TRIACYLGLYCEROL SECRETION IN VERY LOW DENSITY LIPOPROTEINS BY ISOLATED RAT LIVER PARENCHYMAL CELLS[1]

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SUMMARY

Enzymatically isolated rat liver parenchymal cells secreted labeled triacylglycerols when incubated with [^{3}H]glycerol or [^{3}H]oleic acid. The presence of albumin or serum did not affect the secretion, but it was strongly inhibited by cycloheximide, colchicine, EDTA and by incubating at 4°C instead of at 37°C. Analyses of incubation media by agarose gel electrophoresis and by ultracentrifugation showed that the labeled triacylglycerols were in particles with the properties of very low density lipoproteins.

INTRODUCTION

The liver contributes the major part of the non-chylomicron triacylglycerols of blood plasma in the form of VLD[2]-lipoproteins (1). The experimental systems most frequently used in studies on their formation and secretion by rat liver are the intact animal, the perfused organ and tissue slices. In studies on the biosynthesis of lipids by enzymatically isolated rat liver parenchymal cells (2,3), it was of interest to know whether this preparation retains the capacity of lipoprotein formation and secretion, especially since the system is metabolically superior to liver slices and have experimental advantages compared to organ perfusion. Suspended rat liver cells were therefore incubated with labeled glycerol or oleic acid and the release of labeled lipids into the medium was examined.

MATERIAL AND METHODS

Cell isolation and incubation. Male Sprague-Dawley rats (Anticimex AB, Sweden) weighing 200-350 g were fed a balanced diet ad libitum. Liver parenchymal cells were prepared according to Berry and Friend (4) as described elsewhere (2). Bicarbonate-free Hanks' solution buffered with 10 mM phosphate (pH 7.4) was used as the basic incubation medium. Where indicated 2 % delipidated (5) and dialyzed bovine serum albumin (Fraction V, Serva Heidelberg), with or

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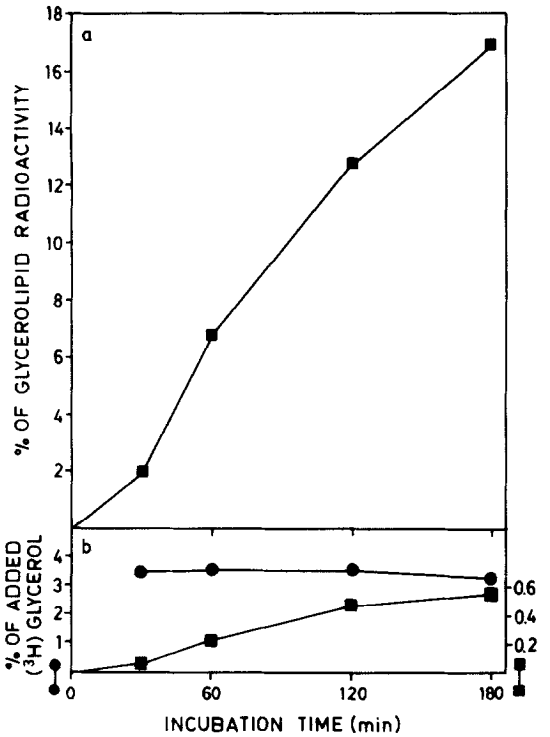
[2]VLD(L), very low density (lipoproteins), d < 1.006.



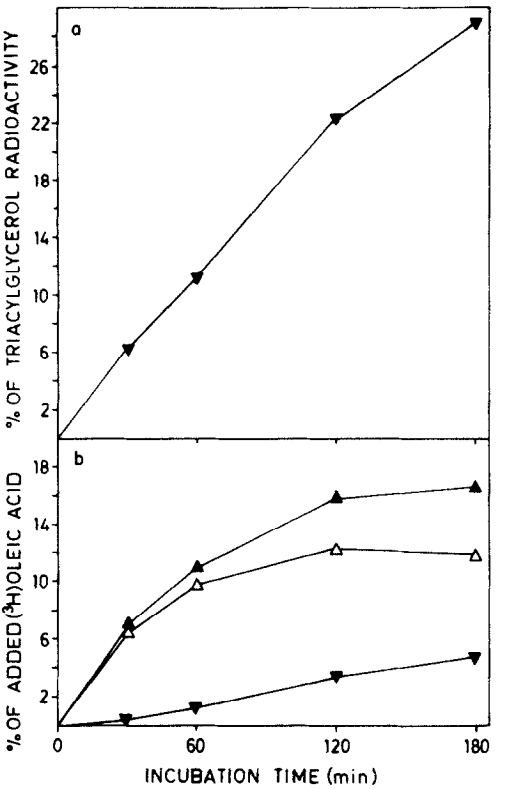


Figure 1 (left). Rat liver parenchymal cells were incubated with [^{3}H]glycerol for the time indicated. The incubation medium contained 10 % rat serum. The figure shows the proportion of total glycerolipid radioactivity in the medium (a) and the total incorporation of added [^{3}H]glycerol into glycerolipids of medium (■—■) and cells + medium (●—●) (b). The results shown are typical of about ten separate experiments.

Figure 2 (right). Rat liver parenchymal cells were incubated with [^{3}H]oleic acid in 2 % albumin in modified Hanks' buffer. The figure shows the proportion of total triacylglycerol radioactivity in the medium (a) and the total triacylglycerol radioactivity in medium (▼—▼), cells (△—△) and cells plus medium (▲—▲) (b).

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without bound oleic acid (6), or 10 % dialyzed rat serum previously heated for 30 min at 56°C was included in the incubation medium. Each incubation contained approx. 5 x 10^6 cells (2.9-5.0 mg protein) and 0.1 µmole [1(3)-^{3}H]glycerol or 0.5 µmole [9,10-$^3H_2$] oleic acid (Radiochemical Centre, Amersham) in a final volume of 1 ml in a 25 ml siliconized Erlenmeyer flask. The flask was shaken (60-80 oscillations per min) at 37°C for 180 min, unless otherwise stated and the incubation was terminated by cooling on ice. Rat plasma (0.1 ml) was added and the cells were sedimented by centrifugation (150 x g for 5 min). An aliquot (3/4) of the supernatant was sucked off for analysis.

TABLE I. Distribution of [^{3}H]glycerol among lipid classes in cells and media.

TG, PE, PC and LPC represent triacylglycerols, phosphatidylethanolamines, phosphatidylcholines and lysophosphatidylcholines, respectively.

Lipid class	Incubation medium							
	Modified Hanks' buffer		+ 2 % albumin and 1 mM oleic acid		+ 10 % serum		In vivo experiment[1]	
	Cells	Medium	Cells	Medium	Cells	Medium	Liver	Serum
	per cent of glycerolipid radioactivity							
TG[2]	50.0	94.9	86.2	93.7	70.2	95.6	48.5	97.7
PE	10.4	1.0	2.2	0.6	5.5	0.4	19.4	0.3
PC	39.4	3.5	11.5	2.3	24.0	3.2	28.2	1.7
LPC	0.2	0.6	0.2	3.4	0.3	0.9	0.8	0.3

[1] Lipid extracts of liver and serum from a male rat killed 45 min after the intraperitoneal injection of [^{3}H]glycerol (1.5 mCi).

[2] Including 1.0-2.0 % diacylglycerols.

Lipid analysis. Lipids were extracted by 6 vol. chloroform-methanol (1:1 v/v). The chloroform phase, obtained by adding to the extract 1 % NaCl (labeled glycerol) or 0.1 M acetate buffer (pH 5.0) (labeled oleic acid), was washed three times with methanol-1 % NaCl (1:1 v/v) saturated with chloroform. Lipids were separated by thin-layer chromatography on silica gel H; developing solvents: light petroleum (b.p. 40-60°C)-diethyl ether-acetic acid (75:25:1 by vol.) for neutral lipids and chloroform-methanol-4 M NH_4OH (60:30:5) for polar lipids.

Analysis by centrifugation. All analyses were performed in a 3 x 5 ml swing-out rotor in an MSE Superspeed 50 TC instrument at 4-6°C. For the flotation of lipid droplets (S_f > 3000) from incubation media, 0.3 ml of the low-speed supernatant (see above) was mixed with 0.1 ml rat serum and 0.1 ml of a suspension of hepatic lipid droplets (7). NaCl, 0.9 % and 1 M sucrose was added to give a final sample volume of 2 ml, 0.2 M in sucrose. The sample was overlayered with 1.5 ml distilled water and centrifuged at 20,000 x g_{av} (15.000 rpm) for 20 min. Three fractions were collected from above (0.5, 0.5 and 2.5 ml) with a syringe equipped with a bent needle. When [^{3}H]glycerol-labeled lipid droplets or rat serum (see below) were analyzed, they were substituted for the corresponding nonlabeled addition.

For the isolation of VLD-lipoproteins 0.2 ml incubation medium was mixed with 0.2 ml rat serum and diluted to 1.2 ml with a solution of KBr (final density = 1.063). The sample was underlaid with 0.5 ml KBr (d = 1.21) and overlayered with 3.2 ml 0.9 % NaCl containing Na_2EDTA (0.1 mg/ml). After centrifugation at

TABLE II. Flotation of lipid droplets from cell homogenates and incubation media after incubation with [^{3}H]glycerol.

Fraction	Incubation medium						
	Modified Hanks' buffer		+ 2 % albumin and 1 mM oleic acid		In vivo experiment		
	CH[1]	M[2]	CH[1]	M[2]	Liver homogenate[3]	Serum	Hepatic droplets
	per cent of glycerolipid radioactivity						
1 (Top)	14.0	0.3	10.9	0.6	12.6	0.3	86.8
2	4.0	4.4	2.2	8.5	-	4.8	3.7
3	82.0	95.3	86.9	90.9	87.4	94.6	9.5

[1]Cell homogenate. The cell pellet was vigorously shaken in a Vortex mixer for 5 min and subjected to recentrifugation (150 x g for 5 min). The supernatant was designated cell homogenate.

[2]Incubation medium.

[3]Total liver homogenate in 0.2 M sucros.

100,000 x g_{av} (34,000 rpm) for 16 hours, the top fraction (1 ml) was collected by tube slicing. The remainder was sucked off in three fractions (1.3 ml each).

Gel electrophoresis. An aliquot of the incubation medium was mixed with an equal vol. of rat plasma containing Na_2EDTA (pH 7.0; 1 mg/ml) and was subjected to agarose gel electrophoresis (8) as described by Johansson (9). After fixation in 5 % trichloroacetic acid five bands were cut out from the gel, corresponding to albumin, α-protein, the region between α- and β-protein, β-protein and the origin, respectively. Lipids were extracted from the gel as described above.

Preparation of [^{3}H]glycerol-labeled hepatic lipid droplets and rat serum. A rat was injected intraperitoneally with 1.5 mC [^{3}H]glycerol and killed 45 min. later. The liver was homogenized in 10 vol. 0.25 M sucrose and lipid droplets were isolated from the homogenate by centrifugation (20000 x g_{av} for 60 min) (Ref. 7). Of the lipid radioactivity in this fraction 98 % was in triacylglycerols, 1.4 % in diacylglycerols and 0.6 % in polar lipids. Blood was collected from the aorta and serum was prepared by centrifugation for 20 min at 3000 rpm. Sodium EDTA (pH 7.0, 1 mg/ml) was added and the serum storec at 2-4°C.

Other methods were as previously described (2).

TABLE III. Flotation of VLD-lipoproteins from incubation media after incubation with [^{3}H]glycerol.

Fraction	Incubation medium (in modified Hanks' buffer)	
	10 % serum	2 % albumin and 1 mM oleic acid
	per cent of triacylglycerol radioactivity	
1 (d < 1.006)	93.1	93.3
2	3.3	1.2
3		2.3
4	3.6	3.2

TABLE IV. Analysis of incubation media by agarose gel electrophoresis. Rat liver parenchymal cells were incubated with [^{3}H]glycerol for 150 min.

Zone	Incubation medium			In vivo experiment
	Modified Hanks' buffer	+ 2 % albumin and 1 mM oleic acid		serum
albumin	8.7[1]	8.6[1]	0.2[2]	6.7[1]
α	5.7	5.6	3.9	2.6
α-β	58.8	68.5	80.6	59.5
β	11.6	8.7	7.8	19.5
origin	15.2	8.6	7.5	11.7

[1]Per cent of total glycerolipid radioactivity.

[2]Per cent of total triacylglycerol radioactivity.

RESULTS AND DISCUSSION

As shown in Figure 1 a sizeable portion of the lipids synthesized from labeled glycerol by the liver cells appeared in the incubation medium. By using a low concentration of glycerol (0.1 mM) maximal incorporation into cellular lipids was reached within 30 min. Experiments with higher amounts of labeled glycerol showed that the rate of glycerolipid synthesis is linear over at least 2-3 hours (3). A comparable release of labeled triacylglycerols were seen after incubation with albuminbound [^{3}H]oleic acid (Fig. 2). Triacylglycerol was the principal labeled glycerolipid released into the medium after incubation with [^{3}H]glycerol (Table I) as well as [^{3}H]oleic acid, in accordance with what is found *in vivo*.

In the experiments with labeled glycerol the proportion of total glycerolipid

TABLE V. Effect of agents and of incubation temperature on glycerolipid secretion from isolated rat liver parenchymal cells.

Rat liver parenchymal cells were incubated with [^{3}H]glycerol and the glycerolipid secretion was determined as the proportion of total glycerolipid radioactivity in the medium. Data from at least two separate experiments are included. The figures shown represent mean percentage ± S.D. of corresponding controls (number of observations). Incubation time was 60-180 min after isotope addition.

Addition	[^{3}H]glycerol in cellular + medium lipids (% of control)	Glycerolipid secretion (% of control)
Cycloheximide[1] (0.035 mM)	102.2 ± 12.8	27.2 ± 4.2 (6)
Colchicine[1] (0.025 mM)	101.5 ± 7.8	22.2 ± 6.9 (4)
Na_2EDTA (3 mM)	145.8 ± 8.7	14.3 ± 3.4 (4)
CaEDTA (1.5 mM) + MgEDTA (1.5 mM)	109.7 ± 14.3	93.9 ± 12.2 (4)
Incubation at + 4°C[2]	105.5 ± 21.7	7.2 ± 4.6 (4)

[1]The cells were preincubated for 60 min in the presence or absence of the inhibitor, prior to [^{3}H]glycerol addition.

[2]Incubation with [^{3}H]glycerol at 37°C for 30 min then at +4°C for 150 min.

radioactivity found in the medium has been used as a measure of the glycerolipid release. This proportion was the same whether the cells were incubated in buffer, 2 % bovine serum albumin or 10 % rat serum. The composition of labeled lipids in the medium was also the same in these media (Table I), except for a certain release of labeled lysophosphatidylcholine in the presence of albumin. Incubation under a gas phase of oxygen did not enhance the release of labeled lipids.

Different approaches were applied to ascertain that the release of labeled lipids was due to secretion of plasma lipoprotein and not to cell disintegration. Centrifugation at 100,000 x g for 90 min of the low-speed supernatant from cell incubations led to the sedimentation of less than 5 % of the labeled lipids, indicating that contamination with labeled cellular membranes was small. This conclusion is further supported by the fact that triacylglycerols were the major labeled lipids in the medium, irrespective of the distribution of [^{3}H]glycerol among cellular lipids (Table I). Another effect of cell disintegration could be the release of triacylglycerol-rich lipid droplets, which occur in the cytoplasm of liver cells and appear as a floating layer after centrifugation of a liver homogenate (7,10). These droplets are larger than the lipoproteins

secreted by liver and therefore flotate more rapidly during centrifugation. A procedure in which the droplets are flotated through a layer of distilled water (7) was adopted for the analysis of incubation media. Under conditions where lipid particles of S_f > 3000 would move into the upper part of the water layer (Table II and Ref. 11) only a negligible fraction of the labeled lipids in incubation media or in serum did so. Disrupting the cells after incubation, on the other hand, released labeled lipid droplets in amounts similar to those in homogenized rat liver labeled with glycerol *in vivo* (Table II). Thus, labeled cell components formed due to cell disintegration do not seem to contribute significantly to the labeled lipids recovered from incubation media.

Analysis of incubation media by density equilibrium centrifugation (Table III) showed that labeled triacylglycerols released from the cells were almost entirely associated with particles of d < 1.006. This fact and the S_f-value of less than 3000 strongly indicate that the particles were VLD-lipoproteins. In accordance with this the majority of them, like plasma VLDL, had pre-β-mobility on agarose gel electrophoresis (Table IV).

Direct evidence that the release of VLDL was due to active secretion was obtained by the use of inhibitory agents (Table V). Cycloheximide inhibits protein synthesis and the secretion of VLD-lipoproteins by the perfused liver, without inhibiting glycerolipid synthesis (12). Neither in the isolated hepatocytes was the incorporation of labeled glycerol into cellular lipids depressed after preincubation with cycloheximide, but the proportion released into the medium was drastically lowered. Colchicine had a similar effect on the release of labeled lipids, in accordance with recent suggestions of a role for the microtubular system in lipoprotein secretion (13,14). Furthermore, the binding of bivalent cations by Na_2EDTA strongly inhibited glycerolipid release. When equimolar amounts of Ca^{2+} and Mg^{2+} were bound to the EDTA prior to addition the inhibition was completely reversed.

The formation and secretion of intact plasma lipoproteins *in vitro* has previously been demonstrated in perfused liver and in liver slices (1), while synthesis of the protein moiety occurs in less complex systems requiring cofactor additions, such as mechanically dispersed rat liver cells (15), rat liver microsomes and isolated ribosomes (16). The present results indicate that plasma VLDL are synthesized and secreted by enzymatically isolated rat liver parenchymal cells. Furthermore, the rate of VLDL secretion, as judged from the fractional release of labeled lipids (Figures 1 and 2), is of the same order as that found in liver perfusion experiments (17-19).

REFERENCES

1. Robinson, D.S. (1970) in Comprehensive Biochemistry (Florkin, M. and Stotz, E.M., eds), Vol.18, pp 51-116, Elsevier, Amsterdam.

2. Nilsson, Å., Sundler, R. and Åkesson, B. (1973) European J. Biochem., in press.
3. Sundler, R., Åkesson, B. and Nilsson, Å., to be published.
4. Berry, M.N. and Friend, D.S. (1969) J. Cell. Biol. 43, 506-520.
5. Michael, S.E. (1961) Biochem. J. 82, 212-218.
6. Spector, A.A. and Hoak,J.C. (1969) Anal. Biochem. 32, 297-302.
7. Bar-on, H., Roheim, P.S., Stein, O. and Stein, Y. (1971) Biochim. Biophys. Acta 248, 1-11.
8. Noble, R.P. (1968) J. Lipid Res. 9, 693-700.
9. Johansson, B.G. (1972) Scand. J. Clin. Lab. Invest. 29, suppl. 124, 7-18.
10. Stein, O. and Stein, Y. (1967) J. Cell. Biol. 33, 319-339.
11. Dole, V.P. and Hamlin, J.T. (1962) Physiol. Rev. 42, 674-701.
12. Bar-on, H., Kook, A.I., Stein, O. and Stein, Y. (1973) Biochim. Biophys. Acta 306, 106-114.
13. Stein, O. and Stein, Y. (1973) Biochim. Biophys. Acta 306, 142-147.
14. Orci, L., LeMarchand, Y., Singh, A., Assimacopoulos-Jaennet, F., Rouiller, C. and Jeanrenaud, B. (1973) Nature 244, 30-32.
15. Capuzzi, D.M. and Margolis, S. (1971) Lipids 6, 609-616.
16. Marsh, J.B. (1969) in Structural and Functional aspects of Lipoproteins in Living Systems (Tria, E. and Scanu, A.M., eds), pp 447-464.
17. Weinstein, I., Dishmon, G. and Heimberg, M. (1966) Biochem. Pharmacol. 15, 851-871.
18. Heimberg, M., van Harken, D.R. and Brown, T.O. (1967) Biochim. Biophys. Acta 137, 435-445.
19. Topping, D.L. and Mayes, P.A. (1972) Biochem. J. 126, 295-311.