Behavior of Polymer-Supported Digitonin with Cholesterol in the Absence and Presence of Butter Oil

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Digitonin was bound covalently to carboxypolystyrene and carboxymethylpolystyrene via an ester linkage. Polymers were prepared by reacting the polymer acid chloride in pyridine with digitonin or by coupling digitonin directly to the carboxy polymer with dicyclohexylcarbodiimide. Samples containing 0.04–0.23 mmol of digitonin/g of polymer complexed cholesterol from hexane solutions of butter oil or cholesterol. Cholesterol was completely stripped from the polymer samples by room temperature benzene extraction, yielding a reactivated polymer with essentially the same cholesterol-complexing ability as the original polymer. The cholesterol–digitonin complex is stable to isobutyl alcohol extraction.

INTRODUCTION

Dairy products are wholesome and nutritious foods that may contain undesirable amounts of cholesterol. Processing milk to butter and cheese simply concentrates cholesterol in the lipid phase. The human system acquires cholesterol via normal liver processes and ingestion of foods of animal origin. Present studies show that under rather restricted conditions elevated plasma cholesterol levels exhibit a positive correlation with cardiovascular disease (Lipid Research Clinics Program, 1984). Reducing cholesterol intake in our foods appears to be the simplest approach to lowering bodily cholesterol levels and the apparent associated disorders.

Scant information is available describing methods to separate cholesterol from dairy products. Present methods applicable to the problem are (A) formation of an insoluble digitonin–cholesterol complex (Schwartz, 1967) during passage of a hexane solution of butter oil through a column of digitonin adsorbed on Celite, (B) formation of an inclusion complex between cholesterol and β-cyclodextrin (Courregelonge et al., 1987) followed by aqueous extraction of the complex and excess cyclodextrin, and (C) supercritical carbon dioxide extraction of butter oil followed by passage of the gas-laden oil through silica gel (Shishikura et al., 1986). The last two methods result in partial removal of cholesterol, require recycling of the sample, and give low recoveries of extracted oil. On the other hand, the first method gave complete selective removal of cholesterol and full recovery of the extracted butter oil. At a 7/1 weight ratio (2/1 molar ratio) of digitonin to cholesterol, complete removal of cholesterol was achieved and residual butter oil was washed from the column with hexane. The same principle was employed to separate 3β-hydroxysterols by TLC using digitonin-impregnated silica gel (Taylor, 1971). In neither approach was the substrate reusable. However, this would be possible in theory if digitonin were covalently bound to the insoluble substrate.

Digitonin consists of the aglycon digitogenin linked to a pentasaccharide composed of xylose, galactose, and glucose. The molecule contains only hydroxy groups and ether linkages. Thus, reaction between a functionalized polymer and a hydroxy group, forming a stable bond, would permanently bind digitonin to the support. The basic premise is that a single bond between digitonin and the polymer matrix would not interfere with the ability of digitonin to complex with cholesterol. Furthermore, low polymer functional group densities would ensure isolation of these groups and reaction of only one hydroxy group of digitonin with the polymer support. This would be the least hindered group, thereby reducing steric distortions within the digitonin molecule.

Insoluble functionalized polymer supports have found extensive use in organic synthesis (Mathur et al., 1980). Usually a multifunctional molecule is bonded to the polymer support. Desirable subsequent reactions are performed on the immobilized molecule, which can then be removed from the support by breaking the anchoring bond. Cross-linked styrene–divinylbenzene copolymers containing 0.1–2.0% divinylbenzene (DVB) have found wide use because of their insolubility, ease of functionalization, and stability. The present program was undertaken to develop a method to selectively remove cholesterol from butter oil. Carboxy popcorn polymer (0.3% DVB) and carboxymethylpolystyrene (2% DVB) resin were esterified with digitonin and treated with hexane solutions of pure cholesterol or butter oil. The extent of cholesterol uptake and a means of regenerating the polymer-supported digitonin were determined.

At this writing, two groups (Shishikura et al., 1986; Kim, 1989) have refined a continuous supercritical carbon dioxide fluid extraction process to the point where they removed 90% of the cholesterol and recovered 85% of the extracted butter oil. The approach is now economically feasible but still lacks selectivity.

MATERIALS AND METHODS

Caution. Benzene is a suspected carcinogen. Exercise extreme care in its use. Styrene and divinylbenzene were distilled prior to use. Dicyclohexylcarbodiimide was distilled at reduced pressure and stored under nitrogen in a desiccator. Pure 4-vinylbenzoic acid was recrystallized from 20% ethanol and vacuum-dried prior to use. Pyridine was distilled from calcium hydride and stored over 4-Å molecular sieves under dry nitrogen. Digitonin was obtained from ICN Biomedicals, Inc., Costa Mesa, CA 92626. Cholesterol and chloromethylated polystyrene–2% divinylbenzene (1.04 mequiv chlorine/g), Merrifield resin, were obtained from Sigma Chemical Co., St. Louis, MO 63178. The same type resin containing 3.5 mequiv of chlorine/g of polymer was obtained from Lancaster Synthesis Ltd., P.O. Box 1000, Windham, NH 03087. Phthalaldehyde, anhydrous dimethylformamide, and dimethyl sulfoxide were obtained from Aldrich Chemical Co., Metuchen, NJ 08840. Infrared spectra were obtained by using KBr disks with a Perkin-Elmer 1310 microprocessor controlled infrared spectrophoto-

meter. Visible spectra were obtained with a Beckman DU Series 70 UV-vis spectrophotometer using 1-cm matched cells. Cholesterol was determined by using the *o*-phthalaldehyde–sulfuric acid colorimetric method (Bachman et al., 1976). With a molar absorptivity of 24 100 L mol^{-1} cm^{-1}, the method is sufficiently sensitive to accurately determine 0.1 μmol of cholesterol. On the basis of absorbance variations of calibration values from 40 to 100 μg of cholesterol, the relative error of the cholesterol concentrations in Tables I through V is ±13%. This error is of the same order of magnitude as found for the colorimetric method. Butter oil was prepared from a sample of sweet unsalted butter purchased at a local supermarket. The butter was liquified and centrifuged. The oily layer was separated and filtered to yield a clear yellow filtrate of anhydrous butter oil which assayed at 0.27% cholesterol. The sample was stored under nitrogen at −20 °C.

Carboxy Polymers. Samples of carboxypolystyrene (popcorn polymer) containing 0.38 and 0.54 mmol of carboxylic acid/g of polymer were prepared essentially by the method of Letsinger et al. (1964, 1966). After pulverization in a Waring Blendor and passage through a No. 20 microsieve, the polymer samples were Soxhlet-extracted overnight with benzene, washed with acetone, and dried to constant weight. Attempts to prepare polymers containing higher carboxyl concentrations resulted in the formation of unusable glassy polymer.

Carboxymethylpolystyrene, Merrifield resin, samples containing ~0.90 and 3.5 mmol of carboxylic acid/g of polymer were prepared by the method Kusama et al. (1970). Polymer carboxylic acid concentration was determined by heating an appropriate size sample for 0.5 h on the steam bath in a solution of 25 mL of reagent dioxane and 10 mL of 0.1 N NaOH and backtitrating the excess base with 0.1 N HCl to a phenolphthalein endpoint. The values in dioxane were about 20% higher than those obtained with ethanol because dioxane is a polymer-swelling solvent.

Synthesis of Digitonized Polymer. *Acid Chloride Method.* This is a modification of procedures (Letsinger et al., 1967; Exocoffier et al., 1972; Wong et al., 1972) to form polymer esters. Polymer carboxylic acid (1.54 g, 0.81 mmol of CO_2H) was added to 30 mL of benzene and dried azeotropically, leaving 20 mL of solvent in the flask. A catalytic amount of dimethylformamide was added to the polymer suspension under dry nitrogen followed by 2.9 g (23 mmol) of oxalyl chloride in 5 mL of benzene. The mixture was stirred at gentle reflux overnight. Benzene and excess oxalyl chloride were removed at 5 mmHg/1 h/25 °C and at 0.05 mmHg/2 h/45 °C. Digitonin 1.0 g (0.81 mmol) was dried at 0.05 mmHg/1 h/100 °C and dissolved in 15 mL of dry pyridine. The solution was added to the dry polymer acid chloride and stirred 24 h at 110 °C. The suspension was treated with 2 mL of methanol and stirred overnight at 25 °C. Solvent was removed with a filter stick. Residual polymer was stirred 10 min with 15 mL of warm pyridine and solvent removed in a like manner. The washing procedure was repeated five times with pyridine and six times with 15-mL portions of methanol. The polymer residue was dried at 0.05 mmHg and 45 °C to constant weight of 1.67 g, giving polymer containing 0.07 mmol of digitonin/g of polymer. An IR spectrum of the polymer showed a broad intense OH band at 3440 cm^{-1} and a carbonyl band at 1730 cm^{-1}.

Dicyclohexylcarbodiimide Method (Buzas et al., 1962; Yip et al., 1971). A sample of polymer carboxylic acid 1.50 g (1.43 mmol of CO_2H) was dried 1 h/100 °C/0.05 mmHg. Digitonin 0.61 g (0.50 mmol) was dried under the same conditions and dissolved in 15 mL of dry pyridine. Under dry nitrogen 0.60 g (2.86 mmol) dicyclohexylcarbodiimide was added to the polymer followed by the digitonin in pyridine. The pale yellow suspension was stirred 24 h at 5 °C and then treated with 20 mL of water and stirred overnight at 25 °C. The supernatant was removed with a filter stick, and 10 mL of warm solvent was added. The polymer was stirred 5 min, and solvent was removed as before. The washing procedure was conducted by using a total of 30 mL of each of the following solvents: pyridine, benzene, benzene–ethanol (2:1, 1:1, 1:2), and ethanol. The off-white residue was finally washed with methanol and dried to constant weight of 1.70 g. The product contained 0.11 mmol of digitonin/g of polymer. An IR spectrum showed the broad OH band at 3440 cm^{-1} and an ester carbonyl at 1730 cm^{-1}.

Polymer-Supported Digitonin Treatment with Butter Oil. Solution A, 20 g of anhydrous butter oil diluted to 50 mL with hexane; solution B, 50 mg of cholesterol and 20 g of tricaprylin diluted to 50 mL with hexane. To a screw-capped centrifuge tube (15-mL capacity) were added 0.40 g of digitonized polymer (0.13 mmol of digitonin/g of polymer), a stirring bar, and 5 mL of solution A. Samples containing only 5 mL of solution A, 0.40 g of carboxymethylpolystyrene plus 5 mL of solution A, and only 5 mL of solution B were run simultaneously. The samples were stirred at least 24 h at 25 °C and centrifuged. A 1-mL aliquot of supernatant was pipetted into a 25-mL volumetric flask and diluted to the mark with hexane. A 5-mL aliquot of diluted supernatant was pipetted into a screw-capped centrifuge tube and solvent evaporated on the steam bath under nitrogen. The residue was treated with 3 mL of EtOH and 2 mL of 50% KOH (w/v) and stirred at 63 °C for 25 min. The tube contents were cooled and treated with 3 mL of water and 5 mL of hexane. The mixture was shaken vigorously and allowed to stand for the separation of two layers. A 1-mL aliquot of the hexane layer was pipetted into a 10-mL volumetric flask and solvent evaporated on the steam bath under nitrogen; 4 mL of color reagent, *o*-phthalaldehyde (50 mg/100 mL of glacial acetic acid) was added. After standing 10 min, the sample was treated with 2 mL of concentrated sulfuric acid. The sample was shaken, and absorbance was measured within 10–90 min at 552.5 nm by using 1-cm matched cells.

Polymer-Supported Digitonin Treatment with Cholesterol. To a screw-capped centrifuge tube (15-mL capacity) were added 0.40 g of digitonized polymer (0.13 mmol of digitonin/g of polymer), a stirring bar, and 5 mL of cholesterol in hexane (1 mg/mL). The mixture was stirred at least 24 h at 25 °C and centrifuged. A 1-mL aliquot of supernatant was pipetted into a 50-mL volumetric flask and diluted with hexane. A 2-mL aliquot of diluted supernatant was pipetted into a 10-mL volumetric flask. Solvent was evaporated under dry nitrogen on the steam bath. The color reaction as described above for butter oil was performed. A control containing polymer carboxylic acid and a calibration curve (0–100 μg of cholesterol) were run simultaneously. The same basic procedure was used to evaluate cholesterol uptake by digitonized polymers using solvents other than hexane.

Cholesterol Removal from Digitonized Polymer. A polymer sample, 0.40 g, treated with cholesterol in hexane and found to contain 3.1 mg of cholesterol/g of polymer was centrifuged and the hexane supernatant discarded. The residue was treated with 5 mL of benzene, stirred vigorously for 15 min, and centrifuged. The supernatant was carefully decanted into a screw-capped vial, and the polymer residue was treated with another 5-mL aliquot of benzene. The sample was stirred and centrifuged as before. This procedure was repeated six times. The first benzene extract was diluted to 50 mL with benzene. The remaining extracts were used as obtained. A 1-mL aliquot of each extract was pipetted into a 10-mL volumetric flask and all solvent evaporated on the steam bath under nitrogen. The color reaction as described in the butter oil procedure was performed on each extract, and a calibration curve (0–80 μg of cholesterol) was determined. The first four extracts contained all the cholesterol present in the sample (5.4 mg of cholesterol/g of polymer).

A check on complete cholesterol removal from the extracted polymer was performed by quantitatively transferring the polymer to a Büchner funnel with methanol and drying to constant weight. Fifty milligrams of polymer was weighed into a 10-mL volumetric flask. A blank sample containing 50 mg of carboxymethylpolystyrene was run simultaneously. The color reaction was performed as described in the butter oil procedure. After the necessary time for color development, the samples were filtered and absorbance measurements made on the filtrates at 552.5 nm. Cholesterol content was determined from the calibration curve. Invariably, benzene-extracted polymer samples contained no cholesterol.

Table I. Cholesterol Uptake by Polymer-Supported Digitonin in Hexane

sample[a]	mmol of CO_2H/g of polymer	mmol of digitonin/g of polymer	mg of cholesterol uptake[b]/g of polymer	wt of digitonin/wt of cholesterol
1	0.38	0.04	4.3	12
2	0.53	0.05	3.9	15
3	0.87	0.06	3.4	22
4	0.53	0.07	4.3	20
5	0.87	0.10	4.7	26
6	0.87	0.11	5.4	25
7	3.5	0.15	8.6	22
8	0.95	0.05	1.2	50
9	0.95	0.11	2.0	60
10	0.85	0.12	3.1	48
11	0.85	0.13	4.1	12
12	0.85	0.14	2.8	61
13	0.85	0.15	3.6	42
14	3.5	0.23	0.4	720
15	control[a]		1.1	

[a] Samples 1–7 from acid chloride, remaining samples from DCC coupling; control is polymer carboxylic acids containing 0.85 and 3.5 mmol of CO_2H/g of polymer. [b] The total cholesterol concentration is equal to cholesterol uptake plus control value. The relative error in these values and those in Tables II–V is estimated to be ±13%.

RESULTS AND DISCUSSION

Polymer carboxylic acid was converted to the acid chloride with oxalyl chloride in anhydrous benzene. After removal of the solvent and excess oxalyl chloride at reduced pressure, the acid chloride was treated with an equimolar amount of digitonin in dry pyridine for 24 h at 110 °C. Excess acid chloride was eliminated with methanol, the digitonized polymer being isolated after repeated solvent washings. Consistent conversion to the acid chloride occurred with ~20 molar excess of oxalyl chloride at reflux overnight. Pyridine affected complete solution of digitonin, optimum polymer swelling, and neutralization of generated HCl. Digitonin addition to the polymer acid chloride was reduced below 100 °C and did not occur at ambient temperatures. Reported polymer contamination (Southard et al., 1971) by sulfur when thionyl chloride is used prompted the use of oxalyl chloride in this study.

The addition of digitonin to polymer carboxylic acids occurred directly with dicyclohexylcarbodiimide (DCC) in dry pyridine at 5 °C. Equimolar concentrations of DCC and polymer carboxylic acid gave about the same digitonin content as those reactions performed with a 20-fold excess. Less than equimolar DCC concentrations gave decreased reaction with digitonin. Reaction times greater than 24 h did not significantly increase the addition of digitonin. No reaction or retention of digitonin occurred in the absence of DCC.

Popcorn polymer carboxylic acids were characterized by carbonyl bands at 1730 and 1680 cm^{-1}, which are associated with free and hydrogen-bonded carboxyl groups, respectively. Merrifield resin derived carboxylic acid showed a broad carbonyl absorption at 1700 cm^{-1}. Digitonized polymers exhibited a gain in weight, a broad intense OH absorption in the 3430-cm^{-1} region, and an enhanced ester carbonyl band at 1730 cm^{-1}.

Table I shows the cholesterol uptake after 72 h in hexane of various digitonized polymer samples obtained via the acid chloride (1–7) or with DCC coupling (8–14). The total cholesterol concentration in Tables I–V is the sum of the control and the sample values. Polymers containing 0.4–3.5 mmol of CO_2H/g of polymer bind digitonin at 0.04–0.23 mmol/g of polymer, which is equivalent to ~10% of the available carboxyl groups. This level of reactivity is attributed to the bulkiness of the digitonin molecule and thus the inaccessibility to most of the carboxyl groups. Only a nominal increase in bound digitonin occurred by increasing the carboxyl level to 3.5 mmol. However, sample 7 retains its cholesterol-complexing ability, while sample 14 shows almost a complete loss of this capacity. This behavior suggests the existence of a critical digitonin level above which steric factors become prominent in reducing cholesterol uptake or the onset of multiester bond formation between a digitonin molecule and the polymer matrix. An earlier study (Schwartz et al., 1967) showed that digitonin on Celite completely binds cholesterol at about a 7/1 weight ratio. The Table I ratios show that the digitonized polymer from the acid chloride are more efficient than those from DDC coupling, neither of which is equivalent to digitonin on Celite. The reason for the efficient cholesterol uptake by sample 11 is not known. Different cholesterol complexing efficiencies from two series of presumably identical digitonized polymers suggest that a digitonized polymer equal in efficiency and solvent stability to digitonin on Celite can be made. Polymer sample numbers in Tables II–V correspond to the same samples described in Table I.

Cholesterol reacts with digitonin to form a stable 1:1 complex insoluble in organic solvents and which can only be dissociated (Issidorides et al., 1962) by treatment with dimethyl sulfoxide or pyridine at 100 °C. Table II summarizes the results of solvent removal of cholesterol complexed on digitonized polymers prepared by both methods using popcorn and Merrifield derived resins. Sample 1 required numerous hexane extractions to remove

Table II. Cholesterol Removal from Polymer-Supported Digitonin by Solvent Extraction

digitonized polymer sample[a]	solvent	mg of cholesterol/g of polymer present[b]	mg of cholesterol/g of polymer extracted
1	hexane	4.3	4.4
7	hexane	8.6	7.4
5	cyclohexane + benzene	4.8	6.9
3	isobutyl alcohol + benzene	1.8	2.2
6	benzene	5.4	6.6
10	benzene	3.1	5.4
8	benzene	1.2	2.9

[a] Polymer sample numbers correspond to the same samples shown in Table I. [b] Values do not include control polymer carboxylic acid of ~1.1 mg of cholesterol/g of polymer.

Table III. Solvent Effects of Cholesterol Uptake by Polymer-Supported Digitonin

digitonized polymer sample	solvent[a]	mg of cholesterol/g of polymer sample	mg of cholesterol/g of polymer control
4	hexane	4.3	1.1
	aq EtOH	2.4	0.5
	aq EtOAc	0.6	0.4
7	hexane	8.6	1.1
	aq EtOH	7.0	0.5
	aq EtOAc	0.4	0.4
13	hexane	3.6	1.1
	aq EtOH	1.9	0.8
	aq EtOAc		
	EtOAc		
	aq C_6H_6		
	C_6H_6		
1	isobutyl alcohol		
5	isobutyl alcohol		
11	isobutyl alcohol		

[a] Solvents are 84% aqueous ethanol, water saturated ethyl acetate, and benzene.

Table IV. Reusability of Digitonized Polymer To Remove Cholesterol from Hexane

sample[a]	mmol of digitonin/g of polymer	mg of cholesterol uptake/g of polymer for polymer series					
		A	B		C		
		after 72 h	after 24 h	after 114 h	after 24 h	after 48 h	after 114 h
1	0.04	3.8	2.1	2.7	2.0	2.3	2.1
5	0.10	4.4	3.0	3.8	2.3	3.0	2.9
11	0.13	4.1	3.7	4.4	3.1	3.3	3.4
control	0	1.1	0.9	1.3	1.4	0.9	1.7

[a] 0.40 g of polymer used in each series.

Table V. Reusability of Digitonized Polymer To Remove Cholesterol from Butter Oil in Hexane

sample[a]	mmol of digitonin/g of polymer	mg of cholesterol uptake/g of polymer for polymer series							
		A			B		C		
		after 24 h	after 48 h	after 72 h	after 72 h	after 96 h	after 24 h	after 48 h	after 114 h
11	0.13	1.1	1.1	1.6	1.5	1.8	1.5	1.3	0.9
13	0.15	0	1.3	1.5	1.0	1.1	0.8	1.3	1.1
10	0.12				1.2	1.1	0.5	1.0	0.9
4	0.07				0.8	1.6	0.9	0.9	0.6
control	0	0.3	0.3	0.3	0.1	0.5	0.4	0.3	0.6

[a] 0.40 g of polymer used in each series.

all complexed cholesterol, while with sample 7 the observed results are a composite of hexane extract values, DMSO treatment plus hexane extract values, and direct treatment of the extracted polymer with the color reagents to determine retained cholesterol. Cyclohexane extraction removed half the cholesterol; complete removal was obtained only after benzene extraction. Isobutyl alcohol, sample 3, was the least active solvent, removing only free cholesterol equal to 0.25 mg of cholesterol/g of polymer. With samples 6, 10, and 8 complete removal of cholesterol occurred with less than six extractions using benzene. Complete removal of cholesterol from the various samples was verified by running the color reaction on the actual extracted polymer samples. These results indicate (a) a stable complex (presumably 1:1) is formed between cholesterol and polymer-supported digitonin, (b) the complex is considerably weaker than that formed between free digitonin and cholesterol, (c) the decreasing order of cholesterol removal was found to be benzene > hexane > cyclohexane > isobutyl alcohol, and (d) benzene removed all cholesterol from polymer-supported digitonin while isobutyl alcohol removed only free cholesterol.

The effect of solvents on cholesterol uptake by digitonized polymer is shown in Table III. Both types of polymer obtained by the acid chloride method and by DCC coupling are included. In the first three series, hexane with low solvating ability and polarity leads to the highest cholesterol uptake followed by aqueous ethanol and ethyl acetate. The latter samples in series 13 and those in samples 1, 5, and 11 showed no cholesterol uptake within experimental error after the control value was corrected for, even though all the samples were treated with cholesterol for at least 24 h. Cholesterol uptake is obviously solvent dependent, with the poorest interacting solvent leading to the highest cholesterol uptake. It is interesting that isobutyl alcohol, showing no activity in dissociating the cholesterol-digitonized polymer complex, should interact sufficiently with cholesterol and the polymer to prevent complex formation, presumably by solvent H bonding.

Two very important aspects of this program were the reusability of the digitonized polymers and their ability to remove cholesterol from butter oil. These results are summarized in Tables IV and V. The results in Table IV include cholesterol uptake by three different digitonized polymers and a blank carboxymethylpolystyrene. Series A shows the cholesterol uptake by the original polymers after a reaction time of 72 h. The polymers and the blank were stripped of cholesterol by benzene extraction (Materials and Methods) and dried. The loss during this operation of 15–30 mg plus the 25 mg of polymer used for cholesterol assay was replaced either with original or regenerated polymer. Cholesterol uptake was performed again as shown by series B after 24 and 114 h. The regeneration was repeated and cholesterol uptake determined as shown in series C after 24, 48, and 114 h. The results show that sample 1 does exhibit a decrease in cholesterol uptake with two regenerations; however, this sample was obtained very early in the program. Samples 5 and 11, however, show excellent retention of cholesterol uptake ability with two regenerations. The data also indicate that essentially complete uptake of cholesterol occurred within 24 h.

Table V shows the same type data as applied to cholesterol removal from butter oil. Sample 11 in Table V occurs also in Table IV. A comparison of these samples shows that cholesterol uptake from butter oil is only ~30% of that observed with cholesterol in hexane. Samples 10 and 4 were not included in the series A determinations; thus, the results in series B are values for the original polymer samples. The data show that samples 11 and 13 are completely regenerated on two evaluations, giving the same cholesterol uptake for series A–C. Furthermore, within 24 h maximum uptake of cholesterol has occurred. Samples 10 and 4 show similar reusability after a single regeneration.

Polymer carboxylic acids derived from popcorn and 2% cross-linked poly(styrenedivinylbenzene) contain considerable quantities of contiguous carboxyl groups even at very low degrees of ring substitution (Mathur et al., 1980; Letsinger et al., 1964, 1966; Crowley et al., 1973). This is evident from the associated carboxyl band at 1685 cm^{-1} and the ready conversion to polymer anhydride. The low level of isolated carboxyl groups is attributed primarily to chain mobility in the solvent-swollen state and to a lesser extent to nonrandom substitution on the polymer chain. The proximity of functional groups on the polymer chain could promote multiple linkages with digitonin, thus increasing molecular distortion and reducing the ability for complex formation.

The results to date indicate (1) hexane is the solvent of choice to promote complex formation between cholesterol and polymer-supported digitonin, (2) only 10% of the digitonin on the polymer is utilized in complex

formation with cholesterol, (3) all cholesterol can be removed from polymer-supported digitonin by benzene extraction, (4) the polymers can thus be regenerated, (5) regenerated polymers retain the same cholesterol-complexing ability as the original polymers, and (6) polymer reusability is effective both for hexane solutions of butter oil and for hexane solutions of cholesterol. This approach offers potential for development if the concentration of cholesterol binding saponin can be increased and a saponin of lower toxicity found.

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