Further Observations on the Attachment of Carbohydrate to Lipoproteins by Rat Liver Golgi Membranes[1] (37677)

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Rat liver Golgi preparations have been shown to incorporate labeled glucosamine from UDP-*N*-acetyl glucosamine-^{14}C into lipoproteins (2). When rat serum was used as a carrier, as in earlier work with amino acids (3), most of the label appeared in the HDL[3] fraction. Recent work has shown that VLDL and HDL can exchange peptides which have been identified as small peptides containing sialic acid (4, 5). It seemed likely that the preponderance of label in HDL previously observed might be due to the exchange phenomenon.

The present experiments were undertaken to investigate further the nature of the carbohydrate-containing lipoproteins which can be extracted from Golgi membranes under mild conditions. Sialic acid, the last carbohydrate to be attached (6), was chosen as the labeling agent. The results of these experiments indicate some peptide exchange but in addition they have revealed the presence of labeled sialopeptides within the Golgi vesicles which are not bound to sufficient lipid to float at density 1.21 but which bind strongly to HDL in serum. These peptides co-chromatographed on DEAE-cellulose with the sialic acid-containing peptides of rat apo-HDL.

Materials and Methods. Golgi membranes were prepared from livers of male Holtzman rats, 200–350 g, fed *ad libitum* on laboratory chow as previously described (2). The Golgi pellet was suspended in 0.1 *M* Tris–malate buffer pH 6.1 with the aid of a Dounce homogenizer (Kontes Glass Co.). CMP-sialic acid-4,5,6,7,8,9-^{14}C was obtained from New England Nuclear Corp. (lot #686-006). After incubation for 1 hr at 37°, the Golgi membrane suspension was centrifuged at 100,000*g* for 30 min at 2° and the pellet suspended in 2×10^{-4} *M* Tris (pH 8.6) and left overnight at 0°. After recentrifugation, the combined supernatants were used for lipoprotein isolation. The incubation medium prior to extraction of the Golgi vesicles contained less than 4% of the labeled lipoprotein. Lipoproteins were isolated, with or without the addition of rat serum after the extraction process, by successive ultracentrifugation at solution densities of 1.006, 1.06, and 1.21 obtained by the addition of NaBr. Centrifugation was carried out at 5° for $2 \times 10^6 g\cdot$hr (VLDL and LDL) and $2.5 \times 10^6 g\cdot$hr (HDL) in the Spinco 50 Ti rotor in 9-ml polycarbonate tubes. The top 1 ml was carefully removed with the aid of a 0.5-ml Lang-Levy micropipet. Lipoprotein recovery was 90 ± 5%, so that LDL and HDL fractions were contaminated to a maximum of 10% from the preceding fraction. In the experiment in which HDL peptides were separated, HDL was purified further by recentrifugation before study.

Measurement of protein-bound radioactivity was carried out after dialysis against 0.15 *M* NaCl and the addition of either 2 mg of bovine serum albumin or an amount of unlabeled lipoprotein corresponding to the fraction in question, isolated from a separate centrifuge tube. After precipitation and re-

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[3] Abbreviations used: VLDL, very low density lipoproteins, $d < 1.006$; LDL, low density lipoproteins $1.006 < d < 1.06$; HDL, high density lipoproteins, $1.06 < d < 1.21$.

TABLE I. Effect of Extraction Technique on Recovery of Labeled Lipoprotein.

Expt no.	Extraction method	Total $d < 1.21$ lipoprotein labeling[a] No serum (cpm)	2 ml serum (cpm)
1	2 *M* LiCl	124	463
2	Sonication in 2 M LiCl	199	538
3	2×10^{-4} *M* phosphate, pH 8.6	1355	2502
4	2×10^{-4} *M* Tris, pH 8.6	1090	3731

[a] In these experiments, approximately 0.6 mg Golgi membrane protein was incubated for 1 hr at 37° with 0.5 μCi of CMP-sialic acid-^{14}C.

centrifugation three times with 1% phosphotungstic acid–5% trichloroacetic acid at 0°, lipids were removed by recentrifugation three times with 3:1 (v/v) ether–95% ethanol. The proteins were then dissolved in 0.5 *N* NaOH, transferred to counting vials, acidified, and counted in Aquasol for a minimum of 3,000 total counts. In control experiments, 90% of the label was released after hydrolysis in 0.1 *N* H_2SO_4 at 80° for 1 hr.

Delipidation of HDL was carried out according to Lux *et al.* (7). Chromatography was carried out on a DEAE-cellulose column as described by Shore and Shore (8). The column was 1.4 × 12 cm, the flow rate 10 ml/hr, at 5°. The starting buffer was 0.03 *M* Tris–6 *M* urea, pH 8, and the limiting buffer 0.5 *M* Tris–6 *M* urea. Fractions were analyzed for protein by the Lowry method (9) and for carbohydrates by the methods previously employed (10).

Results. The results shown in Table I indicate that the extraction technique, derived from the principles of Steck *et al.* (11), gave a satisfactory yield of labeled lipoproteins in comparison with the older method of sonication. The soluble Golgi extract contained 34% of the total protein-bound sialic acid, of which 10% could be recovered as lipoprotein.

The addition of whole rat serum (or 1.5–3.0 mg HDL in some experiments) increased the total amount of labeled lipoprotein which was recovered (Table I). The additional label was found in the HDL fraction (Table II) and it persisted after recentrifugation. The smaller amount of label in carrier-free HDL was not due to a protective effect of protein during isolation since addition of 60 mg of albumin did not alter the recovery, and purified HDL, but not VLDL or LDL, could substitute for

TABLE II. Distribution of Labeled Lipoprotein Extracted from Golgi Membranes.

			Lipoprotein labeling (cpm)					
			VLDL		LDL		HDL	
Expt no.	Golgi protein incubated (mg)	^{14}C-CMP-sialic added (μCi)	No serum	Plus serum	No serum	Plus serum	No serum	Plus serum
1	0.55	1.0	364	215	247	744	787	1500
2	0.43	1.0	177	125	124	114	453	1100
3	0.53	1.0	572	521	108	225	410	2985
4[a]	0.82	1.0	1688	438	468	248	510[b]	1214[b]
5[a]	1.88	3.0	5572	3435	1193	2008	3840[c]	12,100

[a] In these experiments, HDL was recentrifuged before measurement of radioactivity.

[b] After the first centrifugation at *d* 1.21, 799 and 1577 cpm, respectively, were recovered in the HDL fraction.

[c] Addition of 1.5 mg of HDL to the $d > 1.21$ fraction resulted in an additional recovery of 7500 cpm at *d* 1.21.

whole serum. In control experiments, we have been unable to demonstrate differential losses of labeled lipoprotein as a function of concentration during isolation.

The distribution of label in VLDL, LDL, and HDL was examined in the presence and absence of carrier serum (Table II). In the nine experiments reported in Tables I and II, the increase in total recovered label was a factor of 2.33 ± 0.36 (significantly different from 1.0, $p < 0.005$). In only one experiment did we fail to observe the effect, and in this experiment (Table II, Experiment 4) a very great decrease in label in VLDL was noted. In the absence of serum, roughly 42% of the label was in VLDL, 14% in LDL, and 44% in HDL. About one-third of the label in VLDL was lost after the addition of serum, but this effect was quite variable.

The apo-HDL peptides from HDL isolated in the presence of serum were separated on DEAE-cellulose. Eighty-three per cent of the labeled peptides were found in areas 6–9 (Fig. 1). The labeling pattern was identical, though at different levels, whether or not serum had been added during the lipoprotein isolation. Analysis of areas 6–9 indicated that they were the only areas which contained measurable quantities of sialic acid; and the sialic acid content of these areas accounted for the total sialic acid content of the starting apo-HDL solution. Based on the protein content, the percentage of sialic acid was 0.42, 0.49, 2.16, and 1.10%, respectively, in areas 6–9. The percentage of sialic acid in apo-HDL based on the analysis of these four peaks and a total protein recovery from the column of 85%, was 0.36%, in agreement with the sialic acid content of the starting apo-HDL and the value previously reported (10). No carbohydrate was detected in Fraction 3, the major HDL peptide.

Discussion. Golgi membranes from rat liver are capable of transferring labeled sialic acid from CMP-sialic acid to endogenous lipoprotein acceptors, a result which was anticipated from the earlier work with UDP-*N*-acetyl glucosamine (2). Approximately equal amounts of the newly synthesized glycolipoprotein is VLDL and HDL. Only relatively small amounts (14%) of the label appeared at *d* 1.06.

The addition of carrier serum to the Golgi extract did cause a decrease in the amount of label found in VLDL, which could have been anticipated from the VLDL–HDL peptide exchange (4). However, addition of serum increased the total amount of labeled lipoprotein which was recovered. The extra label could not be removed by recentrifugation at *d* 1.21. After separation of apo-HDL peptides on DEAE-cellulose, the distribution of label

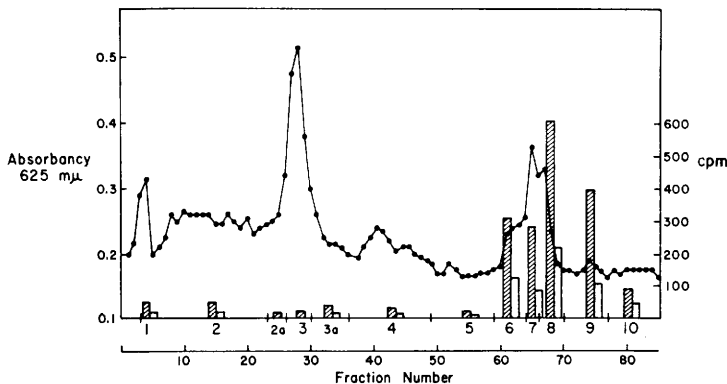

FIG. 1. Chromatographic separation of apo-HDL on DEAE-cellulose in 6 *M* urea. Abscissa—tube number or combined fraction number. Ordinate (left)—absorbancy in the Folin-Lowry protein reaction, carried out on 0.5 ml of the 4 ml in each tube. Ordinate (right)—cpm in each combined fraction. The cross-hatched bars represent the sample to which serum has been added prior to HDL isolation and the stippled bars, the sample in which serum was added after HDL isolation. Both samples were obtained from the same Golgi membrane incubation. It should be emphasized that carrier serum was added after extraction of the Golgi membranes.

coincided with that found when no carrier serum had been added during isolation, and only very small amounts of label were found in regions of the chromatogram which contained no measurable amounts of sialic acid. This suggests that sialic acid-containing peptides of VLDL and HDL are present as a precursor pool within the Golgi. It is not possible to estimate the size of this precursor pool from the present experiments. Further work is required to determine whether or not such precursors could be released *in vivo*.

Summary. After *in vitro* incubation of rat liver Golgi membranes with ^{14}C-CMP-sialic acid, the membranes were extracted by treatment with 2×10^{-4} *M* Tris, pH 8.6, and lipoproteins were isolated by ultracentrifugation. The distribution of labeled lipoprotein was found to be 42% in VLDL, 14% in LDL, and 44% in HDL after sequential isolation of each density fraction. The addition of unlabeled whole rat serum to the extracted Golgi resulted in a decrease in label recovered in VLDL which was attributed to exchange of sialopeptides between VLDL and HDL. However, the addition of serum doubled the amount of recovered labeled lipoprotein and the extra label was associated with HDL. The labeled peptides were similar to those normally found in HDL as judged by DEAE-cellulose chromatography of the apo-HDL peptides. These results suggest the presence of a precursor pool of apolipoproteins within Golgi vesicles.

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