Labeling of high density lipoproteins with [^{3}H]acetic anhydride

Julian B. Marsh

Department of Physiology and Biochemistry, The Medical College of Pennsylvania, Philadelphia, PA 19129

Summary Rat serum HDL was labeled by reaction with [^{3}H]acetic anhydride at pH 7.2 for 30 min at room temperature by a modification of the method of Montelaro and Rueckert (1975. J. Biol. Chem. **250:** 1413). Protein specific activities of 60 dpm/ng were achieved. Seven percent of the label was in lipid, of which 92% was recovered in phospholipid. The labeled HDL migrated as a single band as seen by electrophoretic or column chromatographic analysis. When the labeled HDL was injected into rats without re-isolation, the biological half-life was not significantly different from HDL labeled in vitro with ^{125}I or in vivo with amino acids. All of the apoproteins were labeled; their specific activities were closer to one another than those obtained with ^{125}I. For some applications, acetylation may provide a useful alternative to the ^{125}I labeling procedure.

Supplementary key words In vitro labeling · lipoprotein turnover · phospholipid labeling

The drawbacks to the radioiodination of proteins are well known and include the necessity of a tyrosine residue in the protein, the requirement of a high pH and oxidizing conditions, and the radiation hazards of ^{125}I. Therefore, other labeling methods have been sought. Montelaro and Rueckert (1) have described the acetylation of proteins with [^{3}H]- or [^{14}C]acetic anhydride. Murthy, Monchesky, and Steiner (2) radiomethylated human and pig LDL at pH 9 using either [^{3}H]- or [^{14}C]formaldehyde followed by reduction with borohydride. The present work represents an adaptation of the acetylation method of Montelaro and Rueckert (1) to the labeling of rat HDL. [^{3}H]Acetic anhydride of high specific activity was employed to label HDL at neutral pH and room temperature. Preliminary experiments indicate that VLDL and LDL are also readily acetylated.

Materials and methods

Rat serum HDL was isolated as previously described (3) and purified by recentrifugation at d 1.21 g/ml for 24 hr. After dialysis against 0.15 M NaCl the protein in a concentration of 0.2–1.1 mg/ml (Table 1) was reacted with [^{3}H]acetic anhydride as follows. 2.5 mCi (in 0.6 mg of benzene, sp act 400 mCi per mmol, New England Nuclear Co., Boston, MA) in a sealed ampule was placed in dry ice and an infrared lamp was used to heat the top of the ampule for 30 min in order to concentrate the material at the bottom of the tube (1). After opening the ampule, 0.5 ml of 0.1 M sodium phosphate, pH 7.2, was added. The contents were rapidly mixed and added to 0.5 ml of the HDL solution. After incubation for 30 min at room temperature, the reaction mixture was dialyzed at 4°C against at least three changes of 1 liter of 0.15 M NaCl–0.002 M EDTA for at least 8 hr each. The resulting labeled HDL had a sp act of 14–21 cpm/ng. About 7% of the label was in lipid (**Table 1**).

Abbreviations: HDL, high density lipoproteins; VLDL, very low density lipoproteins; ARP, arginine-rich protein; TCA, trichloroacetic acid; sp act, specific activity.

TABLE 1. Properties of [^{3}H]acetylated HDL

Expt. No.	Protein Concentration	Sp Act	Acetylation Density		TCA-Soluble	Lipid-Soluble
	mg/ml	*cpm/ng*	*µmol acetyl/mg*[a]	*µmol acetyl/µmol*[b]	%	%
1	0.21	14	0.14	28	1.6	7.6
2	0.22	23	0.17	34	0.8	5.5
3	0.28	21	0.16	32	1.1	8.3
4	1.17	21	0.16	32	0.5	6.3

[a] Calculated from the sp act of the protein and the sp act of the acetyl moiety of the labeled acetic anhydride, which was 100 × 10^6 cpm/µmol.
[b] Calculated from the µmol acetyl/mg assuming a protein mol wt of 200,000 for rat HDL.

Ninety-two percent of the lipid label was in phospholipid, as shown by the fact that it was soluble in the chloroform phase after extraction with chloroform–methanol 2:1 and equilibration with water, and that it remained at the origin after chromatography on silica gel G in hexane–ether–acetic acid 85:15:1. Further thin-layer chromatography of acetylated phospholipids[1] revealed that 92% of the label cochromatographed with acetyl phosphatidylethanolamine. The degree of acetylation can be varied at will by changing the concentration or sp act of the labeled acetic anhydride (1).

Protein sp act was determined by the addition of 5 mg of carrier albumin, precipitation with 10% trichloroacetic acid, and extraction of the precipitate with ether–ethanol 3:1. Protein concentrations prior to adding carrier were determined by the method of Lowry et al. (4). Radioactivity was measured by dissolving the protein in 1% SDS and counting was

[1] We are indebted to Dr. George Chacko for these observations.

Downloaded from www.jlr.org by guest, on June 19, 2012

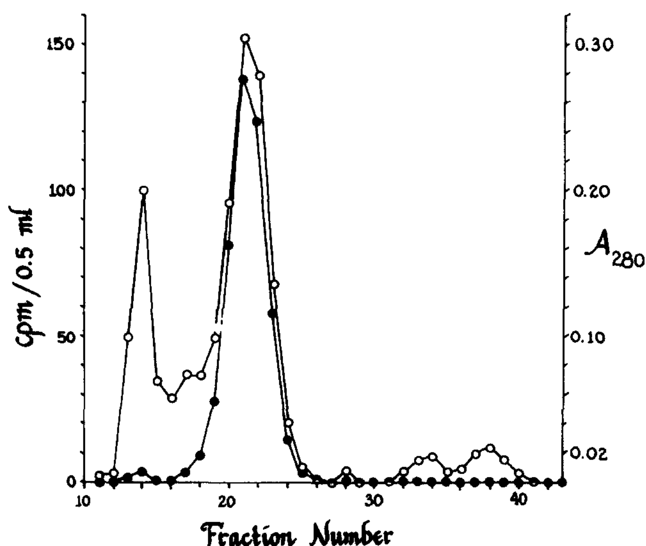

Fig. 1. Chromatography on Sepharose CL-6B of [^{3}H]acetyl HDL in the presence of unlabeled lipoproteins. Open circles, A_{280}; closed circles, cpm per 0.5-ml aliquot of each fraction. The column was 1.5 × 90 cm and 4.0-ml fractions were collected every 30 min. The first A_{280} peak represents carrier VLDL in the void volume; the second A_{280} peak corresponds to carrier LDL; and the main peak in fractions 20–23 corresponds to HDL. The peak at fraction 38 corresponds to the total column volume.

done in 10 volumes of Aquasol in a liquid scintillation spectrometer. Quench corrections were made by the internal standard technique and all samples were counted to a ±1% probable error.

SDS–polyacrylamide gels were run, stained with Coomassie Blue, and scanned by densitometry as previously reported (3). The bands corresponding to the four apoprotein areas named were cut out, digested in H_2O_2, and counted (3). The entire C protein (mol wt 8,000–12,000) area was taken. The cpm divided by the area of the stained band was calculated for each and the relative sp act was calculated, assigning a value of 1.0 to the A-1 apoprotein.

^{125}I-Labeling was carried out by the procedure of Bilheimer, Eisenberg, and Levy (6). The degree of iodination was between 0.6 and 0.8 μmol per μmol of protein, assuming a mol wt of 200,000 for rat HDL protein. ^{125}I radioactivity was assayed directly in a gamma counting spectrometer (Searle Analytic, Des Plaines, IL). HDL labeled with amino acids was isolated from dialyzed rat serum 2 hr after intraperitoneal injection into each of two rats of 0.5 mCi of a mixture of 15-[^{3}H]L-amino acids (New England Nuclear NET-250).

TABLE 2. Relative labeling of HDL apoproteins

Labeling Agent	Relative Specific Activity			
	A-IV	ARP	A-1	C
[^{3}H]Acetic anhydride	0.9	0.6	1.0	1.4
(4)[a]	±0.13	±0.09		±0.40
^{125}ICl	0.25	0.20	1.0	1.0
(2)[a]	0.48	0.22	1.0	1.3

[a] The number in parentheses represents the number of experiments ± SEM.

Results

The labeled HDL migrated as a single band on electrophoresis in 5% polyacrylamide gels. When added to an unlabeled lipoprotein mixture and chromatographed on a Sepharose CL-6B (Pharmacia Co., Piscataway, NJ) column (5), essentially a single peak was obtained (**Fig. 1**) which cochromatographed with HDL. When the apoproteins were separated on SDS-gels (3), all of the stained bands were labeled and negligible radioactivity was found in nonstaining areas of the gel. The relative labeling of four major apolipoprotein bands is shown in **Table 2** in comparison with the results obtained following ^{125}I-labeling. It can be seen that proteins A-IV and the arginine-rich protein were labeled with ^{3}H to a greater degree relative to the major A-1 protein than was the case with ^{125}I. It is not possible to draw conclusions concerning the sp act of the individual labeled apo-

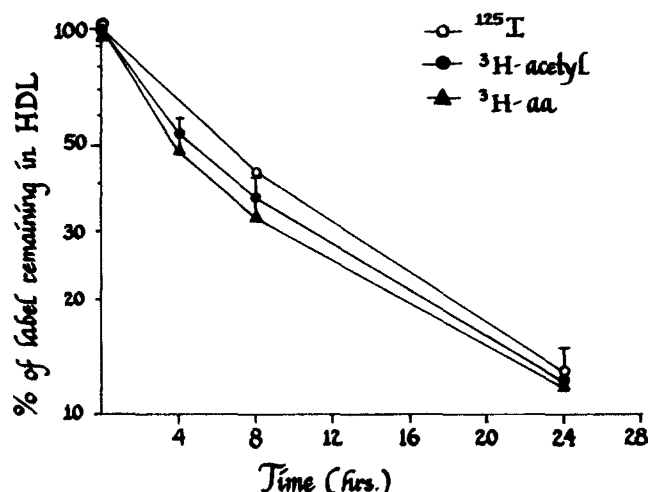

Fig. 2. Turnover of labeled HDL. Open circles, ^{125}I-labeled; closed circles, [^{3}H]acetyl HDL; triangles, [^{3}H]amino acid-labeled. The brackets include one standard deviation above and below the mean values for [^{3}H]acetyl HDL in four experiments. Each experimental point in each experiment has the mean of two rats; two experiments each were carried out with ^{125}I-labeled with [^{3}H]amino acid-labeled HDL. The rats were male albinos of the Fischer strain averaging 250 g and, in each experiment, rat weights did not differ by more than 20 g. Rats were fed ad libitum on standard laboratory chow. The amount of label injected into each rat was 10^5 cpm for [^{3}H]amino acids, 2 × 10^6 for labeled HDL, [^{3}H]acetylated HDL, and 2 × 10^6 for ^{125}I-labeled HDL.

Downloaded from www.jlr.org by guest, on June 19, 2012
ASBMB JOURNAL OF LIPID RESEARCH JLR

proteins since the uptake of Coomassie Blue by each apoprotein may differ.

As a measure of biological stability, the turnover of the acetylated HDL was determined by injection into rats and following the decline of recovered radioactivity in isolated HDL (**Fig. 2**). No significant differences between acetylated HDL and ^{125}I-labeled HDL or amino acid-labeled HDL were detected. When the degree of acetylation was varied over a 160-fold range, from 0.2 to 32.0 μmol/μmol HDL protein (**Table 3**), there was no significant change in the biological half-life as measured between 8 and 24 hr after injection of the labeled HDL. At the very lowest acetylation density (0.05 μmol/μmol protein), a somewhat longer t ½ was observed.

The distribution of radioactivity in four individual apoproteins or groups of apoproteins is shown in **Table 4** as a function of time. The number of observations is too small for statistical analysis but the trend is similar to that found by Eisenberg, Windmueller, and Levy (7) with ^{125}I-labeled HDL. In one respect, however, a significant difference between acetylated HDL and iodinated HDL was found. The distribution of label between d < 1.06 lipoproteins, HDL, and d > 1.21 proteins was not the same as that found with ^{125}I-HDL (**Table 5**); more label was found in the d > 1.21 fraction. This finding was relatively independent of the degree of acetylation; 16% of the label was in the d > 1.21 fraction, even at the lowest acetylation density (0.05 μmol/μmol protein) studied.

TABLE 3. Effect of degree of acetylation on turnover of [^{3}H]HDL

Acetylation Density	No. of Expts.	t 1/2[a]
μmol acetyl/μmol protein		*hr*
0.05[b]	1	14
0.20	1	10
2.20	1	12
32.00	4[c]	10

[a] Calculated from the 8–24 hr time interval only; the calculation was made from a straight line plot on semi-logarithmic graph paper.
[b] Rats were injected with a total of 55,000 cpm in this experiment.
[c] These are the same four experiments shown graphically in Fig. 2.

TABLE 4. Distribution of labeled HDL apoproteins after injection of [^{3}H]acetyl-labeled HDL[a]

Time After Injection	Percent total label in A-IV	ARP	A-I	C
5 min	13.5	8.6	56.9	21.1
4 hr	9.8	9.2	60.0	21.1
8 hr	8.6	11.1	60.5	19.5
24 hr	8.7	11.4	62.7	17.4

[a] From the total HDL shown in Table 3. The SDS-gels were stained, sliced, and counted as previously described (3). The nomenclature for the major band areas is that employed by Swaney, Reese, and Eder (9). The values shown are the means of two experiments, with two rats at each time point.

Discussion

Acetylation of HDL with [^{3}H]acetic anhydride of high sp act results in excellent labeling of the protein, with sp act as high as 60 dpm/ng. About 7% of the label is in phospholipid. Though we have not studied other lipoprotein classes extensively, preliminary experiments indicate that both VLDL and LDL are also readily labeled by this technique. Specific activities of these lipoproteins were 30–60 dpm/ng of protein under the acetylation conditions described above. The degree of lipid labeling, however, was 32% for VLDL and 15% for LDL in two experiments. As with HDL, almost all of the lipid label was in phospholipid.

Since only phospholipid (mainly phosphatidylethanolamine) is labeled, this may be an advantage in certain situations. The modifications of the original method of Montelaro and Rueckert (1) that we have made were the use of a more dilute phosphate buffer, a higher sp act acetic anhydride, and the presence of benzene. We did not find it necessary to replace the benzene in which the [^{3}H]acetic anhydride was supplied with dioxane. The addition of a relatively large volume of aqueous buffer assures that most of the acetic anhydride will be in the aqueous phase. The addition of a constant amount of acetic anhydride produced a reasonably constant sp act over the range of protein concentrations employed (0.2–1.0 mg/ml), as expected (1). It is relatively simple to control the degree of acetylation by varying the amount or the specific activity of the acetic anhydride. The presence of EDTA during acetylation will diminish the degree of protein acetylation, depending on its concentration. Only primary

TABLE 5. Distribution of radioactivity in serum after injection of labeled HDL[a]

Time After Injection	d < 1.06 ^{3}H	d < 1.06 ^{125}I	1.06 < d < 1.21 ^{3}H	1.06 < d < 1.21 ^{125}I	d > 1.21 ^{3}H	d > 1.21 ^{125}I
5 min	6.5	9.2	69.5	85.3	24.1	5.6
4 hr	4.4		76.7		18.9	
8 hr	3.5	11.0	76.9	84.0	19.0	5.0
24 hr	4.4	6.4	73.4	87.3	20.7	6.3

(Columns: Percent of Total Protein Radioactivity in)

[a] Labeled with [^{3}H]acetic anhydride as described in the text or with ^{125}I according to Bilheimer et al. (6). The values shown are the means of two separate experiments in each category.

Downloaded from www.jlr.org by guest, on June 19, 2012

ASBMB JOURNAL OF LIPID RESEARCH

amino groups and tyrosine residues are acetylated in a stable fashion (1). Since residues other than tyrosine are labeled, acetylation produces somewhat more uniform labeling of individual apoproteins (Table 2).

Acetylation did not appear to alter the molecular weight of HDL or to remove any apoproteins since, on molecular exclusion column chromatography, the labeled molecules migrated as a single peak. It is interesting that alteration of the charge by acetylation from 0.05 to 32 μmol/μmol protein (assuming a mol wt of 200,000 for the protein moiety of rat HDL) did not appear to affect its turnover in vivo in comparison with ^{125}I-labeled or biologically-labeled HDL. These data, in conjunction with the studies by Murthy et al. (2) of LDL labeled by amino group methylation, would suggest that many of the primary amino groups are not essential for uptake and degradation of LDL or HDL in vivo. Our values for the t ½ of HDL in the rat are about 10% lower than those reported by Eisenberg et al. (7) with ^{125}I-labeled HDL in the 8–24 hr interval. However, we did not find a single exponential decay curve for HDL disappearance. At present, we have no explanation for this discrepancy. There was also a difference in the distribution of acetylated HDL in the d < 1.06, HDL, and d > 1.21 fractions upon ultracentrifugation. Considerably more label was found in the d > 1.21 fraction with acetylated HDL than was found with ^{125}I-labeled protein. This finding was not a function of the degree of acetylation. It may be related to the relatively higher labeling of the arginine-rich and A-IV proteins, which are also found in d > 1.21 fraction of rat plasma after ultracentrifugation (8).

In vitro labeling of lipoproteins by acetylation with acetic anhydride at neutral pH appears to be a useful addition to present labeling techniques. For example, we have employed this method to label the lipoproteins appearing in perfusates of rat liver. Either ^{14}C or ^{3}H can be employed, and the degree of acetylation can be readily controlled. The fact that the lipid label is confined to phospholipid may also be an advantage in some circumstances.

This work was supported by NIH Grant No. HL19168. The excellent technical assistance of Sarah Massey and William Shriver is gratefully acknowledged. ^{125}I-labeling was carried out in the laboratory of Dr. David Capuzzi. The help of Dr. George Chacko in the chromatography of acetylated phospholipids is also acknowledged.

Manuscript received 18 February 1977 and accepted 18 July 1977.

REFERENCES

1. Montelaro, R. C., and R. R. Rueckert. 1975. Radiolabeling of proteins and viruses in vitro by acetylation with radioactive acetic anhydride. *J. Biol. Chem.* **250:** 1413–1421.
2. Murthy, V. D., T. C. Monchesky, and G. Steiner. 1975. In vitro labeling of β-apolipoprotein with ^{3}H or ^{14}C and preliminary application to turnover studies. *J. Lipid. Res.* **16:** 1–6.
3. Marsh, J. B. 1976. Apoproteins of the lipoproteins in a nonrecirculating perfusate of rat liver. *J. Lipid. Res.* **17:** 85–90.
4. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.
5. Rudel, L. L., J. A. Lee, M. D. Morris, and J. M. Felts. 1974. Characterization of plasma lipoproteins separated and purified by agarose-column chromatography. *Biochem. J.* **139:** 89–95.
6. Bilheimer, D. W., S. Eisenberg, and R. J. Levy. 1972. The metabolism of very low density lipoprotein proteins. I. Preliminary in vitro and in vivo observations. *Biochim. Biophys. Acta.* **260:** 212–221.
7. Eisenberg, S., H. G. Windmueller, and R. J. Levy. 1973. Metabolic fate of rat and human lipoprotein apoproteins in the rat. *J. Lipid Res.* **14:** 446–458.
8. Roheim, P. S., D. Edelstein, and G. G. Pinter. 1976. Apolipoproteins in rat serum and renal lymph. *Proc. Nat. Acad. Sci.* **73:** 1757–1760.
9. Swaney, J. B., H. Reese, and H. A. Eder. 1974. Polypeptide composition of rat high density lipoprotein: characterization by SDS–gel electrophoresis. *Biochem. Biophys. Res. Commun.* **59:** 513–519.

Downloaded from www.jlr.org by guest, on June 19, 2012