notes on methodology

Incorporation of labeled cholesterol esters into chylomicrons in vitro

STEVEN H. QUARFORDT* AND DEWITT S. GOODMAN‡

Department of Medicine, Columbia University College of Physicians and Surgeons, New York, N.Y.

SUMMARY Labeled cholesterol esters were incorporated into chylomicrons in vitro by adding an acetone solution of the cholesterol ester to a suspension of washed chylomicrons. The physical and metabolic properties of the added labeled ester were the same as those of cholesterol esters incorporated into chylomicrons in vivo, except for the added ester being differently distributed in chylomicrons of different size.

KEY WORDS cholesterol ester · in vitro incorporation · chylomicrons · density gradient centrifugation · hepatic clearance

A METHOD IS described here for the in vitro incorporation of labeled cholesterol esters into chylomicrons. With this method, small amounts of labeled cholesterol ester, up to approximately 10% of the cholesterol ester content of the chylomicrons, can be incorporated into chylomicrons. The method is particularly useful for metabolic studies with doubly-labeled cholesterol esters, i.e., esters bearing a label in both the cholesterol and the fatty acid portions of the molecule.

The adequacy of in vitro incorporation was examined by comparing the properties of labeled cholesterol ester thus incorporated with those of differently labeled cholesterol ester incorporated in vivo during the process of chylomicron formation. The properties studied included both physical behavior (centrifugal characteristics, flocculation in a polyvinyl pyrrolidone gradient) and metabolic behavior (rate of plasma clearance and rate of hepatic uptake, after intravenous injection).

Incorporation. Chylomicrons labeled in vivo with either cholesterol-^{14}C or cholesterol-^{3}H (mainly present as cholesterol ester) were obtained by feeding labeled cholesterol to lymph fistula rats (1). The chylomicrons were isolated by flotation (1.03×10^6 *g*-min) and diluted to a concentration of 10 mg total lipid per ml. The labeled cholesterol ester to be incorporated in vitro was then added by slowly injecting an acetone solution of the ester beneath the surface of the chylomicron suspension, via a 100 μl syringe. For each 1 ml of chylomicron suspension, 25 μl of acetone, containing 25 μg of cholesterol ester, was added. The chylomicron suspension was gently shaken at 37°C for 2 hr, after which it was kept at room temperature without shaking for 12–16 hr. The chylomicrons were then equilibrated for 1 hr at 37°C with an equal volume of human whole blood in order to remove readily exchangeable labeled cholesterol (2). The chylomicrons were again isolated and washed by centrifugal flotation through 0.9% NaCl, followed by dispersion and dilution as before.

Physical Properties. The properties of cholesterol ester incorporated into chylomicrons in vivo and in vitro were compared in the same chylomicron preparation. An acetone solution of ^{14}C-labeled cholesteryl oleate was added to chylomicrons labeled in vivo with cholesterol-^{3}H. The chylomicron suspension was incubated as described above, but was not equilibrated with blood. A portion of the chylomicron suspension was layered under 0.9% NaCl and was centrifuged as described. Measured portions of the redispersed washed chylomicrons, and of the chylomicron-free "bottom" portion of the centrifuge tube were then extracted with the mixture described by Dole (3), and the extracts were simultaneously assayed for ^{3}H and ^{14}C in a Packard Tri-Carb liquid scintillation counter. The counting error was less than 2% for each isotope. Ninety per cent of both the ^{3}H and of the ^{14}C was recovered in the chylomicrons. Under these conditions, therefore, the centrifugal characteristics of the in vivo and of the in vitro labels were identical.

Another portion of the chylomicron suspension was subjected to a polyvinyl pyrrolidone gradient according to the method of Gordis (4). After complete flocculation had occurred to the top of the gradient, the primary particles were extracted and assayed for radioactivity. The ratio of ^{3}H/^{14}C radioactivity (cpm) was 0.78 in the primary particles, compared to 0.82 in the whole chylomicron suspension. The behavior of the in vivo and of the in vitro labels was thus virtually identical in the polyvinyl pyrrolidone gradient.

The possibility was then considered as to whether the physical properties described might merely reflect the properties of an acetone dispersion of cholesterol ester. However, when an acetone solution of cholesteryl-^{3}H oleate was added to 0.9% NaCl and incubated as described, ultracentrifugation yielded no radioactivity at the top of the tube. In another experiment an acetone solution of cholesteryl-^{3}H palmitate was added to a 1% (w/v) solution of human serum albumin, followed by the addition of an equal volume of chylomicron suspension and incubation for 1.5 hr at 37°C. After centrifugal flotation of the chylomicrons through 0.9% NaCl only

* Postdoctoral Trainee, Grant T1 AM-5234, National Institutes of Health, Bethesda, Md.

‡ Career Scientist of the Health Research Council of the City of New York under Contract I-339.

15–20% of the ^{3}H was recovered in the packed chylomicron fraction. These experiments indicate that the physical properties described above are not possessed by acetone dispersions of cholesterol ester in either isotonic saline or in 1% serum albumin. In addition, the second experiment indicates that the cholesterol ester added in vitro must be added directly to a chylomicron suspension, and then incubated in the manner described, in order for it to acquire the physical properties of in vivo incorporated cholesterol ester.

Metabolic Properties. Chylomicrons labeled in vivo with cholesterol-^{14}C and in vitro with cholesteryl-^{3}H oleate were injected intravenously into intact rats. The rates of plasma clearance and of hepatic uptake of ^{3}H were considerably greater than those for ^{14}C. In a typical experiment, in which the rats were sacrificed 2.5 min after injection, 42% of the injected ^{3}H vs. 17% of the ^{14}C was recovered in the liver. These differences were due, however, not to differences in the metabolism of cholesterol ester incorporated in vitro and in vivo, but to differences in the distribution of cholesterol esters incorporated in vitro and in vivo into chylomicrons of different size.

Chylomicrons of different size were separated by sucrose density gradient centrifugation as described elsewhere (1), by means of the method originally described by Pinter and Zilversmit (5). Table 1 shows the distributions of in vivo and in vitro incorporated cholesterol ester after sucrose density gradient centrifugation. The ^{14}C distribution was the same in control chylomicrons which had not been exposed to acetone or to cholesterol ester added in vitro. Large differences were observed in the distribution of the two labels among chylomicrons of different size. The larger (lighter) particles were associated with relatively much more ester incorporated in vitro (^{3}H) than were the small particles (at the gradient bottom). This finding suggested that the more rapid clearance of in vitro label noted above might simply reflect the more rapid clearance of larger chylomicrons, which is reported elsewhere (1).

The clearance rates of cholesterol esters incorporated in vivo and in vitro were then compared for chylomicrons of the same size. Chylomicron pools were prepared, as described elsewhere (1), from the top, middle, and bottom portions of sucrose density gradients. These preparations, containing lipids in concentrations of 5.2 (top), 5.0 (middle), and 4.6 (bottom) mg/ml, were injected intravenously into male Sprague-Dawley rats (1 ml of any one preparation per rat). Table 2 summarizes the hepatic recoveries of each of 4 groups of rats. In all instances, most of the injected label not recovered in the liver was found in the plasma. For both labels, the hepatic uptake at 4.5 min was greatest for the large chylomicrons (top), and least for the small chylomicrons (bottom). As noted above, there was a large difference in the rates of hepatic uptake of the two labels after the injection of heterogeneously sized whole chylomicrons. In contrast, the more homogeneous chylomicron preparations which had been separated according to size showed a much smaller difference in the rates of hepatic uptake of the two labels. For the large and the medium size chylomicrons, the difference in uptake of the two labels was not statistically significant. Although there was a significant difference for small chylomicrons, this difference was much smaller than that seen with whole chylomicrons, and probably reflected the fact that the small chylomicron preparation was somewhat less homogeneous in particle size than were the medium and large chylomicron preparations. This latter fact was indicated by a second density gradient centrifugation of portions of similarly prepared chylomicron pools; all pools showed the presence of some heterogeneity which was greatest for the small chylomicron pool. These results demonstrate that the clearance rate is similar for cholesterol esters incorporated in vivo or in vitro for chylomicrons of any given size.[1]

TABLE 1 DISTRIBUTION IN A SUCROSE DENSITY GRADIENT OF IN VIVO INCORPORATED CHOLESTEROL-^{14}C, AND IN VITRO ADDED CHOLESTERYL-^{3}H OLEATE

Distance above Initial Site of Layering	^{14}C	^{3}H	$^{3}H/^{14}C$
cm	*cpm/ml*		*cpm/cpm*
0.5	1415	847	0.6
2.5	243	604	2.5
7.5	81	332	4.1
12.5	80	400	5.0
14.0	89	512	5.8

TABLE 2 HEPATIC RECOVERIES AFTER INTRAVENOUS INJECTION OF CHYLOMICRONS OF DIFFERENT SIZE

Chylomicron Preparation*	Mean Time†	% Recovery of Injected Label in Liver‡	
		^{3}H	^{14}C
Top	4.47	78 ± 2	75 ± 2
Middle	4.65	66 ± 2	61 ± 2
Bottom	4.42	42 ± 2	33 ± 3
Whole chylomicrons	4.51	49 ± 3	25 ± 2

* "Whole chylomicrons" had not been subjected to density gradient centrifugation. Top, middle, and bottom refer to chylomicron pools containing, respectively, large, medium, and small size chylomicrons.

† Individual rats (5 per group) were each sacrificed within 17 sec of the mean time listed.

‡ Chylomicrons were labeled in vivo with cholesterol-^{14}C, and in vitro with cholesteryl-^{3}H oleate. Mean ± SEM.

[1] Previous results with a single isotope (1) did not preclude the possibility that the number of particles cleared per unit of time was the same in all three chylomicron pools, and that the more rapid clearance of larger particles reflected the lesser number of particles injected. The present finding of a large change in isotope ratio during clearance of heterogeneous whole chylomicrons, together with the absence of such change with homogeneous chylomicron preparations, is not consistent with this interpretation.

The evidence herein presented is consistent with the interpretation that labeled cholesterol ester added to a chylomicron suspension in the manner described is actually incorporated into the particles. Although the evidence does not conclusively establish this fact, the assumption of incorporation is supported by the similarity, in physical and metabolic properties, of the added ester compared to in vivo incorporated chylomicron cholesterol ester. In metabolic studies using chylomicrons thus labeled in vitro, the different distribution of the cholesterol ester among chylomicrons of different size, compared to that of in vivo incorporated cholesterol ester, should be recognized. The method described should hence be most useful for studies involving those aspects of chylomicron cholesterol ester metabolism in which there are no major differences between small and large chylomicrons. Preliminary experiments in this laboratory suggest that except for the rates of plasma clearance and tissue uptake there are no major differences in the metabolism of cholesterol esters in large or small chylomicrons.

We thank Mr. T. Shiratori and Mrs. J. Vormbaum for expert assistance.

This work was supported by PHS Research Grant AM-05968 from the National Institutes of Health, U.S. Public Health Service.

Manuscript received 27 January 1966; accepted 2 June 1966.

References

1. Quarfordt, S., and DeW. S. Goodman. *Biochim. Biophys. Acta* **116:** 328, 1966.
2. Goodman, DeW. S. *J. Clin. Invest.* **41:** 1886, 1962.
3. Dole, V. P. *J. Clin. Invest.* **35:** 150, 1956.
4. Gordis, E. *Proc. Soc. Exptl. Biol. Med.* **110:** 657, 1962.
5. Pinter, G. G., and D. B. Zilversmit. *Biochim. Biophys. Acta* **59:** 116, 1962.