

# In vitro labeling of $\beta$ -apolipoprotein with $^3\text{H}$ or $^{14}\text{C}$ and preliminary application to turnover studies

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**Abstract**  $^3\text{H}$ - or  $^{14}\text{C}$ -labeled methyl groups were introduced into apolipoproteins of human and pig low density lipoproteins (LDL). 98% of the label was recovered in the apoprotein of radiomethylated LDL. Such methylated lipoprotein was compared with the corresponding unlabeled LDL with respect to its electrophoretic and immunochemical properties, and its behavior in the analytical ultracentrifuge. The data demonstrated that neither human nor pig LDL underwent gross changes as a result of methylation. The applicability of radiomethylated pig LDL as a tracer for studying the turnover of LDL in pigs was examined. The results showed that the behavior of unscreened and screened  $^3\text{H}$ -labeled LDL was similar. The LDL disappeared with an initial  $t_{1/2}$  of 1.1 hr and a later  $t_{1/2}$  of 30 hr. These values agreed well with those reported for radioiodinated LDL. The technique of radiomethylation of lipoprotein may afford an advantage over radioiodination as it may label peptides that do not have tyrosine.

**Supplementary key words** low density lipoprotein · methylation · biological half-life of  $\beta$ -lipoprotein · lipoprotein metabolism · turnover of LDL

The metabolism of LDL is of considerable interest because increased concentrations of this lipoprotein are associated with atherosclerosis (1, 2). ApoLDL turnover has been studied using LDL labeled in vitro with radioiodine (3–6) or labeled in vivo with [ $^{35}\text{S}$ ]cystine (7). As iodination does not label tyrosine-free peptides, e.g., R-serine of human VLDL (8, 9), we felt it desirable to develop an alternate in vitro method for labeling apolipoproteins. Because its apolipoprotein composition is simpler than that of VLDL, a procedure to methylate proteins by reductive alkylation (10, 11) was adapted to LDL. It produced LDL with  $^3\text{H}$ - or  $^{14}\text{C}$ -labeled methyl groups in the apoprotein.

This paper describes the method of introducing radiomethyl groups into the apoprotein of human or pig LDL. Secondly, it compares the physical and immunological properties of these radiomethylated lipoproteins with those of corresponding unlabeled LDL. Lastly, we report the application of such material in preliminary studies of the

turnover of pig apoLDL. The turnover data are compared with those reported in the literature using radioiodinated LDL. These studies have been presented in part (12).

## MATERIALS

$^3\text{H}$ - or  $^{14}\text{C}$ -labeled formaldehyde was obtained from New England Nuclear Corp., Boston, Mass. All other chemicals were Analar grade (British Drug Houses Ltd., Poole, England). Rabbit antisera to human whole plasma, to human LDL, to pig whole serum, and to pig LDL were made in this laboratory. NCS was obtained from Amersham/Searle Corp., Arlington Heights, Ill.

## METHODS

### Preparation of the lipoprotein

Blood from normal human volunteers or from pigs was collected in flasks containing 1 mg of dipotassium EDTA/ml of blood. Plasma was separated at 4°C. VLDL (d 1.006–d 1.019 g/ml), LDL (d 1.019–d 1.050 g/ml), and HDL (d 1.063–1.21 g/ml) were isolated from the plasma at 12°C in a Beckman L2-65B centrifuge using a no. 40 rotor as described by Havel, Eder, and Bragdon (13). LDL was then washed once through a NaCl-KBr solution of d 1.050 g/ml in an SW 50.1 rotor. It was then dialyzed for about 24 hr under positive  $\text{N}_2$  pressure against a 0.15 M NaCl solution containing 0.05% EDTA solution (pH 7.0), hereafter referred to as NaCl-EDTA solu-

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Abbreviations: LDL, low density lipoprotein or  $\beta$ -lipoprotein; VLDL, very low density lipoprotein; HDL, high density lipoprotein; apoLDL, apolipoprotein of low density lipoprotein; MLDL, methylated low density lipoprotein.

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tion. All lipoprotein preparations were checked by paper electrophoresis (14).

### Radiomethylation of LDL

Labeling was carried out by reductive alkylation of LDL using  $^3\text{H}$ - or  $^{14}\text{C}$ -labeled formaldehyde, according to the method described by Rice and Means (11). All procedures were done at  $4^\circ\text{C}$ . The pH of a solution of 0.5 ml of  $\beta$ -lipoprotein (approximately 3 mg of protein) was adjusted to 8.8. To this, 6  $\mu\text{moles}$  of radioactive formaldehyde was added and mixed. After 30 sec, 75  $\mu\text{g}$  of sodium borohydride in 15  $\mu\text{l}$  was added. The solution was mixed for 1 min and then a further 500  $\mu\text{g}$  of sodium borohydride in 100  $\mu\text{l}$  was added. The methylated LDL was then dialyzed at  $4^\circ\text{C}$  against 3 l of NaCl-EDTA solution in order to remove any unreacted formaldehyde. The dialysate was changed three times and dialysis was continued for a total period of 30 hr. When used for turnover studies, MLDL was stored at  $4^\circ\text{C}$  for not longer than 24 hr, during which time its purity and specific activity were determined.

### Delipidation and determination of specific activity

The LDL or MLDL was lyophilized. It was extracted with ethanol-ether 3:1 (v/v) as described (8). The dry residue was taken up in 0.2 M Tris-HCl buffer, pH 8.2, containing 100 mM sodium decyl sulfate (15). The suspension was gently stirred, and any undissolved particles were removed by centrifugation. Whole lipoprotein or apolipoprotein samples were counted in a Beckman liquid scintillation counter after dissolving about 150  $\mu\text{g}$  of protein in 2 ml of NCS and adding 15 ml of Bray's scintillation mixture (16). Corrections for quenching were carried out using external standard. Protein was determined according to the procedure of Lowry et al. (17). 97% of the LDL protein was recovered at the delipidation step.

### Analytical ultracentrifugation studies

LDL and MLDL were adjusted to  $d$  1.17 g/ml with KBr and dialyzed against KBr solution of the same density at  $4^\circ\text{C}$  for 19 hr. The dialyzed lipoprotein solutions were used for the analytical ultracentrifuge studies. KBr solution ( $d$  1.17 g/ml) was used as reference. These studies were carried out in a Beckman model E analytical ultracentrifuge at  $26^\circ\text{C}$ , using a titanium ANH rotor at 40,000 rpm according to the procedure described by Fisher and his colleagues (18, 19).

### Immunological methods

Immunoelectrophoresis of both human and pig LDL and MLDL was based on the method of Grabar (20), using the Gelman apparatus (Gelman Instrument Co., Ann Arbor, Mich.). Immunodiffusion of pig LDL, pig MLDL, and pig plasma was carried out as described by Ouchterlony (21). The immunoprecipitin lines, obtained

after immunoelectrophoresis, were stained for protein with light green and for lipid with oil red O (14). Radioautography of the immunoelectrophoretic slides containing  $^3\text{H}$ -labeled MLDL was carried out by exposing the slides to X-ray film (Kodak RP Royal X-Omat) for 4 wk.

### Turnover of LDL

Male pigs weighing 13-14 kg, maintained on Master pig feed, were fasted overnight. They were anesthetized with nitrous oxide-oxygen-Fluothane 70:30:1, and the femoral artery and femoral vein of each animal were cannulated. A separate batch of  $^3\text{H}$ -labeled pig MLDL (10  $\mu\text{Ci}$  in 1.5 mg of protein) was injected into the vein of each of three pigs. 1 hr later the pigs were taken off anesthesia, and they were allowed food and water ad lib. Arterial blood samples were collected, as outlined above, at intervals over 52 hr. The VLDL, LDL, HDL, and  $d > 1.21$  infranate were separated, dialyzed, and counted as described earlier. The protein content was assayed and the specific activity calculated. The data were plotted as the fraction of the injected dose remaining at any given time (22), and the kinetics were calculated according to the principles described by Matthews (23).

In another series of three experiments the behavior of "screened"  $^3\text{H}$ -labeled MLDL was examined. Three separate batches of pig LDL were methylated. Each was injected into a separate pig. 5 hr later each pig was exsanguinated, and the "screened" MLDL (2-3  $\mu\text{Ci}$ ) was injected into one of three other pigs. These pigs and their LDL were handled in the same way as that described above for the studies with "unscreened" LDL.

## RESULTS

### Radiomethylation and characterization of human LDL

Human LDL was isolated and labeled with  $^3\text{H}$  as described above. The  $^3\text{H}$ -labeled MLDL had a specific activity of about 5-7  $\mu\text{Ci}/\text{mg}$  of protein. This represented a recovery, in LDL, of 2.5% of the added formaldehyde. Based on the molecular weight of apoLDL being between 27,000 and 64,000 (24), one can calculate, assuming the former molecular weight, that about 1.5  $\mu\text{moles}$  of formaldehyde reacted with each  $\mu\text{mole}$  of apoLDL. Assuming the latter molecular weight, this figure would be 3.5  $\mu\text{moles}$  of formaldehyde per  $\mu\text{mole}$  of apoLDL. An attempt to remethylate the already methylated LDL did not result in any more label being incorporated. Methylation was carried out at pH 8.8, as suggested by Rice and Means (11). Methylation at pH 7 resulted in 85% less label being incorporated. After delipidation, 98% of the label was recovered in apoLDL. The methylated LDL was then subjected to lipoprotein electrophoresis. The strips were scanned for radioactivity in a Packard chro-

matoscanner and were then stained with oil red O. There was a single peak of radioactivity, which corresponded to the single stained band with  $\beta$  mobility, similar to that shown in **Fig. 1**.  $^{14}\text{C}$ -labeled MLDL had the same characteristics as  $^3\text{H}$ -labeled MLDL.

Human LDL and  $^3\text{H}$ -labeled MLDL were both examined in the analytical ultracentrifuge. Each displayed a single peak. The  $S_f$  value of LDL was 8.7 and that of MLDL was 8.9. As the 95% confidence limits of the procedure overlapped, they are probably not significantly different.

Immunoelectrophoresis of human LDL and  $^3\text{H}$ -labeled MLDL was carried out using antiserum to human whole plasma as well as antiserum to human LDL. Whereas whole plasma gave several immunoprecipitin lines with anti-whole plasma serum, the LDL gave only a single arc, showing the immunological homogeneity of the LDL preparation. Similar results were obtained with MLDL. When LDL and MLDL were run against anti-LDL serum, both LDL and MLDL produced a single immunoprecipitin arc, each in a comparable location. On subjecting this immunoelectrophoretic slide to radioautography, a single line corresponding to the immunoprecipitin line of  $^3\text{H}$ -labeled MLDL was obtained. These data were all similar to those illustrated below for pig LDL and MLDL.

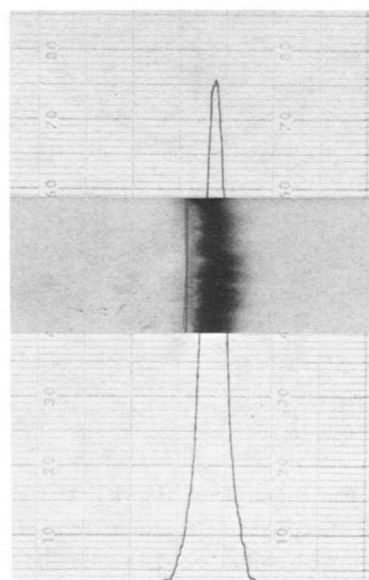
Thus, the electrophoretic, analytical ultracentrifugal, and immunological data strongly suggest that the chemical methylation procedure did not grossly change human LDL.

### Studies on pig LDL

Because the data demonstrated that the methylation procedure did not grossly alter the physical and immunological properties of human LDL, it was of interest to investigate the suitability of radiomethylated LDL for turnover studies. It was felt that initially such studies should be undertaken in experimental animals. The pig was chosen, and its LDL was isolated and methylated. The specific activity and the recovery of radioactivity in pig methylated LDL were similar to those in human methylated LDL. From this and the specific activity of the formaldehyde, we calculated that approximately  $5\ \mu\text{moles}$  of formaldehyde reacted with each 100 mg of pig apo-LDL. Prior to undertaking turnover studies, the properties of pig MLDL were investigated using the same analytical tools described for human MLDL.

Pig MLDL was subjected to lipoprotein electrophoresis and scanned before staining. The pig  $^3\text{H}$ -labeled MLDL (**Fig. 1**) showed a single peak of radioactivity, which corresponded to the single stained band with  $\beta$  mobility.

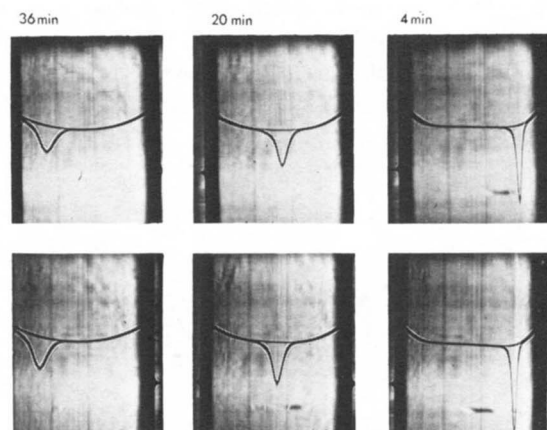
Samples of pig LDL and  $^3\text{H}$ -labeled MLDL were subjected to analytical ultracentrifugation. Data in **Fig. 2** demonstrate that each lipoprotein displayed a single peak with an  $S_f$  value of 11.8. In calculating the  $S_f$  values it



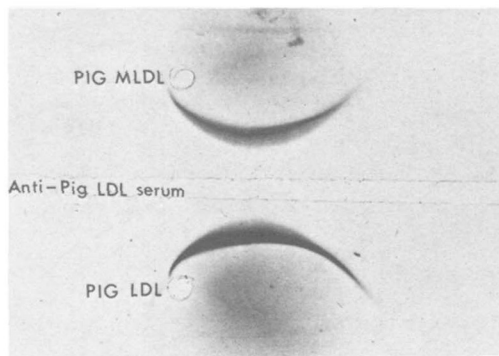
**Fig. 1.** Radioactive scan of the paper electrophoretic strip of pig MLDL. After scanning, the strip was stained with oil red O. The origin is marked with a line.

was assumed that pig LDL and MLDL had the same values for  $\bar{v}$  and  $D$  as human LDL (18, 19).

On immunoelectrophoresis against anti-pig serum, both pig LDL and MLDL showed a single arc, each in a similar location (**Fig. 3**). Radioautography of the slide showed radioactivity in a location corresponding to the immunoprecipitin arc of  $^3\text{H}$ -labeled MLDL (**Fig. 4**). At present we are uncertain whether the suggestion of a double arc on the radioautogram, corresponding to the single stained arc on the same slide (**Fig. 3**), is a photographic artifact or represents the presence of more than one labeled peptide in apoLDL. Immunodiffusion studies (**Fig. 5**) of LDL, MLDL, and pig plasma using anti-pig serum



**Fig. 2.** Analytical ultracentrifugal pattern of pig LDL (*upper row*) and pig MLDL (*lower row*) at different times after reaching full speed. The direction of flotation is from right to left. Experimental details are given under Methods.

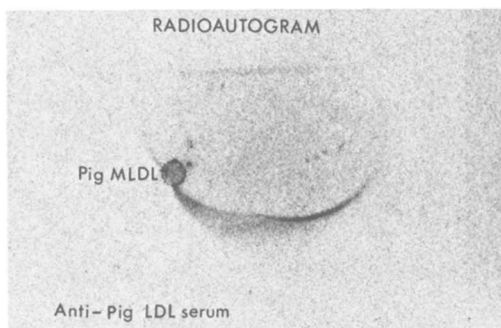


**Fig. 3.** Immunoelectrophoresis of pig LDL and pig MLDL against anti-pig LDL rabbit serum.

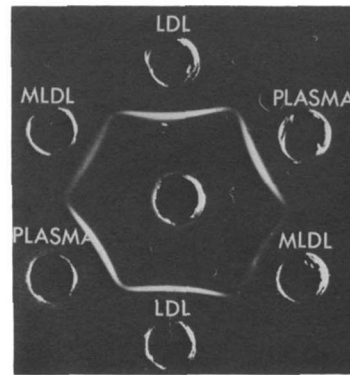
demonstrated the identity of the LDL and  $^3\text{H}$ -labeled MLDL. The difference in the intensity of the band with LDL and that with MLDL represents the difference in the amount of applied antigen.

#### Turnover studies using pig MLDL as a tracer

Some preliminary experiments were undertaken to determine the potential applicability of methylated LDL for turnover studies. Three separate batches of  $^3\text{H}$ -labeled MLDL were prepared and studied in separate pigs. 1.5 mg of LDL protein was injected into each animal. Our own data, and that of Kirkeby (25), indicated the LDL concentration in pig plasma is about 250 mg/dl or about 50 mg of LDL protein/dl. Thus, the injected dose represented less than 0.5% of the approximately 350 mg of circulating LDL protein in pigs weighing 14 kg. The specific activities of the VLDL, LDL, HDL, and  $d > 1.21$  infranate, isolated from the plasmas obtained over 52 hr, were assayed. At all times, 95% or more of the total plasma radioactivity was in LDL. The mean  $\pm$  SEM of the LDL specific activity from the three pigs at each time point was calculated and is plotted in **Fig. 6**. By "curve peeling," the overall disappearance curve was resolved into two components. The individual LDL disappearance curve for each pig also could be resolved into two components; the constants and half-lives are given in **Table 1**.



**Fig. 4.** Radioautogram of the immunoelectrophoretic slide in **Fig. 3**. Conditions are given under Methods.



**Fig. 5.** Immunodiffusion pattern of pig LDL, MLDL, and plasma against anti-pig LDL rabbit serum in the center well.

The fractional catabolic rates calculated from these values (23) are also shown.

In a separate series of experiments, the turnover of screened  $^3\text{H}$ -labeled MLDL was examined. The screening had been carried out as described in the Methods section. Lipoprotein electrophoresis and radioactive scanning of the plasma removed after 5 hr from the pigs used for screening revealed all of the radioactivity to be in the  $\beta$ -lipoprotein band. A separate batch of screened  $^3\text{H}$ -labeled MLDL was injected into each of three pigs in these turnover studies. The amount of LDL injected was less than 2.5% of the plasma LDL pool. The data describing the decay curves and the turnover characteristics are presented in **Table 1**.

## DISCUSSION

We have attempted to develop a new *in vitro* method to label apolipoprotein. Currently, the most commonly used method to accomplish this is radioiodination. However, the possibility of radiation damage has raised problems with a variety of radioiodinated proteins. Furthermore, iodination is not a satisfactory way to label tyrosine-free peptides, such as apoLP-Ser (26). These considerations prompted the endeavor to label apolipoproteins *in vitro* with  $^3\text{H}$  or  $^{14}\text{C}$ . We did this first with LDL, as its apolipoprotein composition is relatively simple compared with that of the other apolipoproteins.

In proteins in which specificity of labeling with formaldehyde has been examined, methyl groups are attached to the  $\text{NH}_2$ -terminal amine function and the  $\epsilon$ -amino groups of lysine (10). Although the entire LDL was subjected to reductive alkylation, the label entered only the apolipoprotein. Calculations indicate that the product had an average of 1.5–2.5 methyl groups/mole of apoLDL. This labeling did not appear to be a nonspecific phenomenon, as it was not increased by remethylation and it was decreased at a lower pH. Reductive alkylation did not seem

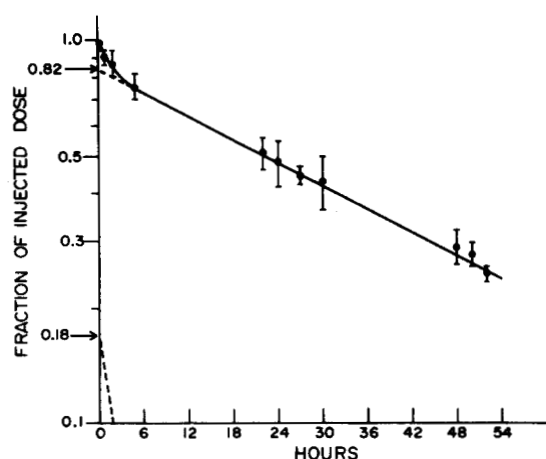


Fig. 6. Disappearance of radiomethylated unscreened pig LDL. Data are pooled from three separate experiments, each using a separate batch of labeled LDL, and each point is the mean  $\pm$  SEM of the fraction of injected dose remaining at the given time (22). The late, linear component has been extrapolated to  $t_0$ . By "curve peeling," a second, more rapid, linear component was obtained.

to alter the immunological identity of the apoprotein, nor did it alter the electrophoretic properties of the LDL. The LDL still retained  $\beta$  mobility on paper, and the radioactivity corresponded to this lipoprotein band. In addition, on immunoelectrophoresis against anti-whole plasma the methylated LDL gave one arc in the same location as native LDL. On immunoelectrophoresis against anti-LDL, human MLDL was shown to be identical with human LDL. Radioautograms of the human and the pig MLDL showed that the label was associated with this lipoprotein only. Reductive alkylation did not change the ultracentrifugal behavior of the LDL.

In view of the similarities of MLDL and LDL described above, we undertook studies to assess the applicability of MLDL to kinetic studies. Three separate studies with unscreened MLDL yielded decay curves that could

TABLE 1. Turnover of unscreened and screened  $^3\text{H}$ -methylated LDL

	$t_{1/2}$ in Components of Decay Curve		Fractional Catabolic Rate (FCR) <sup>a</sup>
	Fast	Slow	
	hr	hr	
Unscreened			
1	1.5	24	0.036
2	1.2	27	0.029
3	0.8	38	0.019
Mean $\pm$ SEM	1.1 $\pm$ 0.2	30 $\pm$ 4	0.028 $\pm$ 0.004
Screened			
4	1.8	27	0.035
5	1.2	25	0.037
6	1.6	32	0.028
Mean $\pm$ SEM	1.5 $\pm$ 0.2	28 $\pm$ 2	0.033 $\pm$ 0.002

<sup>a</sup> Calculated from:  $\text{FCR} = 1/(c_1/b_1 + c_2/b_2)$ , where  $c_2$  and  $b_2$  are the intercept and slope, respectively, of the fast component, and  $c_1$  and  $b_1$  are those of the slow component (23).

be resolved into two components, one with a  $t_{1/2}$  of  $1.1 \pm 0.2$  hr and the other with a  $t_{1/2}$  of  $30 \pm 4$  hr. The overall fractional catabolic rate was 2.8%/hr. These values are not significantly different from those obtained with screened MLDL and suggest that the MLDL was biologically intact. Although our half-lives are slightly longer and the fractional catabolic rates slightly lower, those obtained by Sniderman et al. (27) using iodinated LDL do not differ greatly. This further indicates the applicability of MLDL to biological studies. If anything, the slightly slower turnover of MLDL may suggest that reductive alkylation alters the LDL less than does iodination (28). ■

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