(Table IX). There was no loss in piperacillin activity whether incubated alone or with gentamicin; however, a 47% loss of the initial activity of gentamicin was noted when it was incubated with piperacillin. The data clearly demonstrate that interaction between the two drugs in urine is feasible.

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Potential Tumor- or Organ-imaging Agents XXIV: Chylomicron Remnants as Carriers for Hepatographic Agents

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Abstract □ This paper describes the possible utility of plasma lipoproteins for the site-specific delivery of diagnostic agents. The class of lipoproteins known as chylomicrons was selected for this preliminary study, since they are known to be rapidly metabolized and taken up by the liver. Cholesteryl iopanoate (II), an iodinated analogue of a normal constituent of the hydrophobic core of chylomicrons, was synthesized from cholesterol and iopanoic acid (I) and subsequently radiolabeled with ioidine-125. Whereas intravenous administration of II in physiological saline resulted in the appearance of ~31% of the dose in the liver at 0.5 hr, prior incorporation of II into chylomicrons resulted in an almost threefold (87%) increase in the liver accumulation of II in the same time period. A more gradual appearance of II in steroid-secreting tissues was consistent with the association of II with high-density lipoproteins following administration.

Keyphrases □ Chylomicron—remnants as carriers for hepatographic agents, potential tumor- or organ-imaging agents □ Tumor-imaging agents—potential, chylomicron remnants as carriers for hepatographic agents □ Organ-imaging agents—potential, chylomicron remnants as carriers for hepatographic agents

The early detection of small metastatic lesions in the liver has been a long-term goal of radiology and nuclear medicine. Among the noninvasive diagnostic approaches, radionuclide scintiscanning, ultrasonography, and computed tomography (CT) have all enjoyed variable success (1). Over the past several years, one of the goals of this laboratory has been to devise approaches for the selective delivery of radiopharmaceuticals or radiopaque agents to the liver on the premise that specific uptake of these agents in either normal or abnormal tissue will significantly improve image resolution of small lesions. While others have employed liposomes as delivery vehicles for radiopharmaceuticals (2) and radiopaque contrast agents (3), the focus of this study is on those naturally occurring macromolecules responsible for the transport of lipophilic substances in the plasma—the lipoproteins.

It has been known for many years that the liver plays a major role in lipoprotein catabolism. This is especially true for the class of lipoproteins known as chylomicrons (4, 5). The chylomicrons are synthesized in the intestinal mucosa during fat absorption and are responsible for the transport of dietary fats to sites of utilization and storage. Structurally they are the largest (800–5000 Å) and the lightest (<0.95 g/ml) of the lipoproteins, and consist of an apolar core of lipid surrounded by a phospholipid monolayer (Fig. 1). The lipophilic core is composed of triglycerides and cholesteryl esters. Free cholesterol and apoproteins are associated with the outer phospholipid membrane.

Once in the circulation, these native chylomicrons are acted on by tissue lipoprotein lipase, the enzyme responsible for hydrolyzing triglycerides and providing free fatty acids for cellular metabolism. The resulting triglyceride-depleted, cholesteryl ester-enriched chylomicrons are referred to as chylomicron remnants. In humans, these smaller remnants (300–800 Å) are rapidly taken up by the liver, and their plasma half-life is in the range of 4–5 min (6).

The uptake of chylomicron remnants by liver cells has been shown to occur by a saturable high-affinity process, suggesting the existence of receptors on the surface of liver cells capable of specificially binding these particles (7, 8). Moreover, the presence of apoprotein E on the surface of the remnants has been shown to be important for the recognition and uptake of these particles (9, 10).

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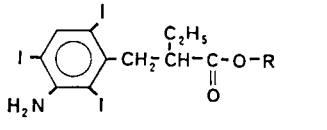
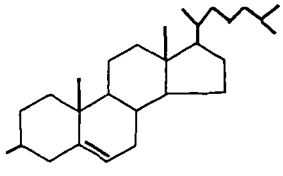
The parenchymal cells, which comprise the majority of cells in the liver, have been shown to be the most intimately involved in the clearance of chylomicron remnants from the circulation (11, 12). Of particular interest was the finding that remnant-incorporated cholesteryl esters are not taken up to any significant extent by nonparenchymal cells (Kupffer and endothelial) of the rat liver (13). Since radioactive colloids currently used for imaging of liver function are phagocytized by Kupffer cells, chylomicron remnants offer an alternate mechanism for the localization of imaging agents in the liver.

Since cholesteryl esters are a major constituent of chylomicron remnants, it seemed reasonable that incorporation of polyiodinated esters of cholesterol into remnant vesicles could give rise to a new class of hepatographic agents. The iodine of these polyiodinated esters would confer sufficient electron density to serve as radiopaques, or the iodine could be substituted with any one of its various radioisotopic forms (*e.g.*, iodine-123) for radionuclide imaging. To test this hypothesis, iopanic acid (I), an established cholecystographic agent was esterified with cholesterol to afford the highly lipophilic cholesteryl iopanoate (II). This new ester was then labeled with iodine-125 to assist in the measurement of chylomicron incorporation and subsequent tissue distribution analysis.



I. R = H = IOPANOIC ACID



II. R =

EXPERIMENTAL[1]

Cholesteryl Iopanoate (II)—A solution of iopanoic acid (I, 571 mg, 1 mmole) and 1,1′-carbonyldiimidazole (170 mg, 1.05 mmoles) in dry tetrahydrofuran (5 ml) was heated at reflux for 10 min. Cholesterol (425 mg, 1.1 mmoles) was added along with a catalytic amount of sodium hydride. The mixture was stirred at room temperature for 1 hr, whereupon the solvent was removed using a rotatory evaporator. Water (10 ml) was carefully added to the residue and the mixture was extracted with benzene. The benzene extracts were combined, the solvent was removed under reduced pressure, and the residue was chromatographed on silica gel (benzene). The fractions containing the ester were combined, the solvent was removed under reduced pressure, and the residue was crystallized from ethanol–water to give II, mp 128–130° (dec) in 58.5% yield. IR(KBr): 3465, 3370, and 1738 cm^{-1}; ^1H-NMR: 4.8 (m, 3 H), 4.85 (s, NH_2), 5.45 (m, 6 vinyl H), and 8.15 ppm (s, Ar 1 H).

Anal.—Calc. for $C_{38}H_{56}I_3NO_2$: C, 48.58; H, 6.01; I, 40.52. Found: C, 48.71; H, 5.97; I, 40.23.

Isotope Exchange of II—The ester (5 mg) and acetamide (100 mg) were placed in a flask containing 10^{-9} *M* NH_4Cl (1.0 eq based on the amount of pH 9 $Na^{125}I$ solution to be added) and 10^{-7} *M* $Na_2S_2O_7$ (1.3–1.5 eq based on $^{125}I^-$ with a specific activity of 2.5×10^9 mCi/mole). $Na^{125}I$ (15 mCi) was added and rinsed in with acetone (0.15 ml). The flask was heated at 170° under a slight positive nitrogen pressure for 1 hr to evaporate the acetone and water present. Heating at 170° was continued for an additional 12 hr, and the flask was allowed to cool. The solid residue was dissolved in benzene, washed with water, and chromatographed on silica gel with benzene to afford [^{125}I]II (3 mg; 2.4 mCi). Radiochemical purity was established by TLC with unlabeled ester as the standard: R_f 0.50 (C_6H_6); R_f 0.08 (CCl_4).

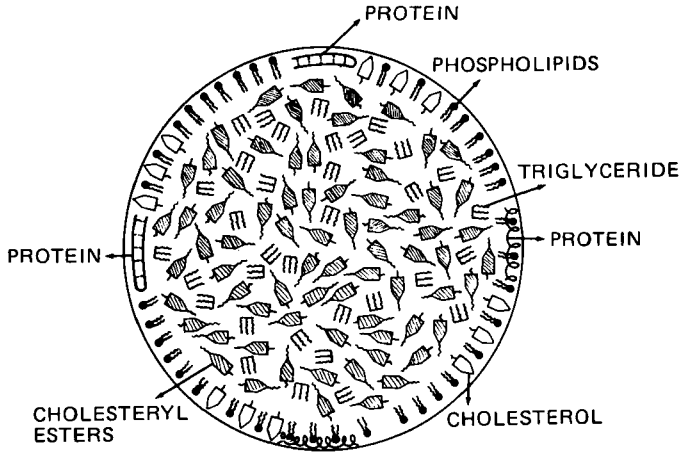


Figure 1—*General structure of a plasma lipoprotein.*

Animals—Sprague–Dawley rats (200–250 g), maintained on a standard rat food, were used for all studies. They were housed in temperature- and light-controlled quarters and had free access to food and water.

Isolation of Chylomicrons—To maximize the yield of chylomicrons from blood samples, the animals were given corn oil intragastrically (2 ml) three times at 3-hr intervals with a fourth dose (3 ml) administered 20 min prior to exsanguination. Blood was drawn by cardiac puncture in heparinized evacuated tubes[2] while the rats were under ether anesthesia (each rat gave approximately 6 ml of blood). The blood was immediately centrifuged at 3000 rpm for 10 min at 4° to separate plasma from packed cells. The plasma was divided into 6.25-ml fractions and layered beneath an equal volume of 0.15 *M* NaCl solution containing 0.002 *M* EDTA [(ethylene dinitrilo)tetraacetic acid] in cellulose nitrate tubes. These tubes were placed in an ultracentrifuge[3] with a Type 40 rotor and spun at 37,000 rpm (114,000×*g*) at 12° for 20 hr. The top, milky white, flocculant layer containing the chylomicrons was carefully separated from the remaining solution and stored at 4°. Lipid analysis of this fraction showed it to contain 281 meq/liter of triglycerides and 15.5 meq/liter of cholesterol[4].

Chylomicron Incorporation—Radioiodinated II (2.0 mg, 420 μCi) was dissolved in benzene (1 ml) to which was added polysorbate 20 (0.2

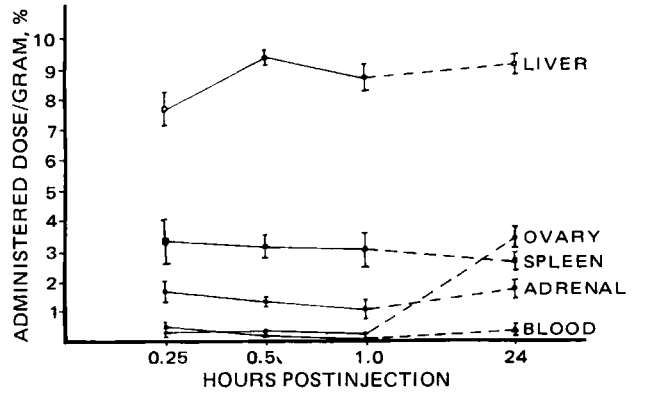


Figure 2—*Tissue distribution of radioactivity following administration of chylomicrons labeled with [^{125}I]cholesteryl iopanoate as a function of time.*

[1] NMR spectra were obtained on a Varian EM360A spectrometer in $CDCl_3$ containing tetramethylsilane as an internal reference. IR spectra were taken in KBr pellets on a Perkin-Elmer 281 spectrophotometer. Elemental analyses were performed by Midwest Microlab, Ltd., Indianapolis, Ind. TLC was performed on Eastman polyethylene-backed silica gel plates with a fluorescent indicator. Cholesterol and 1,1′-carbonyldiimidazolde were obtained from the Aldrich Chemical Co., Milwaukee, Wis. Iopanoic acid was a gift from the Sterling-Winthrop Research Institute, Rensselaer, N.Y. Sodium iodide-125 was purchased from Union Carbide, Tuxedo, N.Y. Polysorbate 20 (Tween 20) was obtained from Sigma Chemical Co., St. Louis, Mo. Rats were obtained from Spartan Research Animals, Inc., Haslett, Mich. Rat food was purchased from Ralston Purina Co., St. Louis, Mo. Agarose gel (Biogel A-50M) was obtained from Bio-Rad Laboratories, Richmond, Calif.

[2] Vacutainer; Bectin, Dickinson and Co.

[3] Beckman LS200.

[4] Analysis provided by Dr. Walter Block of the University of Michigan School of Public Health.

Table I—Distribution of Radioactivity 0.5 and 24 hr after Intravenous Administration of Cholesteryl [^{125}I]Iopanoate in Polysorbate 20–Saline Vehicle or Chylomicrons

Tissue	% Administered Dose/g of Tissue ± *SEM*			
	Polysorbate 20–Saline		Chylomicrons	
	0.5 hr[a]	24 hr[a]	0.5 hr[b]	24 hr[b]
Adrenal cortex	4.903 ± 0.718	11.029 ± 2.172	1.293 ± 0.126	1.921 ± 0.207
Blood	5.191 ± 0.441	0.696 ± 0.048	0.062 ± 0.008	0.231 ± 0.047
Liver	4.035 ● 0.543	6.587 ± 0.316	9.220 ± 0.251	9.151 ± 0.315
Lung	1.340 ± 0.126	0.599 ± 0.078	0.286 ± 0.062	0.252 ± 0.050
Ovary	5.161 ± 0.716	24.542 ± 2.964	0.118 ± 0.019	3.464 ± 0.773
Plasma	10.369 ± 0.862	1.345 ± 0.082	0.133 ± 0.027	0.388 ± 0.104
Spleen	0.777 ± 0.073	2.001 ± 0.134	3.136 ± 0.447	2.735 ± 0.344
Thyroid	0.868 ± 0.098	3.571 ± 0.218	0.075 ± 0.014	1.319 ± 0.317

[a] $N = 5$. [b] $N = 4$.

ml). This mixture was vortexed and the benzene evaporated. Physiological saline (1.0 ml) was added, and the solution was vortexed for 5 min. This solution was added to the chylomicron suspension (2.5 ml) in an Erlenmeyer flask, and the mixture was incubated in a water bath at 37° with shaking for 24 hr. At the end of the incubation period, an aliquot was taken for radioactivity determination and the remainder was placed on an agarose-gel (1.5 × 90 cm) column. The column was eluted with 0.20 *M* NaCl solution containing 0.002 *M* EDTA and 0.02% sodium azide. The column was run at a flow rate of 2.5 ml/min, the fractions were collected in a fraction collector[5] (150 drops/tube). The fractions were assayed for optical density (280 nm) and radioactivity[6] (50-μl aliquots) (Fig. 3). The fractions in the void volume were pooled, concentrated to a final volume of 13 ml in an ultrafiltration unit[7], and analyzed for total radioactivity.

Tissue Distribution—Rats were injected with radiolabeled chylomicrons (0.6 ml, 2.5 μCi per animal) intravenously through the tail vein. Groups of four animals were sacrificed by cardiac puncture under ether anesthesia at 0.25, 0.5, 1.0, and 24 hr. The tissues were excised, placed in preweighed cellulose acetate capsules, weighed, and assayed for radioactivity as previously described (14). The tissue concentrations at 0.5 and 24 hr are tabulated in Table I; the results for five key tissues are graphed in Fig. 2.

Tissue Extraction—Adrenal, liver, and plasma were extracted according to the method of Folch *et al.* (15). The percentage of radioactivity in the aqueous and organic phases was measured. The organic phase was streaked on a TLC plate and chromatographed using a petroleum ether–diethyl ether (7:2) system. The TLC plates were sliced and counted in a γ-counter to determine the amount of original ester remaining in the tissues.

Polyacrylamide Gel Electrophoresis (PAGE)—PAGE analysis of plasma samples was performed according to the method of Narayan *et al.* (16), as previously described (17). The amount of radioactivity associated with each lipoprotein class was determined by sectioning the gels and counting each section in a γ-counter. The radioactivity associated with each lipoprotein class was expressed as a percentage of the total radioactivity applied to the gel (Table II).

RESULTS AND DISCUSSION

As has been shown in previous papers in this series (18, 19), cholesterol is efficiently esterified by acid imidazolides. Using this mild procedure, cholesteryl iopanoate (II) was obtained in 58.5% yield from cholesterol and iopanoic acid. This product was readily radioiodinated with iodine-125 by using the isotope exchange method in acetamide (19).

Incorporation of II into chylomicron remnants also proved successful. Similar to previous studies (20) with radiolabeled cholesteryl oleate, II was found to diffuse from the saline solution into the lipophilic core of the chylomicrons during incubation. Incorporated ester was readily separated from unincorporated ester by gel filtration. The elution profile exhibited two peaks when assayed for radioactivity and absorbance at 280 nm (Fig. 3). Since the molecular mass exclusion limit of the gel was 50×10^6 daltons and the approximate molecular mass of chylomicrons is $\sim 200 \times 10^6$ daltons, the radioactivity appearing in the void volume represented II associated with chylomicrons and the radioactivity in the later fractions represented unincorporated ester. Extraction of the labeled chylomicrons and TLC analysis of the extract demonstrated that all of the radioactivity was still associated with II. Moreover, PAGE analysis was consistent with incorporation of II into chylomicrons. The overall process is outlined in Fig. 4. Following this procedure, ~40% of II was incorporated into chylomicron remnants.

Table II—PAGE[a] Analysis of Rat Plasma at Various Times Postinjection of Cholesteryl [^{125}I]Iopanoate in Polysorbate 20–Saline or Incorporated into Chylomicron Remnants[b]

Preparation	Region[c]	Total Radioactivity in Gel, %				
		0 hr[d]	0.25 hr	0.50 hr	1.00 hr	24 hr
Polysorbate 20–Saline	Stacking gel	80.0	—	1.1	—	5.1
	VLDL/LDL	16.8	—	23.5	—	27.2
	HDL/albumin	2.2	—	75.2	—	66.7
	Below albumin	1.0	—	0.2	—	0.9
Chylomicron remnants	Stacking gel	97.0	90.6	38.2	40.6	32.4
	VLDL/LDL	0.8	5.7	21.2	18.4	15.3
	HDL/albumin	1.1	2.4	21.2	22.3	35.2
	Below albumin	1.1	1.3	19.4	18.7	17.1

[a] PAGE is polyacrylamide gel electrophoresis. [b] $n = 3$–5. [c] LDL = low-density lipid; HDL = high-density lipid. [d] Analysis of dose prior to administration.

The disposition of II administered in saline was compared with II administered in chylomicron remnants. The tissue distribution of radioactivity at 0.5 and 24 hr after intravenous administration of these two preparations is summarized in Table I. When the ester was given in saline, high levels of radioactivity remained in the plasma at 0.5 hr. Plasma levels declined with time and at 24 hr, the adrenal and ovaries were the tissues containing the highest concentration of radioactivity. Lipid extraction and TLC analysis of these tissues demonstrated that the radioactivity was still associated with cholesteryl iopanoate.

Administration of II incorporated into chylomicron remnants, on the other hand, led to a marked increase in the amount of ester reaching the liver within 0.5 hr. Based on organ weights, ~87% of II was present in the liver following administration in chylomicrons as opposed to ~31% when given in saline. This result agrees very closely with that obtained earlier (20) using chylomicron remnants labeled with cholesteryl oleate. A major difference from cholesteryl oleate, however, was that the concentration of II in the liver remained essentially unchanged over the 24-hr period (Fig. 2). TLC analysis of the lipid extracted from the liver revealed that the radioactivity was still in the form of cholesteryl iopanoate (II). Unlike the naturally occurring esters of cholesterol, II is apparently a poor substrate for cholesteryl ester hydrolase (EC 3.1.1.13) present in the liver. In all likelihood, this *in vivo* stability accounts for its ability to persist in the liver.

The increased radioactivity in the steroid-secreting tissues (adrenals and ovaries) with time (Fig. 2) suggests redistribution of II from chylomicron remnants to other lipoproteins. In the rat, the major cholesterol carrier is high-density lipoproteins (HDL), and steroid-secreting tissues have been shown to acquire their cholesterol by an HDL receptor-mediated process (21). Thus, the high levels in the ovary and adrenal may result from the transfer of II from chylomicron remnants to HDL. Support for such a sequence comes from the PAGE analysis of the plasma (Table II). At 0 hr, 97% of II is in the chylomicron band (stacking gel), but by 0.5 hr, 21.2% is found to be associated with HDL. At 24 hr, the amount of II associated with HDL is 35.2%. By contrast, when II is administered in normal saline, it rapidly becomes associated with HDL such

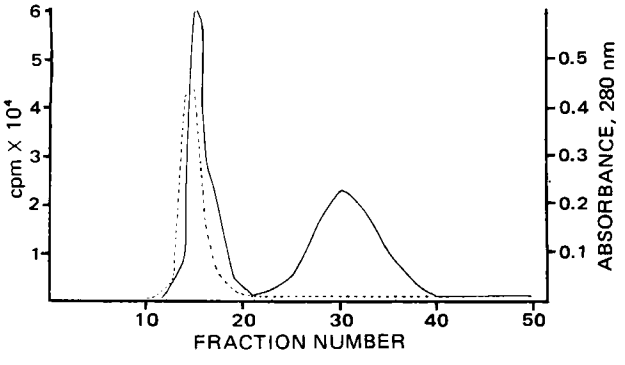


Figure 3—*Gel filtration profile of incubation medium containing rat chylomicrons and [^{125}I]cholesteryl iopanoate. The solid line represents radioactivity in cpm and the dotted line represents absorbance at 280 nm.*

[5] LKB 2070 Ultra Rac II.
[6] Searle 1185 γ-Counter (84.5% efficiency).
[7] Diaflo YM-30; Amicon Corporation, Lexington, Mass.

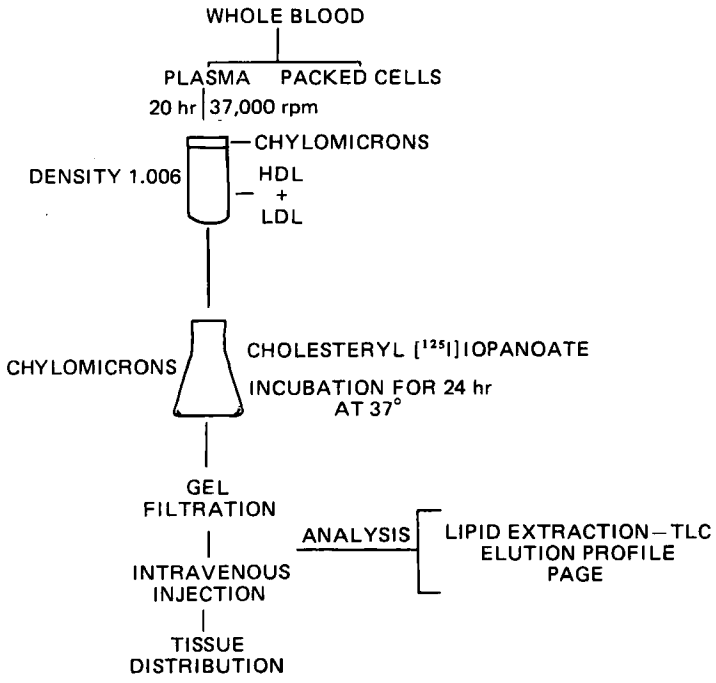


Figure 4—*Outline of procedures employed for the isolation of rat plasma chylomicrons, the incorporation of radioiodinated cholesteryl iopanoate, and the subsequent purification and analysis of the radioiodinated chylomicron preparation.*

that >75% is associated with this lipoprotein fraction at 0.5 hr. This could account for the higher concentration of ester appearing in the adrenals and ovaries following administration of II in saline.

The purpose of this preliminary study was to determine whether foreign lipid molecules could be introduced into chylomicron remnants and delivered selectively to the liver. This study has demonstrated that, at least in radiotracer amounts, cholesteryl iopanoate (II) can be incorporated into chylomicron remnants in a manner similar to that shown previously for cholesteryl oleate. Moreover, it has been shown that such remnant incorporation of II markedly increased its ability to selectively accumulate in the liver. These results suggest a potential use of lipoproteins as carriers of radiopharmaceuticals for liver imaging. Even in this instance, however, the procedures described in this paper would have to be modified in order to use radionuclides such as iodine-123, which has a half-life of 13 hr. Moreover, to be useful as vehicles for the delivery of radiopaques to the liver, it would be necessary to develop methods capable of incorporating radiologic concentrations of radiopaques into chylomicrons. Studies aimed at answering these and related questions are now in progress.

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