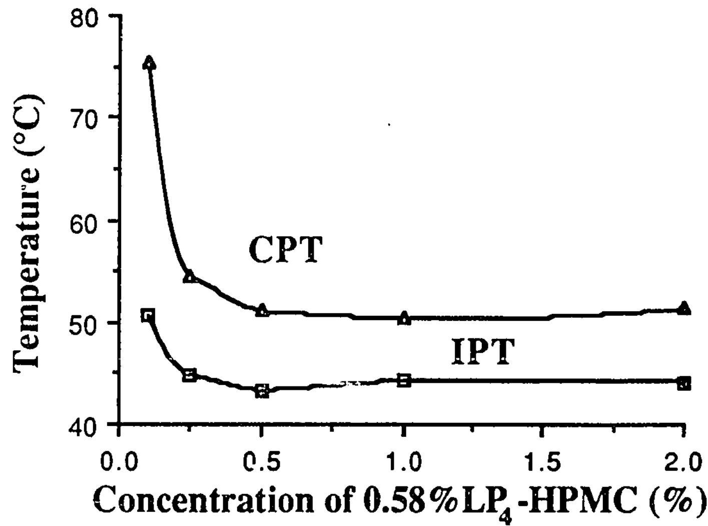


Fig. 25 - Effect of 0.58%LP_4GE/HPMC Concentration on CPT and IPT.

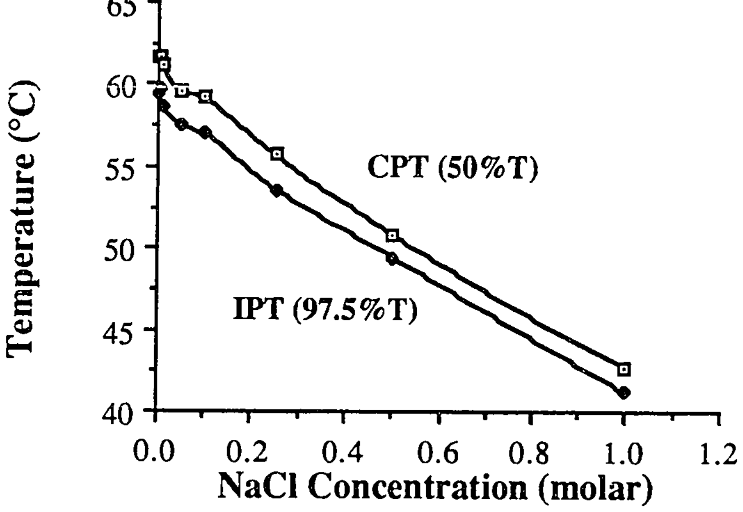


Fig. 26 - Effect of Sodium Chloride on HPMC CPT and IPT.

higher concentrations. This is important in several aspects of understanding the interaction of these polymers with amphiphiles, as presented in the next chapter.

CHAPTER 5

Interaction of Amphiphiles with HM-HPMC

The principle focus of this thesis is to examine the interaction of HM-HPMC with amphiphiles, particularly those present in the intestinal tract. Since much of the published results on the complexation of surfactants with polymers have been performed on commercially relevant detergents we desired to compare what interactions were present between HPMC and a commonly studied amphiphile. We choose SDS since it is one of the most studied anionic surfactants. We have examined the effect of SDS on HPMC solution character using viscometry, fluorescent probe λ_{max} shift and cloud points.

In contrast to the wealth of paper dealing with polymer/commercial detergent interactions, almost no results have been published on the mechanism of the interaction of principle detergents in the GI tract (bile salts and fatty acids) with polymers. Since the primary application of HM-HPMC for this study was its use in this environment we devoted considerable effort to find methods for examining this interaction. The simplest system utilized was the study of how single component micelles containing NaTC interacted with HPMC and HM-HPMC using equilibrium dialysis. We then graduated to examining how two component mixed micelles of NaTC and NaOl interacted with our polymers. Finally, mixed micelles containing the four primary components of mixed micelles (bile salts, fatty acids, lysolecithin (LL), and cholesterol) in the GI tract were studied to lend some relevance to the physiologic application. These studies were performed using equilibrium dialysis and solid phase extraction methods. We also examined using selected *in vivo* experiments the effect of HM-HPMC on fat absorption and showed that the material is tolerated by rats. As an introduction, the following section will review published results on amphiphile interactions with polymers.

5.1. Background on Amphiphile-Polymer Interactions

Recently, a comprehensive review was presented by E.D. Goddard of the Union Carbide Corporation regarding polymer-surfactant interactions [193,194]. In two parts he describes these interactions for uncharged water-soluble polymers and the interaction of poly-electrolytes with surfactants of opposite charge. A variety of methods have been utilized to examine this interaction. These include: surface tension, electrical conductivity, viscosity, electrophoresis, ultracentrifugation, cloud point elevation, solubility, gel filtration, adsorption on solids, surfactant binding studies, solubilization of hydrophobic dyes, fluorescent probes, electro-optics, nuclear magnetic resonance (NMR), small angle neutron scattering (SANS), calorimetry and others. Several general conclusions can be drawn by examination of this literature.

Studies on the interaction of nonionic polymers with surfactants indicate that the more hydrophobic the polymer the greater the interaction [195]. Qualitatively, the surfactant solubilizes the polymer. In addition to this phenomena, it is recognized that anionic surfactants interact more strongly with nonionic polymers than cationic or nonionic detergents. Nonionic surfactants may not interact because of steric hindrance (the "polar head group" of these detergents is usually a polymer). The differences between oppositely charged ionic surfactants is more interesting, since it may indicate some specific interactions between the charged head group and the nonionic polymer.

Several models have been proposed which attempt to explain how a charged surfactant complexes with a non-ionic polymer. Most of these models rely on a two step interaction process. Gilanyi and Wolfram have proposed that as surfactant is added to a polymer solution, no interaction is seen until a critical detergent concentration is reached [196]. This critical concentration is below that for the CMC of the detergent in solution without added polymer. Above this concentration, any added surfactant will go into polymer/surfactant complexes. After saturation of these sites (a second critical concentration) any added detergent will form free micelles in solution. This second critical concentration is higher than the CMC for the detergent in solutions without polymer present.

With regard to this thesis an exciting result has come from the Hercules work on hydrophobically modified hydroxyethyl cellulose (HM-HEC) monolayers at liquid/liquid and liquid/polymer interfaces. It was found that the adsorbed polymer greatly retards the rate of transport of small organic molecules across a liquid/polymer interface [197]. This work was done on polystyrene latex colloids. It was found that the surface coat of polymer slowed the entry of THF from aqueous solution into the "oily" colloid core. We might speculate that this diffusional barrier (caused by adsorbed HM-HEC) would also slow the diffusion of lipids out of a colloid into aqueous solution (as found in the gastrointestinal tract). In addition, the adsorption of a polymer on a lipid droplet would probably retard the activity of enzymes which process lipids (primarily triglycerides) to fatty acids.

In another paper, they concluded that these polymer monolayers can be regarded as insoluble coatings [198]. However, we can speculate that the interaction or SDS with MeC would also play a role. These results were obtained using stress-jump measurements at liquid/air and liquid/liquid interfaces. In these experiments the surface tension is measured as the surface area is suddenly compressed. Depending on relaxation of the surface it is possible to quantitate compliance of the surface to the stress jump. Prior to the Hercules work it was known that MeC adsorbs onto polystyrene latexes [199]. It was found that, in the presence of sodium lauryl sulfate this absorption decreases. This is presumably due to competition for the interface between the MeC and the detergent. These results confirm our expectations that these materials may be useful in trapping lipid emulsions in the intestinal tract by stabilizing the emulsion phase. Recall that one of our proposed reasons for activity of surface active polymers is their ability to reduce intestinal cholesterol absorption by coating the outside of lipid droplets (Section 2.4). The following subsections will review some of the literature on methods we used to examine the interaction of HM-HPMC with amphiphiles.

5.1.1. Rheological Examination of Polymer Detergent Interactions

Viscometric examination of polymeric solutions is an established practice. It can provide information on the extent of polymer-polymer interactions (which can either increase or decrease the viscosity) and the extent of molecule chain unfolding [200,201]. For this reason it is commonly used to investigate the interactions of polymers with amphiphiles. One of the most studied polymers is polyethylene oxide (PEO). Nagarajan and co-workers have completed much of this work. They found that as SDS is added to a solution containing PEO the viscosity increases to a maxima [201]. This was interpretated as indicating polymer molecule unfolding. Without surfactant present the polymer forms a spherical shape in solution. As SDS is added, the polymer chain unfolds as surfactant interacts with regions of the polymer. Ionic repulsion between charged detergent head groups driving chain extension. The resulting structure has been called a "pearl chain" with the polymer (chain) tying SDS micelles (pearls) together.

Lewis and Robinson have studied the interaction of SDS with methyl cellulose (MeC) and PVA [202]. It was determined that a critical concentration of SDS was necessary before any change in the viscosity of aqueous polymer solution was noted. Furthermore, they speculated that SDS caused deaggregation of polymers at room temperature.

5.1.2. GPC Analysis of Polymer Detergent Interactions.

Gel permeation chromatography (GPC) can provide information on molecular size of polymer surfactant complexes. It has not been used much to study the interactions of polymers with surfactants though. Sasaki *et al.* have examined the interaction of SDS with PEO and PVAc [203,204]. It was found that the SDS/PEO complex had a larger apparent molecular weight than the native polymer. This agrees with the viscometric determinations of Nagarajan (chain unfolding would increase molecular size). Szmerekova *et al.* have also studied these interactions [205].

5.1.3. Fluorescent Probe Analysis of Polymer Detergent Interactions.

We have done some limited work on the use of a fluorescent probe to examine the interaction of amphiphilic materials with our polymers. This represents a very interesting and informative method for examining HI. While the use of such probes to examine the structure of lipid membranes for the biological scientist is not new, their use for examining detergent/polymer interactions is fairly recent [206-211]. Fluorescence changes are used to measure the change in environment for the probe. Generally, some spectral change in fluorescence is seen if the probe enters a more hydrophobic area. It is assumed that at least some of the probes will fluoresce while inside micelles or aggregates, if these sites are present.

The probe used in our work was 3-carboxyaldehyde pyrene (CAP). The chemical structure can be found in Fig. 27. We note that this chemical is mostly lipid however it has the aldehyde group which lends some water solubility. Indeed, this probe is generally believed to occupy the surface region of micelles while pyrene resides in the "inner surface" [212]. Excitation of this probe with light of wavelength 360nm leads to a a blue-violet fluorescence the peak of which is found near 469nm (in water) [213]. It has been found that solvent polarity plays a role in the location of this maxima. A linear relationship between the wavelength of maximum emission (λ_{max}) and solvent dielectric constant has been found.

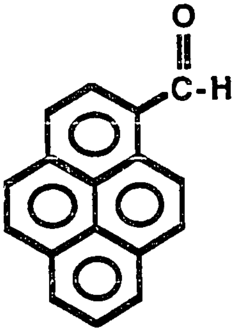


Fig. 27 - Chemical Structure of 3-Carboxyaldehyde Pyrene (CAP).

$\lambda_{max} = 0.52 \times \varepsilon + 431.5$

where:

ε = dielectric constant (from 10 to 75)

Brederick at al. believe this peak is a mixture of two transitional states [214]. For "non-activating" solvents (hydrocarbons) the quantum yield is very low while for "activating" (generally polar) solvents the compound fluoresces well.

Turro *et al.* have presented an idealized schematic of polymer surfactant interactions [209]. This is reproduced in Fig. 28 [209]. We see that two critical concentrations exist. At low detergent concentration no interaction exists (Region I). At a critical concentration of detergent, polymer/surfactant interactions occur which alter the fluorescence property being measured (assuming that the probe occupies this environment). Above a second critical concentration the probe resides for the most part in SDS micelles (region III).

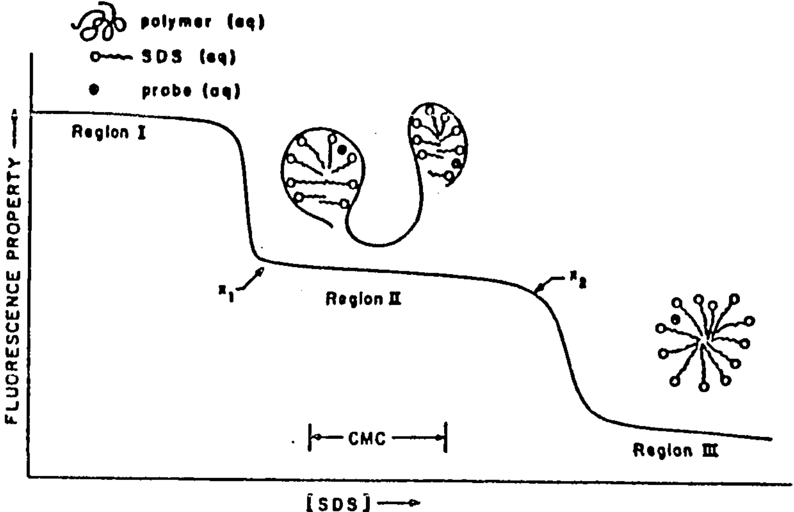


Fig. 28 - Idealized Schematic of Polymer Surfactant Interactions [209].
In Region I the water-soluble polymer, SDS monomers and probe are solubilized in aqueous phase and are not associated. Region II, at a concentration (below the CMC of SDS) association of SDS with the polymer begins at x_1.
By region III micelles (which exist in equilibrium with polymer/SDS complexes) become the predominant species.

5.1.4. Cloud Point Elevation by Surfactant Polymer Complexes

Since polymer surfactant complexes are primarily due to hydrophobic interactions between the detergent and polymer, the water solubility of polymers is enhanced by the presence of detergent. Elevated cloud points have been observed for PVA/PVAc, and PPO/PEO. We were unable to find any papers on cloud point elevation for detergent/cellulose ether complexes. Goddard however, states that the elevation of cloud point is well known for MeC/anionic detergent complexes [193]. It appears that he took the observations of MeC aggregate break-up by Lewis and Robinson [202] to indicate greater solubility of the polymer in solution. We found this to be an incorrect assumption for HPMC/SDS complexes.

5.1.5. Partitioning of Surfactants with Polymers

These tests yield adsorption isotherms for surfactant/polymer interactions. This represents the primary method we used to examine this interaction. Two possible methods exist: equilibrium dialysis, and solid phase adsorption (either chromatographic elution or filtration/centrifugation). We have used dialysis to examine the interaction of NaTC with both HPMC and HM-HPMC while solid phase adsorption has been used for mixed micelles similar to those found in the gastrointestinal tract. We found that the interaction was enhanced as lipid conjugation was increased. This also forms a polymer which is water insoluble.

5.1.5.1. Equilibrium Dialysis of Surfactant/Polymer Complexes

In equilibrium dialysis a polymer sample is either placed in a dialysis bag or on one side of a flat membrane with the surfactant to be studied. The bag is then placed in a solution containing the same concentration of surfactant. After sufficient time has elapsed (several days to weeks), the bag is opened and the concentration of detergent determined. This allows the quantitation of adsorption isotherms. These studies have been the primary means to confirm that polymers interact with detergents at two critical detergent concentrations.

A major source of error using this method is the development of ionic potentials across the membrane. To overcome this, either an electrolyte can be added to solution or the Donnan equation can be used. Some authors have ignored this [202] while others take it into account. Arai *et al.* used 0.1 N NaCl as an electrolyte presumably to overcome this source of error and provide relevance to the desired application [215]. Fishman and Eirich discovered that it was not correct to assume that ions bound to the polymer/surfactant complex did not contribute to ionic activities [216] when using the Donnan equation. It seems that counter-ion binding was not the same for the SDS/polymer complex as for SDS micelles. This makes the use of the Donnan equation difficult to interpret. We have used physiologic buffer to hopefully overcome this source of error.

5.1.5.2. Solid Phase Adsorption of Amphiphiles to Polymers

The easiest method to examine the polymer amphiphile interaction is to use solid phase affinity polymers. Binding isotherms can easily be generated by filtering or centrifugation of the polymer-detergent complex away from the supernatant. Using radiolabeled compounds it is possible to obtain data on small volumes. Since the majority of intestinal cholesterol binding food additives are insoluble this has been the method of choice for research in this area. Some of this is reviewed in Chapter 2.

5.1.6. The Use of Surface Tension for Measuring Polymer Surfactant Interactions

The use of surface tension as a probe of bulk polymer interactions with detergents is good if the polymer does not exhibit surface activity. For non-surface active polymers it can be assumed that the detergents surface activity is much higher than the polymers. It is assumed that the detergent bound to the polymer is not available at the surface. Thus the concentration of the surfactant bound to the polymer can then be determined by comparing samples containing polymer with controls. As the detergent interacts with HPMC (which is surface active) it would not be possible to predict what the complex might do to the surface tension. For this reason we have not

used this tool to examine HPMC/detergent interactions. We have used the measurement of surface tension as an indication of purity for the SDS used and to look at NaTC, and NaOl micelles.

5.2. Interactions of Amphiphiles with HM-HPMC - Methods.

We have studied the interaction of amphiphiles including: SDS, NaTC, NaOl, and mixed micelles of NaTC/NaOl/LL/Chol with HM-HPMC. We found that HPMC (native polymer) interacted strongly with NaTC and were interested in how this polymer compared to published results on SDS interactions with other polymers.

5.2.1. Viscometric Examination of HPMC Interactions with SDS - Method.

For this study we examined the effect of SDS on native HPMC. Samples were made-up using HPMC of varying concentrations in solutions containing SDS which was also varied. These were not filtered and were allowed to stand at room temperature overnight. Viscosities were determined using an Ubbelohde viscometer at 25°C using the same procedure as described in the previous Chapter. The SDS used in this study was electrophoresis quality (Biorad) and was recrystallized from 95% ethanol three times before use.

5.2.2. Fluorescent Probe Studies of HPMC Interactions with SDS - Methods.

The fluorescent probe used in our work was 3-carboxyaldehyde pyrene (CAP) and was purchased from Aldrich (>98% purity). It was purified by three recrystallizations from hot ethanol. 0.46 mgs of this material was placed in a 2 liter volumetric containing deionized/distilled water and stirred for two days. This resulted in a $<10^{-6}$M solution which was filtered through a 0.45μm cellulose acetate filter to remove undissolved CAP. All samples are made up using this water in scintillation vials which are stirred overnight at room temperature (using an orbital shaker). The fluorescence maxima was determined using a Perkin Elmer 650-10S spectrometer.

5.2.3. Cloud Point Curves for HPMC in SDS Solutions - Methods

The cloud point curves for HPMC in SDS solutions were determined using the same set-up as above. The concentration of SDS was altered at various HPMC concentrations.

5.2.4. Sequestration of Bile Acids and Lipids from Aqueous Solution

Very few studies have attempted to systematically examine the interaction between polymers which lower cholesterol absorption in the GI tract and bile salts or mixed bile salt micelles using binding isotherms. Most investigators have used only physiologic concentrations and frequently at only one concentration of probe. This is unfortunate since it precludes determining how the food additive binds the lipids.

5.2.4.1. Equilibrium Dialysis of NaTC and HM-HPMC - Methods.

These experiments represent the simplest system for quantitating the interaction of biologically relevant molecules with HM-HPMC. The amount of polymer is held constant while the concentration of NaTC ranged from 1mM to 100mM. This easily includes the physiologic concentration which is generally near 10mM in the duodenum [18].

The method used for the determination of NaTC equilibrium binding coefficients by HM-HPMC is based on the protocol provided by Spectrum Medical Industries for the use of their equilibrium dialyzers. The equilibrium dialyzer cell is made of Teflon with 1ml volume cell halves on each side. A schematic can be found in Fig. 29. The assembled cell sandwichs a cellulosic dialysis membrane using a spring arrangement that prevents leakage of the contents. The dialysis membranes were either: Spectra/Por 2 which has a M.W. cutoff of 12-14,000 or Spectra/Por 6 (6-8000 MWCO). The dry membranes were hydrated in deionized/distilled water for 1 hr with a change of water every 15 minutes. They were then soaked in 30% ethanol/water for an hour then with deionized/distilled water before equilibration (for 15 minutes) in the buffer solution. Isotonic Sorenson's phosphate buffer was prepared with a pH of 7.35. It was filtered through 0.45μm

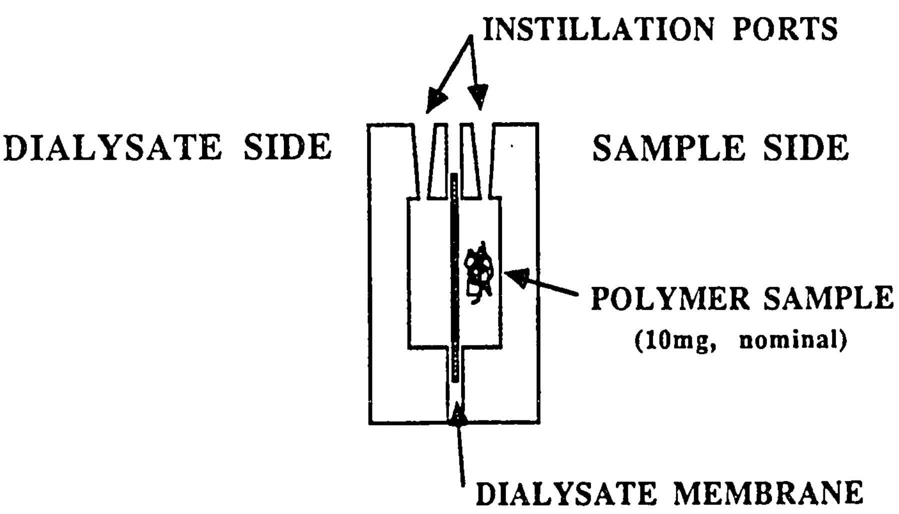


Fig. 29 - Equilibrium Dialysis Apparatus.
(Spectrum Medical Industries)
Initial: 1ml labeled NaTC solution added to both dialysate and sample sides.
Final: Dialysate side sampled after 48 hours 37°C, 20 rpm.

cellulose acetate syringe filters (Corning) which had been conditioned by rinsing with deionized/distilled water to remove most of the surfactants and/or wetting agents.

The cells were washed between each experiment using a mild detergent then rinsed with deionized water. They were dried in a vacumn oven at 60°C for about an hour. Care was taken during the washing and drying operation to prevent scratching the inside of the cell (which might cause a leak). To the male side of the disk nominally 10mgs of HM-HPMC was added and weighed to ±0.01mg using a Mettler H20 balance. It was found that the use of a static eliminator (Polonium 210, an α emitter, StaticMaster®) greatly facilitated the accurate weighing of the samples since the Teflon cells became statically charged easily. The instrument was waved over the Teflon cell half before weighing the polymer into it (kind of magical). The prepared membranes were blotted gently on a KimWipe® and placed over the male half of the cell (which contained the sample). The equilibrium dialysis system was assembled according to the manufacturers recommendation and 1ml

of dialysis solution added to each side of the cell using a glass syringe. Both the dialysate and sample sides were filled with the same concentration of binding probe. This was done to prevent a large change in sample volume which was observed when the dialysate side contained no probe (buffer only) at high concentrations of probe. The dialysis system was then allowed to rotate at 20 rpm for the duration of the experiment in a bath at 37°C.

After the prescribed time of dialysis (about 48 hours for 12-14,000 MWCO membranes) the contents of the dialysate and sample compartments were evacuated using clean glass syringes. Samples were then placed in preweighed scintillation vials. Either 0.5ml was placed into standard 20ml (Wheaton) vials or 0.25ml was placed in 5ml Solvent Saver® (Kimble) vials. Generally, two samples were taken from the dialysate side. This provided the necessary information to determine the binding character. Notes were also made as to whether the polymer had dissolved. It was found that at high concentration of surfactants (e.g. NaTC) the polymer was dissolved while at low levels of surfactant the polymers did not dissolve and formed swollen masses in the sample compartment. The samples were then re-weighed so the activity per gram dialysate solution could be used for subsequent calculations. Either 15ml for the standard vials or 5ml for the Minivials of Aquasol II (DuPont, NEN) was added to the vials which were stirred on a vortex mixer for 15 seconds. The outside of the vials were then wiped with a static eliminator cloth used for clothes drying and with an acetone/KimWipe immediately before placement in the liquid scintillation counter (Beckman LS7000). The samples were counted and the output ported directly to an Apple II+ computer using a serial interface.

It is well known that photoluminescence can spuriously alter scintillation counting results due to excitation by fluorescent lights [217]. This process can last for up to 20 minutes. To reduce this, the fluors were allowed to assume ground state by setting the scintillation counter to count a blank for 50 minutes after the cover of the scintillation counter was closed. Chemiluminescence errors were assumed to be negligible. Background and at least 4 samples of the native probe solution were

also counted. The activity of the samples was determined using quench curves which were either generated using Beckman quench standards or ^{14}C NaTC and Sorenson's buffer as quenching agent. This method is reviewed in the appendix. The activity (nCi/gm) was then determined using the weight of the sample.

The NaTC binding of the sample was calculated using the following method. Initially, the total amount of probe is easily determined by multiplying the concentration by the volume.

$$T = V_i C_i$$

where:

T = total probe $[\mu moles]$

V_i = volume of labeled probe $[ml]$

C_i = concentration of labeled probe $[mM]$

Since 1ml of the same probe solution is placed in each cell half the total amount is equal to twice the concentration. At the end of the experiment, the total amount of probe is given by:

$$T = V_D C_D + V_S C_S + B_P + B_D$$

where:

V_D = volume in dialysate side $[ml]$

V_S = volume in sample side $[ml]$

C_D = free probe concentration in dialysate side $[mM]$

C_S = free probe concentration in sample side $[mM]$

B_P = polymer bound probe $[\mu mole]$

B_D = dialysate appartus bound probe $[\mu mole]$

At equilibrium the concentration of free probe must be equal on both sides of the membrane. This will be the concentration of probe measured in the dialysate side.

$$C_D = C_S$$

By assuming that water bound to the polymer is negligible or that it has the same probe concentration as that in the free solution:

$$V_D + V_S = V_i$$

This was found in our labs not be the case for cross-linked polymers (HM-Sephadex®). We found that if the pore size of the polymer was less than that for the micelle then water was sorbed by the particle, while the micelles were excluded, resulting in an apparent increase of the NaTC concentration in solution. The total concentration can be expressed as:

$$V_iC_i = V_i + B_P + B_D$$

and the polymer bound probe is given by:

$$B_P = V_iC_i - V_iC_D - B_D$$

Finally, The amount bound to the dialysis apparatus is assumed to be negligible.

$$B_P = V_i\left[C_i - C_D\right]$$

It can be shown that the following equation also satisfies the assumptions listed.

$$B_P = \left[V_s + V_d\right]C_i\left[1 - \frac{C_d}{C_n}\right]$$

where:

B_P = NaTC bound to polymer [μmoles]

V_s = volume in sample side [1.0ml]

V_d = volume in dialysate side [1.0ml]

C_i = NaTC concentration in native solution [1–100mM]

C_d = probe concentration in dialysate side [nCi/gm]

C_n = probe concentration in native solution [nCi/gm]

Note that this equation is only valid for the system described with the same concentration of probe placed on both sides of the membrane initially. If the labeled probe is placed on only one side then the equation must be modified as per Smith and Jubiz [218,219].

5.2.4.2. Solid Phase Extraction of Amphiphiles by HM-HPMC - Methods.

For mixed micelles of NaTC/NaOl, we found that they did not cross the dialysis membranes used in the dialysis experiments. This is not surprising since the molecular weight is much larger than that for single component NaTC micelles. After 7 days at 37°C, equilibrium had not been reached for 50 mM (1:1) NaTC:NaOl when dialyzed with no polymer. When polymer was added to the system it was readily evident that equilibrium had not been reached (in this time period) as we observed negative binding which indicates that water was pulled into the polymer, thus raising the concentration of probe on the dialysate side which could not pass through the membrane. GPC-HPLC analysis of NaTC and NaTC/NaOl in distilled water (on the Water's Ultrahydrogel 1000 column) indicated a peak molecular weight of about 6,000 for NaTC while NaTC/NaOl had a peak molecular weight around 14,000. These values are relative to PEG and PEO molecular weight standards. As an aside, we found that when buffer was used as the mobile phase, the NaTC was adsorbed to the column and did not come off until well after the salt volume. Thus we did not use GPC for the analysis on the interactions of NaTC with HPMC as others have done on the interaction of SDS with polymers. To determine binding we used an easier technique (which precludes using solubilized polymers) in which nominally 15 mgs (weighed out to 0.01 mg) of polymer were placed in a vial and 3 ml of solution added. The vial lid was Teflon lined to reduce adsorption of the probe into the lid. Two general solution compositions were used. One contained a 1:1 molar ratio of NaTC and NaOl while the second contained a 1:1:0.15:0.06 molar ratio of NaTC to NaOl to lysolecithin (LL) and cholesterol (Chol). The latter composition is similar to that observed in the duodenum of humans.

The solutions were prepared by first weighing out the necessary components into a volumetric flask. Nominally 16nCi/ml of 24-^{14}C NaTC and 30nCi/ml of 9,10-^{3}H Oleic acid or 7-^{3}H cholesterol were included in the volumetric flasks. Ethanol was then added. This created a slurry of the dry materials (some of which dissolved) and mixed them together. After evaporation of the ethanol

(using a vacumn oven at 60°C for 2 hours) the volumetric was filled with isotonic Sorenson's buffer pH 7.35 which contained 0.1% sodium azide. The solution was then sonicated for one hour at room temperature. This produced a slightly hazy to clear solution. After 7 days incubation at 37°C in a stirring bath, 1 ml of solution was withdrawn and expelled through a 0.22μm syringe filter (to condition the filter). Next, the remainder of the liquid was withdrawn and forced through the same filter unit into a test tube. 0.5ml of this was then counted using a dual isotope counting procedure (Appendix). The quantity of each nuclide was corrected for quench and for adsorption to the filter assembly (this was found to be low). The latter was determined by using control solutions which were also incubated. Only OGE-HPMC was tested at different concentrations of these micelles. It was found that these samples leveled off in binding above the CMC of the system. Using 10mM NaTC (with other components having the same relation as noted above) we examined HPMC hydrophobically modified with CGE and ODGE.

5.2.5. *In Vivo* Study Methods

Encouraged by the ability of the ODGE modified HPMC to bind lipids (such as sudan black) we undertook a simple *in vivo* test to determine the action of this material. In addition, acute studies were used to examine the polymers activity on intestinal cholesterol adsorption. We are also reporting the results of a study performed by Dr. Linscheer of State University of New York, Upstate Medical Center.

5.2.5.1. Chronic *In Vivo* Study Methods

A special atherogenic diet (SAD) was fed to 32 male Sprague Dawley rats (Tyler Labs), all very close to a specified initial weight of 350 gms. This diet is a product of ICN the composition of which can be found in Table 11. Following an equilibration period (8 days - during which the quantity of food consumed and animal weight gain were noted), the animals were fed *ad libitum* the diet with the addition of a test polymer for 16 days. These compositions can be found in Table 12.

Table 11 - Special Atherogenic Diet (SAD) Composition [220].

Composition	
Alphacel	6.0%
Butterfat (Salt Free)	40.0%
Cholesterol	5.3%
Choline Dihydrogen Citrate	0.4%
Salt Mixture W	4.0%
Sucrose	20.3%
Sodium Cholate	2.0%
Vitamin Diet Fortification Mixture	2.0%
Casein Purified High Nitrogen	20.0%

Table 12 - Diets for *In Vivo* Study.

Group	Diet
Group I	Diet with no additive
Group II	SAD plus 5 wt% HPMC (Aldrich) as control
Group III	SAD plus 5 wt% 0.23wt%-ODGE-HPMC
Group IV	SAD plus 5 wt% 0.45wt%-ODGE-HPMC

The ODGE modified HPMC was prepared as detailed in the synthesis sections except that it was repeatedly washed with acetone instead of dialysis. The quantity was too great to allow for dialysis in our laboratory.

The diet (SAD) was stored in a refrigerator until use. The dried food additive was mixed 1:20 weight ratio with SAD using a Kitchen Aid mixer in small lots. The animals were randomly put in study groups and cages in a single bank of wire bottom cages. Each cage was equipped with a feeder which the animal had to crawl into for feeding. The animals weights were measured at least every other day and food consumed was measured daily. Notations were made if the animal had spilled food out of the cage and on general physical appearance. At the completion of the study the animals were sacrificed following overnight fast and blood collected (18G needles) in EDTA centrifuge tubes (200 mg) via cardiac puncture. The blood was stored on ice until cholesterol analysis was complete. The samples were centrifuged at about 2560 rpm for 30 min in an International Clinical Centrifuge (model CL). Plasma was pipetted off and analyzed by the Harborview Lipid Research Lab where triglycerides and plasma cholesterol were determined (using

an Autoanalyzer II).

5.2.5.2. Acute *In Vivo* Study Methods

This study was suggested by Dr. David Saunders (Gastroenterology, U.W.) as a rapid method of observing changes of intestinal adsorption of lipids. In all cases, the animal was located on a heating pad that was controlled to 37°C. 350 gm male rats were anesthetized (sodium pentobarbitol, IP) and segments of intestinal loops (about 10cm) were isolated from one another by ligation starting about 1 cm distal from the duodenal/ileum junction. This is below the region where the gallbladder enters the intestinal tract. Rats do not have a common bile duct between the gallbladder and the intestine as humans do. Care was taken to leave intact the blood supply to each segment. Segments were then flushed with 3 ml of 37°C lactated Ringer's solution. Following the gentle flushing of air through the segment (to clear out Ringer's) the loop was ligated, and infusion solution added that either was control or contained 1.74%oleyl-HPMC as an additive (2mg/ml). The infusion solution contained 10mM sodium taurocholate, 20mM oleic acid (1-^{14}C), 1.5mM lecithin, and 1.5mM cholesterol in lactated Ringer's. In addition, it contained a trace of 4,000 M.W. PEG (1,2-^{3}H) as a marker for normalization of data. The solutions were dispersed by sonicating for 45 min. The resulting solution had an opaque, milky appearance.

After infusion, the intestinal wall was closed and the animal kept at constant temperature for 1 hour. After this time the animal was sacrificed and each segment was rapidly removed and the contents washed into a pre-tarred vial. Each segment was then placed in another tarred vial and baked in the oven overnight at 80°C. This allowed for normalization of the data to dry segment weight.

In one study we also washed each intestinal tract segment with 1ml of buffer five times into pretarred vials. We desired to see how well the washing of the intestinal tract removed the unabsorbed labeled compound.

5.2.5.3. Dr. Linscheer's *In Vivo* Study Methods

In a study performed by Dr. Linscheer of New York, the effect of 0.76%OGE/HPMC on intestinal lipid absorption was examined using a rat model employing an intestinal perfusion method. An overview of this method can be found in Fig. 30. Two consecutive upper small bowel loops are at random simultaneously perfused with either a control solution, containing ^{14}C oleic acid (OA), and ^{3}H cholesterol in a coarse emulsion with NaTC or a test solution.

The test solution contained the same composition except that our polymer (0.4mg/ml) was suspended in it by stirring. The outflow was then analyzed for radiolabeled probe and the intestinal absorption determined. After initial perfusion, the segment perfusion solutions are reversed and the content of the exiting material analyzed for labeled compound. In this way it was possible to determine if the test compound had any effect on intestinal absorption. He has used this method to examine the effect of fatty acid perfusion on intestinal alkaline phosphatase [221], and the effect of

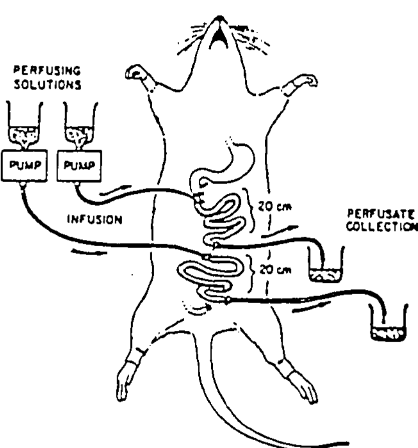


Fig. 30 - Perfusion Method of Dr. Linscheer.
Infused Solution: 20mM Oleic Acid, 1mM Cholesterol, 30mM NaTC. Infusion solution contains ^{3}H cholesterol and ^{14}C oleic acid. It is made-up in lactated Ringer's solution and has a pH of 5.5 and osmolarity of 310 mosmol/kg. The test solution also contained 0.4mg/ml 0.76%OGE/HPMC.

intraluminal pH on cholesterol and oleic acid intestinal absorption [222].

Six male rats were studied, they were fasted overnight with free access to water and anesthetized with pentobarbitol. The small bowel was exposed by abdominal midline incision and sequential loops of upper small intestine (20 cm length each) were isolated. Following placement of inflow and outflow catheters the loops were returned to the abdominal cavity which was then closed. The flow rate for the experiment was 0.4 ml/min for 45 min., each with three 15 min. collection periods.

5.3. HPMC and HM-HPMC Interaction with Amphiphilic Compounds - Results.

5.3.1. Viscometry Results.

The rheological analysis of HPMC as it interacts with SDS has provided some interesting results. We studied HPMC concentrations from 0.05 to 0.5%. Each of these were run over a range of 0 to 20 mM SDS. The results are presented in Fig. 31 where the relative viscosity ($[\eta_r] = [\eta_{meas.}]/[\eta_o]$, polymer (no SDS) = 1) is plotted against SDS concentration. We see that for HPMC concentrations below and including 0.25% the relative viscosity drops at an SDS concentration of 4 mM. Fig. 32 shows the 0.05% polymer concentration sample plotted on an expanded scale. We see that a sharp drop occurs at 4mM SDS while the relative viscosity has leveled off at 10mM SDS. For the 0.5% HPMC sample the viscosity rises dramatically at the same SDS concentration.

It appears that the first critical concentration of detergent interaction (as defined by Wolfram and Gilanyi) is 4mM SDS. Below this concentration little interaction is evident. Lewis and Robinson found very similar results for MeC and SDS. They reported a critical concentration for interaction of 4.2 mM. They also observed a relative viscosity increase for 0.5% solution, while for 0.05% MeC the viscosity dropped. This anomalous behavior was attributed to de-aggregation of polymer aggregates. They referenced Kuhn & Mosers [223] theoretical work and concluded that aggregation can either decrease or increase the measured viscosity. It seems reasonable that at some

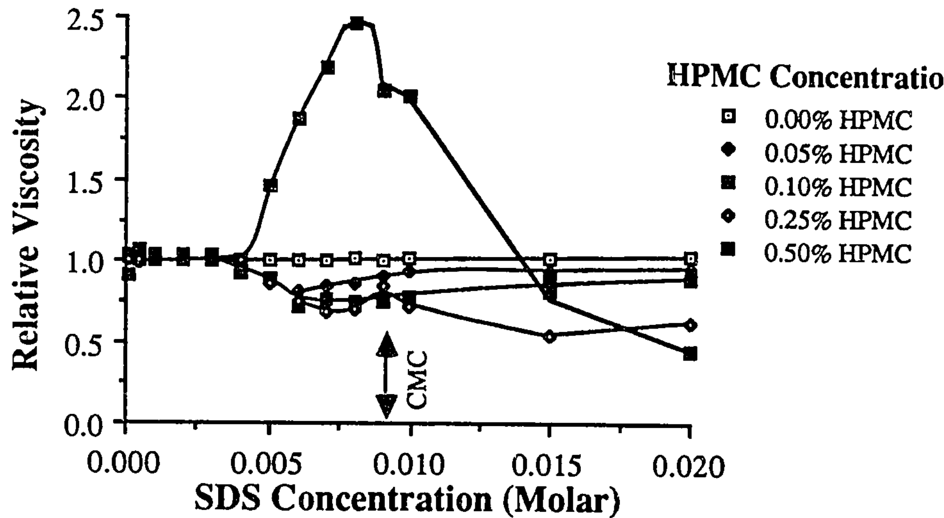


Fig. 31 - Effect of SDS on Relative Solution Viscosity.
($\eta_r = \eta_{meas.}/\eta_{no\ SDS}$)
CMC of SDS shown for no polymer present.

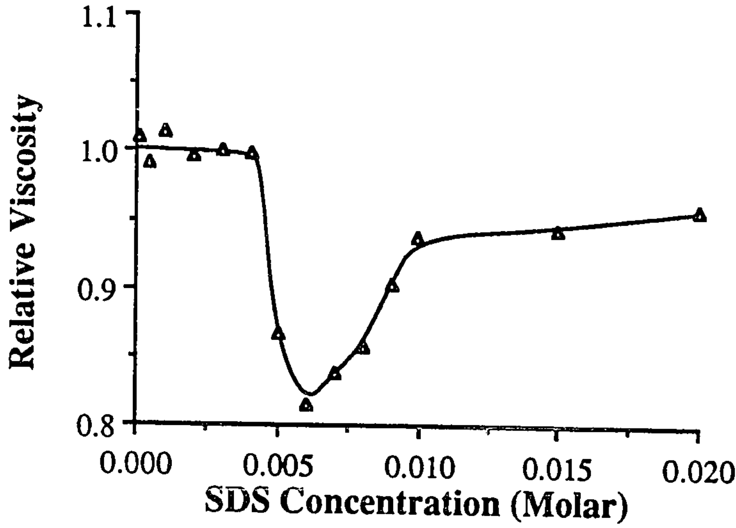


Fig. 32 - Effect of SDS on 0.05% HPMC Relative Solution Viscosity.
Data taken from plot above, ordinate expanded.

concentration of surfactant the HPMC aggregates will be broken up - the hydrophobic regions of the polymer aggregate favoring solvation with SDS. As these aggregate binding sites are broken, new ones become available due to chain unfolding, leading to a gradual break up of the aggregate. Does

this explain the dramatic difference in viscosity between concentrations of 0.25 and 0.5wt%? We believe that at 0.5% the aggregation of HPMC is promoted by SDS until enough SDS is added to break-up the "cross-links" holding the gel together. Conversely at a lower HPMC concentration SDS does not accomplish this because the polymer gel does not establish itself because of the lower likelihood of polymer/polymer (intermolecular) contact which is a consequence of the low concentration.

Remarkably, this disparity occurs near the same concentration as the dramatic change in CPT and IPT for 0.47% LP_4GE/HPMC (Fig. 25). Perhaps at 0.5% HPMC in water the SDS is promoting the aggregation of the polymer molecules much like the conjugation of lipid to the polymer.

Attempts to see if indeed HPMC de-aggregated at 10mM SDS (eluting solvent) using GPC analysis revealed that no change in apparent molecular weight occurred. The HPMC elution profile was the same as when no SDS was present. This suggests that a de-aggregation process may not be responsible for this phenomena. Perhaps the structural integrity of the aggregates is transitory, the aggregates forming and sharing HPMC polymer molecules rapidly. In the GPC this process may be enhanced by the pressure or the aggregates may be stable enough to not go into pores. When SDS is added the polymer/polymer contacts depend on the concentration of polymer and detergent. At high polymer concentration they are more likely to interact with one another; the SDS perhaps providing some unfolding of the polymer chains enhancing microgel formation until enough detergent is added to fully solubilize hydrophobic domains on the polymer. At lower concentrations of SDS, polymer/polymer complexation is decreased by the solubilization of hydrophobic domains by the amphiphile. Our results on cloud points of SDS/HPMC have reinforced this theory (Section 5.3.3).

We also note from Fig. 31 that the final relative viscosity (for 0.5% HPMC) shows a decreasing η_r with respect to SDS concentration above 7.5mM. Nagarajan has observed that the viscosity of SDS/PEO systems maximize as well [201]. He attributed the declining relative

viscosity (after a maxima) to an increase in the concentration of SDS (both monomer and free micelles). This causes a rise in the ionic strength of the solution the consequence of this is a reduction in relative viscosity as seen for polyelectrolyte solutions. We are not certain whether this is the case since low HPMC concentrations did not result in this observation. Further study is warranted in this area. Perhaps careful photon correlation spectroscopy would reveal more information though we found that the diffusions coefficients were very large for a few selected samples.

5.3.2. Fluorescent Probe Results.

Initially, we wished to see if the attachment of lipid to the polymer would produce a material that interacted with CAP in aqueous solution in the form of a micelle. To examine this we used *water soluble* HM-HPMC. When 0.1% solutions of HPMC bound to the lipid LP_4GE (attachment ratios from 0 to 0.85%) were measured we observed no detectable interaction. The peak maxima remained at that for the water/CAP system. We were surprised to see this result. Apparently, the lipid regions of the polymer are not organized enough in solution to provide a hydrophobic environment for inclusion of CAP. If polymeric "micelles" were formed then we would expect some of the probe molecules to occupy these sites, since it is hydrophobic, and fluoresce at a lower wavelength.

This result indicates that our original assumption of a water soluble intramolecular micelle which binds lipid from solution was incorrect for this combination of lipid and polymer. In binding studies of sudan black by our polymer we found that very little sudan black was bound until sufficient lipid was attached to the polymer to make it water insoluble. It may be that when enough ligand is attached it organizes to form a lipid environment which also precipitates the polymer. Fig. 33 shows the binding of sudan black by CGE conjugated HPMC. These samples were prepared by adding sudan black in methylene chloride to small test tubes. The solvent was evaporated and 50mgs of polymer added to each vial. 5ml of 0.91M saline was added and the tubes allowed to

equilibrate for several weeks. The solution and polymer was then withdrawn using a pipet leaving the solid particles of sudan black behind. Sample A and B are controls (A: saline, B: HPMC) and no solublization of the dye was seen. It was found the sample (0.07wt% CGE/HPMC) solubilized some of the sudan black forming a pale blue solution. This polymer sample was "water soluble" it "dissolved" to yield an optically clear solution in the absence of sudan black. As more CGE was attached to the polymer the sudan black was sequestered into the polymer gel forming a very dark blue mass. The amount of polymer in samples B-E was the same, we note that for sample E the gel has collapsed (more so then D). Though it is not obvious from the Figure this gel was also more darkly colored with sudan black then D. We conclude that the lipid conjugated polymer has a degree of detergency.

In a second study we examined the effect of SDS on the native polymer (HPMC) in aqueous solution. SDS concentrations ranging from 0 to 0.5M were used. The control SDS showed a rapid

Fig. 33 - Solubilization of Sudan Black by Cholesteryl Conjugated HPMC.
A: 0.91M NaCl, B: 1% HPMC in 0.91M NaCl,
C: 1% solution of 0.07wt% cholesterol conjugated HPMC,
D: 1% solution of 1.4wt% cholesterol conjugated HPMC,
E: 1% solution of 2.1wt% cholesterol conjugated HPMC.

increase in hydrophobic environment for the CAP emitter which is indicated by a decrease in λ_{max} emission at the expected CMC of this detergent. This result is quite similar to those obtained by other investigators. As an aside, we have discovered that CAP degrades over time in aqueous solution (two peaks appear at 390 and 410 nm). This has not been reported in the literature.

Fig. 34 shows the emission λ_{max} plotted against concentration. We see that for SDS the CMC occurs between 10^{-2} and 2×10^{-2} M. While for the samples containing both HPMC and SDS the CMC has dropped to about 6 mM. From surface tension measurements it is well known that the CMC of SDS is 8.1 mM. It is also known that different methods of analysis yield widely varying values [224]. Our value of about 10^{-2} is quite close to the results of Ananthapadmanabhan et al. [210], who used the same probe for similar studies.

Recall the HPMC/SDS viscosity results revealed that interactions were occurring at 4 mM SDS. This corresponds to the break point in the fluorescence data (when we start from low SDS concentration). This indicates formation of a hydrophobic "micellar" environment. We also observe

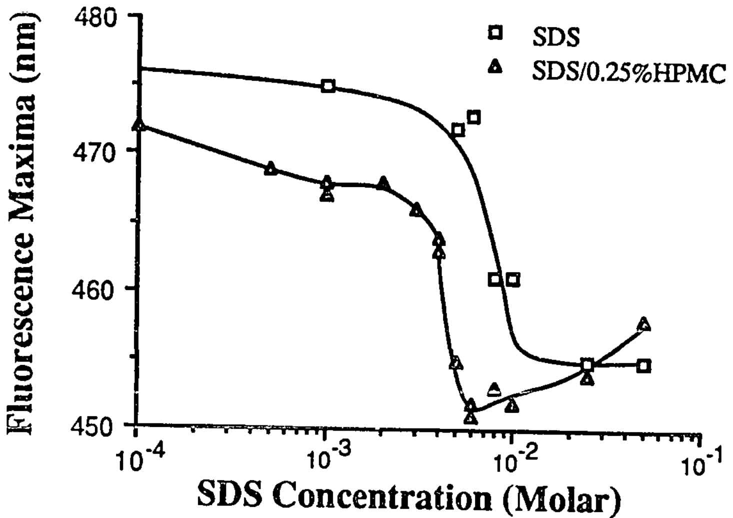


Fig. 34 - Effect of SDS on Fluorescence Maxima for HPMC in Water.
($\lambda_{excitation}$ = 360 nm)

that by 8 mM SDS, "micelle" formation is complete, this also corresponds to the point at which the viscometric results for 0.5% polymer showed a sharp decrease. Using the dielectric constant/wavelength equation provided by Kalyanasundaram and Thomas [213], it is possible to estimate the dielectric constant for this "micellar" environment. We found that the minima for the HPMC/SDS complex has a dielectric constant of about 28. This is below that of methanol ($\varepsilon = 32$). The appearance of a shift in the emission λmax data also suggests that there are SDS/HPMC interactions below 4mM SDS. The HPMC (no SDS) had an identical λ_{max} as that of water. Apparently, the SDS is interacting with the polymer at lower concentrations though the viscometric method of analysis does not indicate this. We can conclude HPMC does not form a "micelle" environment which can bind CAP until a surfactant complexes with the polymer and this complexation becomes very evident at >4mM SDS.

5.3.3. Cloud Point Results

Viscometric and fluorescent probe studies on the interaction of SDS with HPMC produced expected results (as compared to results in the literature). In contrast, we obtained quite surprising results when the IPT and CPT of SDS/HPMC solutions were obtained. Contrary to expected results, we discovered that at intermediate ranges of SDS concentration the polymer came out of solution at a lower temperature than the native polymer. We had expected that SDS would increase the cloud point (keep the polymer in solution). Studies using another LCST polymer (poly(N-isopropyl acrylamide)) in Dr. Hoffman's laboratory, revealed this expected behavior. The LCST for this polymer was increased as SDS was added. All previous work reviewed indicates that the presence of SDS in solution should keep the polymer is solution (raise the cloud point). In addition, we found the presence of a precipitate for the HPMC/SDS complex which did not resemble that found for the native polymer.

A plot of IPT and CPT for HPMC/SDS can be found in Fig. 35. We note that the IPT reaches a minima at about 5 mM (this is close to the break point for the isothermal viscometry

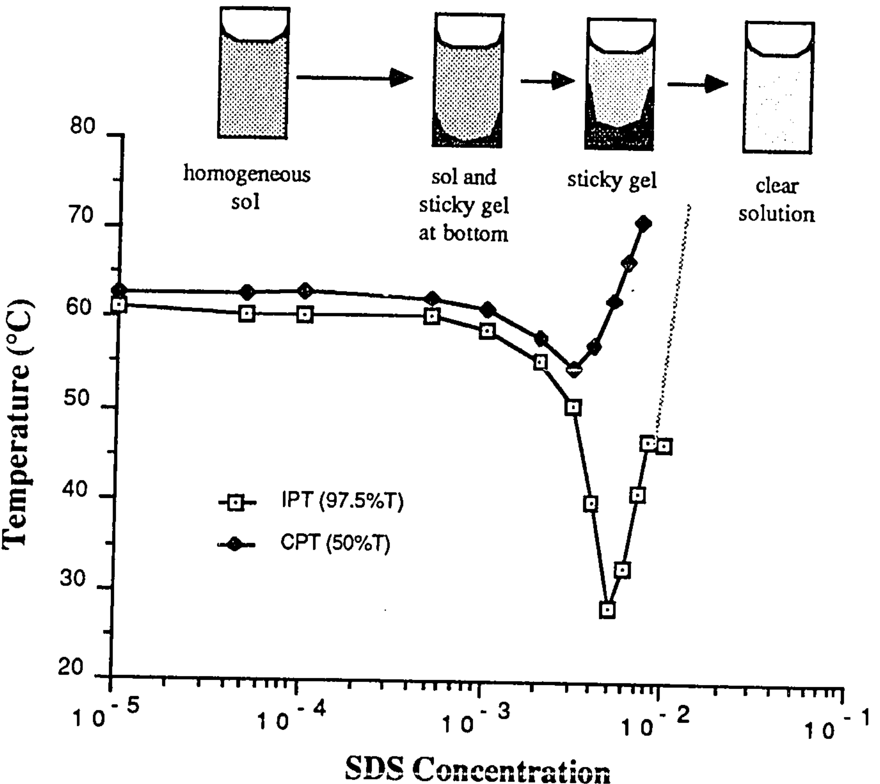


Fig. 35 - Effect of SDS on HPMC Cloud Point.
(Appearance of the precipitate is also shown)
Dashed line indicates the polymer was not precipitated
by higher temperatures (up to 80°C).

data). We found that this minima was the same for all different concentrations of HPMC tested. This result is shown in Fig. 36. Apparently, the SDS is interacting with HPMC to reduce it's stability in aqueous solution. The character of the precipitate is altered by the presence of small amounts of SDS. In the absence of SDS the HPMC precipitates as a sol which is evenly distributed throughout the solution. This precipitates slowly to form an opaque mass. As expected, this dissipates rapidly during cooling. When SDS is added, we observed that the precipitate either stuck to the walls of the cuvette or rapidly sank to the bottom. It appeared to be a gel which contained a lot of water. This would be expected if SDS was precipitating with the polymer - the charged head group "dragging" water with it. Upon cooling, this gel cleared but remained in the bottom of the

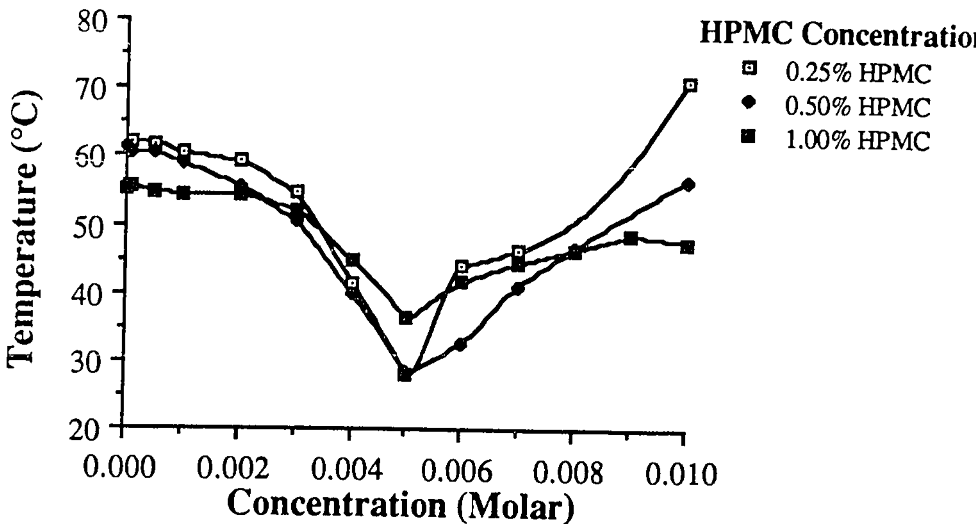


Fig. 36 - Effect of SDS and HPMC Concentration on IPT.

cuvette for weeks, indicating that strong interactions or extreme polymer entanglement had occurred. We found similar results for the polymer with ligand (LP_4GE) attached (no surfactant). Recall that HM-HPMC precipitates as a gel which does not clear rapidly. Perhaps similar phenomena are responsible for both results. We propose that through some type of interaction (most likely HI) the SDS is complexing with the polymer to form hydrophobic cross-links at elevated temperatures. In a similar fashion we observed that HM-HPMC also forms related precipitation structures, the hydrophobic ligands acting to cross-link the polymer chains.

One possibility is that the HPMC has cationic groups (this seems unlikely). Goddard has found using cationic cellulose ethers that at intermediate anionic surfactant concentrations a precipitate forms because the anionic surfactant complexes with the polymer cations [225]. At higher concentrations of surfactant the polymer goes back into solution indicating that the excess detergent is solubilizing the ionically bound SDS/cellulose ether complex.

It should be noted that the CMC for SDS has a minima if the CMC is plotted against temperature. This occurs at 26°C [226]. At 30°C this has not changed much (CMC of 8.22mM vs.

8.16 at 25°C) however, by 60°C the CMC has risen to over 10 mM. We also note from the figures that SDS is altering the IPT below 4mM SDS. Though this is not an isothermal system it indicates, as the fluorescent probe results suggest, that there are interactions as low as 0.5mM SDS.

In conclusion, SDS does interact with HPMC (native) and this interaction causes changes in viscosity, probe fluorescence and the cloud point. Fig. 37 presents the proposed processes involved. At zero or very low SDS concentrations the polymer forms aggregates at room temperature. As SDS is added above a critical concentration (around 4mM) these aggregates are broken up for low concentrations of HPMC. Higher concentrations of HPMC apparently increase aggregability. There

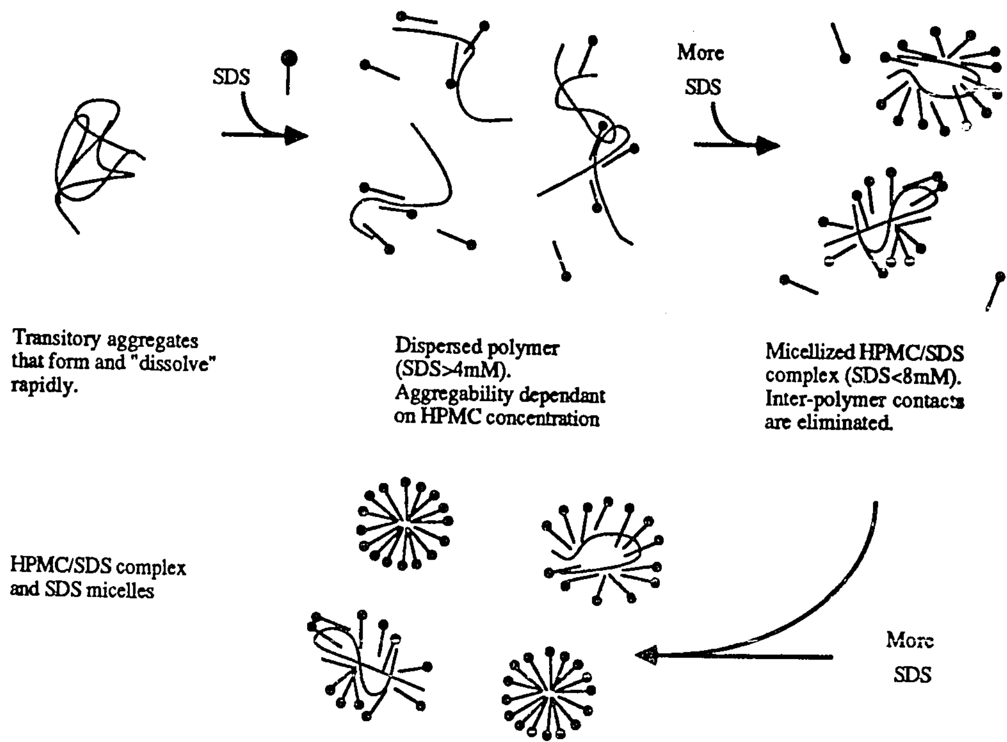


Fig. 37 - Proposed Mechanism of SDS Interaction with HPMC.

are interactions though below this concentration as indicated by fluorescent probe and cloud point data. In the range of detergent concentration up to about 8mM the SDS interacts with the polymer to form an unstable structure that precipitates at elevated temperatures. As more SDS is added the polymer/SDS complex becomes fully micellized and precipitation is not evident at higher temperatures. It is presumed that at still higher SDS concentrations, free SDS micelles will form. This corresponds to the second critical concentration noted by others (see Section 5.1). From our data this point is not evident.

5.3.3.1. Sequestration of Bile Salts and Mixed Micelles Results

5.3.3.1.1. Equilibrium Dialysis Results

An understanding of how bile salts form micelles and solublize lipids provides insight into interpretating the results from these studies. Recall that we quantitated the isothermal sequesteration of the bile salt (NaTC) with HPMC and HM_HPMC using equilibrium dialysis cells at 37°C. It is generally believed that NaTC (and other bile salts) make small micelles in aqueous solution which can have both primary and secondary structures. Recall (Fig. 4) that the charged head group is oriented at the top and bottom of the cylindrical micelle while the three hydrophilic hydroxyl residues are located on the outside wall of the cylinder. This structure leaves a hydrophobic central region.

In 0.15M NaCl (20°C) NaTC has a critical micelle concentration (CMC) near 3.2mM (determined using a spectral shift technique) [22]. In the same system, the aggregation number (Ag#) is 5. For distilled water these values do not change much (CMC: 3.1mM, Ag#: 4). At a very high salt concentration (3.0M NaCl), the CMC drops to 0.7mM with an Ag# of 16.

When sodium cholate (NaC) solubilizes a simple hydrophobic organic, such as cyclohexane, the CMC is 5mM with an Ag# of 4 and 1 solubilizate (cyclohexane) molecule per micelle. The CMC and Ag# are the same as those found for simple mono-component NaC micelles. The

structure of the micelle doesn't change much from that of the simple system with the organic sequestered into the hydrophobic core of the cylindrical structure. Extrapolating these results to NaTC we would expect that this bile salt would solubilize an organic molecule with a CMC near 3.2mM and around 5 NaTC molecules/micelle and 1 solubilizate/micelle. Note that for complex lipids such as cholesterol, solublization by NaTC is not possible, the addition of NaOl or lecithin is necessary to solubilize this lipid.

We found that NaTC was bound to the polymer (both native and OGE/HPMC). Fig. 38 presents this data plotted as NaTC bound (μmoles/mg polymer) against the NaTC equilibrium concentration. We also found similar results for CGE and ODGE modified HPMC. The general appearance of the data suggests a gradual increase in binding at low concentrations of NaTC followed by a plateau at a higher concentration. It was found that near the plateau region the polymers that were solids at low concentrations of NaTC dissolved and the solution became very viscous. While native HPMC started out dissolved in solution it was found that near the plateau

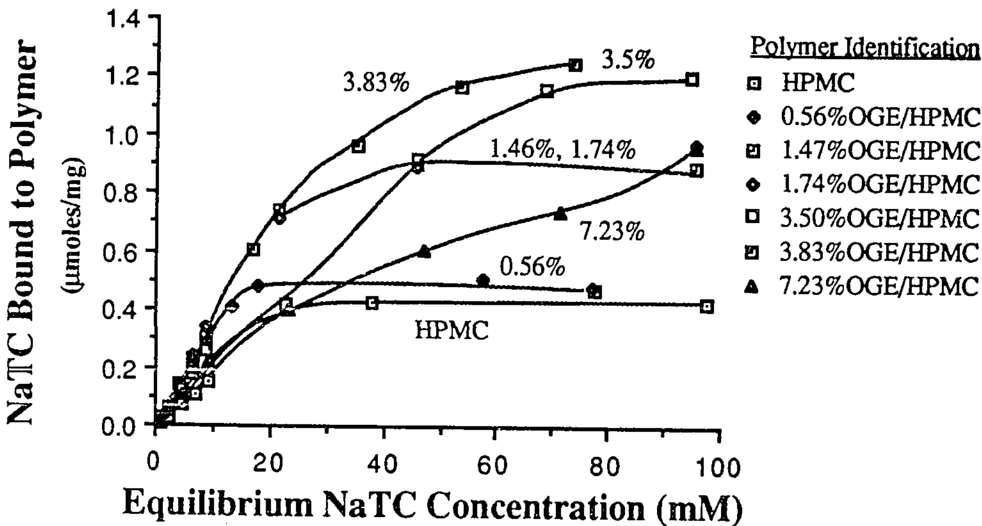


Fig. 38 Binding of NaTC by OGE/HPMC.
(>48 hrs, 37°C, 20 RPM, Spectrum Equilibrium Dialyzer)

region the solution appeared to be more viscous. The following table summarizes the dissolution point of these polymers and the plateau binding of NaTC (Table 13).

We observe that as the amount of lipid attached is increased this plateau occurs at a higher quantity of NaTC bound to the polymer (with the exception of 7.2%OGE, 8.6%ODGE, 4.3% & 4.7%CGE attached to the polymer). The polymers with high attachment have not reached a plateau at 100mM NaTC. If we take the maximum NaTC bound (plateau region) and plot this against the quantity of ligand attached to the polymer (Fig. 39) we observe that a maximum seems to be reached near 4% lipid conjugated to the polymer. However, the points labeled with * have not reached a maximum. It is presumed that they will bind at still higher levels if fully solubilized. We also see that "water soluble" samples (HPMC and low lipid conjugation HM-HPMC) show low binding with maximum binding near 0.4 to 0.5 μmole NaTC/mg polymer, while "water insoluble" HM-HPMC samples show enhanced NaTC sequesteration. Perhaps different mechanisms of binding

Table 13 - Binding of NaTC by Lipid Conjugated HPMC.
Determined using equilibrium dialysis at 37°C.

Sample	Approximate Dissolution Point (mM NaTC)	Maximum Binding (μmole/mg polymer)
Dextran (15,000 MW)	0	0
HPMC	0	0.47
Control HPMC†	0	0.47
0.6%OGE/HPMC	20	0.50
1.5%OGE/HPMC	30	0.90
1.7%OGE/HPMC	40	0.97
3.5%OGE/HPMC	60	1.20
3.8%OGE/HPMC	80	1.26
7.2%OGE/HPMC	>100	>0.96‡
0.23%ODGE/HPMC	0	0.37
0.45%ODGE/HPMC	<20	0.51
5.1%ODGE/HPMC	80-100	1.17
8.6%ODGE/HPMC	>100	1.37‡
2.1%CGE/HPMC	20-40	0.93
2.8%CGE/HPMC	20-40	0.95
4.3%CGE/HPMC	≥100	0.98‡
4.7%CGE/HPMC	>100	0.70‡

† - control HPMC same reaction process as HM-HPMC without glycidyl ether.
‡ - has not reached a maxima.

are involved. It should be emphasized that these maximum values are not in the physiological NaTC concentration region.

Figures 40 and 41 display the moles of NaTC bound per lipid conjugated against ligand attached to the polymer. In Fig. 40, the maximum NaTC bound has been plotted - this includes binding to the backbone polymer - normalized with respect to lipid conjugation to HPMC. We note that native HPMC (which binds NaTC) will yield an infinite value. Remarkably the ligand identity has little effect on this quantity. By subtracting the amount of NaTC bound to the backbone polymer (native HPMC) from the maximum NaTC bound to HM-HPMC we get the curve shown in Fig. 41. This plot shows how many taurocholates are bound to each conjugated lipid assuming that the presence of conjugated lipid has not significantly altered the backbone polymer adsorption of NaTC. A maximum is reached with 10 NaTC molecules are bound to each ligand. We might speculate that at low levels of ligand, the polymer (which is surface active) folds around the attached lipid thus shielding it from interaction with NaTC. At higher attachment levels of lipid, the

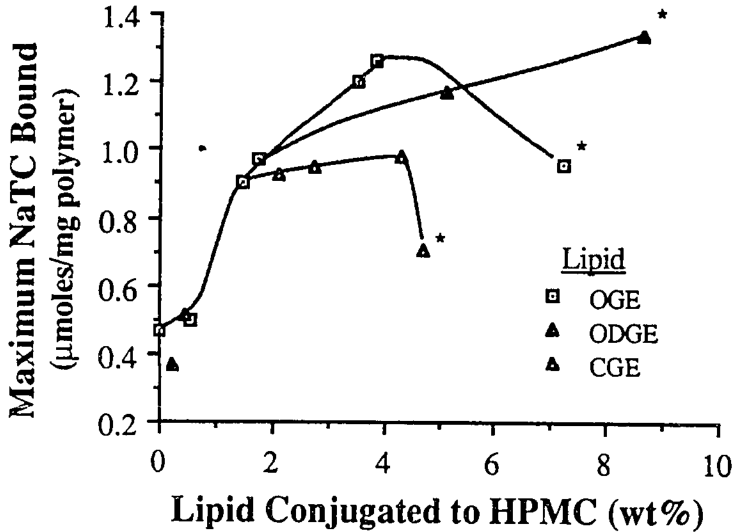


Fig. 39 Maximum Binding of NaTC by OGE, ODGE, and CGE Conjugated HPMC.
* indicates that the polymer has not dissolved and a maxima has not been reached at 100mM NaTC.

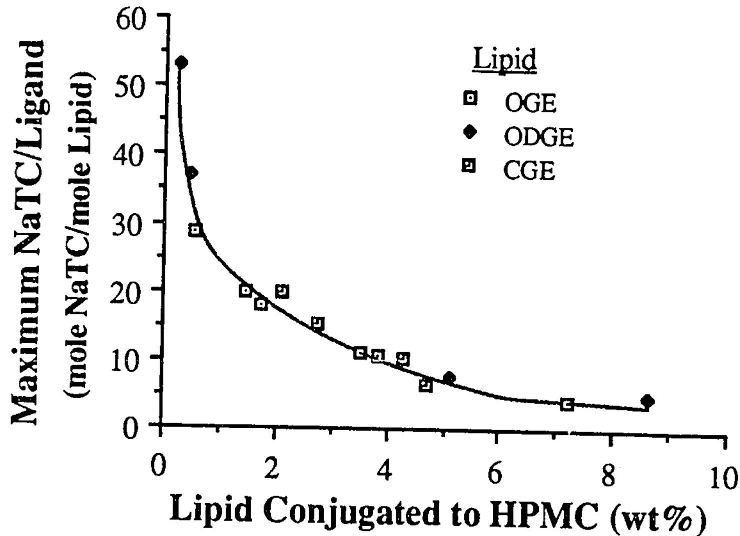


Fig. 40 Maximum NaTC Bound per Ligand Attached to HPMC. (Native HPMC would be infinite.)

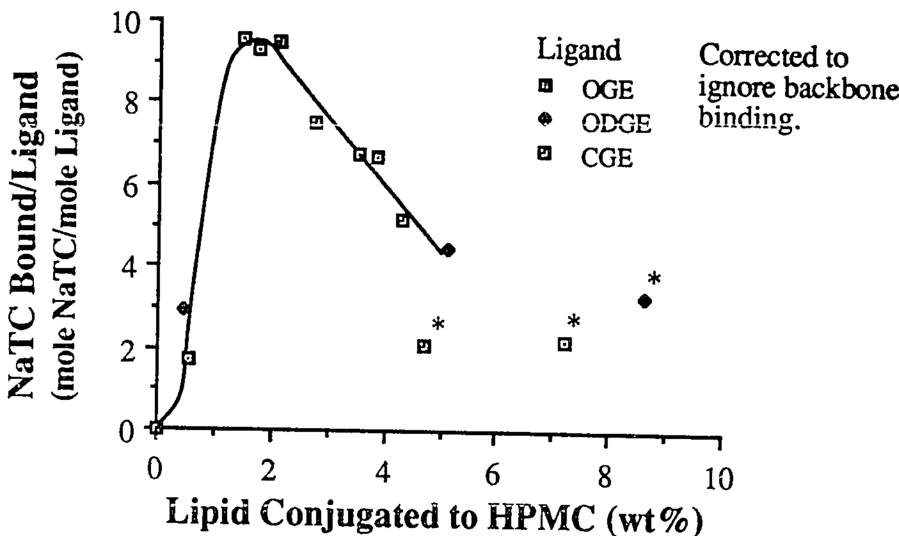


Fig. 41 Corrected Maximum NaTC Bound per Lipid Conjugated to HPMC. Effect of HPMC subtracted from maximum NaTC bound for Ligand/HPMC. HPMC Binding equals 0.
Three points labeled with * have not reached a maxima.

NaTC binds in a 10:1 to 5:1 ratio. We can probably neglect the three high HM-HPMC samples which did not reach maximum binding. Recall, we had expected a binding ratio of around 5:1

(NaTC:Ligand) similar to the solubilization of cyclohexane by NaC (mentioned above). Several mechanisms might explain the difference between the expected and experimental values.

1) The ligand may be enhancing the backbone binding.

2) The ligand may bind aggregates of bile acids which have secondary structure.

3) The presence of the HPMC in solution may be forcing the NaTC to form larger micelles. This process would be similar to the effect of increasing salt concentration on bile salt aggregation.

At higher OGE attachment the ratio of NaTC bound per conjugated lipid is reduced. This process may represent a cooperative binding mechanism where a single NaTC micelle binds several ligands.

In the physiologically relevant range (for the duodenum) of NaTC concentrations (from 10 to 20 mM) it was found that a maximal binding of NaTC is reached when 2-3 wt% ligand is attached to HPMC. At higher levels of lipid attachment the polymers are too hydrophobic and do not wet well. Apparently, the NaTC is unable to overcome the polymer/polymer interactions. As we shall see in the next section this is not the case for mixed micelle binding of NaOl and cholesterol.

Fig. 42 shows a Klotz plot of NaTC binding by OGE/HPMC [227,228]. We note that the inverse of NaTC bound is plotted against the inverse of the equilibrium NaTC concentration. A linear relationship (as we found) is indicative of adherece to a Langmuir adsorption isotherm. This was surprising since many assumptions used to derive the Langmuir equation are not valid for our system. It should be considered an empirical relationship for this reason.

Both HPMC and control HPMC showed the same binding using this technique of analysis, while all HM-HPMC polymers which were water insoluble in aqueous solution (no amphiphile) bind NaTC at low concentration nearly the same. It was found that CGE and ODGE conjugated HPMC also fell in the same area. When very low quantities of lipid are attached to HPMC *and* the resulting polymer is water soluble, binding was between these two extremes. Apparently, the mechanism of binding is very similar for the water-insoluble polymers tested - it is independent of

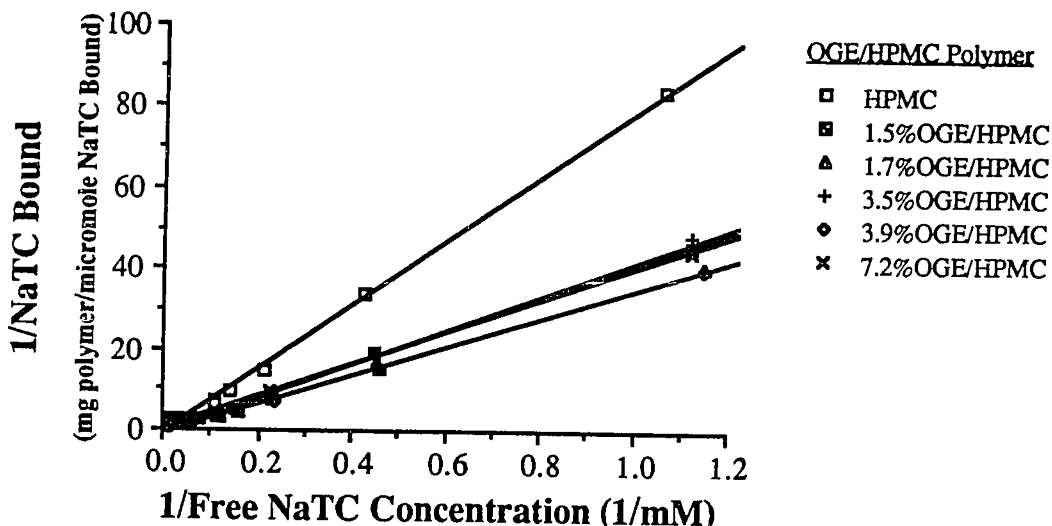


Fig. 42 Klotz plot of NaTC Binding by OGE/HPMC.
Both HPMC and control HPMC fell on the same line, while all water-insoluble OGE/HPMC samples showed very similar binding. This presentation shows binding at low NaTC concentrations, the data was taken from that used for Fig. 38.

the ligand attached. The existence of hydrophobic sites on the surface of the gel provide complexation sites or an interface which binds the same quantity of NaTC from solution. This suggests a simple model for NaTC binding where the NaTC only binds to interfaces - it does not integrate into the lipid regions (like sudan black) until high concentrations of NaTC are present. Binding to HPMC is of a similar nature with the NaTC covering hydrophobic regions. The chemical structure of NaTC would support this theory. The existence of the three hydroxyls on the steroid ring want to orient toward water at a water/oil interface while the lipid side complexes with the oil side. This process is driven by hydrophobic interactions (HI). Recall that HI is more a property of the solvent (water) and its relationship with the solute then of the associating hydrophobes chemically recognizing and binding to one another. At much higher NaTC

concentrations the HI between hydrophobic regions (the conjugated lipid) on the polymer find the environment present in NaTC micelles preferable and the polymer is dissolved.

5.3.3.1.2. Mixed Micellar Binding by HM-HPMC

NaOl does not spontaneously form simple micelles in solution. At very high concentrations it goes through several transitions where the molecules orient in liquid crystalline structures however. In the physiologically relevant range it is not soluble (i.e.: form a clear solution) in water. This made it unlikely that experiments using our technique would succeed since the filtration step would also remove unbound NaOl particulates. In the presence of bile salts NaOl does form clear solutions with oleate solublized in the mixed micelle. Recall that we used a solid phase extraction technique to quantitate the sequesteration of mixed micelles with HM-HPMC.

Characterization of mixed micelles containing biles salts and fatty acids have been studied for only a few cases (Carey and Small have reviewed many of these [22]). For 1:1 ratios of sodium deoxycholate (NaDC) and sodium oleate, the mixed micelles have a molecular weight of around 11,000 with 10 NaDC/micelle and 17 NaOl/micelle. Since the solubility of NaOl is quite low in water we would expect that the majority of this component is solubilized in micelles while the bile salt would be present in solution as both free monomer (about 3.2mM) and in micelles. The structure of mixed micelles can be found in Table 2. We note again that the bile salt forms a cylinder with the fatty acid occupying the central region.

The model system used to determine the binding of components from mixed micelles by water insoluble HM-HPMC was used because the large size of these micelles precluded use of equilibrium dialysis. Recall that binding was determined by incubating mixed micelles containing a 1:1:0.15:0.06 molar ratio of NaTC/NaOl/LL/cholesterol. After about a week the solutions were filtered and the binding determined. This model is the most physiologically relevant and the most complicated of the *in vitro* methods used. Initially, we wanted to determine binding using a simple two component system - 1:1 NaTC/NaOl.

Figures 43 and 44 show the binding of NaTC and NaOl to the polymers using this system. We found that 0.6%OGE/HPMC dissolved (and some went through the filter) above 5mM NaTC,

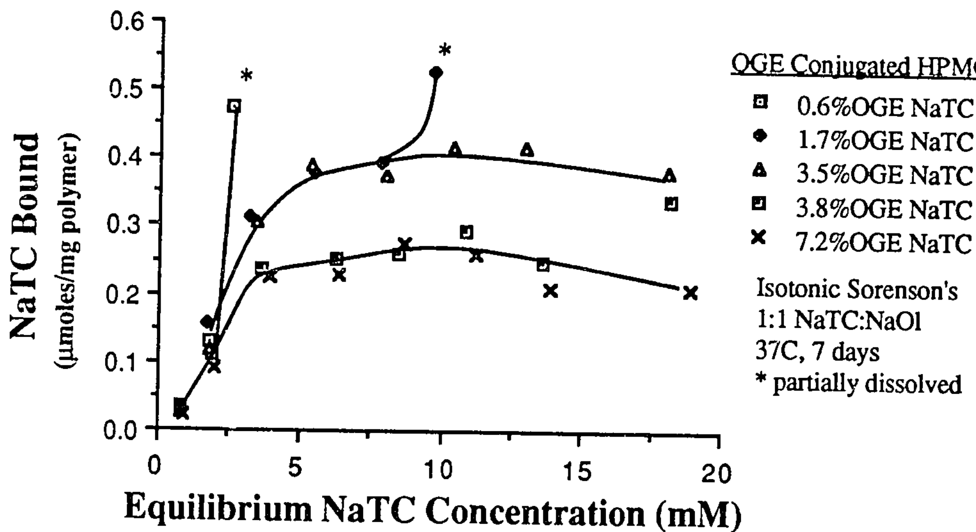


Fig. 43 NaTC Binding by OGE/HPMC From 1:1 NaTC/NaOl.
(^{14}C labeled NaTC)

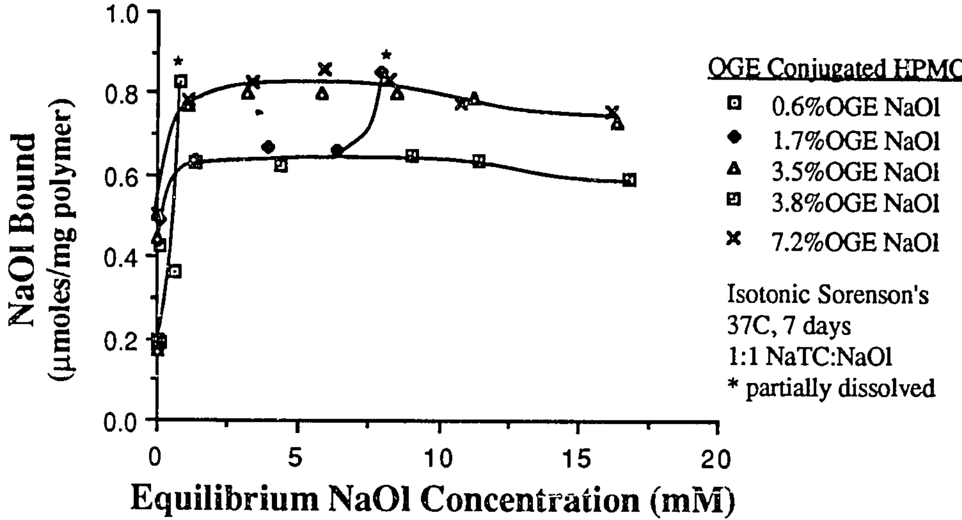


Fig. 44 NaOl Binding by OGE/HPMC From 1:1 NaTC/NaOl.
(^{3}H labeled NaOl)

while 1.7%OGE/HPMC dissolved above 10mM NaTC. For all polymers (but not for the blank) 1mM and 2.5mM solutions showed very high binding (>95%) of NaOl. Plots of the percent binding of these components against concentration can be found Figures 45 and 46. We note that when NaTC binding is expressed as a percentage (Fig. 45) there is a maxima in binding near the CMC of this bile acid. If micelles of bile salt are not present below this concentration then the free concentration will be rising. This apparently binds to the polymer (due to hydrophobic interactions) and the amount bound increases through a simple equilibrium relationship. When bile salt micelles start to form the free bile salt has another "phase" to enter. It will be present as free monomer (3.2mM), sequestered with the polymer, or in the mixed bile salt/ fatty acid micelle. This competition results in the decreasing percentage bound to the polymer as seen in Fig. 45. In Fig. 46 we see that NaOl is bound at nearly 100% below the CMC of the micelles. Above this concentration the binding decreases due to the same competition between the polymer and the mixed micelles.

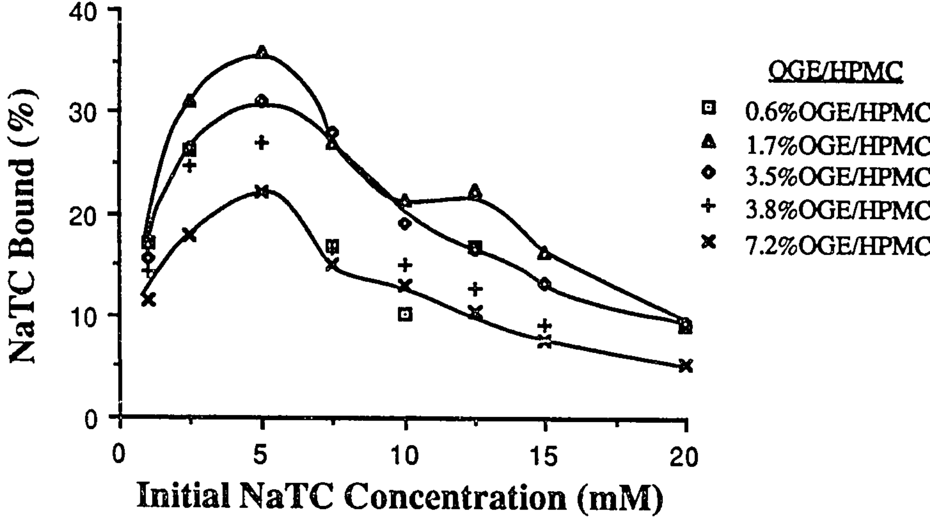


Fig. 45 Percentage Binding of NaTC from NaTC/NaOl Mixed Micelles.
(^{14}C labeled NaTC, 1:1 molar ratio)

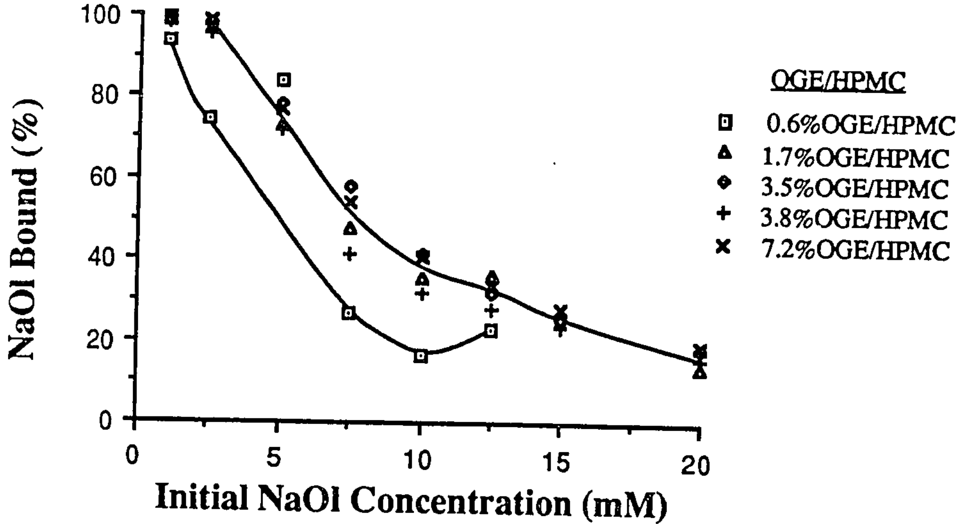


Fig. 46 Percentage Binding of NaOl from NaTC/NaOl Mixed Micelles.
(^{3}H labeled NaOl, 1:1 molar ratio)

Examination of Figures 43 and 44 reveal that there may be two transitions. Initially, NaTC and NaOl binding levels off at values which do not seem to correlate well with OGE attachment. When the polymer dissolves, the binding rises rapidly and may level off at a higher value. Unfortunately, we can't quantitate this binding because the polymer goes through the filter. This second increase may be an illusion and represent an artifact of the filtration method since it was difficult to force these samples thru the filter. The partially dissolved polymer holding up unbound micelles by partially plugging the filter pores. For this reason, we can only hypothesize on the existence of this second binding maximum - the experiment does not allow proof.

The almost complete binding of NaOl seemed to indicate that the NaOl was not being solubilized in bile salt micelles, and found the hydrophobic polymer environment preferable to aqueous solution. To examine this we obtained surface tension measurements of 1:1 NaOl:NaTC solutions in the same buffer system employed for the study. We also determined the surface tension for NaTC and NaOl as single components. NaTC (single component) showed a linear decrease in surface tension until about 0.7mM. This may represent the CMC, but more likely indicates that the

surface was saturated with NaTC since the published CMC (using spectral shift) is found to be 3.2mM [22]. The surface tension then leveled off at about 45 dynes/cm (Fig. 47).

We found that NaOl (single component, saturated solution) had a surface tension of about 27 dynes/cm. We believe that this value represents saturation of the surface with NaOl. Particles of NaOl were observed in the sample for this determination.

When 1:1 molar NaTC:NaOl solutions were run (Fig. 48), we found that the surface was rapidly saturated with NaOl (27 dynes/cm). At about 3mM (remarkably near the published value for NaTC CMC) the surface tension began to rise which indicates that NaOl is being taken off the surface into micelles. We believe that this represents a CMC for the 1:1 molar ratio. Another possibility is that NaOl is not pure and some surface active impurity is being removed from the surface into micelles. A rising surface tension above the CMC is generally assumed to be caused by impurities in single component soaps (e.g. SDS).

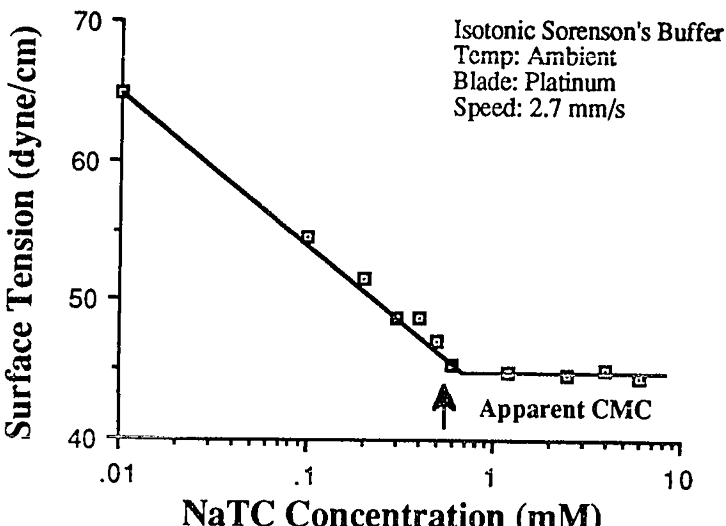


Fig. 47 Surface Tension of NaTC in Sorenson's Buffer.
Indicated CMC is probably saturation of the surface with taurocholate.
Published CMC is 3.2mM.

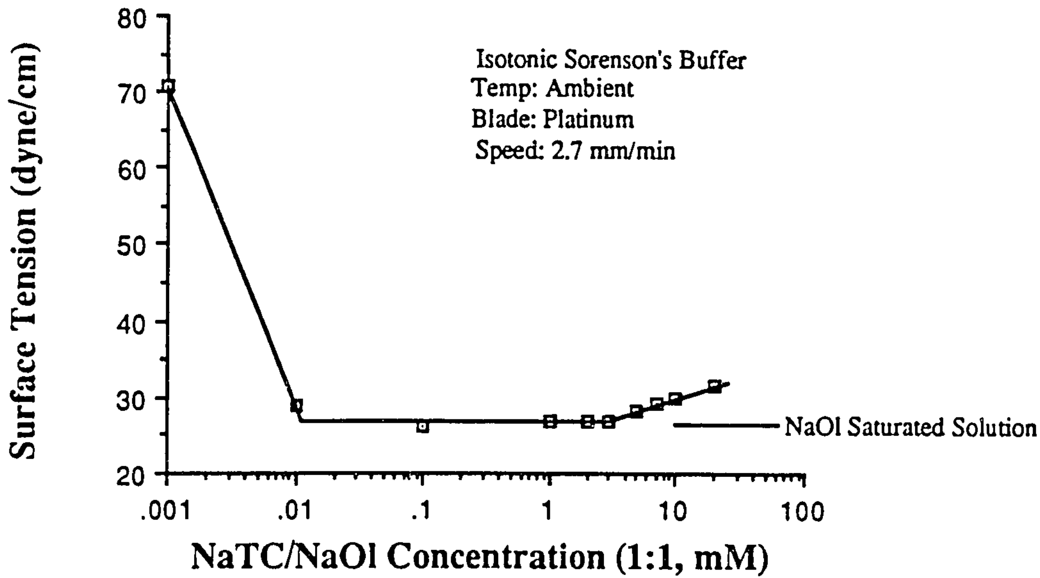


Fig. 48 Surface Tension of NaTC/NaOl (1:1) in Sorenson's Buffer.
CMC of NaTC is 3.2mM.

Fig. 49 presents a concluding mechanism of this binding for 1.7%OGE/HPMC where there are three environments for NaOl to exist in. These include: bound to the polymer, free in solution, and in bile salt mixed micelles. We note that below an initial concentration of 5.0mM almost all of the NaOl is bound to the polymer. We assume that micelles are not available for the NaOl in this region and this environment is not available. 5 mM and above represents a region where NaTC/NaOl micelles are formed, thus competing with the polymer for NaOl. After micelle formation, the free concentration of NaOl should be constant and indeed binding levels off. Finally, after the polymer dissolves binding may increase though we can't be sure of this. We feel that the very high viscosity of these solutions indicate that the micellar regions are acting as cross-links (the solution is almost a gel). This theory would support the early results of SDS/HPMC interactions as measured by viscosity.

Multi-component mixed micelles have the highest relevancy to the anticipated application of HM-HPMC. The composition for our system (1:1:0.15:0.06, NaTC:NaOl:LL:Chol) was chosen due

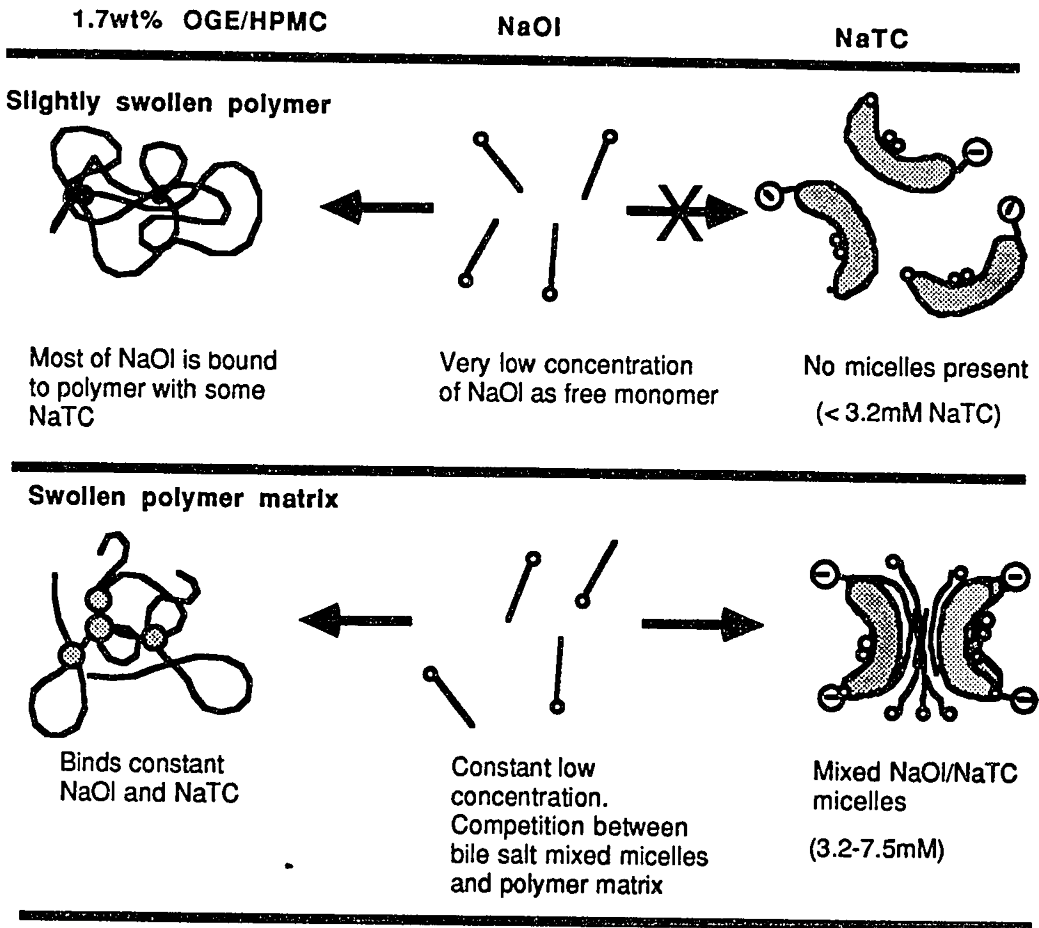


Fig. 49 Binding of NaTC and NaOl by 1.7%OGE/HPMC.

to it's similarity to that found in the duodenum of humans following a meal [18]. LC analysis of filtered distal duodenal fluid on Sepharose 6B has shown that the mixed micelles range in size from 2.3-3.5 nm (27,000-69,000 MW) and that the molar ratio is (1:1.4:0.15:0.06, bile salt:free fatty acid:lysolecithin,cholesterol). Coincident peaks were observed for all four components in this study indicating that indeed mixed micelles were formed). The total bile acid is found to be near 9mM, 0-90 minutes after eating. We choose a 1:1:0.15:0.06 molar ratio since we had problems making optically clear solutions using the composition above.

Figures 50 and 51 show the results of our study examining the effect of mixed micelle concentration on binding to OGE/HPMC. We note (Fig. 50) that NaTC binding is nearly that observed from the NaTC/NaOl binding experiment. This would be the expected result since NaTC and NaOl make up the principle components of the system. Cholesterol (Fig. 51) also appears to be bound in a similar fashion as that observed for NaOl in the NaTC/NaOl study. Initially, very high percentages of the cholesterol were bound and the high OGE attachment polymers showed higher binding than lower attachment levels (with the exception of 3.6% attachment which was also observed for the previous study). Plots of percentage binding for these two components are presented in Figures 52 and 53.

By taking the binding (plateau region) of NaTC, NaOl, and cholesterol and plotting this against the attachment of OGE to HPMC for all binding runs we see that there is a relationship between ligand attachment and the binding of these three components (Figures 54-56). For NaTC binding the results are quite similar to that obtained in the equilibrium dialysis experiments (Fig. 41). The binding maximizes near 2-3 wt% attached then decreases. This is probably related to the wettability of the polymer. At high ligand attachment the polymer becomes very hydrophobic and forms a non-wetting precipitate with a decreased interface or surface for amphiphile interaction. Near 2-3 wt% attachment the polymer is in an intermediate state (not so hydrophobic that it forms a "tight" aggregate) and binds the amphiphile at the interface. For NaOl and cholesterol binding we see an increasing binding as lipid is attached until what appears to be a plateau is reached. It is presumed that these lipids are more hydrophobic then the bile salt and do not require just an interface for binding. They are able to penetrate into the hydrophobic regions of the high conjugation polymer precipitate and are bound through another mechanism. These results indicate that the higher the attachment of ligand the greater the binding of more hydrophobic constituents from mixed micelles.

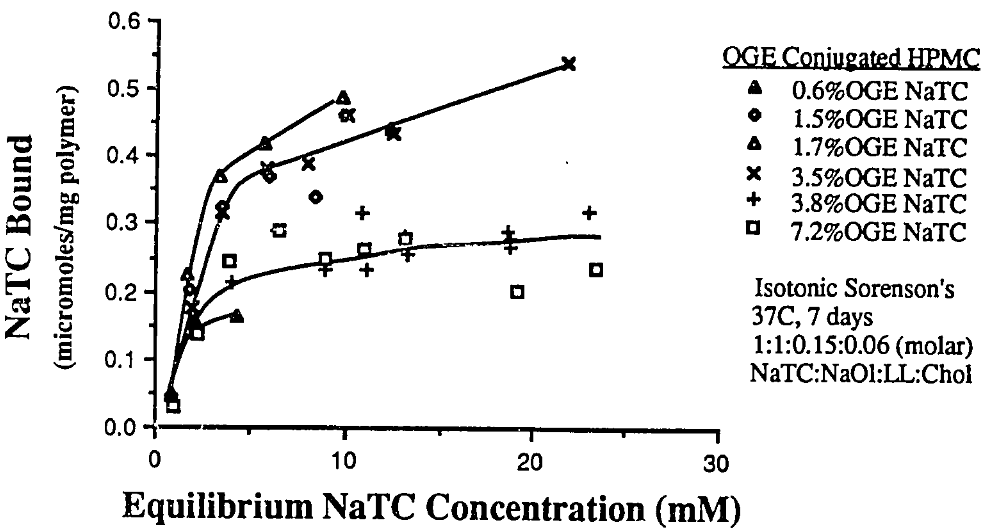


Fig. 50 NaTC Binding from NaTC/NaOl/LL/Chol Mixed Micelles. (1:1:0.15:0.06 molar ratio, ^{14}C labeled NaTC).

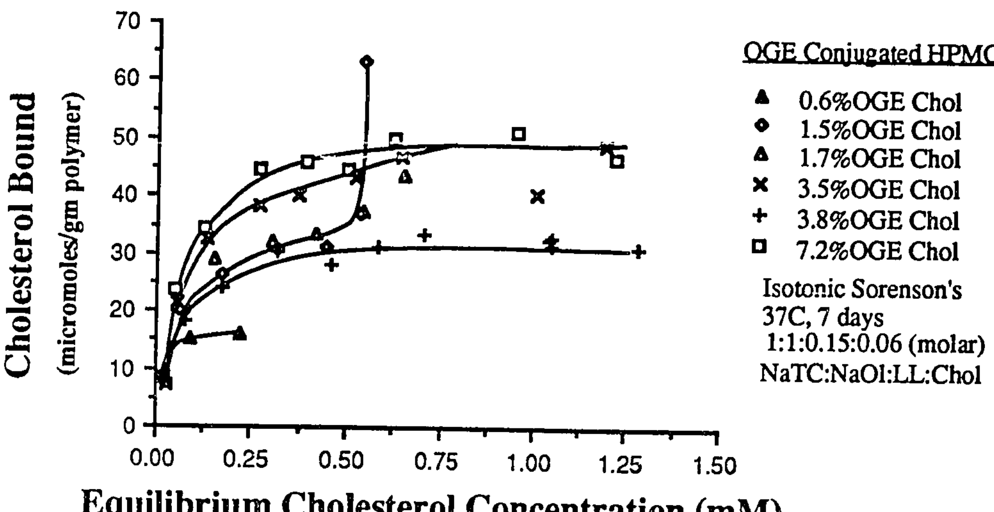


Fig. 51 Cholesterol Binding from NaTC/NaOl/LL/Chol Mixed Micelles. (1:1:0.15:0.06 molar ratio, ^{3}H labeled cholesterol).

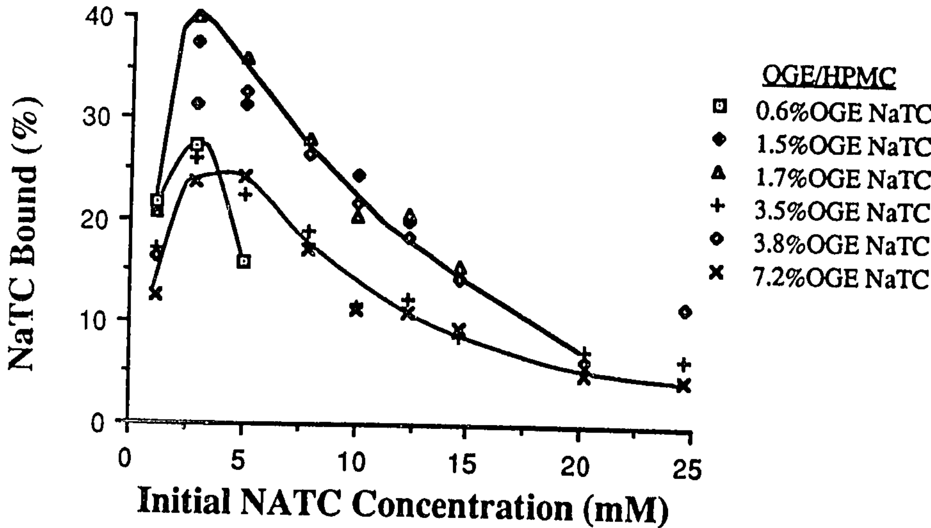


Fig. 52 Percentage Binding of NaTC from NaTC/NaOl/LL/Chol Mixed Micelles. (1:1:0.15:0.06 molar ratio, ^{14}C labeled NaTC).

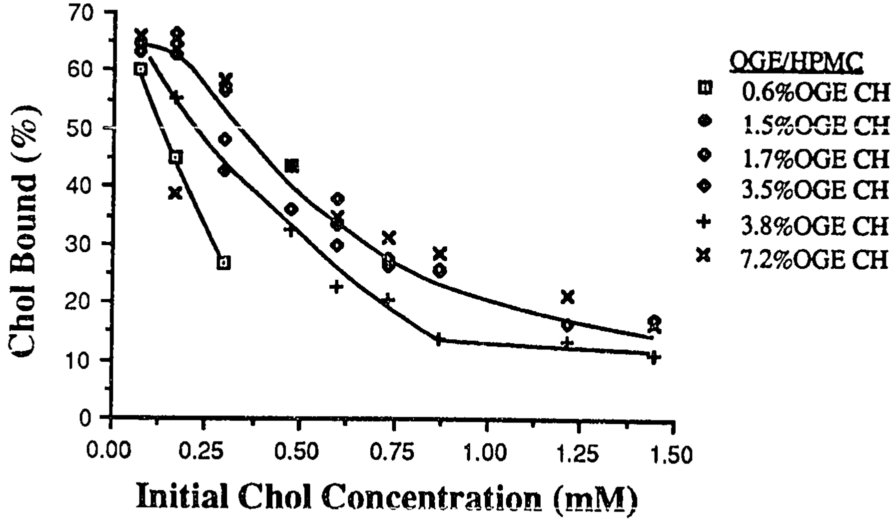


Fig. 53 Percentage Binding of Cholesterol from NaTC/NaOl/LL/Chol Mixed Micelles. (1:1:0.15:0.06 molar ratio, ^{3}H labeled Chol).

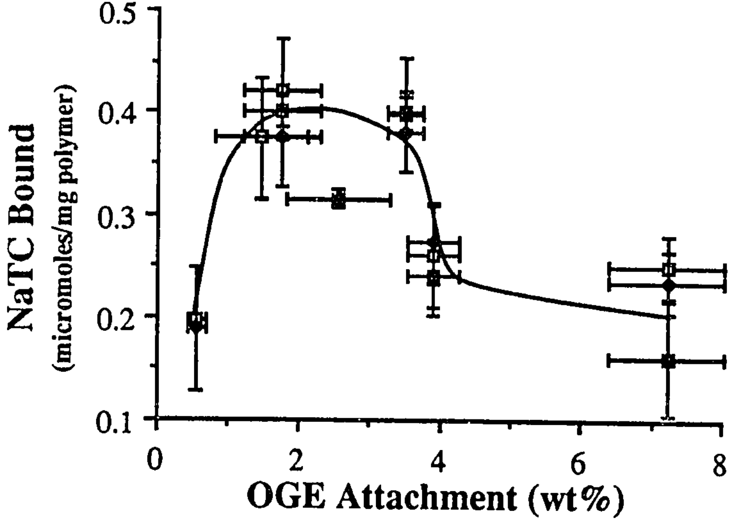


Fig. 54 Plateau Binding of NaTC by OGE/HPMC.
(1:1:0.15:0.06 molar ratio, ^{14}C labeled NaTC).

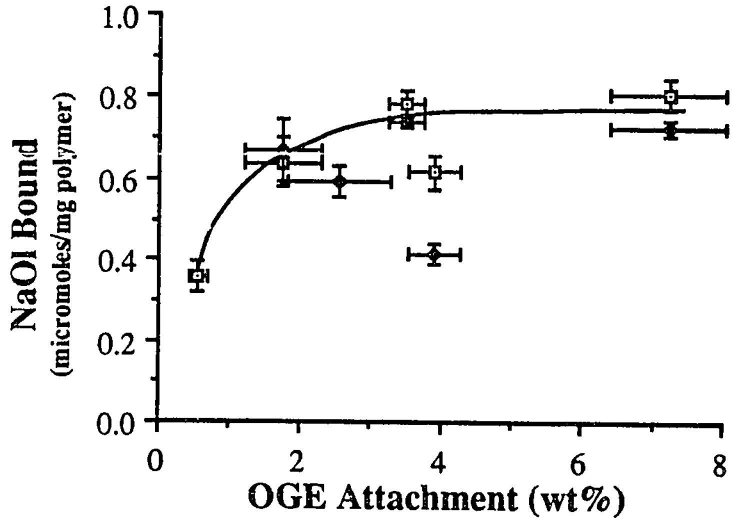


Fig. 55 Plateau Binding of NaOl by OGE/HPMC.
(1:1:0.15:0.06 molar ratio, ^{3}H labeled NaOl).

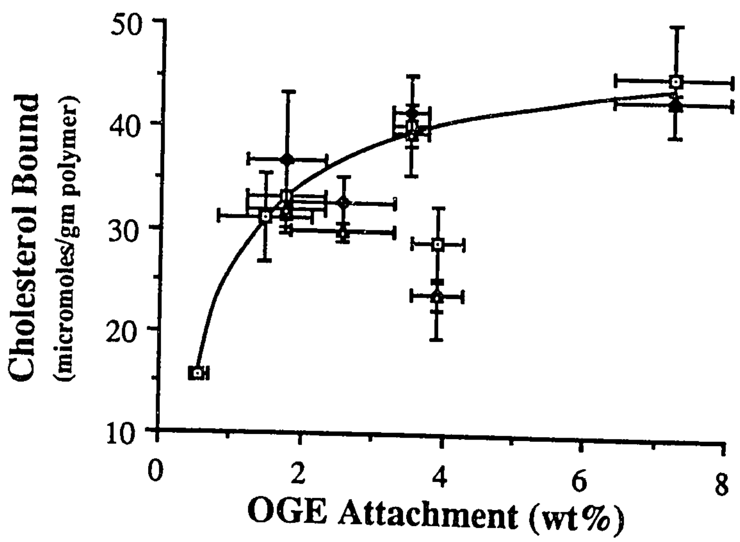


Fig. 56 Plateau Binding of Cholesterol by OGE/HPMC. (1:1:0.15:0.06 molar ratio, ^{3}H labeled cholesterol).

Knowing that binding levels off above 10mM NaTC (with the same relationship to other components) we performed one last *in vitro* study to determine what effect the ligand had on binding. The effect of ODGE and CGE on binding at 10:10:1.5:0.6 mM NaTC/NaOl/LL/Chol reveal that the ligand has little effect on binding (Figures 57 - 59). We see that binding of these probes shows the same trends as OGE/HPMC. Comparing the binding of the lipid components by the three ligands suggests that OGE binds more then ODGE and CGE. This may be related to the liquid crystallinity of this material. Recall that OGE is a liquid at room temperature while ODGE and CGE are solids. Perhaps the hydrophobic domains the HM-HPMC gel are more fluid and allow the penetration of lipids from solution. This suggests that the cholesterol or octadecanol attached to the polymer is probably aggregated at the temperature of the experiment and is not able to open up and bind free materials from solution.

Fig. 60 shows a cross plot of NaOl and cholesterol binding. Remarkably, we see that there is a high correlation which is independent on conjugated lipid identity. Again, we note that OGE binds a higher total amount. The correlation suggests that the binding mechanism for these two

components of biologically relevant lipids is similar. Our theory that hydrophobic domains in the polymer gel acting as sites for sequesteration is upheld. We conclude that the identity of the ligand does not have an effect on relative binding but that is does affect the total amount bound. One explanation of this behavior is that the process of sequestration is being mediated by a process that is independent of the ligand identity. A simple model would suggest that it is hydrophobic interactions that are driving the binding of lipids from micelles into the polymer gel. This would explain the independence observed; the micelle component and it's relationship to water determine to what extent it is bound to the hydrophobic polymer. The hydrophobic polymer provides a nucleation site where conjugated lipids are aggregated together.

Fig. 61 presents the cross plot of NaTC bound to cholesterol bound. We see that there is some correlation, with the outliers being HM-HPMC that have high lipid conjugation. These HM polymers bind more cholesterol then NaTC relative to the correlating polymers. Our description of interface versus lipid phase binding adequately describes the difference.

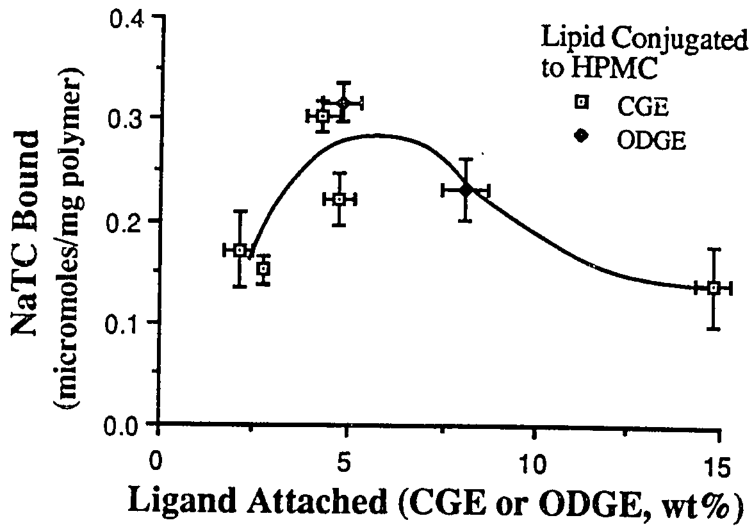


Fig. 57 Binding of NaTC by CGE/HPMC and ODGE/HPMC. (10:10:1.5:0.6 mM, ^{14}C labeled NaTC).

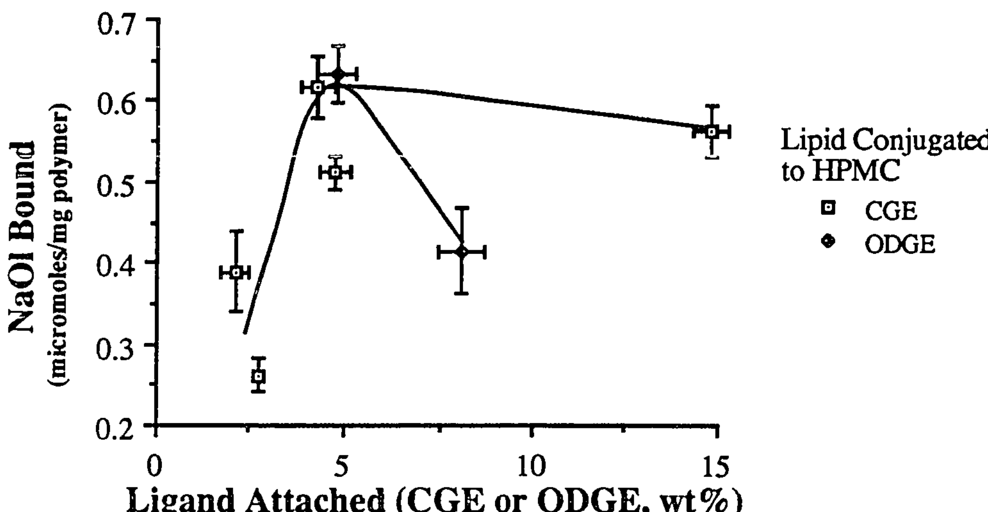


Fig. 58 Binding of NaOl by CGE/HPMC and ODGE/HPMC. (10:10:1.5:0.6 mM, ^{14}C labeled NaOl).

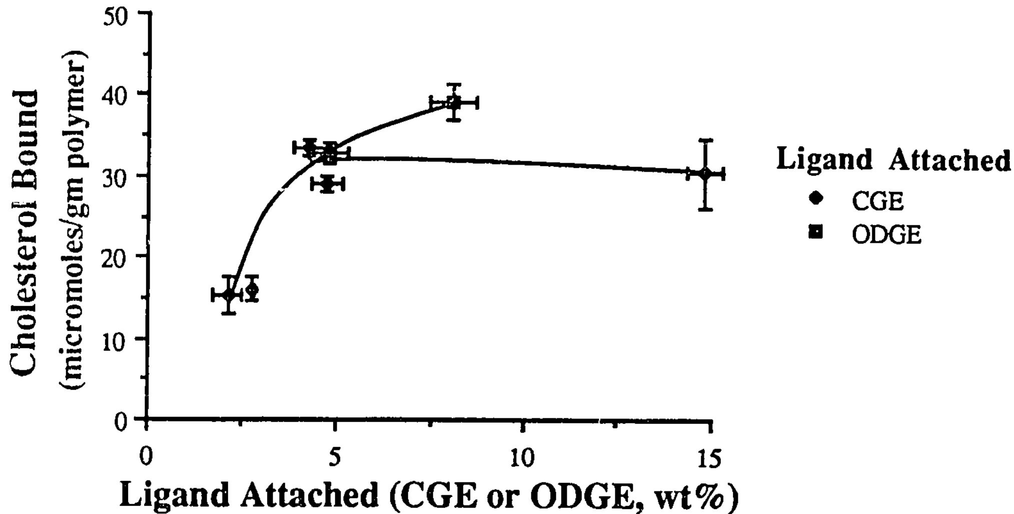


Fig. 59 Binding of Cholesterol by CGE/HPMC and ODGE/HPMC. (10:10:1.5:0.6 mM, ^{3}H labeled cholesterol).

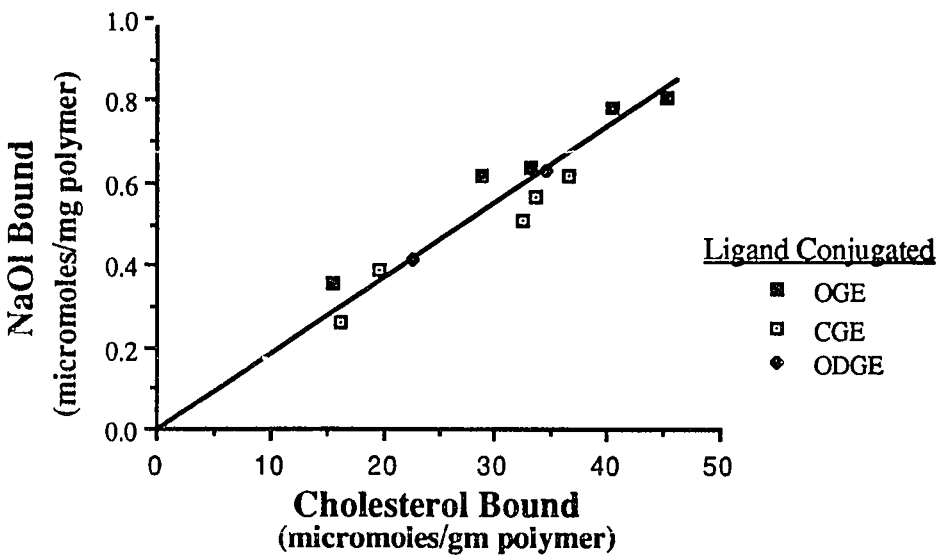


Fig. 60 Cross Plot of NaOl and Cholesterol Binding.
Binding of these components from 10:10:1.5:0.6 mM NaTC/NaOl/LL/cholesterol shows a high correlation with OGE showing higher binding then ODGE and CGE conjugated HPMC.

Overall our theory is that hydrophobic domains of HM-HPMC gels act something like a liquid phase that partitions hydrophobic components. An analogy would be the partitioning of cholesterol between water and oil. Most of the cholesterol will end up in the oil phase. Partitioning into a solid domain will also be less then that observed for a liquid phase. Oleyl is more liquid then either octadecanol or cholesterol (conjugated to the polymer) and binds more lipid by this mechanism. The driving force for this complexation is mediated by hydrophobic interactions with the relationship between the hydrophobic lipid and water or micelles being responsible. There is little or no chemical recognition involved (analogous to a protein binding site). NaTC is a highly surface active component that binds on the surface. Recall that it is always depicted on the surface of mixed micelles. The existence of this interface is dependent on the amount of lipid conjugated to HPMC - the highly conjugated polymer curls up and decreases the interfacial area. We know that this is true for LP_4GE conjugated to HPMC and expect it to be the case for other ligands (Chapter 4 viscosity results).

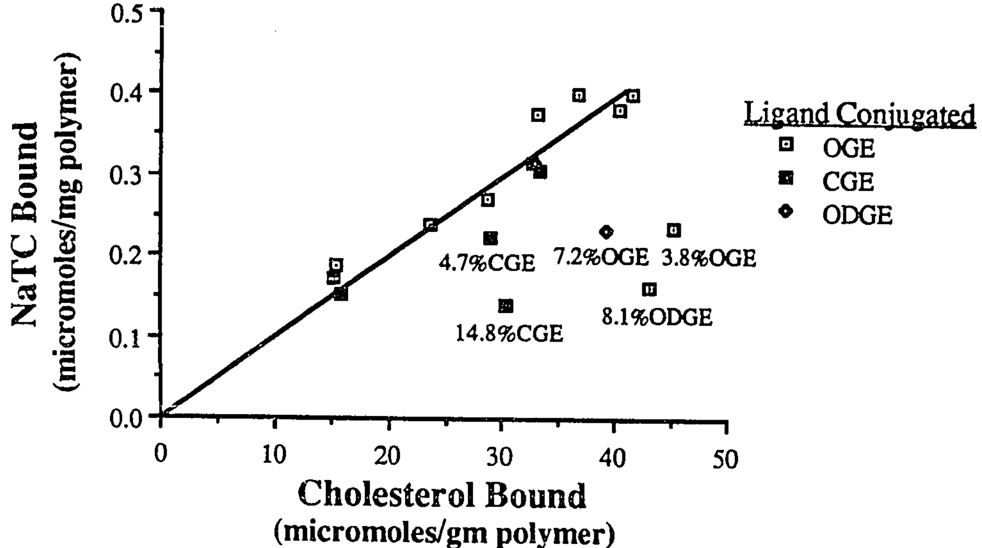


Fig. 61 Cross Plot of NaTC and Cholesterol Binding.
Binding of these components from 10:10:1.5:0.6 mM NaTC/NaOl/LL/cholesterol shows some correlation with higher lipid conjugated HPMC being outliers.

5.3.4. *In Vivo* Results

In vivo results indicate that our polymer does reduce the absorption of lipids from the intestinal tract and increases the ability of rats to tolerate HPMC. The order of presentation was chosen by the chronological events of our work. We performed the chronic study very close to the start while the remaining experiments carried out later. These studies represent only the beginning of work necessary for fruition of this research. Dr. Linscheer is still working with our polymer and will hopefully further it's applicability.

5.3.4.1. Chronic *In Vivo* Results

The diet had the consistency of cookie dough and most of the animals refused at first to consume it. One animal refused the diet completely and was taken out of the study. It was observed that animals in group II (5% HPMC/SAD) as a whole actually lost weight over the course of the study. Recall that there were four groups, **I**: control (no food additive), **II**: 5% of diet composed of HPMC, **III**: 5% of diet composed of 0.23%ODGE/HPMC, and **IV**: 5% of diet composed of 0.45%ODGE/HPMC (Table 12). Fig. 62 illustrates this by showing the change in animal weight divided by the food consumed for the four groups. As is common in animal studies some animals behave differently to a stimulus then others. One animal in this group gained the most weight of all animals in the study and was quite healthy. This group as whole also suffered from redness and sometimes scabs around the anus. Animals in group IV appeared to be the most healthy (appearance and activity) followed by group I, III, and II in decreasing health. This indicates that HPMC (alone) is not well tolerated by the animals. However at high levels of lipid attachment this effect is counteracted.

Struthers has commented on the effect of feeding large quantities of hydrophilic fibers to rats [229]. She notes that intestinal obstruction is rare in humans however can occur in rats. Since the diet for group II contained 5% dry HPMC, the holding capacity of water is quite high and may have caused the poor health of this group. Dow states that Methocel E complies with FDA and USDA

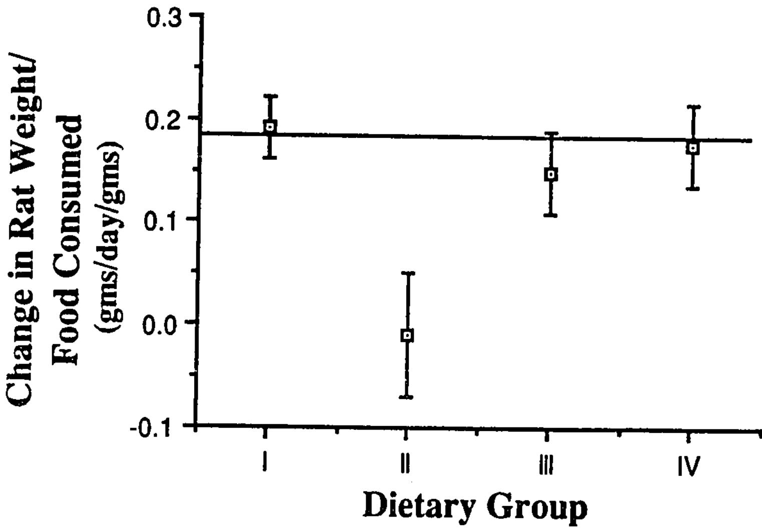


Fig. 62 - Change in Animal Weight for Chronic *In Vivo* Study. Normalized with Respect to Food Consumed for Animal Groups. SAD composition can be found in Table 11.

status to be used as an additive in foods at any level. Our results with rats indicate that 5% of the diet is contraindicated. Plasma cholesterol levels for the four dietary groups are shown in Fig. 63, and triglyceride levels are shown in Fig. 64. We note that there is not much difference between group I and IV. The latter group showing some reduction (though not significant) in plasma cholesterol.

After considering this diet we find it remarkable that any decrease was observed. We feel that the extremely high fat content probably swamped both the absorptive capacity of the intestinal tract (the feces were very fatty) and our polymers ability to influence it. Another study using a different diet and saponins (a material proven to precipitate cholesterol form mixed micelles) found no significant changes in rat serum cholesterol [107]. They noted that "little change in serum cholesterol in response to dietary cholesterol is usually seen in rats" [230]. Another study examining the effect of cholesterol feeding on metabolism in germfree rats found that these animals (like conventional rats) have the ability to compensate for dietary cholesterol by decreasing

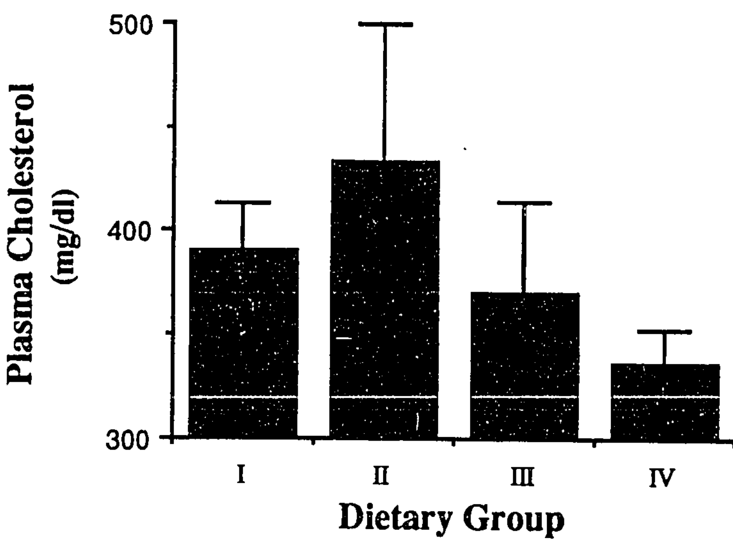


Fig. 63 - Mean Total Plasma Cholesterol Levels for Chronic Study.

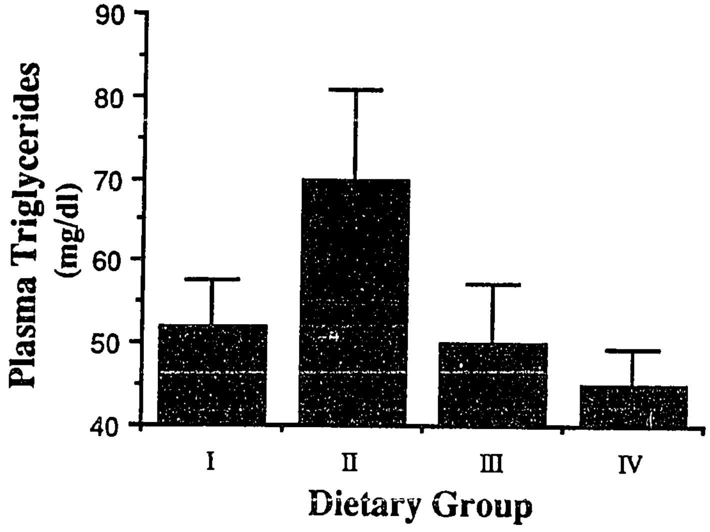


Fig. 64 - Mean Triglyceride Levels for Chronic Study.

cholesterol synthesis and increasing bile salt synthesis (bile salts are their primary method of eliminating cholesterol) [231]. We conclude that for chronic studies, the measurement of plasma cholesterol levels is not an indication of decreased intestinal absorption of cholesterol and that our

diet may have exacerbated the situation.

5.3.4.2. Acute *In Vivo* Study Results

For these experiments, intestinal segments were isolated while leaving the blood supply intact. After flushing each segment with buffer and air they were closed and a test solution injected into the lumen. The test solution contained radiolabeled oleic acid (^{14}C), with or without a sample of our polymer. The quantity of polymer was quite low - only 0.2% of the infused solution.

Three animals were studied, and we found that when the results were normalized with respect to dry weight of tissue divided by the actual time that the segment was exposed to the solution there was a lot of data scatter. These results are displayed in Fig. 65. We have presented the standard error of the mean. Student's T test for unpaired data indicated that the probability of the two populations being the same was 1% ($p = 0.01$). We note that there was a 40% drop in oleic intestinal adsorption from a solution containing 10:20:0.15:0.15mM NaTC/NaOl/lecithin/cholesterol.

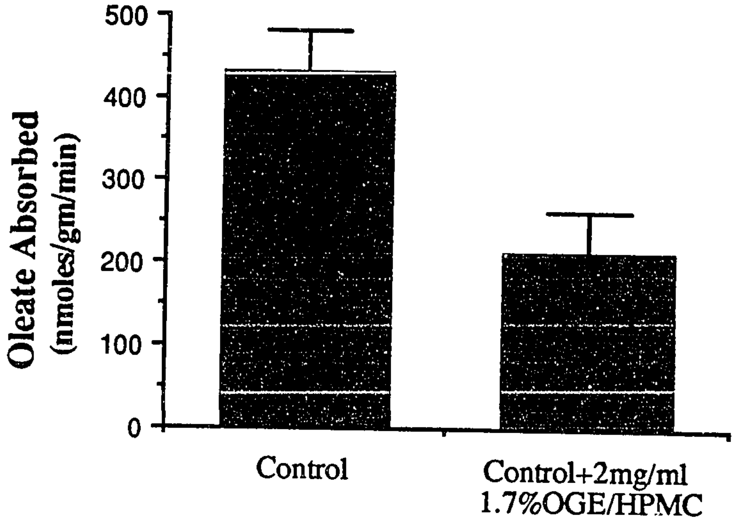


Fig. 65 - Effect of 1.7%OGE/HPMC on Oleic Acid Absorption in the Rat. Absorption determined using simulated intestinal fluid from intact intestinal segments.

Remarkably, this is nearly the same percentage of NaOl bound from a different test solution *in vitro* (Fig. 46, using 10:10 mM NaTC:NaOl).

In one animal we performed a wash-out study and found that for 5 segments there was very good agreement between infusion solutions and the total quantity of oleic recovered. Fig 66 shows the recovery of PEG and oleic acid. We see that recovery of PEG is somewhat dispersed, and not dependent on the addition of HM-HPMC to the perfusion solution, while the recovery of oleic acid is dependent on the segment perfusion solution. Total oleic recovered for the control being significantly different then the sample segments. This may indicate that using PEG as a normalization scheme is not advisable. Since ^3H is a low energy β emitter it is possible that variable quenching between samples (due to a little blood or other factors) may lead to erroneous results.

Overall, the results indicate that there is an effect of HM-HPMC on lipid adsorption in the intestinal tract at low quantities of food additive present. It appears that with experience the test could be make more reproducible. It is fairly easy and provides rapid feedback on the effect of a food additive on fat and cholesterol intestinal absorption. It would be interesting to use this method for comparing several of our food additive formulations with each other and several food additives that have shown activity in man.

5.3.4.3. Dr. Linscheer's *In Vivo* Study Results

Recall that Dr. Linscheer conducted a similar experiment as our own with the advantage of providing a flowing system that allows comparing directly the same segment for both control and sample perfusion fluids. He obtained some remarkable results. Fig. 67 shows the effect of 0.76%OGE/HPMC on oleic acid and cholesterol intestinal absorption using his perfusion technique. We see that inclusion of 0.4 mg/cc of HM-HPMC resulted in a 68% drop of oleic acid adsorption and a 40% decline in cholesterol adsorption. This concentration of polymer is well below the levels we studied in the acute *in vivo* study (2 mg/cc) however, he got greater reduction in fat absorption.

PEG Washout

Oleic Acid Washout

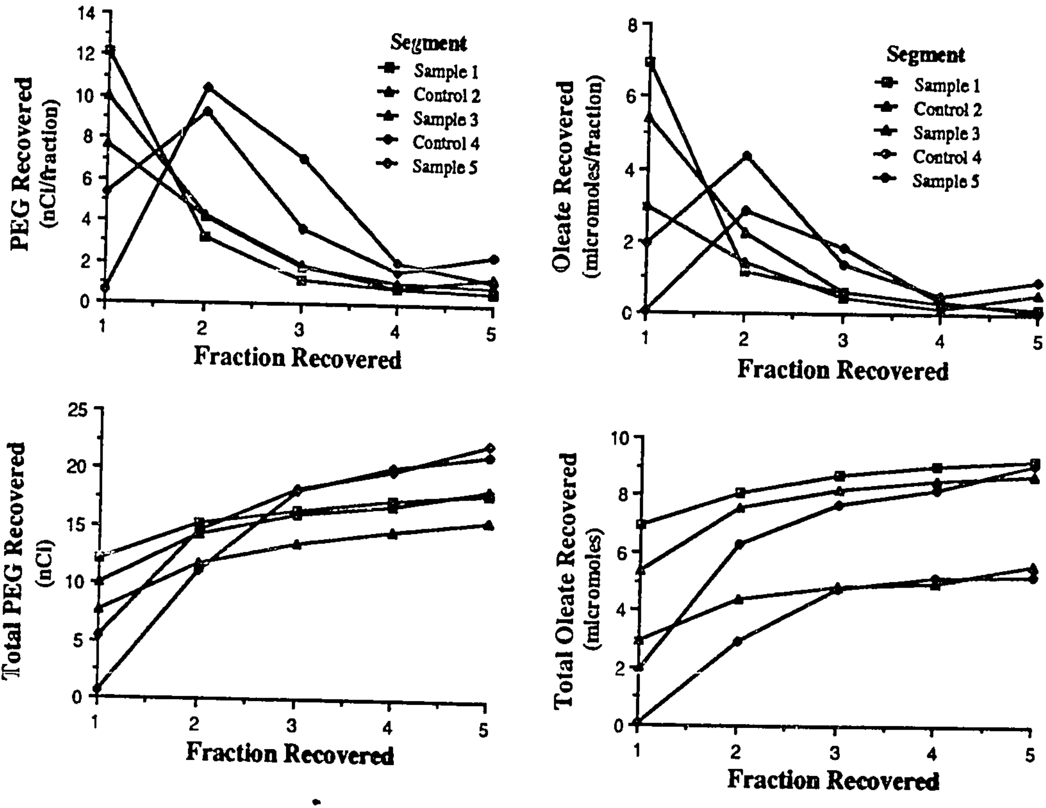


Fig. 66 - Washout of PEG and Oleic Acid From Intestinal Segments.
Note a lack of dependence on segment identification for total PEG recovered while total oleic recovered seems to be highly dependent.
Sample segment contains the same solution as control with 2mg/ml of 1.7%OGE/HPMC added.

He used a flowing system so this might explain the discrepancy since the polymer/lipid complex spends less time in the lumen of the intestinal tract than our static system. We might hypothesize that the HM-HPMC is binding whole mixed micelles, the oleyl ligands acting as anchors.

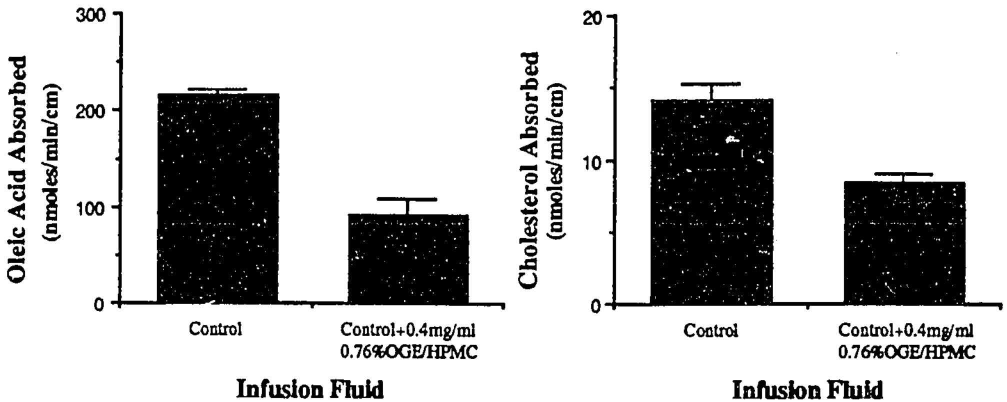


Fig. 67 - Effect of OGE/HPMC on Oleic Acid and Cholesterol Absorption. Results from Dr. Willem Linscheer, Syracuse NY.

CHAPTER 6

Conclusions and Recommendations

This thesis deals with the design, synthesis, characterization, and testing (both *in vitro*, and *in vivo*) of a hydrophobic affinity polymer designed specifically for use in the intestinal tract. We have reviewed the literature on how cholesterol is absorbed into the body and briefly examined how other food additives reduce this. There are primarily five mechanisms for reducing cholesterol absorption. These include: 1) stabilization of the emulsion phase, 2) binding of anionic constituents of the intestinal contents, 3) binding of neutral lipids, 4) decreased activity of enzymes responsible for converting esters of cholesterol into free cholesterol and 5) increasing the viscosity, thereby increasing the UWL. Examination of the Methocel (HPMC - DOW) literature reveals that this polymer may act effectively on both 1) and 5). We have found this polymer also interacts with amphiphiles and binds NaTC by hydrophobic interactions. This makes it an excellent choice as a backbone polymer for the desired application.

The rationale for designing a polymer based on this backbone and enhancing it's activity by covalently attaching lipids is presented as well. This includes reasons for selecting the hydrophobic ligand and methods for conjugating it using biologically stable ether chemistry. We have also seen what effect conjugation of lipid to the polymer has on physical character and ability to bind amphiphiles and lipids in aqueous solution. Finally, the material has shown *in vivo* activity by reducing lipid absorption using selected rat models of the human intestinal tract. This Chapter briefly reviews the important discoveries and innovations produced from the dissertation and discusses some recommendations for future work.

6.1. Conclusions from Synthesis and Characterization

The principal conclusions from this part of the study are outlined in the following numbered paragraphs:

1) It is possible to attach lipids which have a pendant hydroxyl (either primary or secondary) to HPMC using glycidyl ether chemistry. Our approach was to react the lipid-OH with epichlorohydrin then form the glycidyl ether by reaction with NaOH. Following conjugation of the glycidyl ether with hydroxyls on the polymer (using stannic chloride as a catalyst) backbone in dioxane the product is precipitated using hexane. Alternately, the epichlorohydrin could be reacted with HPMC to form pendant glycidyl ethers. These could be reacted with an excess of alcoholic lipid as the second step. We choose the first method because it allowed easy control of the conjugation process and prevented covalent crosslinks which might form by reaction of epoxide-HPMC with free hydroxyls on the backbone. The second synthetic method would be an interesting approach to explore, particularly if one desired a cross-linked gel as for plasmaphoresis or chromatographic applications.

2) The conjugation of lipids can be quantitated using Zeisel cleavage of the ether bonds followed by GC analysis of the resulting iodides. This method was validated by scintillation counting of ^{14}C oleyl glycidyl ether conjugated HPMC both before and after cleavage using hydroiodic acid. In addition, it was found that the method was reproducible and offered the ability to detect small quantities of conjugated lipid (as low as 0.1wt%).

3) Lipid conjugated to HPMC makes the polymer more hydrophobic and decreases the cloud point of the polymer. These are expected results, the attachment of a lipid to a moderately hydrophilic polymer should decrease the hydrophilic/lipophilic balance (HLB). Since hydrophobic interactions are the primary means by which one controls the cloud point, decreasing this balance should (and does) cause the cloud point to decline.

4) Low quantities of lipid conjugated to HPMC produces a polymer which curls up on itself decreasing the intrinsic viscosity until a critical conjugation is reached. We hypothesized that the intrinsic viscosity would decrease as more lipid was attached to the backbone polymer and this was found to be the case. The polymer "hides" the attached lipid from aqueous solution by collapsing.

5) Above this critical conjugation the polymer aggregates and does not form an optically clear solution in water. Presumably this occurs because the HLB is below that possible for dispersion into aqueous solution.

Knowing that cellulose ethers form "microgels" in aqueous solution we can speculate that increased lipid conjugation causes the polymer to form a three dimensional (macro) gel where hydrophobic regions (predominately conjugated lipid) act as crosslinks. We have found that these regions interact with amphiphiles and that as they are increased the interaction is enhanced. It was also discovered that the HLB has an effect on how the polymer interacts with surface binding amphiphiles (such as NaTC) and lipids (e.g. NaOl and cholesterol) that can interpolate into hydrophobic regions.

6.2. Conclusions on the Interaction of HM-HPMC and HPMC with Amphiphiles

It was ascertained that the native polymer (HPMC) interacts with amphiphiles such as SDS producing results similar to those obtained for conjugating lipid to the polymer. We propose that hydrophobic sites on the polymer act as nucleation sites for the formation of micelles below the CMC of the detergent (as evidenced by fluorescent probe). These regions function in much the same way as lipid conjugated HPMC sites - they destabilize the polymer resulting in a lower cloud point and an increased viscosity for 0.5% solution. When these sites form (for both SDS/HPMC and lipid conjugated HPMC) they provide an environment suitable for the inclusion of dyes, fluorescent probes, cholesterol and NaOl. This is particularly evident for HM-HPMC samples that are not soluble in water. These conclusions are summarized in the following paragraphs.

1) HPMC interacts with amphiphiles including SDS and NaTC. This alters the aqueous solution character of the polymer. For SDS/HPMC the viscosity either increases at ~4mM SDS and 0.5% HPMC in water or decreases at the same concentration of SDS and ≤ 0.25% HPMC in water. The concentration of the SDS (4mM) is interpretated as being the first concentration of interaction as defined by others. SDS interacts with the HPMC to either enhance aggregation at higher HPMC concentration (0.5 wt%) or decrease aggregation at lower HPMC concentration (≤0.25 wt%).

2) HPMC lowers the apparent CMC of SDS. The 4mM interaction value is below the generally recognized CMC for SDS of 8.1mM. Apparently, hydrophobic sites on the polymer nucleate the formation of SDS micelles.

3) The interaction of NaTC with HPMC is enhanced by the presence of conjugated lipids. It was found that by conjugating lipid to HPMC the polymer will ultimately sequester more NaTC at equilibrium conditions. We speculate that conjugated lipids act as interfacial sites for the adsorption of NaTC.

4) NaTC acts to solubilize water insoluble lipid conjugated HPMC. As the amount of lipid conjugated to HPMC is increased the polymers become more collapsed in the absence of the detergent NaTC. We have observed that NaTC is able to solubilize these polymers if enough NaTC is present. Presumably, the NaTC "coats" the hydrophobic ligands allowing the polymer chains to unfold.

5) Maximum binding of NaTC to HM-HPMC occurs at higher concentrations of NaTC as lipid conjugation is increased and absolute maximum binding is increased. This supports the coating theory presented above. As the number of lipid sites is increased the potential hydrophobic surface area is also increased. Normally, the polymer chains aggregate together to decrease the interaction with water, however if sufficient surfactant is present it can cause them to deaggregate.

6) In the physiologic region (10-20 mM NaTC) a maximum NaTC binding occurs at about 4% lipid attached to the polymer. At this NaTC concentration, the solubilizing power is moderate. It is able to solubilize some lipids conjugated to HPMC but not enough to "dissolve" the polymer if the lipid attachment is high. Recall that as the amount of lipid conjugated to the polymer is increased the sample forms a gel which tends to collapse into less volume in water. This decreases the interfacial area available for NaTC sequesteration. At 4% lipid attached to the polymer there is a balance of hydrophobic sites available and a polymer which has not collapsed which allows for maximum binding of NaTC.

7) If HPMC backbone binding is neglected then approximately 5-10 moles of NaTC are bound per lipid ligand above ligand attachment of 2 wt%. This is a remarkable result in light of known aggregation values for single component NaTC micelles. The aggregation number is from 2 to 10 NaTC/micelle and indicates that the conjugated lipid is acting like an anchor that binds a whole NaTC micelle.

8) The binding of NaTC to HM-HPMC is very similar for all ligands tested (for OGE, ODGE, and CGE). This suggests that the binding is being driven by hydrophobic interactions not by any type of specific chemical recognition.

When mixed micelles interact with HM-HPMC we have seen that some constituents of the micelle are bound differently.

1) NaOl binding is very high (>95%) when NaTC concentrations are below 5 mM. This indicates that mixed micelles are not available for NaOl and it prefers the polymeric environment to water. This conclusion is supported by surface tension measurements.

2) Cholesterol, NaOl and NaTC binding is a two phase process where NaTC is bound to interfacial regions while cholesterol and NaOl are bound in hydrophobic domains that can be thought of as being a liquid phase.

3) Dissolution of the polymers occurs at lower concentrations of NaTC/NaOl then expected from single component NaTC binding. This indicates that the NaOl is acting to solubilize the polymer by breaking up the hydrophobic "micellar crosslinks". This may indicate that NaOl is helping to break-up the hydrophobic domains that result from high lipid attachment to HPMC.

4) On a molar basis NaOl is bound better then NaTC from the NaTC/NaOl system. This indicates that the NaOl preferential prefers the polymeric lipid regions to that of the mixed micellar NaTC/NaOl regions.

5) Cholesterol binding is very high below NaTC concentrations of 5mM (similar to NaOl in NaTC/NaOl micelles).

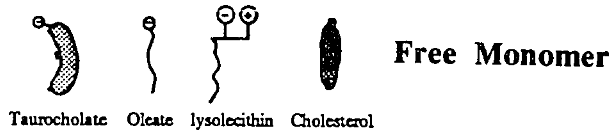
6) Cholesterol binding shows the same trends as that observed for NaOl in NaTC/NaOl binding. The higher the amount of lipid conjugated to HPMC the higher the binding of cholesterol and NaOl from mixed micelles.

The identity of the ligand attached to HPMC has an effect on total binding but not relative binding of lipids from micelles. Oleyl shows higher activity presumably due to formation of liquid domains which solubilize hydrophobic constituents of mixed micelles. Octadecanyl and cholesteryl show similar activity though somewhat diminished. Oleyl is more liquid and allows the lipid constituent of the micelle to enter this environment while octadecanyl and cholesteryl do not allow the lipid to enter easily. NaTC binding is dependent on interfacial area. At high lipid conjugation, the polymers curl-up decreasing this quantity which also decreases NaTC binding. Table 14 presents the relative molar ratios of NaOl, cholesterol, and NaTC in the mixed micellar system investigated and bound to the polymer. We conclude from this data that cholesterol and NaOl are bound by a similar mechanism while NaTC is bound differently. Fig. 68 depicts the phases possible for each of the mixed micellar components. We see at the top the free monomer phase. Since the CMC for NaTC in 1:1 NaTC/NaOl mixed micelles is near 3.2 mM we would expect a free

Table 14 - Binding of NaTC, NaOl, and Cholesterol from Mixed Micelles. This data represents average values for all results from the binding of these probes from mixed micelles containing 1:1:0.15:0.06mM NaTC:NaOl:LL:Cholesterol.

Ratio	Mixed Micelle (mM)	Bound to Polymer (mM)	Conclusion
$\frac{NaOl}{NaTC}$	$\frac{10}{10} = 1$	$\frac{1.75}{1} = 1.75$	Oleate sequestration is enhanced relative to NaTC
$\frac{Chol}{NaTC}$	$\frac{0.6}{10} = 0.06$	$\frac{0.1}{1} = 0.1$	Cholesterol sequestration is enhanced relative to NaTC
$\frac{Chol}{NaOl}$	$\frac{0.6}{10} = 0.06$	$\frac{0.1}{1.75} = 0.06$	Cholesterol sequestration is nearly the same as oleate

concentration near this value. The other three components are not very soluble in water so their concentration should be low. Since the mixed micelles are formed before the experiment begins much of the probes should occupy this phase at the start of the experiment. After the polymer is added to the system we see the existence of two phases for binding. For NaTC the binding is at



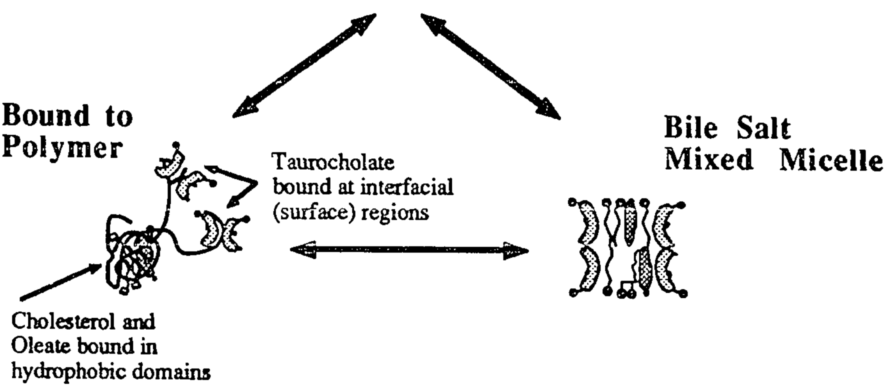


Fig. 68 - Proposed Binding Mechanisms of NaTC, NaOl, and Cholesterol from Mixed Micelles.

interfacial regions either surrounding conjugated lipid or at hydrophobic domains on the polymeric backbone. NaOl and cholesterol are bound in hydrophobic domains presumably with their head groups oriented toward the surface. It may also be possible for whole mixed micelles rich in these components to bind to conjugated lipids with the attached lipid acting like an anchor. However, we have no proof that this is a form of binding.

6.3. Recommendations

As noted the area of research needing the most work is on the effect of HM-HPMC on lipid absorption in animals and man. Results to date are promising, leading us to believe that the food additive may find application as a fat sequestering food additive. Tests that still need to be completed include: the effect of the polymer on lipid absorption in chronic studies. Areas to pay particular attention to are the effect of the polymer on fat soluble vitamin absorption and possible problems related to administering a water swellable polymer in large amounts. As noted in the previous Chapter rats may find the latter to cause gastric blockage as the polymer swells in the aqueous solution.

While this thesis does not specifically address other applications of this polymer there are quite a few. Several have become evident over the course of our work. A few of these are discussed briefly here. One might be the sequesteration of detergents from waste-water streams. It may be possible to formulate a system that would bind detergents or fats from waste water preventing the ecological problems associated with releasing detergents into water supplies. An interesting application might include taking advantage of the LCST behavior of the polymer. The polymer could bind a lipid in aqueous solution then be precipitated by heating.

As noted, HM-HPMC (with low lipid attachment) may find applications where one desires a viscous surface active system such as in the recovery of oil. Obviously the stabilization of emulsions for either industrial (latex) or pharmaceutical applications might be another use. An example might be the delivery of lipophilic drugs in the digestive tract. By loading a gel consisting

of cross-linked HM-HPMC with a lipophilic drug it may be possible to have it released slowly as the drug/polymer matrix passes through the digestive tract.

Finally, the water soluble polymers may find some application in the area of polymer solution partitioning of biologically active proteins and cells. It is well known that PEO and dextran spontaneously form a two phase aqueous system with cells or proteins preferentialy existing in one or the other polymer/water phase. Perhaps HM-HPMC would find an application here as well.

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APPENDIX

Liquid Scintillation Counting

A.1 Method to Determine Quench for Liquid Scintillation Counting (LSC)

Liquid scintillation counting (LSC) quantitatively determines the radioactivity in a sample by measuring a secondary process. When a β-emitting radionuclide decays a part of the kinetic energy of the ionizing particle is transmitted to a solvent molecule. The solvent molecule can then transfer this energy to a special fluorescing molecule (fluor) in the scintillation cocktail which decays releasing a photon. This flash of light is detected by a photo-multiplier tube (PMT) in the device and is counted. The LS counter reports this value as counts per minute (CPM). By determining the efficiency of this process it is possible to quantitate the amount of radioactivity in a sample.

One problem with quantitating the efficiency is the effect of other molecules in the scintillation cocktail. Generally, a sample contains a radionuclide in a solvent. This solvent can be water, organic, or even blood. Depending on the identity and quantity of the sample solvent, which is added to the cocktail, the transfer of energy from the decaying radionuclide to the fluor can be diminished. This is known as variable quenching.

The LS counter used in our study was a Beckman LS7000. This machine has an external method for determining the quench of a sample using a source of γ-rays (^{137}Cs). In a process where the γ-ray interacts with an electron, in the sample, energy is released by compton interactions. Using this process, it is possible to determine the "quench" of each sample. Beckman refers to this as the H number (H#), which is based on an arbitrary scaling of the photon energy measured by the PMT. The reader can refer to the User's manual or [232] for a more complete description of this process. By generating a relationship between H# and efficiency of counting it is possible to easily

calculate the amount of radionuclide in a sample. It should be noted that many other processes can interfere with the process of counting, particularly when some other source of energy is present in the sample. Examples include: photoluminesence, chemiluminesence, and static discharge. Care must be taken in preparing and counting samples to reduce the effect of these sources of error.

We used two methods to accomplish the generation of the efficiency versus H# relationship. It is possible to use quenched standards either available from many suppliers or homemade. These are made-up by putting a known quantity of radionuclide in a series of vials which are then quenched by the addition of an agent which decreases the efficiency of the β-decay to fluor process. We used quenched standards from Beckman for both ^{3}H and ^{14}C determinations. By counting these samples one obtains the H# and CPM. Since all samples contain the same amount of radionuclide, the counting efficiency can be easily obtained by the following equation:

$$\text{Counting Efficiency} = \frac{CPM_{Sample} - CPM_{Blank}}{DPM_{Manuf}} \times 100$$

CPM_{Sample} = counts per minute

CPM_{Blank} = counts per minute in blank

DPM_{Manuf} = disintegrations per minute (from known activity of sample)

By plotting this against H# we obtain the curves shown in Figures 69 to 72 for different user defined programs on the counter. The programs determine the windows of energy that the counter uses to assign the photons. Program 4 is used to count ^{14}C labeled samples while program 5 counts ^{3}H radionuclides. Program 8 provides two windows which are used to count dual labeled samples which contain both ^{14}C and ^{3}H. The appropriate third order equations relating the counting efficiency for program 4 and the H# were found to be:

$$Eff^{1}_{^{14}C} = 95.9899 - 0.0077(H\#) - 3.350X10^{-4}(H\#)^2 - 5.398X10^{-7}(H\#)^3$$

$$Eff^{2}_{^{14}C} = 80.2076 - 0.0558(H\#) + 2.809X10^{-4}(H\#)^2 + 1.554X10^{-6}(H\#)^3$$

where:

$Eff^{1}_{^{14}C}$ = efficiency of ^{14}C in channel 1

$Eff^{2}_{^{14}C}$ = efficiency of ^{14}C in channel 2

While the relationship found for counting efficiency and H# for program 5 are:

$$Eff^{1}_{^{3}H} = 60.9116 - 0.3601(H\#) + 2.297X10^{-4}(H\#)^2 + 1.811X10^{-6}(H\#)^3$$

$$Eff^{2}_{^{3}H} = 52.3693 - 0.2699(H\#) + 1.122X10^{-4}(H\#)^2 + 3.716X10^{-7}(H\#)^3$$

where:

$Eff^{1}_{^{3}H}$ = efficiency of ^{3}H in channel 1

$Eff^{2}_{^{3}H}$ = efficiency of ^{3}H in channel 2

In our experiments we automated the process for determining the actual radioactivity in a sample from CPM and H# data. The CPM and H# were ported directly to an Apple II+ computer using a serial interface. By performing a third order polynomial fit of the standard H# and counting efficiencies it was possible to program the Apple II+ so it would automatically determine the radioactivity in samples. Finally, the equations relating program 8 counting efficiency and H# for dual labeled samples containing ^{3}H and ^{14}C are:

$$Eff^{1}_{^{3}H} = 42.67 - 0.201(H\#) - 5.165X10^{-4}(H\#)^2 + 2.504X10^{-6}(H\#)^3$$

$$Eff^{2}_{^{3}H} = 3.0485 - 0.0474(H\#) + 4.173X10^{-4}(H\#)^2 - 9.362X10^{-7}(H\#)^3$$

$$Eff^{1}_{^{14}C} = 7.4711 + 0.0099(H\#) - 3.372X10^{-4}(H\#)^2 + 7.438X10^{-7}(H\#)^3$$

$$Eff^{2}_{^{14}C} = 79.847 - 0.0645(H\#) + 4.764X10^{-4}(H\#)^2 - 2.185X10^{-6}(H\#)^3$$

Where we have used the same definitions as above.

Near the end of our research a PMT on the LS counter failed and the machine was recalibrated. This necessitated making new quench standards. For this process we used the following protocol.

1) Twenty scintillation vials for each radionuclide were filled with 10cc of scintillation cocktail (Aquasol II) and then counted for background.

2) Ten of the vials received equal amounts of the radionuclide (^{14}C-NaTC or ^{3}H-Oleic acid). These were counted again to determine unquenched activity.

3) In matched pairs the ten vials containing radionuclide and ten background vials received equal amounts of quench (Sorenson's phosphate buffer) over the range that samples are normally prepared. The samples are counted again and the counting efficiency determined using the equation above.

This provides the same relationship between counting efficiency and H# as the Beckman quench standards. As an aside, Beckman uses CCl_4 as the quenching agent. These results are presented in Fig. 73 and 74 for program 8.

We found that over the limited range of H#'s obtained it was not necessary to use a third order polynomial, a simple first order fit to the data was sufficient.

$$Eff^{1}_{^3H} = 38.0616 - 0.191(H\#)$$

$$Eff^{2}_{^3H} = 0.1863 + 0.0091(H\#)$$

$$Eff^{1}_{^{14}C} = 8.7196 - 0.0165(H\#)$$

$$Eff^{2}_{^{14}C} = 75.0079 - 0.0141(H\#)$$

In addition, the spillover of ^{3}H into channel 2 is nearly zero.

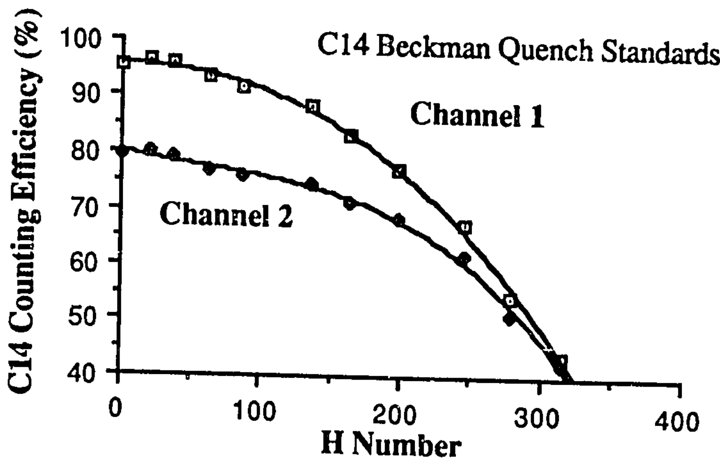

Fig. 69 - ^{14}C Counting Efficiency for Program 4, Beckman Standards. (Window: Channel 1: 0-655, Channel 2: 397-655, LS7000 Counter)

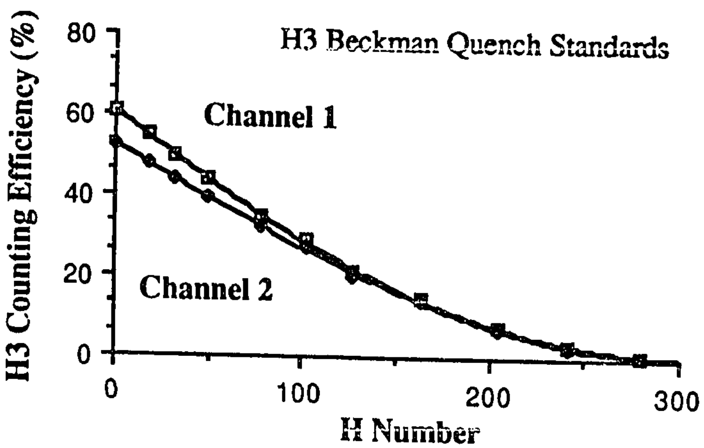

Fig. 70 - ^{3}H Counting Efficiency for Program 5, Beckman Standards. (Window: Channel 1: 0-397, Channel 2: 180-397, LS7000 Counter)

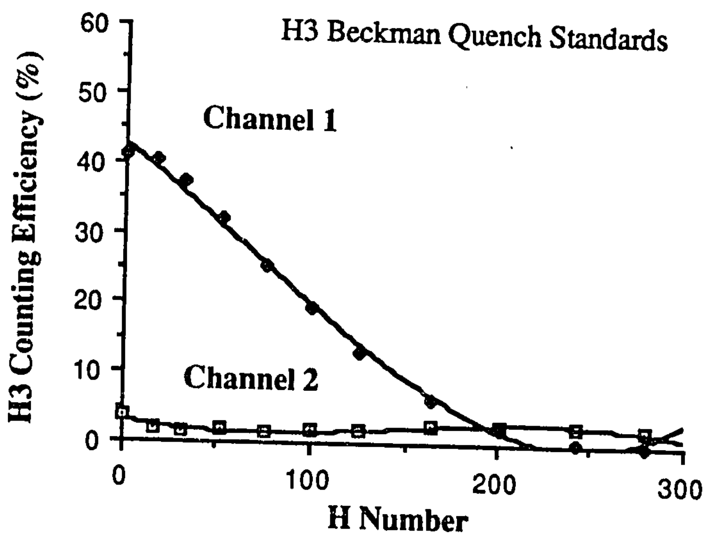

Fig. 71 - ^{3}H Counting Efficiency for Program 8, Beckman Standards. (Window: Channel 1: 0-317, Channel 2: 397-655, LS7000 Counter)

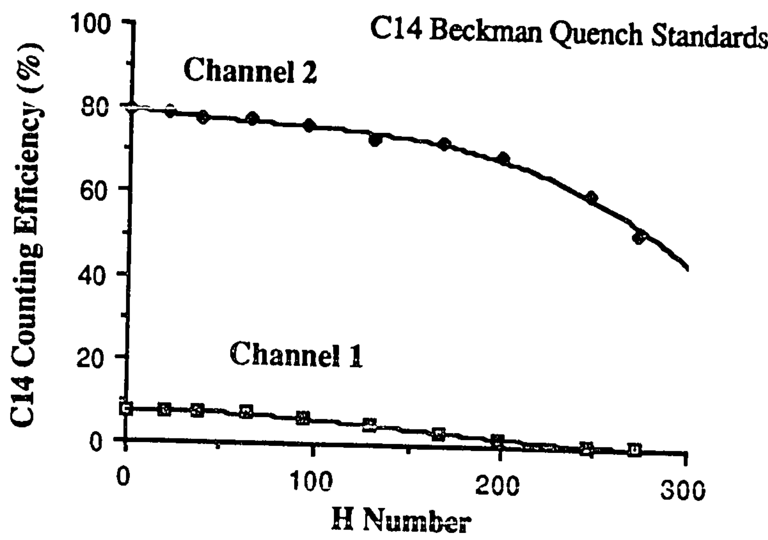

Fig. 72 - ^{14}C Counting Efficiency for Program 8, Beckman Standards. (Window: Channel 1: 0-317, Channel 2: 397-655, LS7000 Counter)

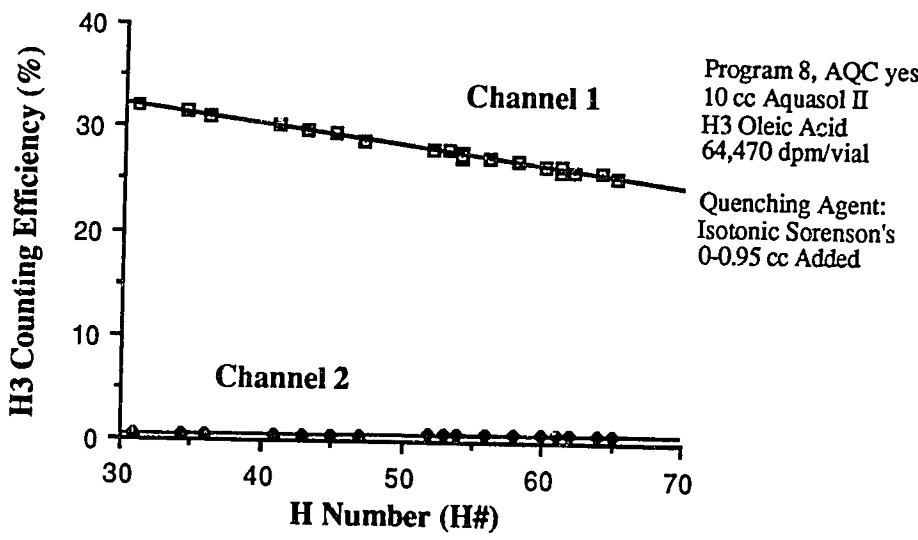

Fig. 73 - ^{3}H Counting Efficiency for Program 8.
(Window: Channel 1: 0-317, Channel 2: 397-655, LS7000 Counter)

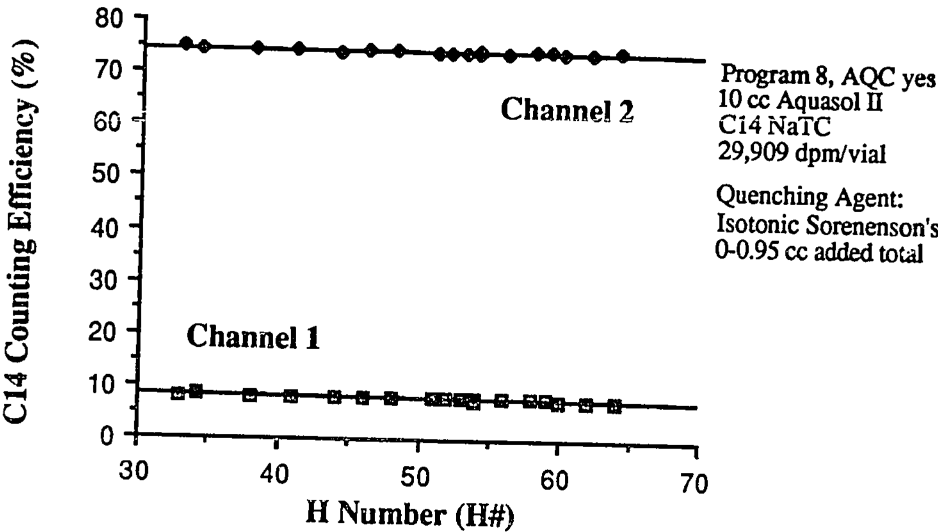

Fig. 74 - ^{14}C Counting Efficiency for Program 8.
(Window: Channel 1: 0-317, Channel 2: 397-655, LS7000 Counter)

A.2 ^{14}C and ^{3}H in Dual Labeled Samples

By knowing the relative contribution of ^{14}C in the tritium window and ^{3}H in the ^{14}C window (this value is low and frequently ignored) it is possible to determine the ^{14}C and ^{3}H in dual labeled samples using the following equations:

$$CPM^{1} = DPM_{^{3}H}Eff^{1}_{^{3}H} + DPM^{14}CEff^{1}_{^{3}H} + BACK^{1}$$

$$CPM^{2} = DPM_{^{3}H}Eff^{2}_{^{3}H} + DPM^{14}CEff^{2}_{^{14}C} + BACK^{2}$$

where:

CPM^{1} = counts per minute in channel 1

CPM^{2} = counts per minute in channel 2

$DPM_{^{3}H}$ = disintigrations per minute due to ^{3}H

$DPM_{^{14}C}$ = disintigrations per minute due to ^{14}C

$BACK^{1}$ = background counts per minute in channel 1

$BACK^{2}$ = background counts per minute in channel 2

By solving for $DPM_{^{3}H}$ and substituting this into the second equation we obtain:

$$DPM_{^{14}C} = \frac{\left[CPM^{2} - \frac{Eff^{2}_{^{3}H}}{Eff^{1}_{^{3}H}}\left[CPM^{1} - BACK^{1}\right] - BACK^{2}\right]}{Eff^{1}_{^{3}H}Eff^{2}_{^{14}C} - Eff^{2}_{^{3}H}Eff^{1}_{^{14}C}}$$

This yields the quantity of ^{14}C in the sample. By substituting this into the first equation we obtain the ^{3}H. Computers generally find tasks like this quite easy so that's what we did.

Vita

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