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Tissue sites of degradation of low density lipoprotein: Application of a method for determining the fate of plasma proteins

(lysosomal degradation/apolipoproteins/lipoprotein metabolism/swine)

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ABSTRACT **A method for determining tissue sites of plasma protein degradation is described as applied to studies of low density lipoprotein (LDL) catabolism in swine. The method is based on the fact that sucrose is not degraded by lysosomal enzymes and thus accumulates in lysosomes. [^{14}C]Sucrose was activated with cyanuric chloride and covalently coupled to the LDL protein. Studies in cultured fibroblasts have established that the sucrose ^{14}C accumulates intracellularly in degradation products at a rate equal to the rate of degradation of ^{125}I-labeled LDL simultaneously measured. *In vivo* the fractional catabolic rate of [^{14}C]sucrose-LDL was the same as that of ^{125}I-labeled LDL. ^{14}C-Labeled degradation products in all major tissues were determined 24 hours after injection of [^{14}C]sucrose-LDL. About 75% of the LDL degraded (calculated from analysis of the plasma decay curve) was accounted for in the ^{14}C-labeled degradation products accumulated in the tissues examined; only 4% appeared in the urine. In three studies, 37.9, 39.6, and 37.8% of the LDL degraded was recovered in the liver. Results were similar at 48 hr (38.7 and 39.9% hepatic degradation), but urinary losses were then about 10% and about 4% was lost in bile. All extrahepatic tissues examined contained ^{14}C-labeled degradation products. The concentration was highest in the adrenal glands—2 to 5 times that in liver and 10 times that in the next most active tissues. In principle this approach should be applicable to studies of the tissue sites of degradation of any of the plasma proteins.**

Little is known about the relative importance of various tissues in degradation of the plasma proteins *in vivo* because there is no validated general method for approaching the problem. Initial uptake rates of the intact proteins by tissues need not correlate with the steady-state degradation rates because uptake may represent in part entry into exchanging extravascular pools. Arteriovenous differences are generally too small to be helpful. Accumulation of degradation products in the tissues is unlikely to be a valid measure of true degradation rate because these products escape rapidly from cells, to be reutilized, degraded, or excreted. Degradation rates can be measured in perfused organs or in cell culture, but these may not accurately reflect the rates *in vivo*. In connection with recent studies in this laboratory attempting to define the sites of degradation of low density lipoprotein (LDL), we have devised an approach to quantify its degradation rate in different tissues, an approach that should have general applicability in defining sites of degradation of other plasma proteins.

The method rests on the fact that sucrose introduced into lysosomes remains trapped there, "leaking" at only a low rate (1). [^{14}C]Sucrose is covalently linked to the protein of interest. When the tagged protein is delivered to the lysosome, lysosomal proteases should degrade the protein moiety but the sucrose moiety should remain trapped, providing a cumulative tally of the number of protein molecules processed through lysosomes. The validity of the method in principle has been demonstrated in cell culture studies (2–4). [^{14}C]Sucrose was covalently linked to the apoprotein of LDL, which was also labeled with ^{125}I in the conventional way (5, 6). It was shown that the amount of sucrose accumulating in normal cultured human skin fibroblasts correlated well with the amount ^{125}I-LDL protein degraded, the latter measured in terms of the amount of trichloroacetic acid-soluble ^{125}I accumulating, predominantly in the medium (3, 4). In the studies described below we have applied this method to determine the tissue sites of LDL degradation in swine.

METHODS

Preparation and Radiolabeling of LDL. LDL in the density range 1.02–1.06 g/ml was isolated from plasma of Duroc or Hampshire swine by preparative ultracentrifugation (7) and dialyzed against 20 mM sodium phosphate buffer/1 mM EDTA/0.15 M NaCl (buffer A, pH 7.2). LDL preparations were electrophoretically pure (8).

Carrier-free [*U*-^{14}C]sucrose (370–390 μCi/μmol; 1 Ci = 3.7 $\times$ 10^{10} becquerels) (Amersham) was activated by reaction with 2 molar equivalents of cyanuric chloride (1,3,5-trichloro-2,4,6-triazine, Aldrich) in aqueous acetone containing 2 eq of NaOH, as described in detail elsewhere (3). After 10–15 sec, the reaction was quenched by addition of excess acetic acid. The resulting activated [^{14}C]sucrose-dichlorotriazine adduct was then added to the protein (1 μmol of sucrose per 25–30 mg of LDL protein in buffer A, pH 7.2). After 1–3 hr, [^{14}C]sucrose-LDL was separated from low molecular weight products by gel filtration and dialysis. The use of slightly acid conditions (buffer A, pH 6.8) during these procedures removed some labile [^{14}C]sucrose moieties from LDL. The final preparation contained 1 μmol of sucrose per 50–150 mg of LDL protein (2.5–7.6 μCi/mg of protein). In some studies the LDL was first labeled with ^{125}I as described (5, 6), dialyzed exhaustively against buffer A, pH 7.2, and then coupled to [^{14}C]sucrose.

Turnover and Tissue Distribution Studies. Young Duroc or Hampshire swine, 12–25 kg, were anesthetized with sodium pentobarbital, and catheters (usually three) were placed in the external jugular veins or branches thereof and advanced to the heart. Distal ends of the catheters were routed subcutaneously to emerge at the nape of the neck. [^{14}C]Sucrose-LDL (30–50 μCi of ^{14}C; 5–12 mg of LDL protein) was injected through one catheter; periodically, blood samples were withdrawn through the others.

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Abbreviation: LDL, low density lipoprotein.

In some experiments the common bile duct was cannulated. The cannula was externalized through the abdominal wall and total bile output was collected. These animals were restrained by suspension in a canvas sling; morphine sulfate (0.2 mg/kg per 3–4 hr) or diazepam (0.2–0.4 mg/kg per 3–4 hr) was administered to relieve discomfort. In all cases total urine output and the bladder contents at the end of the experiment were collected.

At 24 or 48 hr after injection of labeled lipoprotein, the animals were anesthetized and exsanguinated through a catheter inserted into the right atrium. Before cessation of heart action, perfusion of the whole animal with phosphate-buffered normal saline containing EDTA at 0.1 mg/ml was begun through a cannula inserted into the ascending aorta. A total volume of 10–15 liters of buffer was used at a flow rate of about 1 liter/min. The heart and lungs were separately perfused. Discrete organs were removed for analysis and weighed (liver, kidneys, adrenals, aorta, lungs, pancreas, spleen, testes or ovaries, epididymis or uterus). Multiple samples of other tissues (muscle, adipose tissue, skin, brain, large and small intestine) were removed and total tissue weights were estimated from body weight and literature values for organ weights (9).

Determination of [^{14}C]Sucrose-Labeled Metabolic Products in Tissues. In preliminary studies, undegraded [^{14}C]sucrose-LDL (in trapped plasma, in extravascular spaces, or intracellular) was precipitated with 10% trichloroacetic acid. However, the yield of ^{14}C in the acid supernatant fraction was somewhat low compared to the yield of ^{14}C in low molecular weight products separated from intact LDL by gel filtration on 8% agarose. Some ^{14}C was found in particles of modal apparent molecular weight 1000–2000 that were partly precipitable by trichloroacetic acid, possibly representing short peptide chains still attached to the sucrose. Better recovery of degradation products was obtained by adsorbing undegraded LDL with Cabosil (ICN), a silica preparation shown previously to remove lipoproteins from serum (D. B. Weinstein, personal communication; ref. 10).

Tissue samples were homogenized in 5 vol of water by using alternately a Polytron blade-type homogenizer (Brinkmann) and a tightly fitting Teflon-in-glass homogenizer. The homogenate was twice frozen and thawed and again homogenized in the Teflon-in-glass homogenizer. To adsorb undegraded LDL, Cabosil was added (20 mg/ml). The mixture was shaken vigorously for 1 hr and then centrifuged (2000 × *g* for 30 min). In tissues with low ^{14}C content 3–5 ml of the supernatant fraction was lyophilized and redissolved in 1 ml of water for scintillation counting. All samples were corrected for quenching by addition of a ^{14}C internal standard.

The completeness of LDL adsorption by this method was demonstrated by adding ^{125}I-LDL to homogenates of six pig tissues prior to Cabosil treatment (liver, muscle, adipose tissue, kidney, lung, and heart). The supernatant fraction contained only 2.5 ± 1.7%* of the added ^{125}I-LDL. Recoveries were tested by using degradation products prepared by incubating human skin fibroblasts with [^{14}C]sucrose-LDL and fractionating homogenates of the cells by gel filtration on 8% agarose. The pooled retained fraction was added to homogenates of six pig tissues listed above and Cabosil adsorption was carried out. Recovery of added ^{14}C in the supernate was 108 ± 7%. ^{14}C-Labeled degradation products from the liver of a pig injected with [^{14}C]sucrose-LDL were also prepared by gel chromatography; recoveries tested in the same way were 107 ± 2%. Precipitation with trichloroacetic acid was compared with the Cabosil adsorption method in the tissues of a pig killed 24 hr after injection of [^{14}C]sucrose-LDL. The recoveries of ^{14}C in the acid supernatant fraction ranged from 80% to 95% of those in the Cabosil supernatant fractions.

In two 24-hr studies the parenchymal and nonparenchymal liver cells were separated, generally as described by Berry and Friend (11–13).

Analysis of Plasma Decay Data. All plasma decay curves were satisfactorily fit to biexponential functions which were analyzed using the three-compartment open mammilary model described by Matthews (14).

RESULTS

The proposed method for *in vivo* use has been validated previously by preliminary studies in rats by using asialofetuin, a protein known to be rapidly and predominantly degraded in the liver (15). About 85% of injected [^{14}C]sucrose-asialofetuin radioactivity was recovered in the liver 1 hr after injection (3). At 24 hr the ^{14}C in extrahepatic tissues remained essentially unchanged, showing that there was no redistribution of label. Hepatic ^{14}C, however, fell by about 25% and an equivalent amount was recovered in feces and intestinal contents, implying biliary secretion. The present studies show that in swine also some of the ^{14}C-containing metabolites can be secreted into the bile. However, the fraction released into the bile from [^{14}C]-sucrose-LDL in swine was small, corresponding to less than 7% of [^{14}C]sucrose-LDL catabolized over 48 hr.

The plasma disappearance curves for ^{125}I and ^{14}C in doubly labeled LDL were similar and the slopes and intercepts were not significantly different (fractional catabolic rate 0.047 hr^{-1} for ^{14}C; 0.050 hr^{-1} for ^{125}I). In nine studies reported here with [^{14}C]sucrose-LDL, the mean fractional catabolic rate was 0.045 ± 0.012 hr^{-1} (range 0.026–0.061): with ^{125}I-LDL the mean was 0.042 ± 0.012 hr^{-1} (range 0.023–0.065; n = 14) (16). The data indicate that the metabolizing tissues do not to a significant degree distinguish the sucrose-derivatized LDL from iodinated LDL.

Swine were killed 24 or 48 hr after injection of [^{14}C]sucrose-LDL. Tissue distribution of labeled degradation products is shown in Table 1 for five such studies. Results are expressed in terms of the percentage of total [^{14}C]sucrose-LDL catabolized accounted for in each tissue; the amount of LDL catabolized was calculated from the plasma decay curves (14). Recovery in the tissues examined and in urine accounted for almost 80% of the calculated amount of ^{14}C-labeled LDL degraded at both 24 and 48 hr. ^{14}C in the urine accounted for less than 6% of the LDL catabolized at 24 hr and less than 13% at 48 hr. This is in

Table 1. Percentage of catabolized [^{14}C]sucrose-LDL recovered as ^{14}C-labeled metabolites in various tissues

Tissue	$\frac{\text{^{14}C-Labeled degradation products recovered}}{\text{[^{14}C]Sucrose-LDL catabolized}} \times 100$	
	24 hr	48 hr
Liver	39.1 (37.9, 39.8, 39.6)	39.2 (38.7, 39.7)
Adipose tissue	6.5 (3.8, 10.6, 5.0)	4.1 (5.7, 2.5)
Muscle	4.1 (6.5, 4.3, 1.6)	3.7 (6.4, 1.1)
Small intestine	8.7 (10.0, 6.8, 9.2)	5.1 (2.2, 8.1)
Large intestine	3.2 (1.9, 2.6, 5.0)	1.8 (1.7, 1.8)
Lung	4.0 (4.0, 4.9, 3.2)	1.1 (1.2, 1.0)
Skin	3.2 (2.3, 3.1, 4.2)	1.1 (1.8, 0.4)
Remaining tissues	5.5 (6.8, 3.5, 6.1)	5.3 (6.8, 3.8)
Urine	4.0 (5.7, 2.3, 4.0)	10.7 (8.9, 12.5)
Bile	—	4.3 (2.4, 6.2)
Total recovered	78.3	76.4

* All errors are expressed as SD.

contrast to the results with LDL conventionally labeled with ^{125}I, in which case the ^{125}I degradation products are rapidly and almost quantitatively recovered in the urine. Thus, the [^{14}C]-sucrose associated with catabolized LDL is trapped, as anticipated, and leakage is acceptably slow. In three preliminary experiments not shown in Table 1, degradation products in tissues at 24 hr were determined after precipitation of intact LDL with trichloroacetic acid, a method later shown to precipitate some of the products of LDL degradation (see *Methods*). The relative contributions of the various tissues determined in these experiments, however, were indistinguishable from those determined in the experiments shown in Table 1.

As shown in Table 1, the liver accounted for about 40% of the LDL catabolized and the reproducibility was excellent (range 37.9–39.7%). In two experiments the sucrose radioactivity in the liver was shown to be over 90% in parenchymal cells and less than 10% in nonparenchymal cells. Every tissue examined contained ^{14}C-labeled degradation products; i.e., all tissues contributed in some measure to plasma LDL degradation. Adipose tissue, skeletal muscle, and the small intestine made the largest total contributions, after that of liver, to total LDL degradation. The contribution of the intestine, however, may be overestimated because a portion of the [^{14}C]LDL degraded by the liver appeared in the bile and some of this may have been reabsorbed by the intestine or may have contaminated the intestinal samples despite washing. While the contribution of the liver was highly reproducible, the calculated contributions of some of the tissues less active in LDL degradation showed more variation from animal to animal. This may represent true biological variation but may also reflect experimental error in tissues with low specific radioactivity or sampling errors.

The activities of the various tissues in LDL degradation per gram (wet weight) relative to the activity of the liver are shown in Table 2. Expressed in these terms, the adrenal was by far the most active tissue, accumulating 2–5 times as much ^{14}C as the liver and 10 times as much as the next most active tissue, the spleen. Adipose tissue activity was low expressed per g of total wet weight. However, if we take into consideration the fact that 90% of the wet weight represents depot triglyceride, the observed activity should be multiplied by 10 for comparison with other tissues. In these terms adipose tissue activity ranks with that of lung, lymph nodes, kidney, and small intestine. The remaining tissues showed lower activity.

The amounts of ^{14}C accumulating in different tissues over short time intervals after injection of labeled LDL into the plasma need not be a valid reflection of the relative mass of LDL degraded in them. The specific radioactivities of the LDL in the extravascular extracellular pools need not be the same and thus ^{14}C accumulation would not be in proportion to the number of LDL molecules degraded. This consideration applied in the interpretation of tissue uptake of any plasma protein at short time intervals. However, by using stochastic analysis and the Stewart–Hamilton equation (17), it can be shown that the integral to infinity of the time-specific activity curve is the same for all extravascular pools. This analysis assumes that newly synthesized molecules of the material being traced (LDL) enter only into the plasma compartment (17). Thus at sufficiently long time intervals after introduction of the tracer, the integrated uptake of radioactivity in each tissue should be in proportion to the mass uptake.

Table 2. Relative activities of various tissues per gram (wet weight) in degradation of [^{14}C]sucrose-LDL

Tissue	Relative recoveries of ^{14}C-labeled degradation products per gram of tissue	
	24 hr	48 hr
Liver	(100)	(100)
Adrenal	257 (163, 452, 153)	519 (552, 486)
Spleen	28.6 (27.3, 23.2, 35.4)	33.9 (31.8, 36.0)
Lung	18.8 (23.6, 19.7, 13.0)	6.2 (6.0, 6.4)
Lymph nodes	11.3 (15.9, 5.5, 12.6)	28.0 (53.0, 3.0)
Kidney	9.7 (10.3, 6.4, 12.4)	8.8 (10.1, 7.5)
Small intestine	8.3 (9.5, 7.7, 7.8)	6.6 (3.6, 9.6)
Adipose tissue	1.7 (0.9, 3.0, 1.1)	1.8 (2.9, 0.7)
Remaining tissues	<4.0	<3.0

A test was made of whether or not the 24-hr interval chosen was sufficiently long to minimize the source of error discussed above by comparing the results in 24- and 48-hr experiments. As shown in Table 1, the relative values for degradation in liver and in most extrahepatic tissues at 48 hr were comparable to those observed at 24 hr. Only lung and skin showed consistently lower values at 48 hr.

It can be shown by computer modeling, using high estimates for the size of extravascular lipoprotein pools, that a tissue pool exchangeing with plasma LDL so slowly that a difference in relative tracer uptake between 24 hr and 48 hr would go undetected cannot be a quantitatively important net contributor to LDL degradation.

If this method is to be valid *in vivo* there must be a minimal rate of loss of [^{14}C]sucrose-containing products from the cells degrading the labeled protein and there must be little or no redistribution among tissues as a result of such losses. The reasonably close agreement between results at 24 hr and at 48 hr in most tissues suggests that this requirement is met in the case of [^{14}C]sucrose-LDL.

DISCUSSION

The results described here, taken together with the results of *in vitro* studies (3, 4), support the validity of the proposed approach. Derivatization with sucrose did not alter the binding or rate of uptake of LDL by fibroblasts *in vitro* or the rate of catabolism of LDL *in vivo*. The effectiveness of the "trapping" is indicated by the recovery in tissues of ^{14}C-labeled degradation products corresponding to about 75% of the amount of [^{14}C]-sucrose-LDL catabolized at 24 hr and by the similarity in results at 24 and 48 hr. Losses to the urine at 24 hr correspond to only 4% of the total [^{14}C]sucrose-LDL degraded and at 48 hr to only about 11%. Thus, while the trapping is not 100% effective, the apparent rate of leakage is acceptably low relative to the rate of LDL uptake and degradation. In view of the evidence that asialofetuin and LDL are degraded primarily in lysosomes (18, 19) and our demonstration that chloroquine inhibits degradation of [^{14}C]sucrose-LDL in fibroblasts (3), we assume that the labeled degradation products recovered in tissues are localized in lysosomes.

Previous studies attempting to assess the relative importance of liver and of extrahepatic tissues in degradation of LDL have led to conflicting conclusions (20–23). The liver, at least in swine, contains a significant fraction of the total extravascular extracellular LDL pool (6). This pool exchanges fairly rapidly with plasma LDL so that the liver initially takes up a large proportion of labeled LDL injected. However, the rate of irreversible degradation by liver need not correlate with the size of this pool. Studies in cultured cells and in perfused organs have shown some degree of LDL degradation, but the reported rates have varied widely (21, 23, 24). There may be significant species differences and comparisons among species should be made with caution. Sniderman *et al.* (20) established that extrahepatic tissues of swine have a considerable capacity for degrading LDL by showing that LDL degradation continued actively after total hepatectomy. However, from those studies

it was not possible to evaluate quantitatively the relative roles of liver and extrahepatic tissues in the intact animal under physiologic conditions. The demonstration of high-affinity receptors for LDL in several cell types emphasized further the potential for extrahepatic degradation (25). Cultured swine smooth muscle cells degrade LDL at a high rate (26). However, it cannot be assumed that the rates of metabolic processes in cultured cells mirror those *in vivo*. Recently, it has been shown that the rate of LDL degradation by the perfused swine liver approximates the rate of hepatic degradation found in the present studies (27). Bachorik *et al.* (28) have reported the presence in swine liver plasma membranes of a protein that binds LDL with high affinity.

From the data presented we conclude that in swine the sum of LDL apoprotein degradation occurring in all of the extrahepatic tissues examined is approximately the same as that occurring in the liver. If uptake of the protein reflects uptake of intact LDL molecules, as appears to be the case (25, 29), then we calculate that the rate of delivery of cholesterol to the extrahepatic tissues may be as much as 17 mg/day per kg. Extrahepatic tissues cannot metabolize cholesterol, except for the relatively small amounts converted to steroid hormones by the adrenal glands and the gonads, and so cholesterol taken up in the periphery must ultimately be returned to the liver for excretion. Our data now provide a measure of the possible "burden" of cholesterol that must be transported from extrahepatic tissues to the liver.

The carrier(s) for reverse cholesterol transport are not known. Glomset (30) postulated that high-density lipoprotein (HDL) could be a carrier, and there is experimental evidence that HDL can accept cholesterol from tissues (31). Conceivably, LDL could itself be a participant in reverse cholesterol transport by acquiring additional cholesterol, either from other lipoprotein fractions or directly from tissues, before its uptake by liver. Such mechanisms would fit with a proposal previously made to explain the accelerated degradation of LDL after hepatectomy (20) and with recent studies by Sniderman *et al.* (32) measuring arteriovenous differences across the splanchnic bed in humans.

The high activity of the adrenal in LDL uptake (2 to 5 times that of the liver per gram) is of particular interest. The estimated rate of delivery of LDL cholesterol to the adrenals was 1.4 mg/g of tissue per day. Steroidogenesis by the corticotropin-stimulated swine adrenal *in vitro* has been reported to be about 1.2 mg/g of tissue per day (33). Thus LDL could provide all of the sterol precursor in a stressed pig, and by the nature of these studies with catheterized animals, some suspended for 48 hr in canvas slings, we can assume they were indeed stressed. Kovanen *et al.* (34) isolated plasma membranes from bovine tissues and found that the adrenal showed the highest density of high-affinity LDL receptors of any tissue examined. Kovanen *et al.* (35) showed further that corticotropin stimulation increased the number of LDL receptors in adrenal cells in culture. The present results lend strong support to the proposal that the LDL receptor in the adrenal functionally regulates LDL uptake *in vivo*. The relative uptake in other tissues observed here is also, in general, consonant with the relative density of LDL receptors found in isolated plasma membranes, but rankings are not identical. However, LDL uptake will be determined not only by the density of LDL receptors but also by the concentration of LDL to which the cells are exposed. That will be considerably higher in tissues that have a fenestrated capillary endothelium (e.g., liver, spleen, gut, endocrine tissues, etc.).

Before the approach described here can be accepted as generally applicable it will be necessary to evaluate it rigorously, using other plasma proteins. In each case it must be established that derivatization does not alter the metabolism of the protein under investigation. Additional studies are needed to assess the rate of escape of degradation products from specific tissues, and alternative methods for separating degradation products from intact derivatized proteins need to be explored. However, the results to date with LDL are encouraging, consistent with the underlying assumptions. In principle it may now be possible to deal with some unanswered questions regarding the fate of plasma proteins, including other lipoproteins, the polypeptide hormones, and albumin. In extending the approach, to smaller proteins particularly, it may be necessary to turn to markers other than sucrose. Lysosomes are relatively impermeable to a number of other molecules (36, 37). Among these, peptides made up of D amino acids, including D-tyrosine, may allow higher specific activities to be obtained.

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