higher yield of single cells but also reduced the survival. Passing the suspension through an ordinary blood administration filter reduces the mucous contamination and does not substantially reduce the eventual yield.

Proteolytic activity persists in the preparation after differential centrifugation in the absence of stained zymogen (chief) cells probably because the pepsin molecule comes down almost equally well through all layers of the media although the chief cells do not. It is possible that the cytolysis liberates salts which significantly increase the solubility of pepsinogen. Another consideration is that the parietal cells might be coated with pepsinogen which is lost to the suspension solution during incubation and autolysis.

Gastric mucosal cells have been grown in our laboratory in continuous cell culture on well defined media through 14 passages whereupon contamination of the nutrient media forced us to discard the entire preparation and begin anew.

Summary. A technic that provides a suspension of living parietal cells is described. Gastric mucosa is minced, extracted with collagenase and the cells separated by differential centrifugation. Identification of cell types is achieved by histological and histochemical technics. Difficulties encountered in purification are discussed. The suspension is suitable for metabolic studies of the parietal cell.

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Effect of Hypocholesterolemic Agents on Intestinal Cholesterol Absorption.* (28087)

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Recently, several compounds, administered by the oral route, have been reported to be effective in lowering blood cholesterol levels in man and experimental animals. Stern and Treadwell† observed that cholesterol trimethylacetate, a branched-chain fatty acid ester of cholesterol, inhibited hydrolysis of cholesterol butyrate by pancreatic cholesterol esterase *in vitro*. It was also found that this branched-chain ester reduced blood cholesterol levels in rats when added to a diet containing cholesterol butyrate. According to Vahouny and Treadwell(1), cholesterol trimethylacetate itself is not absorbed in lymph-fistula rats and is not hydrolyzed by intraluminar cholesterol esterase.

Tennant *et al.*(2,3) have reported that MK-135 (cholestyramine), a bile acid-binding polymer, causes a marked reduction of blood cholesterol levels and a lower incidence of atheromatosis in cockerels. No toxic effects, either local or systemic, were detected in dogs fed MK-135 for as long as one year. This compound was also found to be effective in reducing blood cholesterol levels in hypercholesteremic patients(4).

Keys *et al.*(5,6) reported that a diet containing undigestible, or difficultly-digestible polysaccharides, such as cellulose, hemicellulose, or pectin, reduced blood cholesterol levels in humans. In rats, addition of pectin to a cholesterol diet largely counteracted any increase in plasma and liver cholesterol due to cholesterol feeding(7).

Nicotinic acid has been shown to be effective in lowering blood cholesterol levels in rabbits and humans; however, nicotinamide did not have a comparable effect(8,9). A study of the effect of nicotinic acid on hepatic synthesis of cholesterol showed a reduc-

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† Unpublished observations.

TABLE I. Composition of Intragastric Test Emulsions.

Emulsion No.	mg	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Albumin	50	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Oleic acid	292	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Sodium taurocholate	288	+	+	+	+	+	+		+	+	+	+	+	+	+	+
Cholesterol-4-C^{14}	50		+	+		+		+	+		+		+		+	
Cholesterol-trimethylacetate	61			+	+											
MK-135	50					+	+									
"	238								+	+						
Pectin	250										+	+				
Nicotinuric acid	100												+	+		
Pyridine-3-acetic acid	100														+	+

All emulsions except emulsions 10 and 11 were made to 3 ml with 0.9% saline.
Emulsions 10 and 11 contained all components and one-half the pectin in 3 ml, and the remaining pectin in a second 3 ml of saline.

tion in incorporation of labeled acetate into cholesterol(10,11). However, the actual mechanism by which nicotinic acid exerts its entire observed cholesterol-lowering effect has not been clarified. Parson *et al*(12), and Miller *et al.*(13) have suggested that nicotinuric acid, a metabolite of nicotinic acid, might play a role in cholesterol metabolism. In fact, addition of nicotinuric acid to the diet did cause a reduction of blood cholesterol levels in humans(14). Also pyridine-3-acetic acid which is structurally related to nicotinic acid was reported to have a blood cholesterol-lowering effect in humans (15). When this substance was administered orally, there was a striking and consistent reduction of total blood cholesterol levels.

Although several mechanisms have been suggested as explanations for the effects of these various compounds on blood cholesterol levels, little definitive data have been presented. In some cases, as with nicotinic acid, the test substance may reduce blood cholesterol, at least in part, by interference with hepatic sterol synthesis. However, it is also possible that interference with dietary cholesterol absorption may be a factor in the observed depression of blood cholesterol levels.

Essentially all absorbed cholesterol enters the blood stream *via* the thoracic lymph duct (16,17). Thus by analysis of the thoracic duct lymph of rats, it is possible to quantitatively determine the direct effect of each of the mentioned compounds on intestinal absorption of cholesterol-4-C^{14} from aqueous homogenates.

Experimental. Adult male rats of the Carworth Farms Wistar strain were maintained on standard laboratory chow‡ and water *ad libitum* prior to operation. The thoracic duct was cannulated by a modification of the procedure of Bollman *et al.*(18), and the animals were subsequently fasted for 18-24 hr with free access to 0.9% saline.

The aqueous test emulsions shown in Table I were prepared immediately before use, as described by Vahouny *et al.*(19). The amounts of oleic acid (292 mg), sodium taurocholate (288 mg), and cholesterol-4-C^{14} (50 mg) used were in an optimum molar ratio of 8:4:1, respectively(20). Albumin was included in the preparation of all test emulsions.

The rats in all groups except those receiving pectin (Groups 10 and 11) were given 3 ml of the appropriate test emulsion intragastrically using light vinyl ether anesthesia. Due to the insolubility of pectin, the rats in Groups 10 and 11 were given 3 ml of the test emulsion containing all the components and only half the pectin, and one hour later were given a second 3 ml feeding containing the remainder of the pectin.

Throughout this study, Group 1 which received only albumin, oleic acid and taurocholate was used as the control group for endogenous sterol absorption. The absorption of fed cholesterol-4-C^{14} under optimal conditions was determined in Group 2. The effect of each test substance on endogenous cholesterol absorption was measured by inclusion of the test compound in the control

‡ Purina Laboratory Chow, Ralston Purina Co., St. Louis, Mo.

TABLE II. Effects of Test Compounds on Cholesterol Absorption from Intestine in Lymph-Fistula Rats.

Number of rats	Lymph cholesterol/24 hr			Cholesterol-4-C^{14} absorbed	"Extra" cholesterol esterified
	Free	Total	Ester		
	(mg)	(mg)	(%)	(%)	(%)
Group 1—Control*					
7	5.0 ± .1†	14.9 ± .8	66.1 ± 1.1		
Group 2—Cholesterol-4-C^{14}					
7	6.8 ± .3	30.8 ± 1.3	77.7 ± .8	37.7 ± 1.8	89.4 ± 1.8
Group 3—Cholesterol-4-C^{14}, cholesterol trimethylacetate					
5	6.8 ± .4	31.5 ± 2.8	77.9 ± 1.7	32.5 ± 4.9	90.2 ± 2.6
Group 4—Cholesterol trimethylacetate					
5	5.3 ± .5	15.4 ± 1.9	64.4 ± 1.6		
Group 5—Cholesterol-4-C^{14}, MK-135 (50 mg)					
4	5.8 ± .4	25.7 ± 1.9	77.5 ± .2	27.7 ± 4.0	94.0 ± 1.8
Group 6—MK-135 (50 mg)					
4	5.1 ± .2	13.3 ± .3	61.6 ± 1.7		
Group 7—Cholesterol-4-C^{14}‡					
6	6.0 ± .3	21.4 ± 1.6	71.8 ± 1.5	16.6 ± 1.9	
Group 8—Cholesterol-4-C^{14}, MK-135 (238 mg)					
6	6.0 ± .3	15.0 ± 1.7	71.0 ± .1	11.0 ± 2.0	87.7 ± 4.1
Group 9—MK-135 (238 mg)					
6	3.5 ± .4	8.7 ± 1.0	58.9 ± 1.8		
Group 10—Cholesterol-4-C^{14}, Pectin					
6	5.9 ± .2	26.1 ± .7	77.3 ± .6	30.7 ± 2.4	92.4 ± 1.4
Group 11—Pectin					
6	5.1 ± .4	14.9 ± 1.1	66.1 ± 1.1		
Group 12—Cholesterol-4-C^{14}, nicotinuric acid					
4	6.5 ± .5	32.4 ± 2.3	79.9 ± .6	32.4 ± 4.4	88.1 ± 1.2
Group 13—Nicotinuric acid					
4	4.1 ± .5	12.7 ± 1.2	67.9 ± 1.3		
Group 14—Cholesterol-4-C^{14}, pyridine-3-acetic acid					
4	6.6 ± .4	34.4 ± 1.0	80.9 ± .7	36.3 ± 2.4	89.4 ± 1.4
Group 15—Pyridine-3-acetic acid					
4	4.2 ± .5	12.1 ± 1.3	65.3 ± 1.0		

* All groups received saline, albumin, oleic acid and sodium taurocholate, as described in Table I, in addition to the indicated supplements.

† Values represent mean ± S.E. S.E. $= \sqrt{(x-\bar{x})^2/n\,(n-1)}$

‡ This group did not receive sodium taurocholate.

emulsion lacking cholesterol-4-C^{14} (Groups 4, 6, 9, 11, and 15). After administration of the test emulsion, lymph was collected for 24 hr in vessels containing 0.5 ml heparin (1:1000), and was subsequently frozen till analysis.

Free and esterified cholesterol were determined on acetone-ethanol (1:1) extracts of the lymph samples by the procedure of Sperry and Webb(21). Free and total cholesterol-4-C^{14} were determined as the digitonides, as described by Swell *et al.*(22).

The experimental data are summarized in Table II, and are expressed as mg free or esterified cholesterol per 24 hr lymph collection, and as percentage of the total lymph cholesterol. "Extra" cholesterol represents the chemical difference in total lymph cholesterol between the corresponding control and experimental groups. The percentage absorption of cholesterol was calculated from the total cpm administered and the cpm in total lymph cholesterol per 24 hr.

All differences were analyzed for significance by the "t" test(23); probability values of $>.05$ were considered significant, and $>.01$ as highly significant.

Results and discussion. Experiment 1. Cholesterol trimethylacetate. This experiment was designed to study the specific effect of cholesterol trimethylacetate on endogenous and exogenous absorption of cholesterol. According to Stern and Treadwell(1),† the addition of cholesterol trimethylacetate to a diet containing cholesterol butyrate produced a decrease in the proportion of the esterified fraction in the total blood cholesterol within 1 week. However, by the end of the third

week of feeding, the proportion of esterified cholesterol in the experimental animals had increased and was the same as in the control group. Furthermore, cholesterol trimethylacetate itself was not absorbed in the rat(1). Thus, it appears that this branched-chain ester may inhibit the hydrolysis of cholesterol butyrate by competition for cholesterol esterase in the intestinal lumen. Addition of 61 mg of cholesterol trimethylacetate (which is equivalent to 50 mg of free cholesterol) to the test emulsion containing free cholesterol-4-C^{14} (Group 3) did not alter the percentage absorption of exogenous cholesterol ($p>.05$) (Table II). There was also no significant change in total lymph cholesterol levels (compare Groups 2 and 3) during the 24 hr period after administration of the test emulsion. The data from these groups indicate that cholesterol trimethylacetate does not inhibit absorption of exogenous free cholesterol from the intestine. As is shown in Group 4, which did not receive cholesterol-4-C^{14}, this ester also did not affect the endogenous lymph cholesterol levels or the lymph cholesterol partition.

Experiment 2. MK-135§ or cholestyramine, has been shown to be effective in binding bile acids *in vitro*(3). According to Tennant *et al.*(2), feeding MK-135 to rats does not lower blood cholesterol levels as it does in other species. This species difference may be due to a greater ability of rats to synthesize hepatic cholesterol(24). However, the results of the present study show that this bile acid-binding polymer markedly inhibits absorption of fed cholesterol from the intestine.

Two levels of MK-135 were used in this experiment. In Groups 5 and 6, 50 mg of the polymer were included in the emulsion, an amount equal to the cholesterol fed. In Groups 8 and 9, 238 mg of MK-135 were added to the emulsions. This amount is 2 molar equivalents with respect to the bile salt administered.

As is shown in Table II, addition of 50 mg of MK-135 to the test emulsion containing cholesterol-4-C^{14} decreased absorption from 38% (control level in Group 2) to 28%; furthermore, administration of 238 mg of the polymer with cholesterol (Group 8) further reduced absorption to 11%. The chemical data show that total lymph cholesterol of rats in Group 8 was essentially the same as in Group 1, which received no exogenous cholesterol in the emulsion.

It is apparent from the data obtained in Group 9 (received 238 mg MK-135 but no cholesterol-4-C^{14}) that the large dose of MK-135 also markedly lowered total lymph cholesterol derived from endogenous sources (compare Groups 9 and 1). This depression was probably due in large part to an inhibition of endogenous sterol absorption by effective removal of circulating bile salts.

Evidence that MK-135 is effective in the binding of endogenous bile salts is shown by comparison of cholesterol absorption in Groups 7 and 8 with Group 2. Group 7 received no exogenous bile salt and absorption of the administered cholesterol-4-C^{14} was 16.6%, in comparison with 37.7% in Group 2. In Group 8 addition of MK-135 to the complete emulsion, which contained 288 mg sodium taurocholate, absorption of labeled cholesterol was 11%. This decrease from 16.6 to 11% absorption suggests that the test substance not only effectively eliminated the stimulation of cholesterol absorption by the exogenous bile salt, but also interfered with the effect of endogenous bile salt.

The results of this study suggest that the blood cholesterol-lowering effect of MK-135 is due in part, if not completely, to impaired cholesterol absorption from the intestine, and that the effect is due to binding of bile salts in the intestinal lumen by the resin.

Experiment 3. Pectin‖ feeding at a level of 5% of the diet has been reported to counteract the increase in plasma and liver cholesterol due to cholesterol feeding in rats(7). In the present study, pectin was included in preparation of the emulsions at a level of 250 mg, or 5 times the amount of cholesterol-4-C^{14} used. However, due to the bulk of this compound, it was necessary to feed 125 mg

§ MK-135 was kindly supplied by Dr. Jesse W. Huff, Merck Inst. for Therapeutic Research.

‖ Pectin (polygalacturonic acid, methyl ester) was obtained from Sunkist Growers, Inc., Ontario, Calif.

of the substance with the other components of the test emulsion, and to administer the remainder of the pectin (125 mg) one hour later. Administration of this amount of pectin in an emulsion lacking cholesterol (Group 11) had no effect on total lymph cholesterol or on the cholesterol partition (compare with Group 1) (Table II). However, when pectin was given in a complete emulsion containing cholesterol-4-C^{14} (Group 10), absorption of labeled cholesterol was depressed from 38% in Group 2, to 31% ($p<.01$). Chemical data also showed a significantly lower total lymph cholesterol level, due primarily to a decrease in the esterified cholesterol fraction.

The actual mechanism by which pectin exerts its blood cholesterol-lowering effect is not well defined. Data from the present study indicate that the observed effect of pectin on blood cholesterol is due in part to an inhibition of intestinal absorption of dietary cholesterol.

In Experiment 4, 100 mg of either nicotinuric acid¶ (Groups 12 and 13), or pyridine-3-acetic acid (Groups 14 and 15) were employed. These amounts were twice that of the cholesterol-4-C^{14} administered.

It is apparent from the data summarized in Table II that there is no change in total lymph cholesterol levels due to administration of nicotinuric acid or pyridine-3-acetic acid, either in control groups lacking dietary cholesterol (compare Groups 13 and 15 with Group 1), or in the cholesterol-fed groups (compare Groups 12 and 14 with Group 2). Also, analysis of lymph cholesterol-4-C^{14} showed no significant depression of labeled cholesterol absorption due to administration of either test substance. It was observed that soon after administration of the test compounds, severe diarrhea developed; in addition, survival of the test animals in these groups was only 50% compared to almost 90% in all other groups in the present study.

The results of this experiment suggest that the site of action of nicotinuric acid and pyridine-3-acetic acid in lowering blood cholesterol levels is not on intestinal cholesterol absorption.

Throughout all groups in the present study in which a net increase in lymph cholesterol occurred, the percentage esterification of the "extra" or absorbed cholesterol ranged between 88% and 93%. As has been previously discussed(25), it is apparent that esterification of cholesterol during absorption is independent of amount of cholesterol absorbed, and these data support the importance of esterification of natural sterols in the absorptive mechanism.

Summary. 1. The direct effect of several blood cholesterol-lowering agents on intestinal absorption of cholesterol-4-C^{14} was studied in lymph-fistula rats. 2. MK-135 (cholestyramine) and pectin caused significant reductions in lymph total cholesterol and absorption of cholesterol-4-C^{14}. 3. With MK-135, the larger of 2 doses studied also reduced absorption of endogenous cholesterol from the intestine. 4. Cholesterol trimethylacetate, nicotinuric acid and pyridine-3-acetic acid had no effect on cholesterol absorption or lymph cholesterol levels. 5. In all groups where significant cholesterol absorption occurred, the percentage esterification of absorbed cholesterol was constant (88-93%), irrespective of the extent of absorption.

¶ Nicotinuric acid and pyridine-3-acetic acid were purchased from California Corp. for Biochemical Research.

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Metabolism of Chlorpromazine. IV. Identification of 7-Hydroxychlorpromazine and Its Sulfoxide and Desmethyl Derivatives.* (28088)

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Urinary excretion of unidentified glucuronic acid conjugates of chlorpromazine has been reported by many workers(1-5). Lin *et al.*(2) isolated 4 such conjugates from human urine by column and paper chromatography. Posner(3) found 3 phenolic metabolites which he suspected were the 3-hydroxy or 3,7-dihydroxy derivatives and/or their sulfoxides. Huang and Kurland(5) briefly reported isolation of 6 chlorpromazine glucuronides and 2 hydroxychlorpromazines, but it is not clear whether these compounds were ever identified, and if so, by what criteria. According to Forrest and Piette(6), "After 10 years of intensive clinical use of . . . chlorpromazine, the major urinary drug metabolites of the phenolic, polar fraction, have not been unequivocally identified, despite the fact that this drug was the subject of many metabolic investigations in numerous laboratories, and more data on its metabolic fate and type and amount of oxidative derivatives are available than for any other phenothiazine compound."

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Our earlier reports(7,4,8) were primarily concerned with the nonpolar metabolites of chlorpromazine. We now turn to its phenolic metabolites. As shown below, 7-hydroxychlorpromazine and 3 closely related derivatives have been identified in both human and dog urine by chemical and paper chromatographic methods.

Experimental. Human subjects included in this study were patients at the Hillside Hospital who were on a daily oral regimen of 300-1200 mg of chlorpromazine HCl. For the animal studies, female mongrel dogs used were fed chlorpromazine HCl or promazine HCl, 4 mg/kg b.i.d. Urine collections were made in the preliminary control period and again during medication.

Isolation of metabolites. Unconjugated metabolites of chlorpromazine (Fraction AB) were extracted from urine at pH 9 using 3 volumes of ethylene dichloride (Eastman). The organic extract was washed with dilute ammonia buffer, pH 9, dried over anhydrous sodium sulfate and taken to dryness. The residue was dissolved in methanol and fractionated by paper chromatography. The residual urine containing the glucuronide conju-