Colestipol-induced changes in LDL composition and metabolism. II. Studies in humans

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Abstract We investigated the effect of the bile acid sequestrant, colestipol hydrochloride, on the composition and metabolism of human low density lipoprotein (LDL). Colestipol treatment produced a disproportionate decrease in LDL cholesterol compared to LDL apoB, resulting in a significant decrease in the LDL cholesterol/apoB ratio. Electron microscopy revealed that LDL particles were smaller in size and analytical ultracentrifugation demonstrated that colestipol therapy selectively depleted larger, more buoyant LDL particles of $S_f°$ 6–7. Thus, colestipol therapy produced LDL that were smaller in size, more dense, and characterized by a decreased cholesterol to protein ratio. To determine whether the altered LDL had different metabolic properties, autologous LDL was isolated from subjects before and during colestipol therapy and their fractional catabolic rates (FCR) were then simultaneously determined in the same patient while on therapy. Eight LDL turnover studies comparing the catabolism of LDL isolated during therapy (Rx-LDL) and LDL isolated off therapy (Con-LDL) were performed in six subjects. All subjects responded to colestipol treatment, with an average 29% fall in LDL cholesterol. In four of six subjects, and in six of eight studies, the FCR of Rx-LDL was substantially slower than that of Con-LDL. These studies demonstrate that a drug intervention may alter subpopulations of LDL particles in such a way that overall LDL composition is changed. This alteration may independently affect the intrinsic metabolic behavior of the LDL. We suggest that such drug- (or dietary-) induced changes in LDL composition need to be considered in kinetic studies designed to assess the overall impact of the perturbation being studied.—**Young, S. G., J. L. Witztum, T. E. Carew, R. M. Krauss, and F. T. Lindgren.** Colestipol-induced changes in LDL composition and metabolism. II. Studies in humans. *J. Lipid Res.* 1989. **30:** 225–238.

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Bile acid sequestrant resins are widely used to lower plasma LDL-cholesterol levels in humans, and recent clinical trials have provided evidence that their use is associated with beneficial effects on coronary artery disease (1–3). Current evidence suggests that they lower LDL levels by induction of hepatic LDL-receptor activity, thereby enhancing LDL removal from plasma by an LDL-receptor-dependent process (4–10). We (11, 12) and others (2, 10, 13) have shown that the composition of LDL is altered by bile acid sequestrant therapy. The most noteworthy change is a decreased LDL cholesterol/protein ratio, a result of a greater decrease in levels of LDL-cholesterol than levels of LDL-apoB. In a recent study (8) we showed that bile sequestrant resins effectively lowered LDL levels in guinea pigs, and that the LDL isolated from cholestyramine-treated guinea pigs was altered in a manner similar to that noted in treated humans. We also showed that the altered LDL had different metabolic properties compared to control-LDL, isolated in the absence of drug treatment (8). In this report, we document that the bile sequestrant colestipol hydrochloride significantly alters the composition, size, and density of human LDL, and we demonstrate that LDL isolated during bile sequestrant therapy has different metabolic properties than control-LDL. Potential implications of these observations are discussed.

MATERIALS AND METHODS

SUBJECTS AND EXPERIMENTAL PROTOCOLS

Studies on colestipol-induced changes in LDL composition

Subjects 1–8 were studied while housed in the General Clinical Research Center (GCRC) of the Washington University School of Medicine, St. Louis, MO. All subjects gave written consent for the studies, which were approved by the Human Studies Committee of Washington University, School of Medicine. Seven of the eight subjects had familial

Abbreviations: LDL, low density lipoproteins; Rx-LDL, LDL isolated during colestipol therapy; Con-LDL, LDL isolated off colestipol therapy; FCR, fractional catabolic rate.

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hypercholesterolemia (**Table 1**). For each subject a 12-hr fasting blood sample was obtained for baseline lipoprotein values as an outpatient while on ad-lib diets (lipid results shown in Table 1). Each subject was then instructed on an NIH Type II diet. After following this diet as an outpatient for a minimum of 8 weeks, each subject was housed in the GCRC and fed an isocaloric solid food diet of fixed composition consistent with NIH Type II guidelines. After 10–14 days, blood was obtained for baseline studies of LDL composition (Control in Table 2). Colestipol HCl was then begun, 20–30 g/day, and blood was obtained after 5–7 days for determination of early changes in LDL composition (Acute in Table 2). Subjects were then discharged and followed for a minimum of 2 months. Each subject was then readmitted to the GCRC and the isocaloric Type II diet was resumed for a minimum of 10 days. Blood was then obtained to evaluate changes in LDL composition produced by long-term colestipol therapy (Chronic values in Table 2). Subjects 9 and 10 followed a similar protocol in San Diego, in studies designed to evaluate the effect of colestipol on LDL size as determined by electron microscopy.

Studies on colestipol-induced changes in LDL metabolism

Subjects 11–19 (Table 1) were studied while housed in the Special Diagnostic and Treatment Unit (SDTU) of the Veterans Administration Hospital, La Jolla, CA. All patients gave informed consent for the study, which was approved by the Human Studies Committee of the University of California, San Diego. Patient characteristics and baseline lipid values are provided in Table 1. Patients 11, 12, 14, and 16 followed NIH Type II diets and were on chronic colestipol therapy prior to studies.

Each subject was fed an isocaloric, NIH Type II diet. Caloric intake was adjusted daily to maintain constant body weight throughout the study period. Two patients with coronary artery disease (#12, 13) received metoprolol, 50 mg bid, and isosorbide dinitrate, 20–40 mg tid, throughout the study. Each subject received oral KI for 5 days prior to injection of radiolabeled lipoprotein and throughout the period of radioactive decay.

To determine whether the changes in LDL composition induced by colestipol therapy were associated with a change in the metabolic properties of the LDL, we simultaneously compared the turnover of autologous LDL preparations isolated during colestipol treatment and during a control period (no drug treatment). The basic protocol for these studies is illustrated in **Fig. 1A.** During period A, all subjects received colestipol-HCl, 20–30 g per day, in divided doses. After a minimum of 10–14 days of colestipol therapy, plasma lipid and lipoprotein levels were measured, and 75–250 ml of plasma was obtained by phlebotomy or plasmaphoresis. Colestipol treatment was then discontinued.

TABLE 1. Characteristics of study subjects

Subject	Age	Sex	Total Chol	LDL-Chol	TG	Presence of FH[a]	Comments[b]
	yr			*mg/dl*			
1	52	F	436	353	104	+	
2	22	M	326	260	117	+	son of subject 1
3	44	M	271	223	110	+	
4	28	M	297	243	121	+	
5	40	F	361	293	63	+	
6	56	M	303	258	104	+	
7	24	M	322	253	239	+	CABG
8	55	M	276	190	243	–	
9	29	M	349	297	64	+	
10	26	F	379	308	92	unknown[c]	CABG
11	38	M	308	244	148	+	
12	53	M	337	271	102	–	CAD, PVD, CVD
13	63	M	237	159	141	–	CAD
14	48	M	314	227	177	–	CAD
15	63	M	176	123	94	–	normal
16	60	F	315	212	90	+	
17	48	M	283	211	259	+	CAD, S/P CABG
18	36	M	127	73	61	–	normal
19	45	M	264	175	301	+	CAD

All patients had undergone routine clinical laboratory tests and were free of thyroid, renal, hepatic, or other metabolic disorders such as diabetes. Presence of associated cardiovascular disorders is noted. Lipid values refer to baseline studies.

[a]FH refers to familial hypercholesterolemia as defined by LDL-chol > 190 mg/dl and one or more of following criteria; tendon xanthomata in patient, and/or first degree relative with hypercholesterolemia and early cardiovascular disease.

[b]CAD, coronary artery disease; PVD, peripheral vascular disease; CVD, cerebrovascular disease; CABG, coronary artery bypass graft.

[c]Patient is an orphan and family history is unknown.

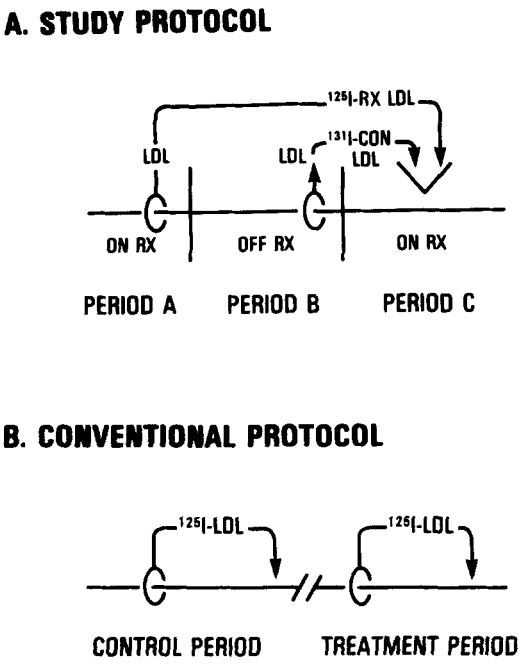

Fig. 1. Protocols for kinetic studies. A: Demonstrates the protocol for kinetic studies used here. These studies were designed to simultaneously compare the rates of clearance of autologous Rx-LDL and Con-LDL in a human subject. After 10–14 days of colestipol treatment (period A) plasma was taken and Rx-LDL was isolated. Colestipol was then discontinued (period B) and after 7 days Con-LDL was isolated. Colestipol treatment was then reinstituted (period C). The two LDL preparations were labeled with ^{125}I and ^{131}I and simultaneously injected. Plasma radioactivity disappearance curves were determined over the ensuing 14 days and FCR was calculated. B: Demonstrates the traditional protocol for assessing the metabolic effects of drug therapy. In this protocol, a kinetic study is performed in a control state using an autologous LDL tracer isolated in the control state. Then, after a period of drug treatment, autologous LDL is again isolated and a repeat kinetic study is performed in the presence of drug treatment.

LDL isolation (Rx-LDL) was carried out immediately by ultracentrifugation using sterile techniques as previously described (14). The preparation was then stored at 4°C. Seven days following discontinuation of colestipol (period B, Fig. 1), plasma was again obtained, and control LDL (Con-LDL) was isolated immediately in an identical fashion. All patients then resumed their previous dose of colestipol therapy (period C).

For each subject, the Rx-LDL was labeled with ^{125}I and Con-LDL with ^{131}I. For subjects 11 (study 1) and 16, labeling was performed immediately after isolation of each LDL fraction, while in all other studies labeling of both LDL preparations was performed at the same time, immediately after isolation of Con-LDL. Then, 2 days after iodination and 5–6 days after each subject had resumed colestipol therapy (period C), ^{125}I-labeled Rx-LDL and ^{131}I-labeled Con-LDL were simultaneously injected intravenously and plasma decay curves were determined over the ensuing 14 days as previously described (14). Subjects 11 (study 1), 12 (study 2), 13 (study 2), 14, and 16 participated in this protocol. In subject 13 (study 1) the entire protocol was repeated except that he did not resume colestipol during period C.

Control studies

The protocol outlined above (Fig. 1A) was developed in order to allow the simultaneous determination of LDL turnover of two autologous LDL preparations isolated under different experimental situations, i.e., on and off drug treatment. Of necessity, this protocol introduced a potential experimental artifact in that the LDL isolated first was held in vitro 1 week longer than the LDL isolated subsequently. A priori, we predicted that the sample isolated first might be more likely to be altered and thus experience an artifactual acceleration of in vivo clearance when reinjected. In order to test this possibility, turnover studies comparing the simultaneous clearance of autologous LDL preparations isolated 1 week apart were performed in subjects 11, 12, 17, and 19. For subject 11 (study 2) the protocol shown in Fig. 1A was repeated with the exception that he remained on colestipol therapy during the entire time, i.e., both LDL preparations were Rx-LDL. For subjects 12 (study 1), 17, and 19 the protocol shown in Fig. 1A was followed except they were not on colestipol at any time (i.e., both LDL preparations, isolated one week apart, were Con-LDL, and no colestipol was given during period C.

Finally, to determine whether the order of isolation of the two LDL preparations was an important variable, a third LDL turnover study was performed in subject 13 (study 3) in which the order of isolation of the LDL preparations was reversed. Thus, Con-LDL was isolated first and then, after 1 week of colestipol treatment, Rx-LDL was isolated. Colestipol treatment was continued during period C when the two labeled LDL tracers were injected. This modified protocol was also followed for the study of subject 15.

In our previous experience with LDL turnover in human subjects (14) and in animals (8, 14–16), we have not noted any consistent isotope effect on the in vivo catabolism of LDL tracers. To again exclude an isotope effect as a signficant factor in these experiments using our current methodology, LDL was isolated from subject 18 and divided into two aliquots. One was labeled with ^{125}I and the other with ^{131}I and the turnover of the two autologous tracers was simultaneously determined in this subject. The results (**Fig. 2**) clearly show that both tracers had virtually identical rates of clearance.

ANALYTICAL TECHNIQUES

Compositional studies of LDL

Routine measurements of lipoprotein levels were performed by the standardized methods of the Lipid Research

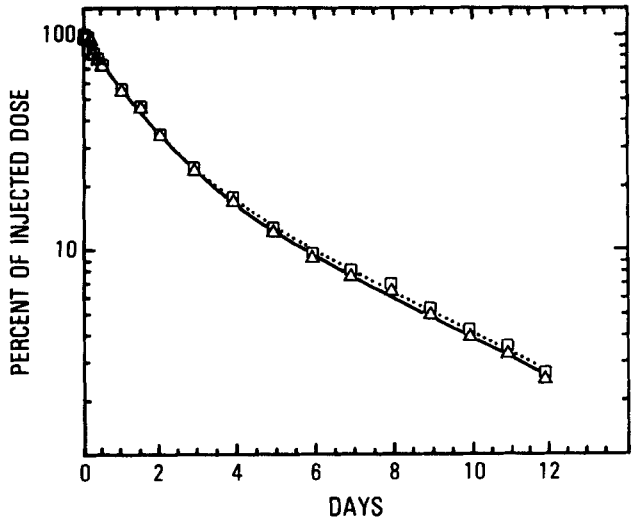

Fig. 2. Demonstration of lack of isotope effect. In this study a direct comparison of the catabolism of two aliquots of the same autologous LDL preparation, labeled with ^{125}I and ^{131}I, was made in normal subject #18. Note that the fractional catabolic rate for ^{131}I-labeled LDL (△—△) (0.428 pools/day) is virtually identical to that of ^{125}I-labeled LDL (□...□) (FCR = 0.417 pools/day).

Clinic System (17) at either the St. Louis or La Jolla laboratory. To avoid possible artifacts caused by the multiple spins required to isolate lipoproteins by ultracentrifugation, 3-ml aliquots of the same plasma sample were centrifuged a single time at d 1.006, d 1.019, d 1.063, and d 1.21 g/ml with appropriate overlay. Each aliquot was spun in a 50.3 rotor for 48 hr at 40,000 rpm in a Beckman L8-55 ultracentrifuge. The top 1.5 ml of each tube was collected by tube-slicing and dialyzed. An aliquot of each was then frozen at −70°C for subsequent apoprotein analysis. Lipid content of each lipoprotein fraction was then determined by difference. For example, the composition of LDL was determined as the difference between values obtained in the d 1.019 g/ml top and the d 1.063 g/ml top. The overall recovery of mass for all lipoprotein classes was 96.7% ± 2.9% (n = 20) for cholesterol, 90.2% ± 3.7% (n = 10) for phospholipid, and 95.7% ± 8.0% (n = 17) for triglycerides. Total and esterified cholesterol were determined enzymatically (Boehringer Mannheim Kits #124087), as were triglycerides (Boehringer Kit #126012). Phospholipids were determined by a modification of the Bartlett procedure (18). ApoB and apoE content were determined in single assays by radioimmunoassay in the laboratory of Dr. G. Schonfeld (19, 20). Preliminary studies established that there was no significant difference between the measured apoB content of fresh and frozen samples under the conditions and time intervals employed in this study. Each sample of plasma was also analyzed by analytical ultracentrifugation at the Donner Laboratory using standardized techniques (21). A difference plot of computer-corrected schlieren patterns was prepared from samples obtained during the control period and during the 5–7th day of colestipol therapy, and during control period versus the plasma sample obtained after 2–3 months on therapy. In some subjects LDL apoprotein composition was studied by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis in gradients of 4–20% (22). Lipoprotein electrophoresis was performed in 1% agarose gels using the Corning Electrophoresis System. To determine the size of LDL particles, LDL was isolated by standard techniques between densities d 1.019 g/ml and d 1.063 g/ml with a wash spin at upper and lower densities. The size distribution of LDL particles was then determined by measuring 200 free-standing particles on electron micrographs of negatively stained preparations as described (23). These were then plotted as a frequency distribution of particle sizes.

LDL turnover studies and analysis

For the turnover studies, LDL was isolated under sterile conditions by sequential ultracentrifugation exactly as previously described (14). LDL was isolated between density of 1.025 g/ml and 1.063 g/ml. Plasma was adjusted to 1.025 g/ml with NaCl-NaBr and spun at 59,000 rpm at 10°C for 18 hr in a 60-Ti rotor. The infranate was collected and adjusted to d 1.070 g/ml and again ultracentrifuged at 59,000 rpm for 20 hr in a 60-Ti rotor. The concentrated supernatant was isolated, adjusted to d 1.063 g/ml, and a final spin was performed at 39,000 rpm for 24 hr at 10°C in a 50.3-Ti rotor. The concentrated LDL was then dialyzed against 0.15 M NaCl with 0.01% ethylene diamine tetraacetic acid (EDTA), pH 7.4 (EDTA-saline) and stored in a sterile, pyrogen-free container at 4°C. LDL was iodinated by the iodine monochloride method as modified by Bilheimer et al. (24) and the molar ratio of iodine to protein was less than 1. Free radioiodide was removed by extensive dialysis for 36–48 hr against EDTA-saline. For both control and Rx-LDL, free iodide averaged less than 0.2% and the degree of lipid-labeling averaged less than 3%. Prior to injection, each LDL preparation was centrifuged at 20,000 *g* for 30 min, diluted with 5% sterile human serum albumin, and double-filtered through a 0.22-μm Millipore filter. Aliquots were set aside for culture and pyrogen testing.

As indicated in the protocol, the ^{125}I-labeled Rx-LDL and the ^{131}I-labeled Con-LDL (1–4 mg of LDL protein, total dose of 50–100 μCi) were simultaneously injected into an antecubital vein, and blood was serially collected from the opposite arm at 10, 30, 45, 60, and 90 min, and at 3, 6, 9, 12, 18, 24, 36, and 48 hr, and daily (fasting) for 14 days. Determinations of plasma radioactivity were made in a double-channel gamma spectrometer. Except in studies on subjects 11 (study 1) and 15, urine specimens were collected in bottles containing an alkaline preservative and were pooled during the following hourly time intervals after injection of labels: 0-time to 1, 1–3, 3–6, 6–12, 12–24, and for every 24-hr period thereafter. Values for urinary radioactivity were normalized to a constant creatinine excretion.

In order to document a steady state LDL composition during the turnover period, the LDL cholesterol to protein ratio was determined three times per week from LDL isolated by sequential ultracentrifugation of a 4-ml plasma sample. LDL protein was determined by the method of Lowry et al. (25) and LDL cholesterol content was determined enzymatically. In each subject studied the absolute LDL-cholesterol level remained within 8% of the average LDL-cholesterol level determined during the period of the LDL turnover.

To determine whether the difference in clearance of Con-LDL and Rx-LDL was due to different rates of clearance via the LDL-receptor, we injected the pair of Con-LDL and Rx-LDL preparations from subject 13 (study 2) into three cholestyramine-treated guinea pigs, as previously reported (8). Both Con-LDL and Rx-LDL preparations were glucosylated, a procedure that we have previously shown abolishes the ability of LDL to interact with the LDL-receptor (15, 16). Then the rates of clearance of glucosylated Con-LDL and glucosylated Rx-LDL were simultaneously determined in the three cholestryamine-treated guinea pigs. The numerical result of this experiment was previously reported as experiment 2, and Table 5, in reference 8; in the present report we show examples of actual plasma decay curves from this experiment.

The apparent fractional catabolic rates for Rx-LDL and Con-LDL turnover were calculated from the plasma radioactivity decay curves as previously described (8, 14), using an interactive curve-peeling program developed by Dr. William F. Beltz and Dr. Thomas E. Carew based on methods originally described by Matthews (26) and later adapted to LDL turnover studies by Langer, Strober, and Levy (27). These calculations make the assumption that there is kinetic homogeneity of the radiolabeled LDL tracer.

RESULTS

Effects of colestipol therapy on LDL composition

In subjects 1–8, LDL was isolated during periods when the subjects were housed on a metabolic ward and were consuming a constant diet, so that observed changes in LDL composition could be reliably attributed to colestipol therapy (**Table 2**). The mean decrease in LDL-cholesterol was 22% (232 mg/dl to 182 mg/dl). Plasma triglycerides were not significantly different and HDL cholesterol did not change. Although we have previously noted relative TG enrichment of LDL isolated from some colestipol-treated subjects (11, 12), we did not observe this change in these subjects. Whereas total LDL-cholesterol fell by 22%, LDL-apoB levels fell only 16%. These changes resulted in a significant decrease in the LDL-chol/apoB ratio, in confirmation of previous reports (4–13). Of interest, although the absolute levels of apoE in LDL (determined by radioimmunoassay) were very low, there was a small but consistent increase in the apoE content within the LDL density range on therapy (8/8 subjects), so that the ratio of apoE to apoB in the LDL fraction increased in all eight subjects.

SDS-polyacrylamide gel electrophoresis of apoLDL isolated by ultracentrifugation during control and treatment periods did not reveal any consistent differences in apoprotein content. In particular, apoA-I was not noted in any of the LDL preparations examined, nor was apoE detected by this technique. Similarly, lipoprotein electrophoresis of the different LDL preparations did not reveal any differences.

Differences in lipoprotein flotation patterns during diet and colestipol treatment periods were studied by analytical ultracentrifugation. In all eight subjects, there was a specific

TABLE 2. Effect of bile sequestration on LDL composition

Fraction	Control	Colestipol-HCl		% Change (Chronic from Control)
		Acute	Chronic	
		mg/dl		
Phospholipid	146 ± 15	118 ± 14**	111 ± 14**	−24
Triglyceride	55 ± 13	47 ± 9	42 ± 11	−23
Free cholesterol	66 ± 7	48 ± 7**	50 ± 6**	−20
Cholesteryl ester	166 ± 16	128 ± 12**	133 ± 17**	−24
Total cholesterol	234 ± 22	176 ± 19**	182 ± 23**	−22
ApoB	136 ± 18	113 ± 11	115 ± 18	−16
ApoE	2.3 ± 0.3	2.7 ± 0.2	3.4 ± 0.5**	+44
Total cholesterol/apoB	1.86 ± 0.12	1.59 ± 0.15	1.58 ± 0.06*	−15
ApoE/apoB	0.019 ± 0.004	0.025 ± 0.004	0.029 ± 0.005**	+52

Values for lipid and apolipoproteins expressed as mg/dl (mean ± SEM) determined in subjects 1–8. Acute refers to values obtained after only 5–7 days of therapy; chronic refers to values obtained after 2–3 months of therapy. Different from control: $^{*}P < 0.05$; $^{**}P < 0.01$.

decrease in the mass of more buoyant, large LDL of $S_f°$ 6–7 while there was no decrease in mass of denser, small LDL particles in the $S_f°$ 2–4 range. **Fig. 3** displays a difference plot for the computer-corrected schlieren patterns of lipoprotein flotation during the control period and during the chronic treatment period for two subjects. An analysis of the difference plots comparing lipoprotein flotation patterns during control and acute and chronic colestipol treatment periods demonstrated that the changes in lipoprotein flotation were evident after only 5 days of treatment in almost all subjects. **Fig. 4** shows a computer-derived average difference plot for all six men studied (panel A) while panel B displays an averaged difference plot for the two women. Clearly there was a selective decrease of large LDL particles during colestipol treatment.

The finding of the different flotation pattern and the decreased LDL-cholesterol/apoB ratio during colestipol treatment suggested that LDL particles isolated from plasma during colestipol treatment would be smaller in size. In subjects 9 and 10, we examined electron micrographs of the LDL particles and determined the particle size distribution of LDL preparations isolated during diet and colestipol

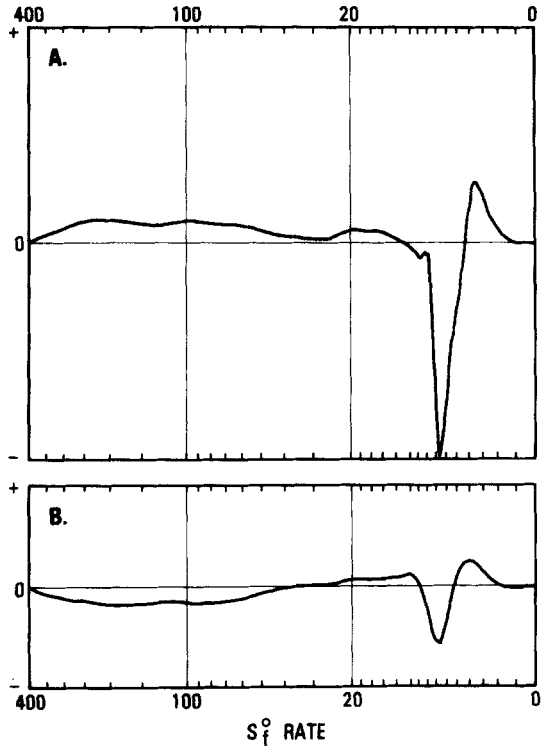

Fig. 3. Difference plots of the computer-corrected schlieren patterns of lipoprotein flotation from plasma samples obtained during the control period and after 2 months of colestipol treatment, for two subjects (panels A and B). Deviation below the horizontal line represents net loss of mass at a given density while deviation above the horizontal line represents a net increase in mass at a given density. In both subjects, A and B, there was a significant decrease in mass of the most buoyant LDL, $S_f°6–7$. Studies revealed that a similar pattern was established after only 5 days of colestipol treatment in most subjects.

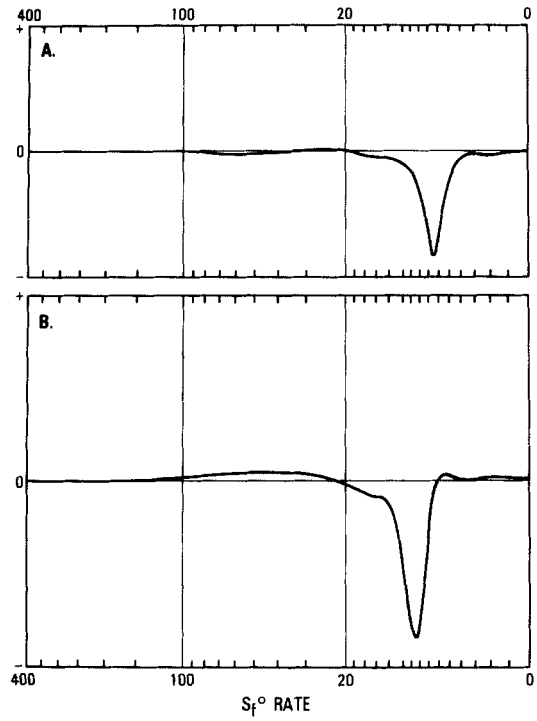

Fig. 4. Computer-derived difference plot for all six men studied (panel A, subjects 2–4, 6–8) and for the two women (panel B, subjects 1 and 5). The plots display the averaged difference of computer-corrected schlieren patterns of lipoprotein flotation during the control period and after 2 months of chronic colestipol treatment. Deviation above the horizontal axis indicates a net increase in mass at a given density while negative deviations indicate a specific decrease in mass at that density. The S_f 0–400 range was divided by computer techniques into seven fractions and the masses before and during therapy were compared. Only the S_f 6-7 fraction showed a significant decrease ($P < 0.01$).

treatment periods (**Fig. 5**). Attention is called to the selective decrease in larger LDL particles, and the relative enrichment of small particles during colestipol treatment. Subject 10 (Fig. 5B) had a rather dramatic colestipol-induced change in LDL particle size distribution. These data were similar to that observed previously in cholestyramine-treated guinea pigs (8).

Validation of experimental protocol

In a previous study, we demonstrated that when LDL isolated from cholestyramine-treated guinea pigs was injected into normal guinea pigs it had a slower FCR when compared to LDL isolated from control chow-fed animals. In order to determine whether a similar phenomenon occurred in man, we designed a protocol that allowed us to simultaneously compare the catabolism of two autologous LDL preparations, one isolated during colestipol treatment and the other isolated in the absence of drug treatment (Fig. 1). Since a week elapsed between sampling and isolation of the two autologous LDL fractions (Fig. 1), it was important to demonstrate that the protocol itself did not result in (artifactual) changes in the FCRs of the respective LDL tracers.

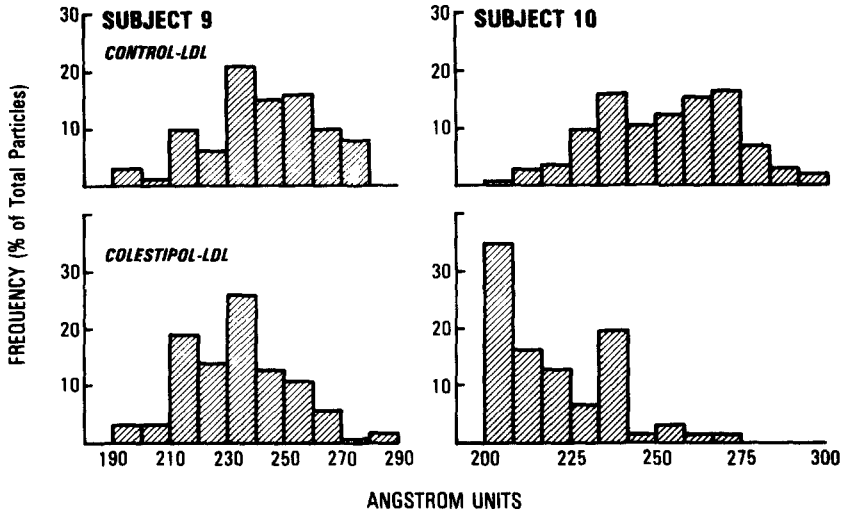

Fig. 5. Particle size distribution for Con-LDL and Rx-LDL in two subjects, determined by electron microscopy. The size distribution of particles within the LDL density range was determined by counting 200 free-standing particles on electron micrographs of negatively stained LDL preparations. Con-LDL was isolated in the absence of drug therapy; Rx-LDL was isolated after 1 month of colestipol treatment. For both subjects 9 and 10 there was a shift in LDL particle size distribution toward smaller particles during colestipol treatment. The differences in the distributions for subject 10, panel B, were highly significant by Chi-square analysis, $P < 0.001$. Differences in patient 9, panel A, were of borderline significance, $P \approx 0.10$.

To document that the FCR of LDL could be reproduced under the experimental conditions used, the protocol shown in Fig. 1 was carried out in three individuals (subjects 12, 17 and 19) except that no drug intervention occurred at any point. As shown in **Fig. 6**, in two of the subjects the plasma disappearance of the two LDL tracers was identical and in the third differed by only 10%. These experiments suggested that it was possible to obtain a reproducible FCR for LDL even after 7 days storage when no perturbation of the steady state occurred.

Colestipol-induced changes in metabolism of LDL

Six subjects participated in metabolic studies as outlined in protocol 1. During colestipol therapy there was a mean fall of LDL-cholesterol of 29%. **Fig. 7A** shows the simultaneously determined plasma radioactivity decay curves for the Rx-LDL tracer and the Con-LDL tracer in subject 11 (study 1). The FCR of the Rx-LDL was 0.341 pools/day, and the FCR of Con-LDL was 0.561 pools/day. As a further control study, a second turnover study was performed in this subject (study 2), except that he took cholestyramine throughout the study. Thus, study 2 compared the catabolism of two Rx-LDL preparations isolated 1 week apart. As shown in Fig. 7, the FCRs of both of these Rx-LDL tracers were similar to the FCR of the Rx-LDL in the earlier experiment (0.273–0.360) and all three were nearly 40% slower than the FCR for Con-LDL determined in study 1 (0.561 pools/day).

A large difference between the FCR of Con-LDL and

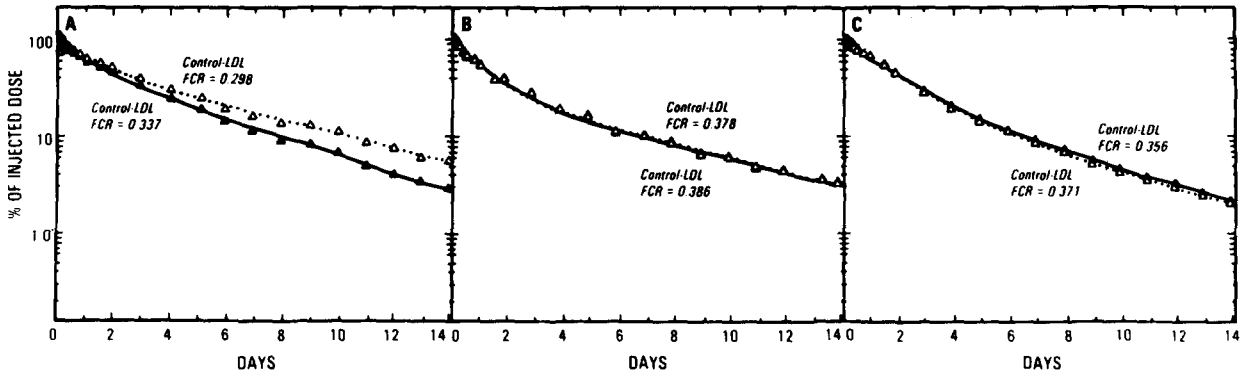

Fig. 6. Comparison of plasma disappearance curves for two Con-LDL preparations isolated 1 week apart and then injected simultaneously as explained in Methods. Studies in subjects 12 (study 1), 17, and 19 are shown in panels A, B, and C, respectively.

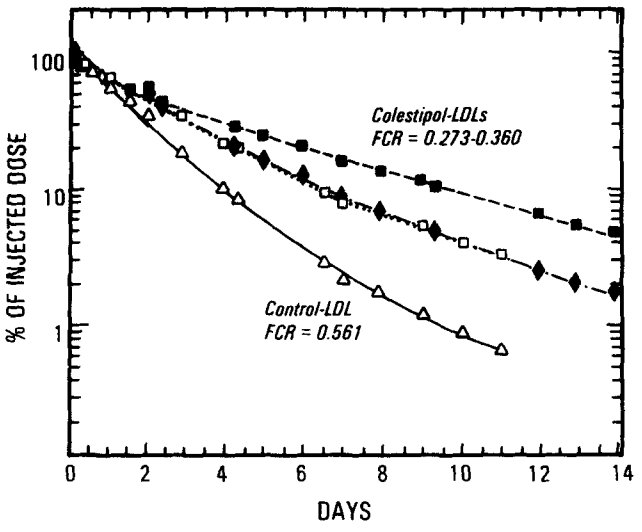

Fig. 7. Comparison of plasma clearance rate of Con-LDL and Rx-LDL in subject 11. In study 1, the plasma disappearance of Rx-LDL (·□·) and Con-LDL (-△-) were simultaneously determined while the subject was on colestipol therapy. The FCR of Rx-LDL (0.342 pools/day) was considerably slower than the FCR of Con-LDL (0.561 pools/day). In study 2 the catabolic rates for two Rx-LDL preparations which were isolated 1 week apart were simultaneously determined while the subject was on colestipol therapy. The FCR of the Rx-LDL preparation which was isolated first (·■·) was 0.273 pools/day, while the FCR of the Rx-LDL preparation isolated 1 week later (·♦·) was 0.360 pools/day. Note that the FCR of all three Rx-LDL tracers are much slower than the FCR of the Con-LDL.

Rx-LDL tracers was also seen when subject 12 underwent the experimental protocol shown in Fig. 1A. In this study, colestipol was administered during period C (study 2, Table 3). The plasma radioactivity decay curves for the two LDL tracers as well as the corresponding daily urine/plasma (U/P) ratios are shown in **Fig. 8**. Analysis of the plasma decay curves (Fig. 8A) yielded an FCR of Rx-LDL of 0.252 pools/day, while the clearance of Con-LDL was 0.403 pools/day. Estimates of the FCRs of the two LDL tracers determined from the daily U/P ratios were in close agreement to estimates determined from analysis of the plasma decay curves. However, precise determinations of FCR from the U/P ratios were not possible, as the ratios did not reach a plateau. Indeed, in all turnover studies similar U/P ratios were noted, i.e., the tracer with a faster FCR had a higher U/P ratio. In general, as shown in Fig. 8B, the U/P ratio plot for the Con-LDL tracer did not plateau and in fact was quite curvilinear, suggesting considerable metabolic heterogeneity. The U/P ratios for the Rx-LDL were in general much flatter, suggesting less heterogeneity.

Bile sequestrant therapy is known to induce hepatic LDL receptors in animals (4–8) and to increase LDL-receptor-mediated clearance of LDL in humans (9, 10). To determine whether the induction in LDL-receptor activity could increase the clearance of both Con-LDL and Rx-LDL, and to assess intrinsic differences in the metabolism of Con-LDL and Rx-LDL, several turnover studies were performed in subject 13. During the first study, the protocol in Fig. 1A was followed except that he was not treated with colestipol during period C, when the turnover was performed; in a repeat study performed several months later, the protocol shown in Fig. 1A was followed exactly (i.e., he resumed colestipol therapy during period C). Thus, in these studies, simultaneous determinations of the FCR of Con-LDL and Rx-LDL during a control period (**Fig. 9A**) and during colestipol treatment (Fig. 9B) were made. In both turnover studies, Rx-LDL was cleared more slowly than the respective Con-LDL. However, the FCRs of both Rx-LDL and Con-LDL were greater during colestipol treatment (Fig. 9B) than the FCRs of their respective tracers in the absence of drug therapy (Fig. 9A), i.e., the FCR of Rx-LDL during the control period was 0.313 pools/day, and increased to 0.423 pools/day when determined while the subject was on drug treatment. In a similar manner, the FCR of Con-LDL also increased in response to treatment (0.527 to 0.627). One further aspect of this pair of studies deserves attention. In this subject, colestipol treatment lowered the LDL-protein pool size

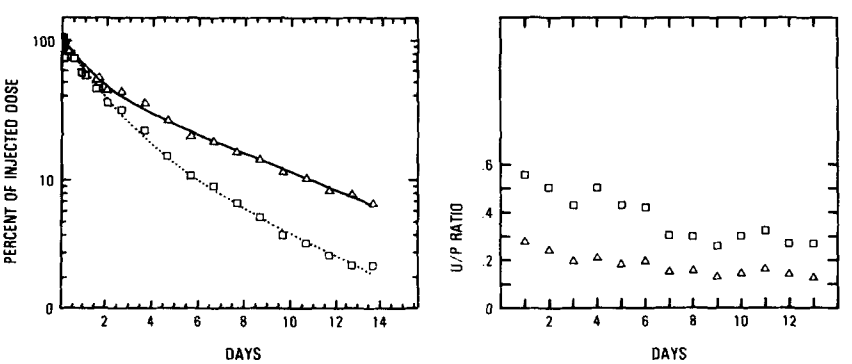

Fig. 8. *Comparison of the plasma clearance and U/P ratios of Rx-LDL and Con-LDL in subject 12 (study 2).* The protocol for this experiment is shown in Fig. 1A. Panel A demonstrates plasma radioactivity disappearance curves for Rx-LDL (-△-) and Con-LDL (·□·). Note the slower clearance of Rx-LDL (0.252 pools/day) compared to Con-LDL (FCR = 0.403 pools/day). Panel B demonstrates the daily U/P ratio for the same study. Note that the daily U/P ratio for Rx-LDL (△) is less than that for Con-LDL (□), reflecting a lower FCR.

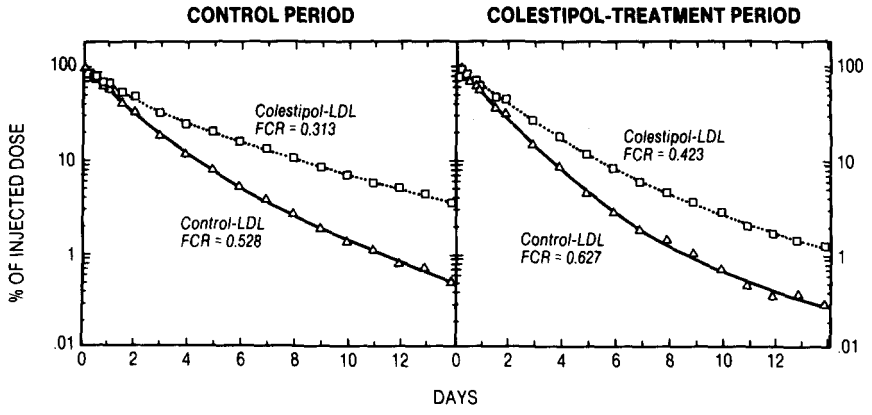

Fig. 9. Comparison of the plasma clearance of Rx-LDL and Con-LDL in subject 13 in the absence and presence of colestipol therapy. Panel A shows study 1 in which the clearance of Rx-LDL (--□--) and Con-LDL (-△) were compared while the subject was not on colestipol therapy during the turnover period (period C in Fig. 1A). The FCR of Rx-LDL was 0.313 pools/day, while the FCR of Con-LDL was substantially faster, 0.527 pools/day. The LDL cholesterol/protein ratio for the Con-LDL was 1.25 and was 0.90 for the Rx-LDL. Panel B shows study 2 in which the subject was on colestipol treatment during the turnover period. The FCR of Rx-LDL was 0.423 pools/day, while the FCR of Con-LDL was faster, 0.627 pools/day. In this study the LDL cholesterol/protein ratio for Con-LDL was 1.24 and was 0.99 for Rx-LDL.

25%. Yet the FCR of Con-LDL during the control period (0.527 pools/day) was actually higher than the FCR of Rx-LDL during the treatment period (0.423 pools/day). Using traditional reasoning, one would then be forced to conclude that steady state LDL levels were reduced because of a 40% decrease in LDL-apoB production.

A third study was performed in subject 13 to ascertain whether the order of treatment and control periods influenced the outcome. The Con-LDL preparation was isolated first, and the Rx-LDL preparation was isolated exactly 1 week later. It is important to note that, using this modified protocol, Rx-LDL was isolated after only 7 days of colestipol treatment, rather than after ~14 days in the standard protocol. However, the results of this study were similar in direction to the first two studies (Fig. 9A). Again, the FCR of the Rx-LDL (0.448 pools/day) was slower than that of the Con-LDL (0.551 pools/day).

In two other subjects studied (#15 and 16) we did not find that the Rx-LDL was cleared more slowly; in fact, it was cleared slightly faster than Con-LDL, despite a modest fall in LDL levels in response to therapy. The reasons why these subjects differed in their response are not known, but it is of interest that these two had the slowest clearance of Con-LDL (0.231 and 0.275 pools/day).

Mechanisms of enhanced clearance of Con-LDL

Because the clearance of the Con-LDL was greater than that of Rx-LDL we sought to test the possibility that the difference was due to an enhanced ability of Con-LDL to interact with the LDL-receptor. To test this possibility we injected the same pair of tracers used in study 2 of subject 13 into three cholestyramine-treated guinea pigs. (We have previously shown that cholestyramine treatment in guinea pigs enhances hepatic LDL receptor activity and LDL-receptor-mediated clearance of plasma LDL (8).) In the guinea pig, as in the patient, the FCR of Con-LDL was greater than that of Rx-LDL (0.189 ± 0.02 vs. 0.153 ± 0.02 pools/hr, $P < 0.03$) (**Fig. 10A**). Each label was then glucosylated, a procedure that we have previously shown abolishes the ability of LDL to interact with the LDL receptor (15, 16). Glucosylated Con-LDL and glucosylated Rx-LDL were then simultaneously injected into three other cholestyramine-treated guinea pigs. The clearance of both glucosylated tracers was now much slower and equal (FCR = 0.059 pools/hr) (Fig. 10B). A similar result was found when another pair of Con-LDL and Rx-LDL isolated from another patient was used (8). These data strongly suggest that the difference in clearance of Con-LDL and Rx-LDL is due to an enhanced ability of Con-LDL to interact with the LDL-receptor.

DISCUSSION

In this report, we document that colestipol therapy in humans results in significant decreases in the LDL cholesterol/protein ratio (Table 2), decreases in LDL particle size (Fig. 5), and a specific decrease in the subpopulation of larger, more buoyant LDL particles (Figs. 3 and 4). These changes in LDL are similar to those found in the guinea pig during bile sequestrant therapy (8).

In the guinea pig, the changes in LDL were accompanied by important changes in the intrinsic metabolic

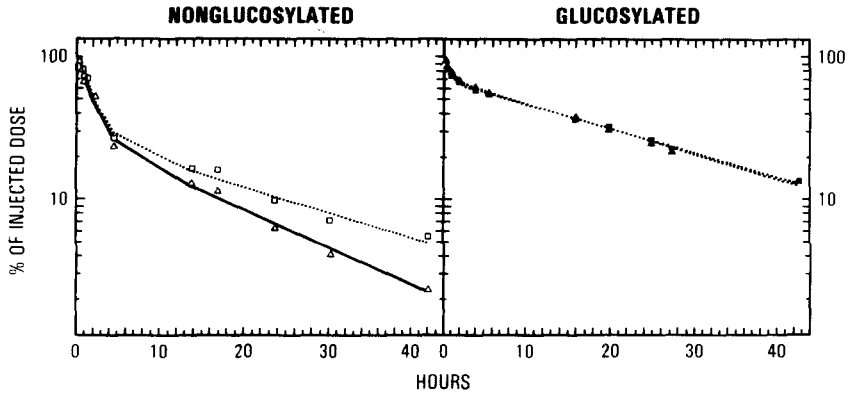

Fig. 10. Clearance of nonglucosylated and glucosylated Rx-LDL and Con-LDL tracers in cholestyramine-treated guinea pigs. Radiolabeled Rx-LDL and Con-LDL preparations from study 2 of subject 13 were injected into three cholestyramine-treated guinea pigs (8). In all three animals the FCR of Con-LDL was greater than that of Rx-LDL; a representative example in one guinea pig is shown in panel A. Each label was then glucosylated, and then injected into three other cholestyramine-treated guinea pigs. The clearance of the glucosylated Rx-LDL and the glucosylated Con-LDL were much slower and equal. Panel B shows a representative example in one cholestyramine-treated guinea pig.

properties of the LDL (8). To determine whether colestipol treatment in human subjects would also alter LDL in a similar manner, we simultaneously compared the catabolism of LDL isolated during colestipol treatment with that of LDL isolated during a control period. However, since the study utilized human subjects, the experimental protocol was designed using only autologous LDL samples for tracers (Fig. 1A). Since the 7-day interval between isolation of the Con-LDL and Rx-LDL preparations may not have been sufficient to allow a new steady state to be fully expressed, one must be cautious in making quantitative judgments based on these studies. Nevertheless, this experimental design did allow for a qualitative assessment of the hypothesis that the compositional changes in LDL produced by colestipol could alter the intrinsic metabolic properties of LDL. In 4/6 subjects tested, and in 6/8 studies the FCR of the Rx-LDL was slower than that of the Con-LDL (**Table 3**), consistent with our previous results in guinea pigs (8). Numerous control studies make it highly unlikely that the differences in clearance of Con-LDL and Rx-LDL are due to experimental artifact. However, in two human subjects, the FCR of the Rx-LDL was actually slightly faster than Con-LDL. The reason for this finding is unclear; however, it is noteworthy that in both of these subjects the FCR of even the Con-LDL was very slow, and we speculate that the 7-day interval between isolation of the Con-LDL and Rx-LDL preparations was not long enough to allow sufficient replacement of the Con-LDL population of particles with Rx-LDL particles. Another possibility is that because of the prolonged residence time (presumably due to lowered hepatic LDL receptor activity) there was significant remodeling of the tracers after their injection. Indeed, we recently showed the metabolic interconversion of LDL subfractions (28).

TABLE 3. Comparison of fractional catabolic rates for Rx-LDL and Con-LDL

Subject	Protocol for Turnover Study	FCR (pools/day)	
		Rx-LDL	Con-LDL
11, Study 1	Fig. 1A	0.342	0.561
12, Study 2	Fig. 1A	0.252	0.403
13, Study 1	Period C with no colestipol treatment	0.313	0.527
13, Study 2	Period C with colestipol treatment	0.423	0.627
13, Study 3	Period C with colestipol treatment, control-LDL isolated first	0.448	0.551
14, Study 1	Fig. 1A	0.480	0.560
15	Control-LDL isolated first	0.365	0.275
16	Fig. 1A	0.274	0.231

In each of the six subjects noted above a simultaneous comparison of the rates of clearance of a Con-LDL and an Rx-LDL tracer was performed using the protocol shown in Fig. 1A, except as noted above. These subjects also participated in numerous control studies as discussed in the Results.

These metabolic studies are unique because they used a protocol (Fig. 1A) designed to assess the metabolic importance of drug-induced LDL compositional changes. Previous investigations of the metabolic effects of bile sequestrants have used a traditional protocol (Fig. 1B), where autologous Con-LDL is used as a tracer in the absence of drug therapy and then, after a period of drug treatment, autologous Rx-LDL is isolated for use as a tracer during drug treatment. A major assumption is that Con-LDL and Rx-LDL tracers have equivalent intrinsic metabolic properties and thus any change in rate of clearance is ascribable to changes in the person's metabolic pathways. Although this assumption may be appropriate for some patients, as shown in this study, this is not the case for all subjects treated with a bile sequestrant. Perhaps because of the traditional protocol's inherent inability to assess simultaneously effects on cellular degradation pathways and metabolic changes related to intrinsic differences of the LDL particles used as tracer, kinetic studies on bile sequentrant therapy have shown inconsistent results. Levy and Langer (29) and Shepherd et al. (10), using the traditional protocol, showed that cholestyramine increased the FCR of LDL. However, Sigurdsson and Kjartansson (30), also using a similar traditional protocol, did not find an increase in the FCR of LDL during cholestyramine therapy and therefore concluded that cholestyramine must lower LDL levels by decreasing LDL synthesis. Huff and coworkers (31) also concluded that cholestyramine lowered LDL in swine by decreasing LDL production. A similar conclusion could be drawn from our studies in guinea pigs (8). Furthermore, in preliminary studies using the traditional protocol (Fig. 1B), we have observed an apparent heterogenous effect of bile sequestrant therapy on LDL clearance in humans (S. G. Young, A. Y. Kesaniemi, S. M. Grundy, J. L. Witztum, unpublished observations). We suggest that these differing results may be due to an inherent inability of the traditional protocol to assess both the effect of increased hepatic receptor activity and the altered metabolic properties of Rx-LDL. In subjects (or animals) not showing an apparent increase in LDL FCR, perhaps a major effect of therapy was the change in the intrinsic metabolic property of LDL, leading to slower catabolism of the Rx-LDL tracer, in spite of increased hepatic LDL-receptor activity. In other studies, such as that of Shepherd et al. (10), in which only a narrow cut of LDL (d 1.030–1.050 g/ml) was used as tracer, it is possible that the narrow LDL cut minimized the compositional and thus the intrinsic metabolic differences between Rx-LDL and Con-LDL, making the effect of increased hepatic degradation easier to detect. In addition, different study subjects with differing degrees of heterogeneity in their LDL populations could contribute to the diversity of results seen.

The first two studies in subject 13 (Fig. 9) clearly demonstrate the two effects of colestipol treatment. In study one (Fig. 9C), the effect of the drug on the intrinsic metabolic properties of LDL is demonstrated. In study 2 (Fig. 9B, when colestipol was administered during the turnover period, the clearance of both tracers was more rapid than the clearance of the respective tracers in study 1 (Fig. 9A) when no drug was given during the turnover period. This effect is consistent with induction of hepatic LDL-receptors during drug treatment.

In this pair of studies, another point deserves attention. If one used the traditional protocol (Fig. 1B) to calculate the effect of therapy on LDL turnover, one would compare the FCR of Con-LDL during the control period (0.527 pools/day) with the FCR of Rx-LDL during the treatment period (0.423 pools/day). In this subject, the LDL apoB pool size fell by 25% during drug treatment in spite of the slower FCR of Rx-LDL during drug treatment. Since cholestyramine clearly induced hapatic LDL-receptors (as noted above), this finding is apparently paradoxical. This same paradox was apparent from our studies in guinea pigs.

How can this paradox be explained? While our current data do not provide definitive answers, we believe that there are several possible explanations for the significant fall in LDL levels without an apparent increase in the FCR of Rx-LDL. If one assumes that both Rx-LDL and Con-LDL are kinetically homogeneous, it follows that LDL levels fell during treatment because of reduced LDL production rates. Indeed, when LDL synthetic rates are calculated during drug treatment in subject 13, they are reduced by nearly 40%. This explanation for lower LDL levels is quite plausible in light of the findings of Kita and coworkers (32) on LDL production rates in the WHHL rabbit. In this rabbit, which is deficient in the LDL receptor, there is a diminished clearance of VLDL and IDL, leading to increased LDL production. Perhaps, in the opposite situation, with enhanced hepatic LDL-receptor activity, as occurs with colestipol treatment, VLDL and IDL clearance by the LDL receptor is increased (i.e., an increase in the shunt pathway), leading to a lower LDL production rate and a fall in LDL levels, in spite of an intrinsically slower catabolism of Rx-LDL.

However, LDL is probably not kinetically homogeneous and a second possible explanation for decreased LDL levels is that LDL levels fall because of enhanced LDL degradation within a subfraction of LDL (28, 33). We suggest that larger, more buoyant LDL particles (which are more abundant in Con-LDL) are removed at a greater rate by receptor-mediated processes than are smaller, more dense LDL particles. In response to enhanced receptor activity there is preferential removal of larger, cholesterol-enriched subfractions of LDL, leaving behind an LDL that is now relatively enriched in smaller, more dense particles. Rapid catabolism of LDL within the subfraction of large particles could explain decreased plasma LDL levels, the decreased LDL-cholesterol/pro-

tein ratio and the selective depletion of large, more buoyant particles. According to this model, Rx-LDL has a slower clearance because its population of particles consists of smaller, more dense LDL particles that interact less well with the hepatic LDL-receptor. The experiments with glycosylation of the two fractions to inhibit their binding to LDL receptors (Fig. 10) strongly support this formulation.

We recently reported direct evidence in support of the hypothesis that large LDL are cleared more rapidly than smaller LDL. In five human subjects we demonstrated that larger, more buoyant LDL particles (d 1.025–1.040 g/ml) were removed from plasma more rapidly than were denser LDL subfractions (d 1.050–1.063 g/ml) (28). However, the situation is even more complex, for our studies also demonstrated that there was metabolic interconversion from one LDL subfraction to the other. Teng and colleagues (34) have also reported similar observations.

The two explanations for bile sequestrant action, decreased LDL production, and enhanced LDL removal from an LDL subfraction are not mutually exclusive. Indeed, as shown in **Fig. 11**, we believe that both mechanisms are probably operative (perhaps to differing degrees in different people). Furthermore, this model would predict that in any situation where hepatic LDL receptor activity was enhanced, there should be a preferential removal of larger, cholesterol-enriched LDL particles, leaving behind LDL characterized by a decreased cholesterol:protein ratio. In fact, this is what is found in a number of situations, for example, as occurs in response to thyroid hormone (35). In the opposite situation, as in response to weight loss in hypertriglyceridemic subjects, the LDL cholesterol:protein increases (36).

Decreased LDL production could be the consequence of still another mechanism. In response to bile sequestration there is an acute, increased input into plasma of large triglyceride-enriched VLDL (11, 12), and even at steady state, triglyceride turnover is increased (37, 38). Thus, in response to the increased hepatic cholesterol (39) and triglyceride synthesis produced by bile sequestration, it is likely that VLDL production and turnover are also increased. Several groups have shown that large VLDL enter and leave the plasma compartment without ever being converted to smaller VLDL, or LDL particles (40, 41). Such a mechanism might further contribute to an increased shunt pathway and decreased LDL production (Fig. 11).

The metabolic consequences of LDL heterogeneity may be even more complex than indicated above. In recent studies we found that the very dense LDL subfractions found in patients with marked hypertriglyceridemia have decreased binding to LDL receptors in cell culture, yet when injected in vivo (in guinea pigs or humans) have a much faster rate of plasma clearance than a more

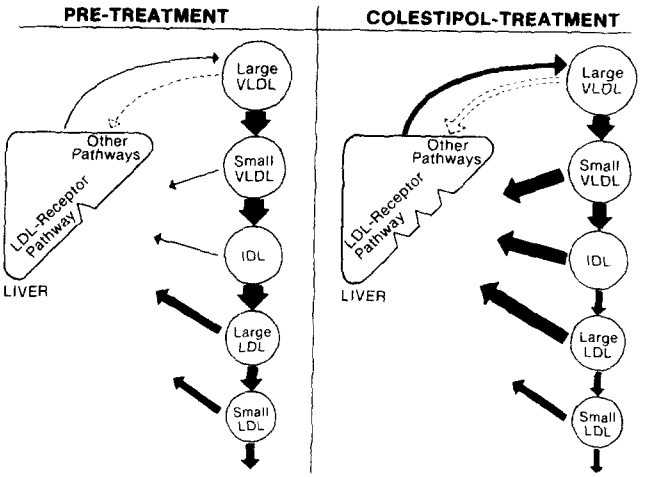

Fig. 11. Proposed model showing mechanisms whereby bile-acid sequestrant resin therapy affects lipoproteins containing apoB. This model assumes that therapy induces hepatic LDL-receptors, and in turn these receptors interact with apoB-containing lipoproteins at a number of different sites in the lipoprotein cascade. It also assumes that there is enhanced turnover of VLDL particles (see discussion for details).

buoyant LDL subfraction, implying enhanced clearance due to a nonLDL receptor mechanism. Vega and Grundy (42) have also reported similar in vivo observations. Thus it may be that there is a spectrum of LDL particles in which the most buoyant, larger LDL particles are cleared rapidly because of enhanced binding to LDL receptors, while smaller, more dense LDL particles (as preferentially accumulate in cholestyramine therapy) are cleared more slowly because of decreased LDL receptor binding. However, still smaller and even more dense LDL particles, found in severe hypertriglyceridemia, or possibly such states as hyperapobetalipoproteinemia (43, 44) may be cleared more rapidly but by a nonLDL receptor-dependent process. Of course, it should be noted that size alone may not be the only (or even most important) factor determining the difference in rates of clearance of such LDL subfractions. For example, the relative content of core lipid, principally triglyceride, appears to have an important impact on apoB conformation (45–47).

The representative U/P data shown in Fig. 7, as well as that previously reported by us (14, 28) and others (48, 49), is consistent with metabolic heterogeneity of LDL. This possibility was previously suggested by Berman (33), based on analysis of plasma decay and U/P data in human subjects, as well as by studies of Fisher (50), Krauss and Burke (51), and Austin and Krauss (52) based on physical heterogeneity. Because kinetic heterogeneity exists among particles within the LDL density range, calculation of synthetic rates from the traditional kinetic studies and models cannot be performed with full confidence, since kinetic homogeneity of LDL particles is the key assumption underlying such calculations. Future studies will have to determine when the traditional models

give acceptable approximations of turnover and when the kinetic heterogeneity is too great to permit meaningful calculations.

Our studies emphasize the fact that drug (or dietary) interventions may have an effect on LDL composition that independently may affect metabolic behavior of LDL. Recently, Grundy and Vega (53) also suggested that such drug-induced alterations in LDL composition may explain, in part, the failure to see an increased FCR of LDL in many patients treated with lovastatin. Thus, we suggest that future kinetic studies designed to investigate effects of a given perturbation on lipoprotein metabolism must evaluate the effect of the perturbation on the intrinsic kinetic properties of the tracer particles, as well as any effect on the host.

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