Biotinylation of low density lipoproteins via free amino groups without loss of receptor binding activity

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Abstract Low density lipoproteins were biotinylated via free amino groups using carbodiimide-activated biotin or D-biotin-N-hydroxysuccinimide ester. The receptor binding activity of the biotinylated LDL was determined by their ability to displace ^{125}I-labeled LDL from the rat hepatic LDL receptor in the liposome filtration assay. LDL biotinylated with either of the two reagents was able to compete effectively with ^{125}I-labeled LDL providing less than twenty biotin moieties were incorporated per lipoprotein particle. When more than twenty biotins were linked there was a marked loss of activity. The following conditions were adopted as standard for the biotinylation of LDL via free amino groups: 0.3 μmol of D-biotin-N-hydroxysuccinimide ester was incubated with 2 mg of LDL for 1 hr at room temperature. These conditions reproducibly yielded 11.3 ± 0.6 biotins per LDL particle. With the biotinylated LDL and a preformed streptavidin-biotinylated peroxidase complex, the hepatic LDL receptor from rats treated with 17α-ethinyl estradiol was visualized as a single band on electroblots. Finally, the biotinylated LDL was used in an enzyme-linked sorbent assay for the LDL receptor. When solubilized liver membrane proteins from rats treated with 17α-ethinyl estradiol were fixed to the wells with 1.6% paraformaldehyde, a specific binding greater than 0.4 absorbance units was observed which was about ninefold higher than with solubilized proteins from normal rats. We therefore suggest that D-biotin-N-hydroxysuccinimide ester can be used to biotinylate LDL reliably without destroying the lipoprotein's ability to bind specifically to its high affinity receptor. —**Roach, P. D., and S-P. Noël.** Biotinylation of low density lipoproteins via free amino groups without loss of receptor binding activity. *J. Lipid Res.* 1987. **28:** 1508–1514.

Supplementary key words LDL receptor • soluble receptor assay • liposome filtration assay • ELSA • ligand overlay • ligand blotting • Western blotting • receptor visualization • electroblot • carbodiimide • ^{125}I-labeled LDL • 17α-ethinyl estradiol • D-biotin-N-hydroxysuccinimide ester

Macromolecules linked to biotin (1) have been used for cytochemical localization studies (2), visualization of antigens on nitrocellulose (3), and enzyme-linked immunoassays (4). These methods take advantage of the particularly high affinity ($K_d = 10^{-15}$ M) that exists between biotin and avidin (5), a protein isolated from egg white (6). To permit detection of the biotinylated substances, avidin can be labeled with radioactive, electron opaque, or fluorescent compounds or linked to enzymes that give colored products. However, because avidin is glycosylated and has a pI of 10 (6), nonspecific binding can be a problem (7). Streptavidin, a protein produced by *Streptomyces avidinii,* is very similar in structure to egg white avidin and binds biotin with the same high affinity. It has been touted as a better biotin detection agent than avidin (2) because it is not glycosylated and has a neutral pI (8).

D-biotin-N-hydroxysuccinimide ester is the most commonly used reagent for the biotinylation of proteins (1, 9). With it, the biotin is covalently linked to free amino groups. The reagent could therefore be inappropriate for the biotinylation of low density lipoproteins (LDL) because the free ε-amino groups of the lysine residues are implicated in the binding to its high affinity receptor. Weisgraber, Innerarity, and Mahley (10) have shown that modification of the ε-amino groups results in a total loss of receptor recognition when 15 to 20% of the lysine residues are modified.

For fear of affecting the ability of LDL to recognize its receptor, Wade, Knight, and Soutar (11) chose not to use D-biotin-N-hydroxysuccinimide ester for the biotinylation of LDL. They chose instead, first, to oxidize the sialic acid residues of LDL with sodium metaperiodate (12) and then to incubate it with biotin hydrazide (1). The LDL thus biotinylated via sialic acid residues retained its biological activity and, in particular, the authors showed that it was useful for visualizing the LDL receptor on nitrocellulose paper.

Abbreviations: LDL, low density lipoproteins; HDL, high density lipoproteins; 17α-EE, 17α-ethinyl estradiol; EDTA, ethylenediamine tetraacetic acid (disodium salt); BSA, bovine serum albumin; DAB, 3,3'-diaminobenzidine-HCl; ELSA, enzyme-linked sorbent assay.

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We present here the biotinylation of LDL via free amino groups using either D-biotin-N-hydroxysuccinimide ester or biotin activated with a carbodiimide. We show that it is possible to biotinylate LDL in this fashion without destroying its receptor binding activity. When less than twenty biotins were incorporated per particle, the LDL retained most of its activity as judged by its ability to compete with ^{125}I-labeled LDL for the solubilized rat hepatic LDL receptor in a liposome-filtration assay. The biotinylated LDL was used, along with a preformed streptavidin-biotinylated peroxidase complex, to visualize the LDL receptor blotted onto nitrocellulose paper and to detect it in an enzyme-linked sorbent assay (ELSA).

METHODS

Materials

Male Sprague-Dawley rats weighing 250–300 g were obtained from Charles River Canada Inc. (St. Constant, QC). Carrier free sodium [^{125}I]iodide, D-[carbonyl-^{14}C]biotin, and the preformed streptavidin-biotinylated peroxidase complex were purchased from Amersham Canada Ltd. (Oakville, ON). Bovine serum albumin (BSA) fraction V, 17α-ethinyl estradiol (17α-EE), egg yolk phosphatidylcholine type V-E, and 4-chloro-1-naphthol were from Sigma Chemical Co. (St. Louis, MO). We obtained D-biotin-N-hydroxysuccinimide ester from Boehringer Mannheim Canada (Dorval, QC), 3,3'-diaminobenzidine-HCl (DAB) from Canlab Chemical (Mount Royal, QC), and sodium suramin from Mobay Chemical Corp. (New York, NY). 1-Cyclohexyl-3-(2-morpholinoethyl) carbodiimide metho-*p*-toluene-sulfonate, hereafter referred to as the carbodiimide, was obtained from Aldrich Chemical Co. (Milwaukee, WI). Schleicher and Schuell nitrocellulose paper was purchased from Spectrex Ltd. (Montréal, QC). Immulon 2 Removawell strips and holders (Dynatech Lab.), paraformaldehyde, and O-phenylenediamine were from Fisher Scientific Ltd. (Montréal, QC). Human blood, 3 to 5 days old, was obtained from the Montréal Blood Transfusion Center of the Canadian Red Cross (Montréal, QC).

Lipoproteins and iodination

Human LDL (density 1.025–1.05 g/ml) and high density lipoproteins-3 (HDL_3, density 1.125–1.21 g/ml) were prepared by sequential ultracentrifugations (13) as described by Brissette and Noël (14). Phenylmethylsulfonylfluoride (10 μM), EDTA (1 mM), and NaN_3 (0.02% w/v) were added to all plasma samples before centrifugations. The isolated lipoproteins were dialyzed against a 154 mM NaCl solution, pH 7.5, containing 1 mM EDTA.

LDL was iodinated using a modification (15) of the iodine monochloride method of McFarlane (16) as fully described previously (17). Protein was measured by the method of Lowry et al. (18) using BSA as standard.

Biotinylation of LDL with D-biotin-N-hydroxysuccinimide ester

To 2 mg of protein of LDL (0.2 to 0.4 ml) diluted to 1 ml with distilled water, the desired amount (0.02 to 4 μmol) of D-biotin-N-hydroxysuccinimide ester dissolved in dimethylformamide (2 to 20 μl), was quickly added while agitating on a Vortex mixer. The lipoproteins were then incubated under nitrogen for 1 hr at room temperature and dialyzed overnight against a 154 mM NaCl solution, pH 7.5, containing 1 mM EDTA.

Biotinylation of LDL with carbodiimide-activated biotin

For activation of the biotin, 45 μmol of biotin and 90 μmol of the carbodiimide (19) were dissolved in 100 μl of hot dimethylformamide (tube was dipped in boiling water) and then left for 3 hr at room temperature. In one experiment, 0.4 μmol of [^{14}C]biotin was also added.

For the biotinylation, 2 mg of protein of LDL (0.2 to 0.4 ml) diluted to 1 ml with distilled water was incubated for 1 hr at room temperature with the desired amount (0.05 to 9 μmol) of the carbodiimide-activated biotin which was quickly added while agitating on a Vortex mixer. The lipoproteins were then dialyzed overnight against a 154 mM NaCl solution, pH 7.5, containing 1 mM EDTA.

Liposome binding assay for soluble LDL receptor

To induce their hepatic LDL receptor, male rats were given daily subcutaneous injections of 17α-EE, 5 mg per kg of body weight, for 3 consecutive days. Liver membranes from normal and treated rats were prepared by the method of Kovanen, Brown, and Goldstein (20), solubilized in Triton X-100, and the detergent was removed as previously described (17). The solubilized proteins were incorporated into phosphatidylcholine liposomes and LDL receptor activity was assayed using ^{125}I-labeled LDL as described by Schneider, Goldstein, and Brown (21). The ability of biotinylated LDL and unlabeled LDL to displace ^{125}I-labeled LDL was assessed using this assay.

Electroblotting of soluble liver membrane proteins

The nomenclature of Gershoni and Palade (22) is used when referring to protein electric transfer (electroblotting), incubation with BSA to block free sites (quenching), and incubation with ligand (overlay).

The buffers were as follows: membrane solubilization buffer, 125 mM Tris-maleate, pH 6.0, 2 mM $CaCl_2$, and 1 mM phenylmethylsulfonylfluoride; quenching buffer, 60 mM Tris-HCl, pH 8.0, 25 mM NaCl, 2 mM $CaCl_2$, and 40 mg/ml BSA; incubation buffer, as quenching buffer

except 20 mg/ml BSA; washing buffer, as quenching buffer except 10 mg/ml BSA.

Electrophoresis was done on 4–18% polyacrylamide gradient slab gels, 1.5-mm thick, containing 0.1% (w/v) sodium dodecyl sulfate as described by Laemmli (23). The proteins were electrotransferred from the gels onto 0.2 μm Schleicher and Schuell nitrocellulose paper according to Burnette (24) using a Bio-Rad Transblot apparatus at 70 V for 20 hr with water cooling. The gels and electroblots were calibrated with high molecular weight standards (Bio-Rad Lab. Canada Ltd., Mississauga, ON).

Electroblots were incubated for 30 min at 37°C in quenching buffer and then overlaid with the biotinylated LDL or dried and stored at −70°C for use later. The electroblots stored in this fashion were further incubated for 30 min at 37°C in quenching buffer before overlaying with biotinylated LDL.

LDL overlays

Electroblots were incubated for 1 hr at room temperature in the presence of biotinylated LDL, 20 μg of protein/ml in incubation buffer. A 25-fold excess of either unlabeled LDL or unlabeled HDL_3, 10 mM EDTA, or 2 mM suramin was included in some incubations. The electroblots were then rinsed five times for 5 min in washing buffer, followed by a 30-min incubation at room temperature in the presence of the streptavidin-biotinylated peroxidase complex which was diluted 800-fold with the washing buffer. They were then rinsed three times for 5 min in washing buffer, followed by a 30-min incubation at room temperature in the presence of the streptavidin-biotinylated peroxidase complex which was diluted 800-fold with the washing buffer. They were then rinsed three times for 5 min in washing buffer and finally incubated with 4-chloro-1-naphthol (0.4 mg/ml) and H_2O_2 (0.03%) (25) or DAB (0.05 mg/ml) and H_2O_2 (0.02%) in the presence of cobalt chloride and nickel ammonium sulfate (26).

Enzyme-linked sorbent assay for the LDL receptor

The buffers used for the ELSA were the same as those used for the electroblot-overlay method except that the washing buffer did not contain any BSA. The solubilized liver membrane proteins from normal rats or rats treated with 17α-EE, 200 μg, in 100 μl of membrane solubilization buffer, were incubated for 1 hr at 4°C in Immulon 2 Removawells in the presence of 1.6% paraformaldehyde (27). The wells were rinsed three times with washing buffer and incubated for 1 hr at room temperature with quenching buffer. They were then rinsed three times with washing buffer and incubated for 1 hr at room temperature with biotinylated LDL, 100 μg protein/ml in incubation buffer. A 25-fold excess of either unlabeled LDL or unlabeled HDL_3 or 10 mM EDTA was added to some of the wells. The wells were rinsed three times with the washing buffer and incubated for 30 min at room temperature with the preformed streptavidin-biotinylated peroxidase complex which was diluted 800-fold in the washing buffer (+10 mg/ml BSA). They were then rinsed three times with the washing buffer and finally incubated with 100 μl of 0.43 mg/ml *o*-phenylenediamine and 0.002% (v/v) H_2O_2 in citrate–phosphate buffer, pH 6, as described by Campbell (28). The reaction was stopped with 50 μl of 2 M H_2SO_4 and the absorbance at 492 nm was read with a Titertek Multiskan Spectrophotometer (Flow Laboratories, McLean, VA).

Enzyme-linked sorbent assay for biotinylated LDL

Biotinylated LDL, 40 ng in 100 μl of 10 mM Tris-HCl buffer, pH 7.5, containing 154 mM NaCl were incubated for 1 hr at room temperature in Immulon 2 Removawells. The biotinylated LDL bound to the wells was then detected as described in the ELSA for the LDL receptor.

RESULTS

We have investigated the biotinylation of LDL via the free amino groups. We incubated LDL in the presence of increasing amounts of carbodiimide-activated biotin; by including [^{14}C]biotin of known specific radioactivity, we were able to quantify the biotins we were incorporating per LDL particle. As expected, the degree of biotinylation increased with the amount of carbodiimide-activated biotin used (**Fig. 1**). Most of the biotins were linked to protein, presumably via the ε-amino group of lysine residues; only

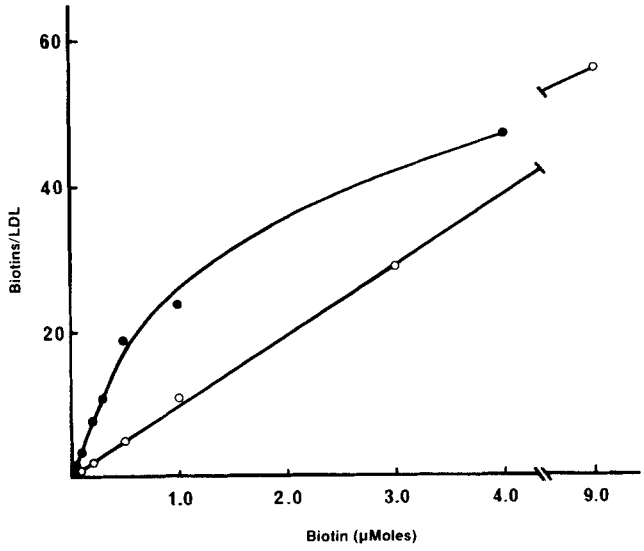

Fig. 1. Correlation of the number of biotins linked per LDL particle with the amount of activated biotin used. LDL was biotinylated using the indicated amounts of D-biotin-N-hydroxysuccinimide ester (●) or carbodiimide-activated biotin (○). The degree of biotinylation was determined using [^{14}C]biotin in the latter case and the ELSA for biotinylated LDL in the former. Values are means of duplicate determinations.

6.1 ± 1.1% (mean ± SEM, n = 7) of the radioactivity was extracted with the lipids, probably linked to phosphatidylethanolamine, and no radioactivity was soluble in trichloroacetic acid. We also incubated LDL with increasing amounts of D-biotin-N-hydroxysuccinimide ester. To quantify the biotins linked per LDL, we used the ELSA for biotinylated LDL. A standard curve of coloration versus number of biotin molecules per LDL particle was obtained using the [^{14}C]biotin-labeled LDL and the biotins per particle obtained with D-biotin-N-hydroxysuccinimide ester were then estimated from the coloration observed in the same ELSA (Fig. 1). We assumed an M_r = 387,000 daltons for apoB (29) and one apoB per LDL particle (30).

The ability of LDL, labeled with varying amounts of biotin, to compete with ^{125}I-labeled LDL for the rat hepatic LDL receptor was evaluated using the liposome filtration assay. As seen in **Fig. 2,** LDL with less than 20 biotins were able to compete very effectively; they were 85% or more as effective as unlabeled LDL. However, when more than 20 biotins were incorporated, there was a marked loss of activity. When up to 63 biotins incorporated, the LDL were only 10% as effective as unlabeled LDL in competing with ^{125}I-labeled LDL. These results were confirmed with electroblots (**Fig. 3**): with LDL having 5, 10, or 19 biotins/particle, we were able to visualize the rat hepatic LDL receptor; with LDL having 29 biotins, the receptor band was just barely visible; and with LDL having 56 biotins, it was not observed. Fig. 3 also shows that, with increasing amount of biotins, the reactivity with unidentified low molecular weight bands also increased. This was clearly detected with LDL having 19 or more biotins/particle. We have no certain explanation for this phenomenon but these bands may be part of the so-called "scavenger" receptor which is known to interact with LDL that had been chemically modified via its free amino groups (31). In **Fig. 4,** the visualized band, which corresponded to a protein with an M_r = 130,000 daltons, is shown to be due to the LDL receptor. The coloration was prevented by a 25-fold excess of unlabeled LDL, 10 mM EDTA, and 2 mM suramin but not by a 25-fold excess of unlabeled HDL_3.

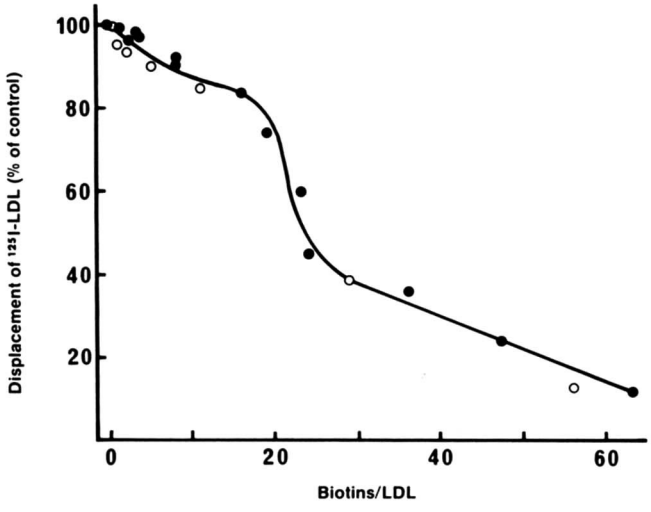

Fig. 2. Receptor binding activity of the biotinylated LDL. Biotinylation was done using D-biotin-N-hydroxysuccinimide ester (●) or carbodiimide-activated biotin (○). The ability of the biotinylated LDL to displace ^{125}I-labeled LDL from the LDL receptor was determined using the liposome filtration assay. Each assay tube contained 60 μg of solubilized liver membrane proteins from rats treated with 17α-EE, 1 μg of ^{125}I-labeled LDL (372 cpm/ng), and a 25-fold excess of non-radiolabeled LDL having the indicated number of biotins per particle. Values are means of duplicate determinations.

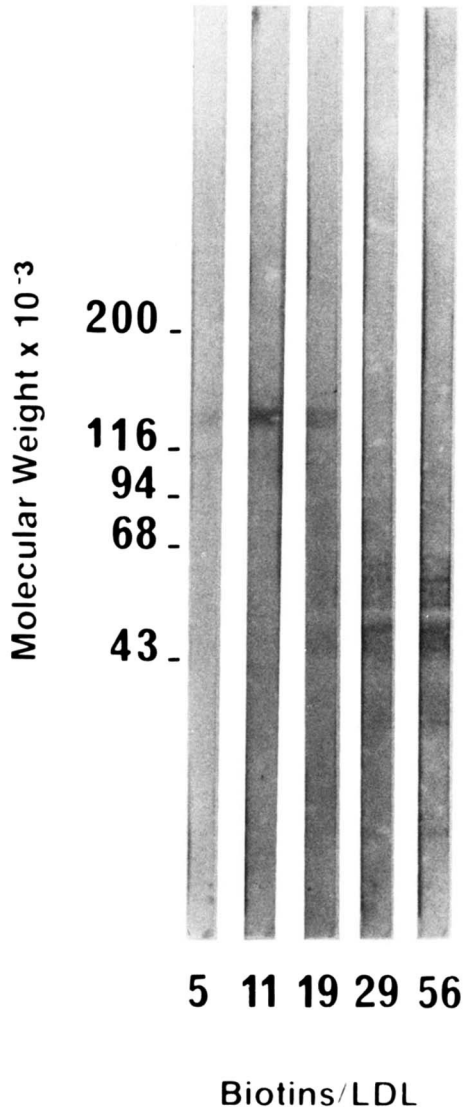

Fig. 3. Overlay of electroblots with LDL having varying amounts of biotin. Solubilized liver membrane proteins (150 μg) from rats treated with 17α-EE were subjected to electrophoresis and electroblotted onto nitrocellulose paper. The electroblots were incubated with 20 μg/ml of LDL having the indicated number of biotins per particle. A preformed streptavidin-biotinylated peroxidase complex and 4-chloro-1-naphthol were used as the detection system.

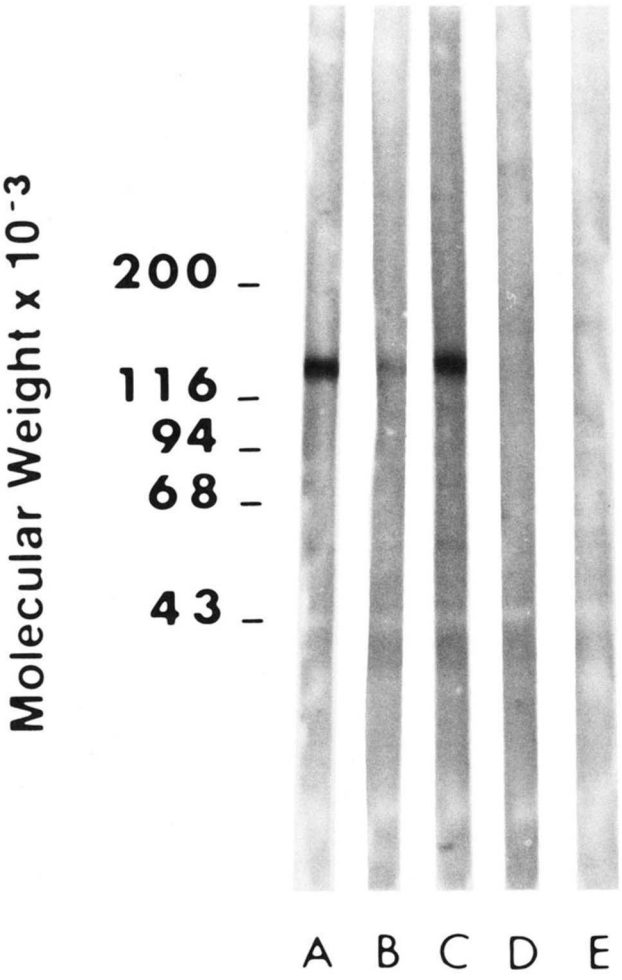

Fig. 4. Demonstration of the properties of LDL binding to the LDL receptor. Solubilized liver membrane proteins (150 μg) from rats treated with 17α-EE were subjected to electrophoresis and electroblotted onto nitrocellulose paper. The electroblots were incubated with 20 μg/ml of biotinylated LDL in the (A) absence or presence of (B) a 25-fold excess of unlabeled LDL, (C) a 25-fold excess of unlabeled HDL_3, (D) 10 mM EDTA, or (E) 2 mM suramin. A preformed streptavidin-biotinylated peroxidase complex and DAB with heavy metal intensification were used as the detection system.

The degree of biotinylation obtained with D-biotin-N-hydroxysuccinimide ester was very reproducible. With 0.17 or 0.3 μmol of the activated biotin, 7.0 ± 0.4 or 11.3 ± 0.6 biotins were incorporated per LDL particle, respectively (mean ± SEM, n = 4). These results were obtained using the ELSA for biotinylated LDL as described above. Furthermore, both results corresponded very well with the results shown in Fig. 1.

Finally, we evaluated the potential of using biotinylated LDL in an ELSA for the LDL receptor. We found that the biotinylated LDL bound to wells that had been incubated overnight at 4°C with solubilized liver membrane proteins from rats treated with 17α-EE, but the binding was nonspecific. Although the biotinylated LDL was effectively displaced by unlabeled LDL, EDTA had no effect. Furthermore, unlabeled HDL_3 was as effective as unlabeled LDL in displacing the biotinylated lipoprotein (data not shown). When the biotinylated LDL was incubated in the presence of a 25-fold excess of HDL_3, all of the binding which was displaced by unlabeled LDL was specific, as evidenced by sensitivity to 10 mM EDTA. This specific binding however, was very low, 0.07 absorbance units. By fixing the solubilized proteins to the wells with 1.6% paraformaldehyde (27), the EDTA-sensitive binding was increased 6-fold to 0.435 absorbance units (**Table 1**). This binding was approximately nine times higher than that observed when the same amount of solubilized liver membrane proteins from normal rats was fixed to the wells with the paraformaldehyde (0.05 absorbance units).

DISCUSSION

It is well known that the free ε-amino groups of some of the lysine residues of LDL are crucial to the lipoprotein's receptor-binding activity (10). However, by linking increasing amounts of biotin to LDL via the free amino groups, we were able to determine that up to 20 biotins could be incorporated per particle without drastically affecting the lipoprotein's receptor binding activity (Fig. 2). However, linking a few more biotins caused a pronounced drop in activity. These results are consistent with those of Weisgraber, Innerarity, and Mahley (10), particularly with one of their carbamylation experiments in which the LDL retained 100% of its activity when 21 lysine residues were carbamylated but only 10% when 27 were modified. In general, their LDL retained close to 100% of its activity when 10 or less lysine residues were modified and lost most of it when more than 40 were affected. Their results were normalized assuming an M_r = 387,000 daltons for apoB (29) and one apoB per LDL particle (30). The loss of receptor binding activity we observed was, therefore, most probably due to the linking of biotin via the ε-amino groups of lysine residues.

Biotinylation of LDL with D-biotin-N-hydroxysuccinimide ester is very easy and fast; one short incubation step is all that is needed. In contrast, biotinylation with either carbodiimide-activated biotin as we have presented here or biotin hydrazide as described by Wade, Knight, and Soutar (11), requires an additional incubation step, the former to activate the biotin with the carbodiimide, and the latter to oxidize the sialic acid residues with metaperiodate. Biotinylation with D-biotin-N-hydroxysuccinimide ester is also easily controlled and very reproducible. This control is important when biotinylating LDL via free amino groups because of the importance of the lysine residues to the receptor binding activity (10). We chose the following conditions as standard for the biotinylation of LDL: 0.3 μmol of D-biotin-N-hydroxy-

TABLE 1. Enzyme-linked sorbent assay for the rat hepatic LDL receptor[a]

Treatment	Normal Rats		Treated Rats	
	Absorbance (492 nm)	Δ Absorbance[b]	Absorbance (492 nm)	Δ Absorbance[b]
No addition	0.458		0.884	
+ 25 × unlabeled LDL	0.366	0.092	0.469	0.415
+ 10 mM EDTA	0.408	0.050	0.449	0.435

[a]Solubilized liver membrane proteins were fixed to wells with 1.6% paraformaldehyde. A 25-fold excess of unlabeled HDL_3 protein (relative to biotinylated LDL protein) was included in all wells during the incubation with biotinylated LDL.

[b]The Δ absorbance was obtained by subtracting the absorbance observed in the presence of a 25-fold excess of unlabeled LDL protein (relative to biotinylated LDL protein) or 10 mM EDTA from the absorbance observed with no addition. Values are means of duplicate determinations.

succinimide ester incubated for 1 hr at room temperature with 2 mg of protein of LDL. These conditions resulted in 11 biotins being incorporated per particle and LDL thus biotinylated retained 85–90% of its receptor-binding activity.

We cannot directly compare the degree of biotinylation we observed with that obtained by the sialic acid method (11) because the incorporation of biotin was not quantified in the latter case. As for the amount of N-acetyl-neuraminic acid found in LDL, the values reported in the literature vary. Vauhkonen et al. (32) found the equivalent of 4 sialic acids per LDL particle, Lee and Breckenridge (33) found 14, while Swaminathan and Aladjem (34) found 22. For the sake of comparison, their results were normalized using an M_r = 387,000 daltons for apoB (29) assuming one apoB per LDL (30). Wade, Knight, and Soutar (11) could, therefore, have incorporated from 4 to 22 biotins per LDL assuming that 100% of the sialic acids were biotinylated.

In addition to being useful for the visualization of the LDL receptor on nitrocellulose paper, biotinylated LDL could also be used in an ELSA type assay for the solubilized rat hepatic LDL receptor (Table 1). We did encounter a problem with nonspecific binding, but it was overcome by including unlabeled HDL_3 during the ligand incubation step as we have previously done in the membrane-centrifugation assay (14, 35). Fixing the solubilized proteins to the wells with paraformaldehyde increased the specific binding approximately 6-fold over simply incubating the solubilized proteins overnight in the wells. The 9-fold higher response obtained with solubilized proteins from rats treated with 17α-EE as compared to normal rats (Table 1) was consistent with the 11-fold increase we have previously observed using the liposome-filtration assay (17) and with the 10-fold increase seen by Windler et al. (36) using the membrane-centrifugation assay. Also as observed in the liposome-filtration assay, all of the binding displaced by unlabeled LDL from solubilized proteins from treated rats was sensitive to EDTA while only about 50% of it was sensitive to the divalent ion chelating agent in the case of the normal rat (17).

The response obtained with the normal rats is fairly low, 0.05 absorbance units. To improve the response, the receptor could be partially purified by chromatography on a DEAE-gel (21). In this purified preparation the receptor would account for a greater proportion of the protein bound to the wells. The best way, however, may be to first coat the wells with an antibody that can bind to the receptor without affecting its LDL binding activity. In effect, the receptor would be preferentially bound and concentrated on the wall of the wells thereby permitting a strong response in the ELSA with biotinylated LDL.

In conclusion, we propose that D-biotin-N-hydroxysuccinimide ester is a useful reagent for the biotinylation of LDL via free amino groups. The incorporation of biotin can be controlled and is reproducible. Care should be taken however, not to incorporate more than 20 biotins per LDL particle, in order to prevent the loss of receptor-binding activity. To this effect, we have suggested a protocol to link 11 biotins per LDL. The biotinylated LDL retained most of its receptor-binding activity and was used to visualize the LDL receptor on nitrocellulose paper as well as to detect LDL receptor activity in an ELSA type assay.

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