Guinea pig low density lipoproteins: structural and metabolic heterogeneity

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Abstract The structural and metabolic heterogeneity of low density lipoproteins (LDL, d 1.024–1.100 g/ml) has been investigated in the guinea pig. Two LDL subfractions, of d 1.024–1.050 and 1.050–1.100 g/ml, respectively, were isolated by sequential ultracentrifugation; while both were enriched in cholesteryl ester and apoB-100, the former was heterogeneous displaying three particle size species of diameters 26.9, 25.6, and 24.7 nm, whereas the denser subfraction was relatively homogeneous containing a single, smaller species (diam. 23.6 nm). The fractional catabolic rates (FCR) of the two LDL subfractions were alike (~0.090 pools/hr) in the guinea pig in vivo. After modification of each subfraction by reductive methylation, the FCRs were reduced similarly and indicated that 70–80% of degradation occurred via the cellular LDL receptor pathway. However, the intravascular metabolism of these LDL subfractions, determined from the radioactive content of density gradient fractions as a function of time after injection of radiolabeled native or chemically modified LDL, tended to be distinct. Thus, while radiolabeled apoB-100 in the lighter subfraction maintained the initial density profile up to 48 hr, the radioactive profile of its methylated counterpart changed, the proportion of radioactivity in the lighter gradient fractions (d 1.027–1.032 g/ml) increasing while that in the denser (d 1.037–1.042 g/ml) fractions diminished. A more marked transformation occurred in LDL of d 1.050–1.100 g/ml, in which the radioactive profile shifted towards lighter particles of the d 1.024–1.050 g/ml species; this shift was partially dependent on the LDL receptor, since it was more pronounced in the methylated subfraction. Furthermore, a net increase in the radioactive content of gradient subfractions 7 to 9 (d 1.032–1.042 g/ml) was found 10 hr after injection of methylated LDL of d 1.050–1.100 g/ml, at which time the bulk of LDL radioactivity had been removed from plasma. Several mechanisms, acting alone or in combination, may account for these findings; among them, some degree of transformation of dense to lighter LDL species appears a prerequisite. In conclusion, our data attest to the structural heterogeneity of circulating LDL in the guinea pig, and suggest that the intravascular processing and metabolism of LDL particle subspecies is directly related to their structure and physicochemical properties.—**Luc, G., and M. J. Chapman.** Guinea pig low density lipoproteins: structural and metabolic heterogeneity. *J. Lipid Res.* 1988. **29:** 1251–1263.

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Low density lipoproteins (LDL) play a fundamental role in the transport of plasma cholesterol in both humans (1) and guinea pigs (2). In normolipidemic individuals, the bulk of circulating LDL appears to represent the catabolic product of very low density lipoproteins (3). These quasi-spherical, plurimolecular complexes are catabolized, in part, by a high affinity specific receptor (4), the LDL apoB,E receptor, and in part by receptor-independent pathways (5).

Low density lipoproteins are typically isolated from human plasma by ultracentrifugal flotation over a wide density range (i.e., 1.019–1.063 g/ml). Heterogeneity has been described in several of the physicochemical characteristics of LDL, which include hydrodynamic properties, chemical composition, molecular weight, and Stokes diameter in both normolipidemic subjects (6–8) and in certain hyperlipoproteinemic states. Indeed, the latter include both homozygous and heterozygous forms of familial hypercholesterolemia (9–12), as well as hypertriglyceridemia and dysbetalipoproteinemia (13–15). More specifically, in normolipidemic subjects Stokes diameter and molecular weight diminished, the proportion of protein increased, and that of cholesteryl ester tended to decrease as the density of the lipoprotein particles became progressively higher in value (6–8).

Considerable evidence is now available to substantiate the kinetic heterogeneity of low density lipoproteins in humans (16–20) and in certain animal species, such as the cynomolgus monkey (21) and rabbit (22). Indeed, these data establish the complexity of LDL metabolism in vivo and reveal potential relationships to the structural heterogeneity of LDL and its subspecies.

The relationship between the defined physicochemical properties of a given molecular species of LDL particle and the intravascular catabolism of this particle in an in vivo model remains largely indeterminate. Teng and colleagues (18) studied the intravascular metabolism of two LDL sub-

Abbreviations: LDL, low density lipoproteins; d, density; EDTA, ethylenediaminetetraacetic acid; EC, ester cholesterol; SDS, sodium dodecyl sulfate; apoB, apolipoprotein B; M_r, molecular weight; FCR, fractional catabolic rate.

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fractions in six normolipidemic males; they observed that the LDL fraction of lower density (light LDL) was not only catabolized faster than that of greater density (heavy LDL), but also that the two fractions were related by a precursor–product relationship. However, neither the physicochemical properties of the injected fractions, nor the proportion of each fraction catabolized by the receptor pathway, were defined (18). More recently, in a heterologous system, radiolabeled light or heavy human LDL were injected into normal rabbits (22). While the fractional catabolic rates of both fractions did not differ significantly, a greater proportion of light LDL was catabolized by the receptor-dependent pathway (22).

Consequently, the catabolism of LDL particles that differ in their physicochemical properties may be distinct. We have examined this question in an animal model, the guinea pig (*Cavia porcellus*). This species exhibits an unusual feature by comparison with other mammals, displaying relatively high levels of LDL and only minor amounts of high density lipoproteins (2). Furthermore, human LDL and guinea pig LDL have similar fractional catabolic rates when injected into guinea pigs (23). However, low density lipoproteins in the guinea pig are distributed up to densities approaching 1.100 g/ml, with average hydrated densities in the range of 1.050 to 1.060 g/ml (2), values that are clearly higher than those typical of normolipidemic man (6). In this report we describe the physicochemical characteristics of two subfractions of guinea pig LDL, of d 1.024–1.050 and 1.050–1.100 g/ml, respectively, and show that, despite similar rates of disappearance from plasma, their intravascular metabolism is quite distinct.

MATERIALS AND METHODS

Animals and diet

Nonconsanguinous male guinea pigs (a total of 36 animals) of the Hartley strain (HA) BR, weighing 0.5 to 0.8 kg, and 55 to 80 days of age, were supplied by Charles River France (St. Aubin-les-Elbeuf, France). Animals were maintained on a diet containing 4% (w/w) fat, 18.5% (w/w) protein, 14.5% fiber, 43% carbohydrate, 8% minerals, and 12% moisture and supplemented with vitamins A, C, and D_3. Water was given ad libitum and, for metabolic studies, potassium iodide was added to drinking water (150 mg/l) 2 days before the injection of radiolabeled lipoproteins; such treatment was maintained until the end of the study in order to block the uptake of radioiodine by the thyroid. Animals used in turnover studies (see Metabolic studies below) were used in a single experiment only.

Isolation of lipoproteins

Blood was removed by cardiac puncture from overnight-fasted animals under light diethyl ether anaesthesia and collected on EDTA (1 mg/ml). Blood samples from 10 to 20 animals were pooled. Plasma was immediately separated by low speed centrifugation (1000 *g*, 20 min, 4°C) and subfractions of LDL were then isolated by flotational ultracentrifugation (24). For this purpose, the density of plasma was increased to 1.024 g/ml with solid KBr (25); such plasma was then ultracentrifuged in polycarbonate tubes (Beckman, tube no. 336091, volume 22 ml) in a Type 60 Ti rotor in the Beckman L8-55 ultracentrifuge at 45,000 rpm for 20 hr at 10°C. The supernatant (top 5 ml, d $<$ 1.024 g/ml) was discarded; the infranatant was pooled and its solvent density was raised to 1.050 g/ml with solid KBr. Low density lipoproteins of density 1.024–1.050 g/ml were then separated under the same ultracentrifugal conditions as those described above. The denser lipoproteins of density 1.050–1.100 g/ml were subsequently separated by a similar ultracentrifugal procedure from the d 1.050 g/ml infranatant after raising its solvent density to 1.100 g/ml with KBr. The LDL subfractions of d 1.024–1.050 g/ml and 1.050–1.100 g/ml were simultaneously washed and concentrated at their respective upper limiting density in a Beckman Type 50 Ti rotor (tube no. 336090, vol. 8 ml) at 45,000 rpm for 20 hr at 10°C. Each LDL subfraction was dialyzed at 4°C against buffer A (0.15 M NaCl, 0.01% EDTA (w/w), pH 7.4).

The electrophoretic mobility and possible presence of additional lipoproteins (of α- or pre-β mobility) were evaluated for each LDL density subfraction by electrophoresis on agarose gels (Corning, Palo Alto, CA), performed according to the method of Noble (26); gradient gel electrophoresis (see below) was used to measure the Stokes diameters of particles in each subfraction, thereby facilitating evaluation of the possible contamination of LDL by particles with sizes characteristic of VLDL and of HDL, respectively.

Characterization of lipoproteins

Chemical analysis. The chemical composition of each LDL subfraction was determined by the following procedures: protein by the method of Lowry et al. (27) using bovine serum albumin as the working standard; phospholipid, by use of the Phospholipids B-Test Wako (Bio-Lyon, Dardilly, France); triglycerides with the Triglycerides enzymatiques PAP 150 (BioMerieux, Charbonnières-les-Bains, France); and total and free cholesterol with the enzymatic kit of Hoffman-La Roche (Basel, Switzerland) (10). Ester cholesterol (EC) was calculated as the difference between total and free cholesterol; cholesteryl ester was then calculated as EC $\times$ 1.67 (10).

Analysis of particle size and heterogeneity. The heterogeneity of particle species and their Stokes diameters were estimated in each LDL subfraction by two procedures. One was lipoprotein electrophoresis on polyacrylamide gradient gels made up to contain a 2 to 16% gradient (PAA 2/16; Pharmacia Fine Chemicals, Uppsala, Sweden) as described by Krauss and Burke (28); 20 μg protein of each subfraction was applied to each well. After electrophoresis, the gels were

fixed in 10% sulfosalicylic acid for 30 min, and then stained in a solution containing 25% methanol, 10% acetic acid, and 0.1% Coomassie blue R 250; destaining was with a solution containing 25% methanol and 10% acetic acid. The diameter of particles in each band was determined from a calibration curve using standards of known diameter; these standards included latex beads of 38.0 nm, thyroglobulin (17.0 nm), and apoferritin (12.2 nm) (Pharmacia Fine Chemicals). A representative correlation coefficient for such a curve was −0.9996. The second procedure to determine particle size was negative stain electron microscopy. Preparations of each LDL subfraction containing about 0.5 mg of protein/ml were negatively stained with 2% (w/v) phosphotungstate at pH 7.4 (29) as previously described (30). Grids were examined with a Philips EM 300 electron microscope.

Electrophoretic analysis of apolipoproteins. The apolipoprotein content of each LDL subfraction was determined by electrophoresis in SDS-polyacrylamide slab gels containing a linear gradient of 3 to 15% (31), using the buffer system of Stephens (32). Lipoprotein subfractions were first dialyzed against 10 mM NH_4HCO_3, then lyophilized, delipidated with ethanol–diethyl ether (peroxide-free) 3:1 (v/v) (33), and subsequently solubilized by incubation for 2 hr at 37°C in 40–60 μl of sample buffer containing 0.0025 M Tris-glycine, 1% (w/v) SDS, and 1% (v/v) β-mercaptoethanol. Forty to 60 μg of protein of each subfraction was applied to individual wells. The molecular weight of each apolipoprotein band was calculated from a calibration curve established with the use of standards of known molecular weight (M_r 56,000–280,000) provided by BDH Biochemicals Ltd. (Poole, UK). A representative correlation coefficient for such a curve was −0.9910. The molecular weight of guinea pig apoB was compared to that of human apoB-100 by electrophoresing samples of each (human apoB-100 as apoLDL) in the same gel.

Guinea pig apoB-100 was purified as previously described (34). Antiserum against guinea pig apoB-100 was then used to detect apoB-100 by immunoblotting. Electrophoresis of apoLDL in SDS gels was first carried out as described above; the protein was then transferred onto a nitrocellulose sheet as described by Towbin, Staehelin, and Gordon (35), with the exception that Tris-glycine buffer lacking methanol was used. The revelation of apoB was carried out as described earlier (10), with the exception that an antiserum to guinea pig apoB was applied.

Metabolic studies

In vivo experimentation. Each LDL subfraction was iodinated with I^{125} or I^{131} (Amersham International PLC, Amersham, UK) by the procedure of McFarlane (36) as modified by Bilheimer, Eisenberg, and Levy (37). Unbound iodine was removed from labeled LDL by chromatography on a PD 10 column (Pharmacia Fine Chemicals) equilibrated with buffer A, and then by dialysis against the same buffer. After dialysis, radioiodinated LDL contained less than 1% unbound iodine as determined by precipitation with 10% (w/v) trichloroacetic acid (final concentration). As determined by extraction with chloroform–methanol 2:1 (v/v), less than 8% of the radiolabel in LDL was in the lipid moiety. By adjusting the quantity of ICl used during radioiodination, no more than 1 mole of iodine/mole of LDL protein (M_r ~500,000) was incorporated. Electrophoresis of the apolipoproteins of radiolabeled LDL in the presence of SDS in 3% polyacrylamide gels, following by quantitation of radioactivity in the stained bands, showed that ~80% (range 76–88%) of radioiodine was incorporated into apolipoprotein B. Modification of I^{131}-labeled LDL by reductive methylation was carried out as described by Weisgraber, Innerarity, and Mahley (38).

The lack of binding of radiolabeled methylated-LDL to the LDL receptor was verified in the porcine adrenal cortex membrane system (39), in which methylated guinea pig LDL could not displace I^{125}-labeled human LDL from the porcine LDL receptor (results not shown).

Animals lightly anesthetized with diethyl ether were injected by cardiac puncture with the various homologous I^{125}- and I^{131}-labeled LDL subfractions at maximal doses of about 500 μg of lipoprotein protein. In certain experiments, only one I^{125}-labeled, native LDL subfraction (of either d 1.024–1.050 g/ml of d 1.050–1.100 g/ml) was injected. In others, we injected two preparations simultaneously; these were either native I^{125}-labeled LDL of d 1.024–1.050 g/ml and its methylated counterpart labeled with I^{131}, or the denser native LDL subfraction (d 1.050–1.100 g/ml) and its methylated counterpart labeled with I^{125} and I^{131}, respectively. Immediately before injection, the respective radiolabeled LDL subfractions were filtered through a 0.45-μm Millipore filter (Millipore, Bedford, MA). Blood samples (0.5–1.8 ml) were withdrawn from the heart at 2 min, and then at ca. seven intervals, each of 10–14 hr, up to 96 hr after injection; blood was taken into tubes containing 2 mg EDTA. In order to permit density gradient studies of the distribution of labeled LDL from the time of injection up to 48 hr, the size of blood samples was progressively increased from 0.5 ml at 0 time to 1.8 at 48 hr; samples of 0.5–0.8 ml of blood were taken at time points from 48 to 96 hr. Samples of plasma were taken either *i)* for direct determination of radioactivity in a Gamma counter (Packard 5650; Packard Instrument Company Inc., Downers Grove, IL) or *ii)* for determination of radioactivity in plasma apoB following initial ultracentrifugal isolation of a d < 1.150 g/ml fraction and selective precipitation and delipidation of apoB by the procedures described below. Counts were corrected both for spillover and for radioactive isotope decay. Results were expressed as a percentage of the value obtained 2 min after injection; fractional catabolic rates (FCR) were estimated as described by Langer, Strober, and Levy (40),

whose analysis was derived from that of Matthews (41).

In order to evaluate any modification in the density distribution of each radiolabeled subfraction as a function of time after injection, plasma samples were layered onto a density gradient; this gradient was slightly modified from that outlined earlier (30). In brief, 2.5 ml of d 1.0240 g/ml NaCl-KBr solution was first placed in the bottom of an Ultraclear tube of the Beckman SW 41 rotor, and successively overlaid with 3 ml of guinea pig plasma adjusted to a density of 1.210 g/ml with solid KBr, 2 ml of d 1.063 g/ml, 2.5 ml of d 1.019 g/ml, and 2.5 ml of d 1.006 g/ml salt solution, respectively. The rotor was centrifuged at 40,000 rpm for 48 hr at 15°C and slowed without the brake. This gradient was then fractionated by the same procedure as described elsewhere (10). The curve of density as a function of volume was determined from gradients containing only NaCl-KBr solutions as before (30), using an NaCl-KBr solution of d 1.210 g/ml instead of plasma at the same density.

As the apolipoprotein B content of guinea pig LDL represents only about 80% of the protein moiety (42), the radioactivity contained in apoB in each gradient fraction was determined after selective precipitation of apoB by 2-propanol (43) and delipidation with ethanol-diethyl ether 3:1 (v/v) (33). Before the addition of 2-propanol, about 100 μg of human LDL protein (d 1.019-1.063 g/ml) was added to each gradient as carrier. The radioactivity per unit volume of plasma was calculated and plotted against the time after the injection of each lipoprotein subfraction. The absolute radioactivity in a given gradient fraction at each time point was calculated by multiplication of the radioactivity contained in 1 ml plasma at each time by the percentage of the total gradient radioactivity present in this same fraction.

Statistical methods

The Student's *t*-test (44) was employed to evaluate the significance of differences between the various metabolic parameters obtained for the respective LDL fractions.

RESULTS

Physicochemical characteristics of guinea pig LDL subfractions

The chemical composition of each LDL subfraction is shown in **Table 1**. The denser LDL subfraction, of d 1.050-1.100 g/ml, was distinguished from its lighter counterpart, i.e., the LDL subfraction of d 1.024-1.050 g/ml, by a greater proportion of protein (34% as compared to 25%). In addition, the denser fraction tended to exhibit a higher proportion of triglyceride and lower proportions of both free cholesterol and phospholipid. As might be expected from its greater density, the d 1.050-1.100 g/ml subfraction contained a higher proportion of surface constituents (protein, free cholesterol, and phospholipid, 54.6%) relative to the higher subfraction (49.7%).

On a quantitative basis, the lighter LDL subfraction typically predominated in our animals, representing 69% of total lipoprotein mass of d 1.024-1.100 g/ml. In absolute terms, the total plasma concentration of this latter subfraction was typically in the range of 32-38 mg/100 ml.

Electrophoresis on agarose gels showed that each LDL subfraction migrated as a single band with β-mobility. Furthermore, when the same amounts of lipoprotein protein of each subfraction were applied, their mobilities were indistinguishable, suggesting their net electrical charge at pH 8.6 was similar.

Particle size and heterogeneity in each of the two LDL subfractions were evaluated by gradient gel electrophoresis (**Fig. 1**) and by negative stain electron microscopy (**Fig. 2**).

By electrophoresis in 2-16% gradient gels, one or more distinct bands were detected in each LDL subfraction whose size decreased with increase in density (Fig. 1). In the LDL subfraction of lighter density, (d 1.024-1.050 g/ml), one band predominated with a mean Stokes diameter of 26.9 ± 0.5 nm. Similarly, in the denser subfraction of d 1.050-1.100 g/ml, a single major band with a diameter of 23.6 ± 0.2 nm was typically found. In contrast to the d 1.050-1.100 g/ml subfraction, in which a single size com-

TABLE 1. Comparison of the chemical composition of two subfractions of low density lipoproteins from guinea pig plasma

Density subfraction[b]	Component[a]				
	Protein	Phospholipid	Free Cholesterol	Cholesteryl Ester	Triglyceride
g/ml			*% by weight*		
1.024-1.050	25.1 ± 0.2	17.7 ± 0.2	6.9 ± 0.5	44.7 ± 1.4	5.6 ± 1.9
1.050-1.100	33.8 ± 0.5	15.3 ± 1.4	5.5 ± 1.0	37.9 ± 0.4	7.5 ± 2.8

[a]Values are the means ± SD of duplicate determinations on each of three preparations of each subfraction.
[b]LDL subfractions were isolated by sequential ultracentrifugal flotation (see Methods).

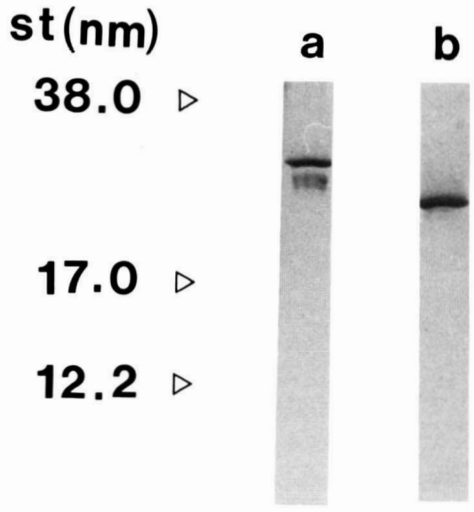

Fig. 1. Gradient gel electrophoresis of guinea pig LDL subfractions. An aliquot (10 μg protein) of each LDL subfraction was applied to the 2–16% PAA (Pharmacia Fine Chemicals) gel; gels were electrophoresed and stained with Coomassie R250. Standards (St) for size calibration were: latex beads, 38.0 nm; thyroglobulin, 17.0 nm; and apoferritin, 12.2 nm. LDL subfractions are: (a), LDL of d 1.024–1.050 g/ml and (b), LDL of d 1.050–1.100 g/ml.

ponent was most frequently found, the lighter subfraction (d 1.024–1.050 g/ml) presented up to two additional minor particle size species, of which the most readily visible displayed Stokes diameters of 25.6 and 24.7 nm, respectively. Clearly then, a greater degree of particle size heterogeneity was seen consistently in the lighter LDL subfraction; in addition, absolute estimation of diameters of corresponding particle species in different preparations of each subfraction showed values to be reproducible to within a range of ± 0.5 nm.

Electron microscopic studies showed that both guinea pig LDL subfractions (of d 1.024–1.050 and 1.050–1.100 g/ml, respectively) displayed similar morphology (Fig. 2), appearing as essentially spherical particles that deformed slightly in packed fields to give polygonal forms. In a representative preparation, the lighter subfraction (d 1.024–1.050 g/ml) contained particles with mean diameter of 23.5 nm, a modal diameter of 24.9 nm, and a range of 21.0–28.0 nm. By contrast, the denser subfraction contained predominantly smaller particles, with a mean diameter of 18.5 nm, a modal diameter of 20.0 nm, and an overall range of 16.0–25.0 nm.

Apolipoprotein content of LDL subfractions

Electrophoresis of the protein moieties of LDL subfractions on SDS-polyacrylamide gradient slab gels (3–15%) revealed the predominance in each subfraction of two high molecular weight components (**Fig. 3**); of these, the major band migrated identically to human apoB-100, the latter presenting an M_r of ~500,000 in this system. Upon electrophoretic transfer to a nitrocellulose sheet, followed by immunoblotting with our sheep antiserum to guinea pig apoB, both forms were identified as apolipoprotein B; application of the centile nomenclature of Kane, Hardman, and Paulus (45) showed that these forms corresponded to B-100 and B-95, respectively. Human apoB-100 also reacted positively with our antiserum (data not shown), confirming the immunological cross-reactivity originally observed between guinea pig and human LDL when reacted with an antiserum to human LDL (2).

Minor apolipoprotein components with molecular weights of ca. 67,000, 48,000, 16,500, and 14,000 were also detected in each subfraction. These data are largely confirmatory of those obtained earlier in our own and other laboratories (34, 42, 46).

Metabolic studies of LDL subfractions

The in vivo disappearance of I^{125}-labeled LDL from the plasma of a guinea pig in a representative experiment is shown in **Fig. 4**. This profile shows the decay of radioactivity in whole plasma (solid lines) and in plasma apoB after precipitation and extraction (dotted lines) following injection of the homologous native LDL subfraction and of the corresponding subfraction modified by reductive methylation as a function of time. Comparison of the decay curve of radioactivity in whole plasma with that obtained from the corresponding precipitated apoB showed them to be typically superimposable in the case of the native LDL (Fig. 4). In the case of the methylated fraction, the decay curve indicated a slightly slower removal of radioactivity in apoB than in whole plasma, the difference representing less than 5% of plasma radioactivity at each point over the entire time course. **Table 2** shows data on the fractional catabolic rates of the various native and reductively methylated LDL subfractions determined from measurements of plasma radioactivity; values for fractional catabolic rates derived from corresponding sets of data (i.e., plasma and apoB radioactivity) were alike. No significant differences were detected upon comparison of the mean FCR values for the native

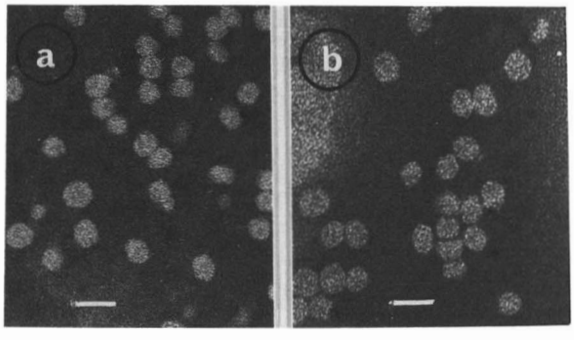

Fig. 2. Electron photomicrographs of negatively stained guinea pig LDL subfractions. Samples are (a), LDL subfraction of d 1.024–1.050 g/ml, and (b), LDL subfraction of d 1.050–1.100 g/ml. A representative electron micrograph of each subfraction is shown. Each horizontal bar represents 50 nm.

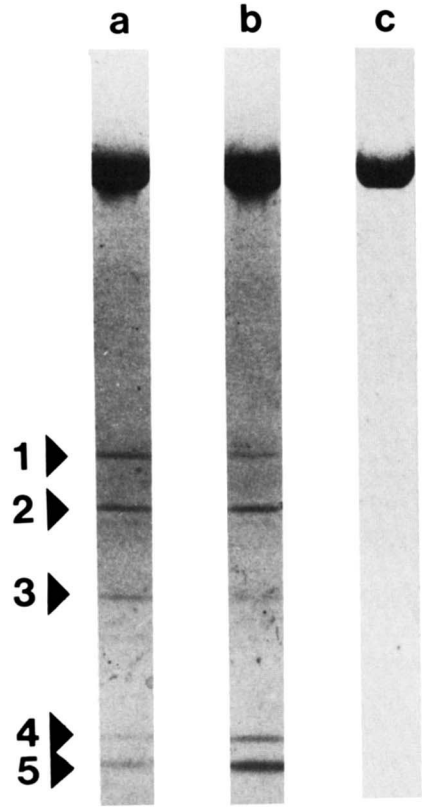

Fig. 3. Electrophoresis of the apolipoproteins of human and guinea pig LDL subfractions on SDS-polyacrylamide gradient gel (3–15%). The LDL subfractions (60 μg protein) were delipidated, solubilized, and electrophoresed as described in Materials and Methods. The molecular weights of respective apolipoprotein bands (1–5) were calculated from a calibration curve derived from a series of molecular weight markers electrophoresed in parallel. Gels were stained with Coomassie R250. In order to detect minor apolipoprotein bands, gels were overloaded with LDL protein; for determination of the M_r of apoB species, similar gradient gels were electrophoresed, but smaller amounts of LDL protein were loaded (gels not shown). The electrophoretic patterns shown are: (a) guinea pig LDL subfraction of d 1.024–1.050 g/ml; (b) guinea pig subfraction of d 1.050–1.100 g/ml, and (c) human LDL of d 1.024–1.050 g/ml. The numbered bands in guinea pig apo-LDL correspond to M_r values of (1) 67,000, (2) 48,000, (3) 34,000, (4) 16,500 and (5) 14,000.

LDL subfractions of density 1.024–1.050 and 1.050–1.100 g/ml. The FCR values for the various native LDL subfractions were 0.0977 pools hr^{-1} for LDL of density 1.024–1.050 g/ml and 0.0925 pools hr^{-1} for LDL of density 1.050–1.100 g/ml (Table 2). Modification of LDL by reductive methylation blocks a number of functionally significant lysine residues of apolipoproteins B and E and thereby inhibits binding of LDL to its specific cellular (apoB,E) receptor (38). This chemical modification of LDL reduced its FCR significantly ($P < 0.01$) (Table 2). The difference between the FCR of native LDL and its methylated counterpart represented that percentage of the catabolism of LDL that occurred by the LDL receptor pathway.

The proportion of LDL catabolized via the specific receptor pathway tended to be higher when the LDL were of lighter density (Table 2) but the differences were not statistically significant. Seventy to 80% of each guinea pig LDL subfraction was catabolized by the LDL receptor pathway (Table 2).

The density distributions of radioactivity in plasma apoB (measured after isopropanol precipitation and lipid extraction (33, 43)) in typical experiments in which a given preparation of radiolabeled native LDL and its methylated counterpart were injected into the same animal are presented in **Fig. 5**.

The radioactivity seen in apoB after injection of the less dense subfraction of native LDL (d 1.024–1.050 g/ml) tended to conserve its initial density distribution as a function of time, i.e., from injection to the fourth point of blood sampling at 48 hr (Fig. 5a). Nonetheless, a small increase in the proportion of gradient fractions 6 and 7 (d 1.027–1.032 g/ml) progressively appeared, although the peak of the percentage of total radioactivity always occurred in gradient fraction 8 or 9 (d 1.037–1.042 g/ml). Indeed, 65% of the total radioactivity present in apoB in this native LDL subfraction was isolated in gradient fractions 8 and 9 at time

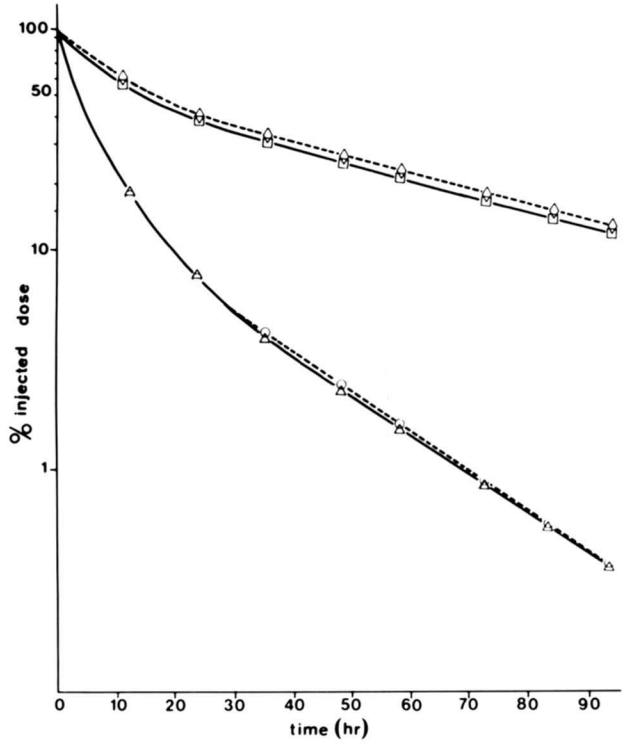

Fig. 4. Plasma decay curves of the radiolabeled guinea pig LDL subfraction of d 1.024–1.050 g/ml and of the corresponding subfraction after reductive methylation in a guinea pig in vivo. After injection of a mixture of control and methylated LDL, the amount of radioactivity (^{125}I and ^{131}I, respectively) was determined in whole plasma (solid lines) and in plasma apoB (broken lines) (apoB was selectively precipitated and delipidated from a d < 1.150 g/ml ultracentrifugal fraction; see Methods) over a period of 96 hr. Values on the ordinate are plotted as percentages of the value 2 min after injection. Values shown are taken from one guinea pig representative of seven.

TABLE 2. Fractional catabolic rates of LDL subfractions in the guinea pig

Lipoprotein Subfraction Density	Fractional Catabolic Rate[a]		% Degradation by Receptor-Specific Pathway[b]
	Native	Reductively Methylated	
g/ml	*pools/hr*		
1.024–1.050	0.0977 ± 0.0202 (n = 7)	0.0250 ± 0.0061 (n = 5)	75.3 ± 6.4 (66.8–82.6)
1.050–1.100	0.0925 ± 0.0122 (n = 7)	0.0259 ± 0.0044 (n = 5)	71.9 ± 3.3 (67.9–75.7)

[a]Fractional catabolic rates (FCR) are expressed as means ± SD and were calculated as described in the Methods section. A portion of each subfraction was chemically modified by reductive methylation, and the native and modified LDLs were labeled differently with either ^{125}I or ^{131}I, and simultaneously injected into control guinea pigs.

[b]The percentage of LDL catabolized by the LDL receptor pathway was calculated for each subfraction as:

$$\frac{\text{FCR, native LDL} - \text{FCR, reductively methylated LDL}}{\text{FCR, native LDL}}$$

and the results are expressed as means ± SD for five experiments, each with different preparations of LDL subfractions. The range in values found is given in parentheses. Data were calculated from whole plasma radioactivity, and were indistinguishable from values derived from plasma apoB radioactivity obtained after apoB precipitation and delipidation (33, 43).

zero and 48% at 48 hr, while 18% of the radioactivity was found in gradient fractions 6 and 7 at time zero and 26% at 48 hr.

By contrast, apoB in the reductively methylated form of this same subfraction (d 1.024–1.050 g/ml) displayed a more marked modification of the density distribution of its radioactivity as a function of time (Fig. 5b). Thus, a net diminution in the percentage of apoB radioactivity in gradient fractions 8 and 9 was seen (from ~61% to ~31%), while that of gradient fractions 6 and 7 increased from ~25% at time zero to ~46% at 48 hr. Moreover, in two animals, a further increase in the percentage of radioactivity in the lighter gradient fractions was observed, since fractions 4 (avg. density 1.022 g/ml) and 5 (avg. density 1.025 g/ml) accounted for up to 20% of the total radioactivity in apoB in the less dense, reductively methylated LDL subfraction at 10 and 20 hr.

The density distribution of radiolabeled apoB in both the native and chemically modified forms of the denser guinea pig LDL subfraction (d 1.050–1.100 g/ml) underwent marked modification during the period from injection to 48 hr (Fig. 5, c and d). Indeed, the profile of the percentage of total radioactivity present in gradient subfractions 6–8 (d 1.027–1.037 g/ml) and 10–12 (d 1.050–1.065 g/ml) shifted markedly at each time point over the period from 10 to 24 hr, in contrast to the data in Fig. 5, a and b for the labeled LDL subfraction of d 1.024–1.050 g/ml, in which shifts in the density profile of radioactivity with time tended to be less pronounced. Thus, in studies of four separate preparations of the native d 1.050–1.100 g/ml LDL (Fig. 5c), an average of 75.9% of the total radioactivity was found in gradient fractions 10–12 and 1% in fractions 6–8 at the initial blood sampling, while at 48 hr, 27.8 and 34.8%, respectively, were present in these same gradient fractions. Furthermore, a still greater proportion of the total radioactivity was located in fractions 6–8 at 48 hr in the case of radiolabeled apoB of methylated LDL (60.9%), and only a trace amount (1.1%) at time zero (Fig. 5d). In contrast, an opposing tendency was seen in the change in radioactive content in gradient fractions 10–12, which decreased markedly from 77.6% at time zero to 16.6% at 48 hr.

We next asked whether these modifications resulted from a faster and preferential catabolism of certain LDL particles in the density range of 1.050–1.100 g/ml at the expense of particles of density 1.024–1.050 g/ml, or from a transformation of LDL particles to other particles of lighter density, or both. We therefore calculated the radioactivity per unit volume of plasma in each LDL gradient fraction (see Methods) and plotted these data against time for each subfraction. These calculations were made using the percentages of total radioactivity measured before extraction by isopropanol, as the material available at each blood sampling was insufficient to allow both gradient subfractionation as well as determination of apoB specific activity in LDL after extraction and precipitation.

It must be added that these calculations were made for time points at 2 min, 10 hr, 24 hr, 34 hr, and 48 hr. Under these conditions, no comment can be made on the possible metabolic relationships between gradient subfractions occurring over the period from 2 min to 10 hr, a period when the bulk of the radioactivity (~70–90%) in native LDL was removed. Slightly less of the injected dose of the reductively methylated LDL subfractions (~35–75%) was removed from the circulation over the same period.

When the lighter LDL subfraction of d 1.024–1.050 g/ml was injected, the radioactivities of gradient fractions 7–9 (d 1.032–1.042 g/ml), expressed as cpm/ml plasma, diminished with a similar slope from 10 hr onwards (**Fig. 6a**). The evolution of radioactivity was, however, more variable in gradient fractions 10–13 (d 1.050–1.071 g/ml) and depended upon the individual animal studied. For example, the rate of reduction in the radioactive content of the latter fractions was similar to that of the lighter fractions (nos. 7–9)

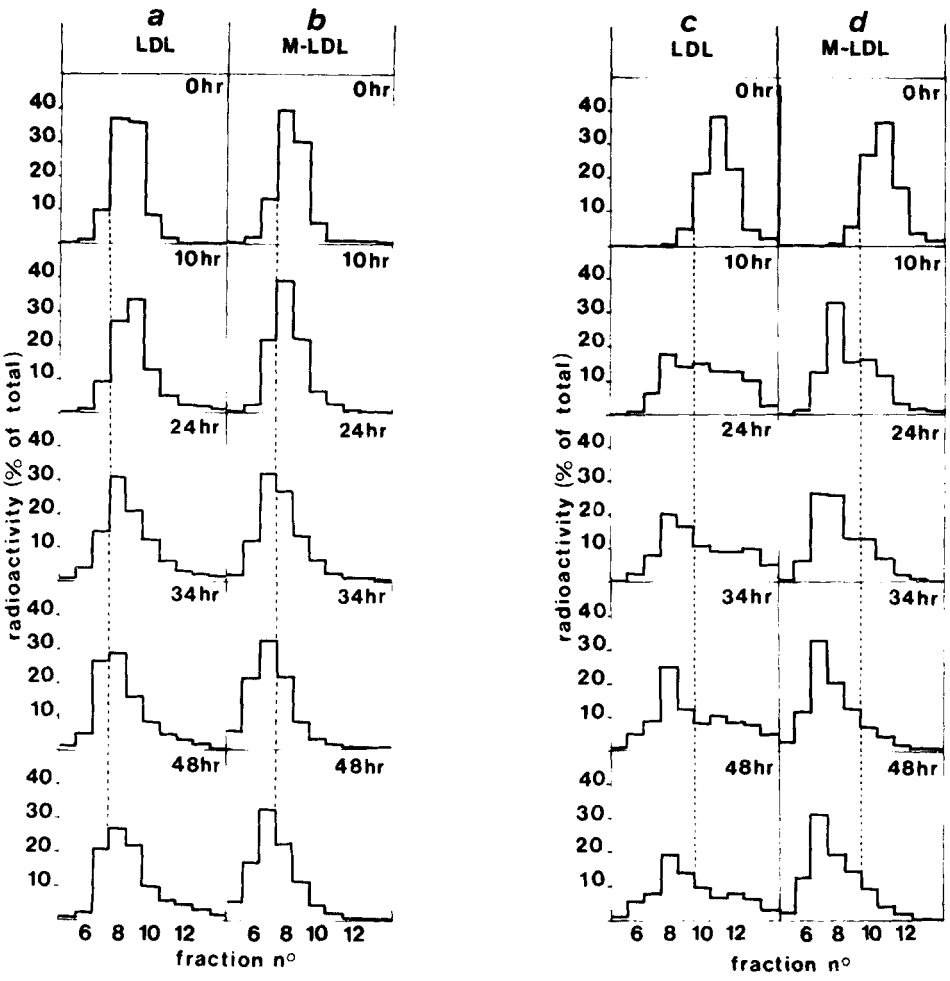

Fig. 5. The plasma profiles of total radioactivity present in apoB in each guinea pig LDL subfraction from injection to the fifth point of blood sampling in three representative experiments; the 0 time sample was withdrawn 2 min after injection, the fifth sample at 48 hr. Plasma samples were first subfractionated by density gradient ultracentrifugation (see Metabolic Studies in Materials and Methods); apoB in each subfraction was then precipitated and delipidated, and its radioactive content was determined. The percentage of total radioactivity at each time point is plotted on the ordinate and the gradient fraction no. on the abscissa (from meniscus downwards). Each vertical column in the histograms represents the shift of the % radioactivity in apoB as a function of time in one animal. In Figs. 5a and 5b, ^{125}I-labeled native LDL of d 1.024–1.050 g/ml and its ^{131}I-labeled, reductively methylated counterpart, respectively, were injected. In Figs. 5c and 5d, the ^{125}I-labeled native LDL of d 1.050–1.100 g/ml and its ^{131}I-labeled methylated counterpart, respectively, were injected. The dotted line constitutes a reference marker in each case, and in (a) and (b) corresponds to d 1.034 g/ml, and in (c) and (d) to 1.046 g/ml, respectively.

in two animals (Fig. 6a), while in two other guinea pigs, either no change or an increase in the radioactive content occurred at the second blood sampling (~10 hr after injection) (data not shown).

Upon injection of reductively methylated LDL (d 1.024–1.050 g/ml), the radioactivity in gradient fractions 7–13 (d 1.032–1.071 g/ml) decreased in a fashion consistent in all animals studied (Fig. 6b); in this case, however, the rate of disappearance of the labeled tracer was less rapid than for the corresponding native LDL (Fig. 6a). It is perhaps noteworthy that the apparent rate of decrease in radioactivity in gradient fraction 7 (d 1.032 g/ml) over the first 12 hr after injection was consistently slower than that in the other fractions.

When the denser native LDL subfraction of d 1.050–1.100 g/ml was injected, the radioactivity in gradient fractions 7 and 8 (d 1.032–1.037 g/ml) was increased considerably at the tenth hour, at which time a peak occurred (Fig. 6c; fraction 8 not shown); subsequently, their radioactive content diminished at a rate similar to that of fractions 9, and 10 to 13 (d 1.042 g/ml and d 1.050–1.071 g/ml, respectively). The latter gradient fractions (nos. 10–13) were distinct from fractions 7 and 8 in their radioactivity profiles, since their radioactive content diminished rapidly from zero time on-

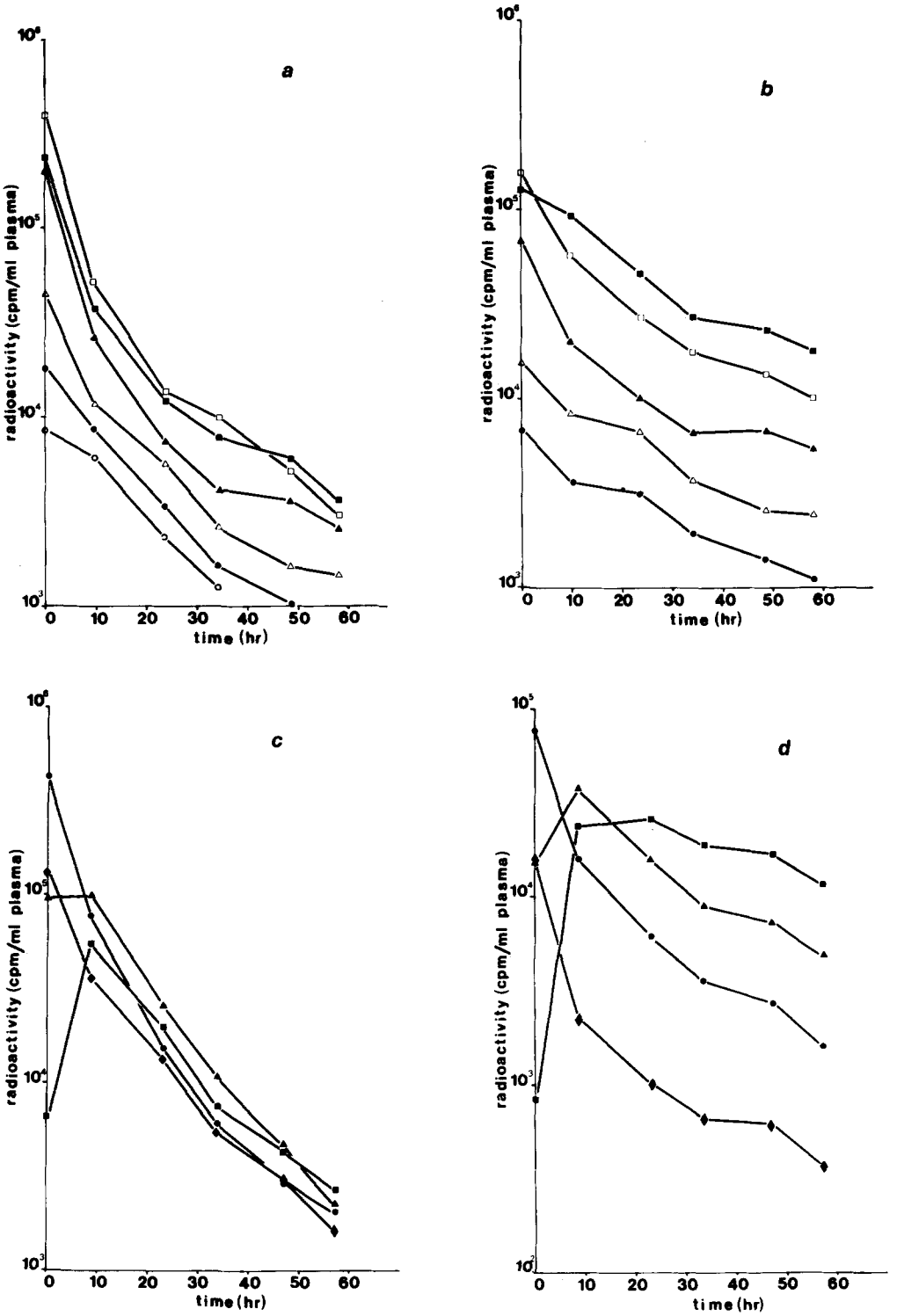

Fig. 6. Radioactivity in individual LDL density subfractions as a function of time after injection in the guinea pig in vivo. The total radioactivity in each LDL subfraction, expressed per unit volume of plasma, was calculated at each time point from the total radioactivity in a given volume of plasma and the proportion of this activity in individual LDL gradient fractions. These data are plotted on the ordinate against time after injection on the abscissa. In each figure, the curves shown are as follows: (■), fraction 7; (□), fraction 8; (▲), fraction 9; (△), fraction 10; (●), fraction 11; (○), fraction 12; and (♦), fraction 13. The radiolabeled LDL subfraction injected in Fig. 6a was the native LDL subfraction of d 1.024–1.050 g/ml, and in Fig. 6b, its reductively methylated counterpart. The radiolabeled LDL subfraction injected in Fig. 6c was the native LDL subfraction of d 1.050–1.100 g/ml, and in Fig. 6d, its reductively methylated counterpart.

wards, whereas that of fraction 9 was similar at both time zero and the 10-hr point.

Examination of the disappearance of radioactivity following injection of the reductively methylated, dense LDL subfraction (d 1.050–1.100 g/ml) revealed profiles for individual gradient fractions that resembled those above for the corresponding dense native LDL. An exception was the retarded appearance of a peak of radioactivity in fraction 7 at 24–30 hr (Fig. 6d), instead of at ca. 10 hr in the native LDL (Fig. 6c). Furthermore, the lesser degree of slope seen in the decrease in radioactive content of all gradient fractions derived from radiolabeled, reductively methylated dense LDL clearly demonstrate their slower rates of disappearance from plasma as compared to the corresponding fractions of the parent native LDL (Fig. 5c).

DISCUSSION

Our present studies have documented the metabolic and structural heterogeneity of low density lipoproteins in normolipidemic guinea pigs. Two populations of particles have been isolated and characterized, i.e., those of lower density, specifically 1.024–1.050 g/ml, and those of higher density, i.e., 1.050–1.100 g/ml. The chemical and physical properties of these LDL subfractions were distinct. Thus, while both were enriched in cholesteryl ester (Table 1), the lighter (d 1.024–1.050 g/ml) subfraction contained proportionally more of this neutral lipid (44.7% as compared to 37.9% in the denser subfraction), and less protein (25.1% as compared to 33.8% in the fraction of d 1.050–1.100 g/ml). However, whereas the chemical composition of the lighter LDL subfraction (d 1.024–1.050 g/ml) resembled that reported by Witztum et al. (47) for LDL of d 1.019–1.063 g/ml, some differences were evident upon comparison with the fraction of d 1.007–1.049 g/ml of Mills, Chapman, and McTaggart (2). More specifically, the content of phospholipid was greater and that of triglyceride some threefold less in LDL of d 1.024–1.050 g/ml in the present investigation. Furthermore, our LDL subfraction of d 1.050–1.100 g/ml contained higher proportions of both phospholipid and free cholesterol, but lesser proportions of both cholesteryl ester and triglyceride than fractions isolated over the density range 1.049–1.077 g/ml by the latter authors (2). These minor dissimilarities may be at least partially accounted for by differences in the density ranges of individual LDL subfractions, as well as by differences in the strain of animal employed, and in the compositions of their diets. The particle sizes of the lipoprotein composing each subpopulation of guinea pig LDL varied as a function of their density range. In assessing particle size and heterogeneity, we have applied two physical methods of analysis, i.e., gradient gel electrophoresis and negative stain electron microscopy, which differ fundamentally in their nature. Thus, the former procedure estimates particle size in the hydrated state and is particularly sensitive to particle heterogeneity. By contrast, the latter procedure involves determination of size in a dehydrated state and averages particle sizes to give a single mean value; it is thereby less sensitive to heterogeneity. As in humans (6, 8), the diameter of the guinea pig LDL particles diminished with increase in density. Thus, electron microscopy showed the lighter subfraction to have a mean diameter of 23.5 nm (range 21–28 nm) and the denser subfraction 18.5 nm (range 16–25 nm). These values are compatible with those described earlier using the same type of analysis; for example, Witztum et al. (47) found that particles d 1.019–1.063 g/ml from control animals displayed a mean diameter of 21.5 nm (range 15.8–21.8 nm), a similar value (21.4 ± 2.5 nm; range 12.5–28.0 nm) having been reported earlier by Chapman and Mills (34) for LDL of d 1.007–1.100 g/ml from normolipidemic control animals. By contrast, a substantially higher mean diameter for a d 1.019–1.063 g/ml LDL fraction of 25.9 ± 2.7 nm was reported by Sardet, Hansma, and Ostwald (48). However, on the basis of hydrodynamic measurements derived from analytical ultracentrifugal studies, Mills et al. (2) indicated that the principle component of guinea pig LDL possessed a hydrated density of 1.056 g/ml and a Stokes diameter of 16.8 nm. Clearly then, differences in lipoprotein particle size estimation may derive from the nature of the analytical procedure used. Indeed, in the present study, the average size of the major component determined in the hydrated state (gradient gel electrophoresis) was uniformly larger than the corresponding value obtained in the dehydrated state (electron microscopy) for each LDL subfraction. The discrepancy between these diameters for each subfraction was, however, notably less when the modal diameter seen by electron microscopy was compared to the diameter of the major component as seen by the electrophoretic method (24.9 nm and 26.9 nm for the d 1.024–1.050 g/ml subfraction and 20.0 nm and 23.6 nm for the denser subfraction, respectively). Such differences in size estimation as determined by these two procedures may not only reflect dissimilarities in hydration state, but also methods for size calibration; in particular, the electrophoretic procedure relies on protein standards whose physical nature is quite distinct from that of complex macromolecular particles such as lipoproteins.

While the denser guinea pig LDL subfraction appeared relatively homogeneous by gradient gel electrophoresis (single band; Stokes diameter 23.6 nm), the lighter LDL of d 1.024–1.050 g/ml were typically heterogeneous, displaying up to three bands of Stokes diameter 26.9, 25.6, and 24.7 nm, respectively, the former predominating.

Despite the above distinctions in the physicochemical properties of our two subfractions of guinea pig LDL, however, LDL-apoB in both subfractions disappeared at apparently similar rates from the plasma in vivo, at least as reflected by similarities in their fractional catabolic rates, which varied from 0.092 to 0.098 pools · hr^{-1}. FCR values

resembling our own have previously been reported by Witztum et al. (47) and by Weech, McTaggart, and Mills (49) (0.110 and 0.092 pools · hr^{-1}, respectively). Inasmuch as reductive methylation of lysine residues in apoB inhibits the binding and degradation of the LDL particle by the LDL (apoB,E) receptor pathway (38), this provided us with a practical approach to the determination of the proportion of guinea pig LDL that is catabolized in vivo by the LDL receptor pathway; thus methylated LDL are apparently catabolized by receptor-independent pathways, the difference between the FCR of native LDL and that of the corresponding methyl-LDL representing the FCR of LDL catabolized by the receptor pathway (5). In our study, the proportion of LDL catabolized by the LDL-receptor pathway (65–80%) was clearly similar to that (~70%) measured by Witztum et al. (23) by a procedure involving glycosylation of LDL.

The profile of the density distribution of radiolabeled apoB in LDL, and of the radioactivity in each gradient fraction derived from it, as a function of the time after injection (Figs. 5 and 6) showed that our two density subfractions of LDL differed fundamentally in certain aspects of their in vivo metabolism. Thus, the lighter guinea pig LDL subfraction of d 1.024–1.050 g/ml underwent only a minor shift in the density profile of radioactivity prior to disappearance following injection in the native form (Fig. 5a). Indeed, the overall radioactivity profile of LDL-apoB across the density gradient was maintained, at least up to 48 hr, with only a minor increase (10%) in the proportions of fractions 6 and 7 (d 1.027–1.032 g/ml). The bulk of this LDL subfraction would appear then to have disappeared from plasma at densities corresponding to those at which it was initially isolated (i.e., d 1.024–1.050 g/ml, corresponding approximately to gradient fractions 7–10 in Fig. 5a). A small but detectable increase did, however, occur in the proportion of material in the denser fractions (nos. 11–13, d 1.055–1.071 g/ml) with time, although the total contribution of such fraction always represented less than 10% of total gradient radioactivity (Fig. 5a). These changes may reflect a moderate influx of transformed, lighter LDL particles, a phenomenon described earlier in both guinea pig (50) and humans (18).

When the intravascular metabolism of the corresponding methyl-LDL subfraction (d 1.024–1.050 g/ml) was evaluated, the overall gradient radioactivity profile was again largely maintained with time, with the exception of fractions 6 and 7, whose radioactive contents increase as a proportion of the whole; fraction 7 progressively replaced fraction 8 as the peak component (Fig. 5b). Since the proportion of the total radioactive content of the gradient present in fraction 7 also tended to increase in the unmodified, native LDL, then this process would not appear to be LDL receptor-dependent, but rather could reflect either gain of lipid or loss of apolipoprotein(s) (see below). When considered together, these data on the LDL subfraction of d 1.024–1.050 g/ml indicate that only minor changes occurred in its radioactivity profile as a function of density, and support the notion that the catabolism of the bulk of this subfraction was largely independent of that of the denser LDL subfraction; this interpretation is, however, subject to some limitation given the lack of data on the metabolism of the light LDL subfraction from injection to 10 hr.

In marked contrast to our findings with the native and chemically modified light LDL, the more dense d 1.050–1.100 g/ml subfraction underwent marked intravascular modification (Figs. 5c and 5d and Figs. 6c and 6d). Indeed, a significant increase not only in the proportions of the lighter gradient fractions (nos. 6–9 of d 1.032–1.042 g/ml) (Figs. 5c and 5d), but also in their radioactive content (Figs. 6c and 6d) was found 10 hr after injection, during which time the major proportion (> 70%) of the dense LDL had, of course, been removed from plasma. The findings in Figs. 6c and 6d thus indicate that a net increase in radioactive content (of the order of ninefold in gradient fraction 7 of native LDL of d 1.050–1.100 g/ml and of the order of fourfold and threefold in gradient fractions 7 and 9, respectively, of its methylated counterpart) had occurred. Given the magnitude of such changes and the presence of ~80% of the radioactivity of LDL in apoB-100, we interpret these phenomena to result from one or a number of catabolic events, i.e., either from conversion of denser particles to lighter species, or to the preferential removal of certain denser particles, or from exchanges and/or transfer of non-apoB apolipoproteins from LDL particles in certain gradient fractions to others, or from a combination of these three processes. In view of the marked elevations seen in the radioactive content of certain of the lighter gradient fractions, the latter mechanism would not by itself appear sufficient to account for our findings. Indeed, some degree of net conversion of denser to lighter particles seems necessary. It is possible that the mechanism of such a transformation is partially dependent on catabolism via the LDL receptor pathway, since the radioactive profiles of the native and methyl-LDL became distinct with time (Figs. 5c and 5d). Furthermore, since the transformation of heavy to light fractions that we presently postulate apparently occurred to a greater extent in methyl-LDL, it is conceivable that their delayed catabolism results in an accentuated degree of transformation. Again, the underlying mechanisms may involve gain of lipid, or protein loss, or both.

The metabolic heterogeneity of LDL has been documented in both human and animal species (16–22). Indeed, the conversion of lighter LDL particles to those of higher density has been described in man, the cynomolgus monkey, and rabbit (16, 18, 21, 22). However, the potential conversion of heavy LDL into its lighter counterpart has been observed in humans (18), while in vitro studies have also indicated that human LDL may be reversibly modified towards less dense, triglyceride-rich, cholesteryl ester-poor precursors (51). We have presently observed that in the

guinea pig, the predominant tendency is for heavier LDL of d 1.050–1.100 g/ml to be transformed into lighter particles. Nonetheless, a small but detectable transformation of light into more dense LDL also appears to occur. Only the former process seems significantly dependent upon the LDL receptor.

While the precise mechanisms of such intravascular transformations are unknown in the guinea pig, we may hypothesize that the underlying basis involves HDL-cholesterol metabolism in this species. Thus, the guinea pig is HDL-deficient (2, 46), possessing only a small pool of HDL-cholesteryl ester. Despite this, such cholesteryl ester turns over at a rapid rate (52), and is associated with rates of lecithin:cholesterol acyl transferase activity (LCAT) that resemble those of man and other mammals (53); in addition, rates of cholesteryl ester transfer activity in guinea pig plasma are intermediate between those of humans and other animals (54). Since LDL are the major carriers of cholesteryl esters in guinea pig, only trace amounts being transported in VLDL (2), we are led to speculate that cholesteryl esters formed in HDL are preferentially and efficiently transferred to heavy LDL, which in turn are transformed to less dense particles. This hypothesis is entirely consistent with the major difference in the chemical composition of our two LDL subfractions; i.e., a higher cholesteryl ester content in the d 1.024–1.050 g/ml subfraction as compared to its denser (d 1.050–1.100 g/ml) counterpart (~45% and ~38%, respectively), and with the predominant type of plasma transformation (i.e., heavy to light LDL) that we have found.

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