Regulation of production and release of lipoprotein by the perfused rat liver

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ABSTRACT The relationship between protein and triglyceride release into d < 1.007 lipoprotein was studied in the isolated perfused rat liver. Livers were perfused with a medium either high or low in linoleate content. Perfusion with the linoleate-rich medium resulted in a marked increase in the net release of both d < 1.007 lipoprotein triglyceride and lipoprotein protein, and caused a significant increase in amino acid incorporation into the protein moiety. Amino acid incorporation into d 1.008–1.21 protein was not affected by fatty acid concentration, while incorporation into whole perfusate and tissue proteins was depressed by a perfusate high in fatty acid content. Electron microscopic studies demonstrated that the livers with the higher rate of triglyceride release also produced a greater number of lipoprotein particles. The particles they released were also somewhat larger.

These studies suggest that the intracellular concentration of newly esterified triglyceride and (or) some other lipid metabolite can specifically influence the release and perhaps the synthesis of d < 1.007 lipoprotein protein. They also suggest that the liver increases its rate of triglyceride release primarily by producing more lipoprotein particles.

KEY WORDS very low density lipoprotein · fatty acid esterification · triglyceride synthesis · protein synthesis · electron microscopy · lipoprotein particles · linoleate

TRIGLYCERIDE IS RELEASED from the liver while complexed to carrier protein(s) (1) and in this form is transported in plasma (2, 3). Numerous investigations have shown that the rate of triglyceride release depends on such factors as the hormonal and nutritional state of the animal (4, 5), the intrahepatic concentration of glycerol phosphate (6–8), and the concentration of fatty acid in the portal vein (K. C. Richards, N. B. Ruderman, and M. G. Herrera, data submitted for publication; references 9, 10). In contrast, little is known about the synthesis and release of the carrier protein(s). For example, it is not known whether alterations in the rate of hepatic triglyceride release are accompanied by parallel alterations in the release of carrier protein, or whether the latter is released at a constant rate and carries a greater or lesser amount of lipid as the situation demands. In the present study, we have investigated these questions using the isolated perfused rat liver. We perfused livers with media containing either high or low concentrations of free fatty acid, in order to produce different rates of triglyceride release. The production of d < 1.007 lipoprotein protein and lipoprotein triglyceride and the incorporation of amino acid-^{14}C into the protein moiety were then determined. In addition, the number and size of the lipoprotein particles produced by the livers were estimated by electron microscopic techniques recently described (1, 11).

MATERIALS AND METHODS

Male Sprague-Dawley rats, weighing between 300 and 400 g and fed Purina laboratory chow ad lib., were anesthetized with intraperitoneal pentobarbital (35 mg/kg of body weight). The bile duct and portal vein were cannulate in situ, and perfusion with oxygenated Krebs–Ringer bicarbonate buffer (KRB) (12) was immediately started. The liver was then removed from the animal and placed in a modified Miller apparatus (13) in which it was cyclically perfused with 100 ml of a cell-free medium composed of KRB and, per 100 ml: 4 g of fat-poor bovine serum albumin (Mann Research Labs Inc., New York), 5 mg of potassium penicillin, 5 mg of streptomycin, 200 mg

Abbreviations: KRB, Krebs–Ringer bicarbonate buffer; TCA, trichloroacetic acid.

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of glucose, 180 mg of Stuart amino acid mixture (Stuart Co., Pasadena, Calif.), and in several experiments 25 μc of a ^{14}C-labeled algal protein hydrolysate (New England Nuclear Corp., Boston, Mass.). The medium was passed through a Millipore filter (Millipore Filter Corp., Bedford, Mass.) with interstices of 0.45 μ immediately before the perfusion.

One series of livers was perfused with a medium which initially contained 120 μmoles of sodium linoleate (K & K Laboratories, Inc., Plainview, L.I., N.Y.). An additional 120 μmoles per hour of the fatty acid was infused into the medium throughout the experiment. A second series had only 40 μmoles of linoleate in the initial medium and KRB instead of fatty acid was added to the perfusate. One liver from each series was perfused simultaneously in the same isothermal box. All perfusions lasted 180 min, during which time the medium was constantly equilibrated with a humidified gas phase of 95% O_2 and 5% CO_2. Liver viability was assessed on the basis of gross appearance, bile production, and perfusion flow rate. None of the livers in this study had to be discarded because of abnormalities indicated by any of these criteria. Additional details of the perfusion procedure have been previously published (14).

Analysis of Perfusate and Tissue

After the perfusion, about 50 ml of media was centrifuged at 2000 *g* for 10 min; this removed any residual red cells that might have been washed off the surface of the liver during the perfusion. Three 10 ml aliquots of the supernatant fluid (solvent density = 1.008 as determined with a Moore–Van Slyke bottle) were then centrifuged at 105,000 *g* for 18–20 hr at 10°C in a Spinco model L ultracentrifuge (No. 40 rotor), and the upper 1.5 ml from each tube was removed by means of a tube slicer. The pooled supernatant fluids were then diluted to 10 ml with 0.15 M NaCl (Solution A).

Amino Acid Incorporation into Lipoprotein Protein ($d < 1.007$)

To 2 ml of the supernatant fluid described above (Solution A) we added 5 ml of rat serum and 3 ml of 0.15 M NaCl. The resultant solution ($d \cong 1.0062$) was ultracentrifuged as described above. 0.5 ml of bovine serum albumin (40 mg/ml) and 2 ml of 10% trichloracetic acid (TCA) were added to the upper 1.5 ml of this mixture. The mixture was then heated at 90–95°C for 30 min. This caused the denatured lipoprotein to precipitate and eliminated the ether-layering step used by Roheim, Miller, and Eder (15) to precipitate TCA-denatured lipoprotein. The heating step was also found necessary to insure the complete precipitation of the albumin carrier, for it has been shown by Levine (16), and confirmed in the present study, that TCA-precipitated albumin is appreciably soluble in lipid solvents such as methanol, ethanol, and acetone unless it is first heated.

The heat-denatured TCA precipitate was washed using a modification of the method of Radding and Steinberg (17). This involved washing three times with 4 ml of ethanol–acetone 1:1, once at 70°C and twice at 25°C, then once with 2 ml of diethyl ether and twice with 2 ml of 10% TCA at 25°C. Analysis of the final ethanol–acetone wash, the ether wash, and the final TCA wash revealed no significant radioactivity. The washed precipitate was dissolved in 2 ml of 1 N NaOH and a portion of this solution was counted in a liquid scintillation counter in a mixture of Cab-O-Sil (Cabot Corporation, Boston, Mass.) and dioxane containing naphthalene, 2,5-diphenyloxazole, and *p*-bis[2-(5-phenyloxazolyl)]benzene.

Determination of $d < 1.007$ Lipoprotein Protein and Triglyceride

To the remaining 8 ml of the original supernatant fluid (Solution A) was added 2 ml of 0.15 M NaCl. This solution ($d = 1.0067$) was then ultracentrifuged as described above. The upper 1.5 ml was removed and a portion of it was dialyzed twice against 1 liter of KRB for 36 hr. This procedure almost totally eliminated free amino acids. The protein concentration of the dialyzed solution was determined (18).

A second portion of the upper 1.5 ml of the ultracentrifugate was extracted in chloroform, the triglyceride was isolated and hydrolyzed (19), and glycerol was then determined enzymatically (20, 21). In later experiments triglyceride was determined according to Carlson (22) after extraction of the solution with chloroform–methanol 2:1.

Amino Acid Incorporation into d 1.008–1.21 Protein

The specific gravity of a portion of the infranatant fluid from the initial ultracentrifugation ($d = 1.008$) was raised to 1.21 by the method described by Havel, Eder, and Bragdon (23). This solution was then ultracentrifuged at 105,000 *g* for 18–20 hr. The upper 1.5 ml was removed and its protein was precipitated with TCA, washed, and counted as described above.

Other Determinations on the Perfusate and Tissue

α-Amino nitrogen was determined by a modification of the technique of Moore and Stein (24). Radioactivity of TCA-precipitated proteins from tissue and uncentrifuged perfusate was determined as described previously (1).

Electron Microscopic Studies

After centrifugation to remove any residual red cells, 3 ml of final medium was fixed in an equal volume of 2% O_sO_4. After 2 hr of fixation, the mixture was centrifuged at 20,000 *g* for 60 min. The pellet obtained in this way

was dehydrated rapidly and embedded in Epon 812 (25). Sections about 1000 A thick were stained with lead and examined with an RCA EMU 3 G electron microscope. The diameters of the particles seen in the pellets were all determined on the same day, shortly after the microscope was calibrated. Particle volume was calculated on the assumption that the particles were perfect spheres. No correction was made for the fact that some particles were probably cut tangentially, since this would have had only a negligible effect on the differences in mean particle diameter and volume (see Table 4).

All statistical analysis are reported as group comparisons unless otherwise noted. *P* values based on paired comparisons are reported only where they differed from values based on group comparisons. In these instances, livers perfused in the same isothermal box on the same day were paired.

RESULTS

To ascertain whether an increase in hepatic triglyceride release is accompanied by a comparable increase in the release of carrier protein, the concentration of the latter was determined in the perfusate of livers with different rates of triglyceride release. The results are shown in Table 1. Perfusion with a linoleate-rich medium resulted in a 2- to 3-fold increase in the net release of both triglyceride and protein into the d < 1.007 fraction of the final medium. The percentage increase in triglyceride appeared to be somewhat greater, although the difference was only statistically significant when calculated as a paired comparison.

Amino acid incorporation into d < 1.007 lipoprotein was also significantly increased in the livers perfused with the higher concentration of fatty acid (Table 2). The specific activities of the lipoprotein proteins were not significantly different, however.

Amino Acid Incorporation into Other Protein Fractions

A general stimulation of amino acid uptake and(or) protein synthesis by fatty acid could theoretically have accounted for the observed difference in amino acid incorporation. Therefore, net amino acid uptake and the incorporation of amino acid into the proteins of tissue and whole perfusate were also assessed (Table 3). As is readily apparent, net amino acid uptake was identical for the two groups of livers. On the other hand, amino acid incorporation into both tissue and whole perfusate protein appeared to be reduced by a high concentration of fatty acid in the medium. A similar result was obtained when amino acid incorporation into whole perfusate protein

TABLE 1 NET RELEASE OF d < 1.007 LIPOPROTEIN PROTEIN AND TRIGLYCERIDE BY THE PERFUSED RAT LIVER

	Total Linoleate Administered (μmoles)		
	40 (n = 9)	480 (n = 9)	*P*
Triglyceride release (μmoles/10 ml of medium)	1.24 ± 0.17	3.34 ± 0.38	<0.001
Protein release (μg/10 ml of medium)	219 ± 38	435 ± 35	<0.001
Triglyceride/protein (10^3 × μmoles/μg)	5.92 ± 0.45	7.76 ± 0.85	0.1 > *P* > 0.05*

All determinations were performed on d < 1.007 fractions of the 180-min perfusate and represent the mean ± SEM. There was no detectable triglyceride or protein in this fraction in the initial medium.

* *P* < 0.02 when calculated as a paired comparison (see Methods).

TABLE 2 AMINO ACID-^{14}C INCORPORATION INTO d < 1.007 LIPOPROTEIN (LP) PROTEIN

	Total Linoleate Administered (μmoles)		
	40	480	*P*
Label incorporation into LP protein (dpm/10 ml of medium)	9,193 ± 1,381 (*n* = 6)	13,758 ± 1,353 (n = 6)	<0.05*
LP protein release (μg/10 ml of medium)	227 ± 37 (n = 5)	452 ± 62 (n = 5)	<0.02
Specific activity LP protein (dpm/μg)	36 ± 2 (n = 5)	30 ± 4 (n = 5)	NS

See footnote to Table 1. Values are means ± SEM.

* *P* < 0.005 when calculated as a paired comparison (see Methods).

TABLE 3 EFFECT OF LINOLEATE ADMINISTRATION ON NET AMINO ACID (AA) UPTAKE AND ON AMINO ACID-^{14}C INCORPORATION INTO VARIOUS PROTEINS

	Total Linoleate Administered (μmoles)		
	40	480	*P*
Net AA uptake (μmoles/10 ml of medium)	32 ± 2 (n = 5)	32 ± 1 (n = 5)	NS
Label incorporated into tissue protein (10^{-3} × dpm/g of liver)	954 ± 64 (n = 6)	820 ± 77 (n = 6)	NS*
Label incorporated into total perfusate protein (10^{-3} × dpm/10 ml of medium)	564 ± 22 (n = 6)	508 ± 168 (n = 6)	NS
Label incorporated into d 1.008–1.21 lipoprotein protein (10^{-3} × dpm/10 ml of medium)	6.6 ± 0.3 (n = 5)	6.2 ± 1.1 (n = 5)	NS

All determinations were performed on fractions of perfusate or tissue obtained after 180 min of perfusion and represent the mean ± SEM.

* When calculated as a paired comparison $P < 0.05$.

was determined after 90 min of perfusion (data not shown).

In order to determine whether a shift in the disposition of newly synthesized labeled protein (26) might have accounted for the apparent stimulation of d < 1.007 protein synthesis observed in this study, we also measured the incorporation of amino acids into d 1.008–1.21 protein. The results, which are presented in the last line of Table 3, argue against this possibility since amino acid incorporation into this fraction was essentially the same in the two groups. However, the possibility that a protein normally present in the d < 1.21 fraction was incorporated into VLDL could not be ruled out.

Electron Microscopic Studies

Previous studies in which rat livers were perfused with fatty acid have shown that fixation of a portion of final medium with OsO_4 usually yields particles which precipitate as a pellet upon centrifugation (1, 11). Electron microscopic examination of this pellet has revealed a homogeneous population of osmiophilic particles (300–800 A in diameter) which in all likelihood are very low density lipoproteins. In addition, it has been demonstrated that the size of the pellet varies with the amount of lipoprotein released and is not influenced by perfusate free fatty acid concentration per se (A. L. Jones and N. B. Ruderman, unpublished data). In the present investigation, the pellet was examined grossly and microscopically in an attempt to determine whether the increase in triglyceride release is associated with an increase in the number or size of the lipoprotein particles. The findings are presented in Figs. 1–3 and Table 4.

The pellets obtained from the perfusates containing the high concentration of linoleate were obviously larger than those obtained from the perfusate containing the low concentrations of linoleate. Although precise quantification was not possible, the difference in pellet size as visually judged was at least twofold (Fig. 1).

The particles in both pellets were for the most part of the same diameter, although a higher percentage of particles with diameters above 1000 A was found in the group perfused with the high concentration of linoleate (Figs. 2 and 3, Table 4). The mean diameter of the particles was significantly larger in this group; however, this difference in particle size was not sufficient to account for the great difference in pellet volume. If one calculates the volume of 100 particles from each group using the formula

$$V = \int_{r=300}^{r=\infty} 4/3\ \pi r^3 n$$

where r = the radius of the particle in Angstrom units and n = the number of particles with that radius, values of 18.6 ± 10^9 A^3 and 24.5 ± 10^9 A^3 are obtained for particles from the livers perfused with fatty

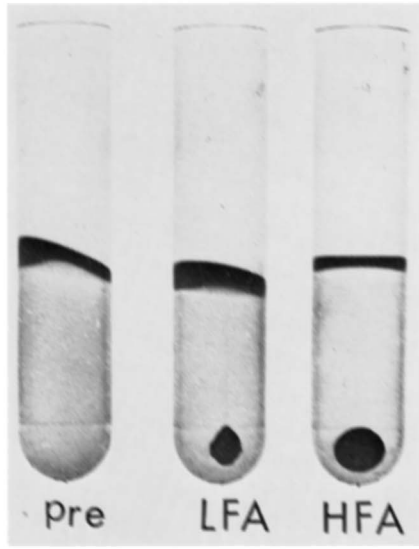


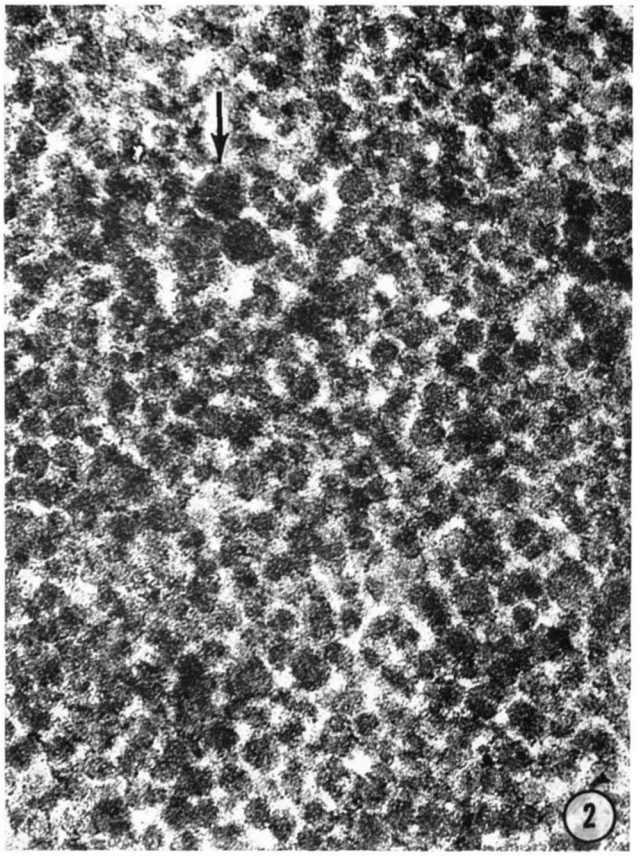
FIG. 1. Lipoprotein pellets, isolated from 5 ml of perfusate, fixed in osmium tetroxide and embedded in Epon (Luft). The horizontal dark band is an artifact due to light refracted at the surface of the embedding medium. pre, initial medium which contained 120 μmoles of linoleate; LFA, 180-min perfusate of livers that received 40 μmoles of linoleate; HFA, 180-min perfusate of livers that received 480 μmoles of linoleate.

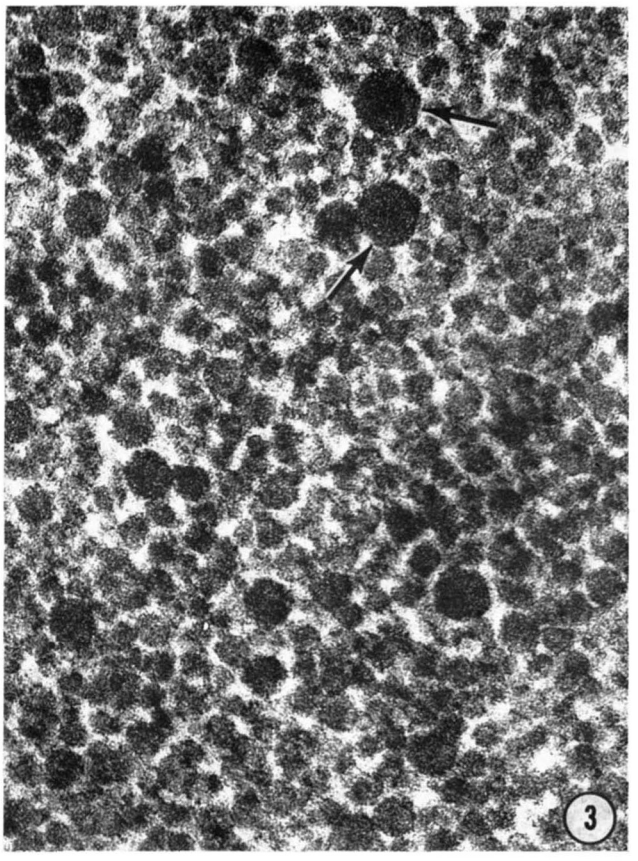
acid-poor and fatty acid-rich media respectively. Since this represents a difference of only about 30% and there was at least 100% difference in pellet volume, it is evident that there were many more particles in the pellet obtained from the linoleate-rich media.

DISCUSSION

The foregoing results show that when the liver increases its rate of triglyceride release, it also increases the release of the latter's carrier protein.The amino acid incorporation data indicate that the additional carrier protein is composed, at least in part, of newly synthesized peptide. This suggests that the enhanced release of d < 1.007 protein might have been secondary to a stimulation of its de novo synthesis. On the other hand, this does not rule out the possibility that the increment in carrier protein release resulted from the stabilization of some unstable peptide precursor of the lipoprotein protein, or from the incorporation into the d < 1.007 fraction of protein that ordinarily would have had another fate. In regard to this latter possibility, Roheim et al. (15) have recently demonstrated the presence of a circulating apolipoprotein in the d > 1.21 fraction of rat serum which under appropriate circumstances could be incorporated into d < 1.019 lipoprotein by the perfused rat liver.

The morphologic data indicate that the liver increased its rate of triglyceride release primarily by producing more lipoprotein particles. This is borne out by the present finding that lipoprotein protein and triglyceride





FIGS. 2 and 3. Electron micrographs of the OsO_4-fixed lipoprotein pellets. × 65,000. FIG. 2. Lipoprotein particles from the perfusate of livers that received 40 μmoles of linoleate (LFA). The diameter of the largest particle (arrow) is 1230 A. FIG. 3. Lipoprotein particles from the perfusate of livers that received 480 mμ of linoleate (HFA). The diameter of the largest particles (arrows) is 1350 A.

release increased in parallel. The observation that the particles produced by the livers receiving the more concentrated fatty acid medium were also slightly larger suggests that these livers may have further increased triglyceride release by incorporating more lipid into the individual particles. This is also suggested by the somewhat higher triglyceride–protein ratio in their $d < 1.007$ fraction.

There have been relatively few reports in the literature that bear directly on the present study. Haft and his collaborators (27) failed to observe alterations in amino acid incorporation into $d < 1.063$ protein when they perfused isolated livers from rats fed a diet high in cholesterol and olive oil. On the other hand, when they perfused livers from normally-fed rats with heparinized blood from the animals fed cholesterol and olive oil, they observed a more than twofold increase in both the net production of $d < 1.063$ protein and in amino acid-^{14}C incorporation into this fraction. They did not present an explanation for these findings; however, since the sera of the animals fed cholesterol and olive oil were probably high in triglyceride, it is probable that heparin induced lipolysis and thereby subjected the livers to high concentrations of free fatty acid similar to those in the present study. It is of interest that in later experiments these workers (28) demonstrated that livers from rats fed cholesterol and olive oil have an increased rate of triglyceride release.

Radding and Steinberg (17), working with rat liver slices, observed no alteration in amino acid incorporation into the protein moieties of a mixture of lipoproteins floated at $d < 1.21$ when the donor animals had been pretreated with Triton WR-1339, which increased net hepatic cholesterol and triglyceride release (3), or had been fed a 1% cholesterol diet to diminish hepatic cholesterol synthesis. In contrast, Merrill, Roels, and Lacy (29), studying the incorporation of leucine-^{14}C into lipoprotein by intact rats, reported that the administration of Triton WR-1339 resulted in a tenfold increase in the specific activity of $d < 1.020$ protein. On the basis of this finding, they suggested that the incorporation of leucine may have been increased in response to a demand for triglyceride transport. The reason for the apparently different effect of Triton WR-1339 in the hands of these investigators and Radding and Steinberg is not clear. It may have been related to the difference in the density fraction studied, or to the fact that one study was done in

TABLE 4 SIZE DISTRIBUTION OF PARTICLES IN LIPOPROTEIN PELLETS

Particle Diameter	Total Linoleate Administered	
	40 μmoles	480 μmoles*
A	*No. of particles*	
450	7	5
600	35	24
750	50	49
900	6	14
1050	1	4
1200	1	1
1350	0	3
Mean particle diameter (A) ± SEM	693 ± 10	754 ± 16†
Mean volume of 100 particles (A^3)	18.6×10^9	24.5×10^9

For both groups of livers the diameters of 100 particles were estimated in two randomly chosen areas from the center of the pellet. Two pellets in each group were studied. Particle diameter was estimated to the nearest 150 A.

* 120 μmoles added to initial medium plus 120 μmoles/hr by infusion.

† The significance of the difference in mean particle diameter was $P < 0.005$.

vivo and the other in vitro. Finally, Wilcox, Fried, and Heimberg (30) measured lysine incorporation into lipoprotein protein by the perfused rat liver. Total amino acid incorporation was not determined, however; hence, their findings are not comparable to those of the present study.

It is well known that substrates and hormones can regulate the synthesis or degradation of enzyme protein (31–34). The idea that triglyceride or some other lipid metabolite can perhaps regulate the production or release of its carrier protein, however, is a relatively novel one. Several substances including iron (35), protoporphyrin (36), and hemin (37–39) have been shown to stimulate specifically the incorporation of amino acids into the globin moiety of hemoglobin. In addition, it has been reported that the intraperitoneal administration of iron enhances leucine incorporation into ferritin in intact rats (40). It has been suggested that these effects are due to a stimulation of protein synthesis at the ribosome level (39) or a facilitation of protein release from the ribosome (33). In the case of ferritin, iron may have both stabilized an unstable precursor of ferritin and retarded the degradation of the completed ferritin molecule (40). Whatever the mechanism, these studies all suggest that the phenomenon of a nonpeptide substance influencing the production of its carrier protein may be a general occurrence.

On the basis of the present study, it appears that the intracellular concentration of newly esterified triglyceride and(or) some other lipid metabolite may be an important determinant of lipoprotein protein release and possibly synthesis. The importance of this regulatory mechanism in normal lipid physiology and in certain disturbances of lipid metabolism remains to be established.

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REFERENCES

1. Jones, A. L., N. B. Ruderman, and M. G. Herrera. 1967. *J. Lipid Res.* **8:** 429.
2. Lindgren, F. T., and A. V. Nichols. 1960. *In* The Plasma Proteins. F. W. Putnam, editor. Academic Press, New York. **2:** 1–58.
3. Scanu, A. M. 1965. *Advan. Lipid Res.* **3:** 63.
4. Morris, B. 1963. *J. Physiol.*, **168:** 564.
5. Mayes, P. A., and J. M. Felts. 1966. Symposium on Experimental Study of the Effect of Drugs on the Liver. Excerpta Medica Foundation. Int. Cong. Series. **115:** 16.
6. Fritz, I. B. 1961. *Physiol. Rev.* **41:** 52.
7. Wieland, O., and F. Matschinsky. 1962. *Life Sci.* **1:** 49.
8. Tzur, R., E. Tal, and B. Shapiro. 1964. *Biochim. Biophys. Acta* **84:** 18.
9. Nestel, P. J., and D. Steinberg. 1963. *J. Lipid Res.* **4:** 461.
10. Heimberg, M., I. Weinstein, G. Dishmon, and M. Fried. 1965. *Am. J. Physiol.* **209:** 1053.
11. Jones, A. L., N. B. Ruderman, and M. G. Herrera. 1966. *Proc. Soc. Exptl. Biol. Med.* **123:** 4.
12. Krebs, H. A., and K. Henseleit. 1933. *Z. Physiol. Chem.* **210:** 33.
13. Miller, L. L., C. G. Bly, M. L. Watson, and W. F. Bale. 1951. *J. Exptl. Med.* **94:** 431.
14. Williamson, J. R., A. Garcia, A. E. Renold, and G. F. Cahill, Jr. 1966. *Diabetes.* **15:** 183.
15. Roheim, P. S., L. Miller, and H. A. Eder. 1965. *J. Biol. Chem.* **240:** 2994.
16. Levine, S. 1954. *Arch. Biochem. Biophys.* **50:** 515.
17. Radding, C. M., and D. Steinberg. 1960. *J. Clin. Invest.* **39:** 1560.
18. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. *J. Biol. Chem.* **193:** 265.
19. Jagannathan, S. N. 1964. *Can. J. Biochem.* **42:** 566.
20. Bergmeyer, H. U., G. Holz, E. M. Kauder, H. Mollering, and O. Wieland. 1961. *Biochem. Z.* **333:** 471.
21. Vaughan, M. 1962. *J. Biol. Chem.* **237:** 3354.
22. Carlson, L. A. 1963. *J. Atherosclerosis Res.* **3:** 334.
23. Havel, R. J., H. A. Eder, and J. H. Bragdon. 1955. *J. Clin. Invest.* **34:** 1345.
24. Moore, S., and W. H. Stein. 1954. *J. Biol. Chem.* **211:** 907.
25. Luft, J. H. 1961. *J. Biophys. Biochem. Cytol.* **9:** 409.
26. Levy, R. I., R. S. Lees, and D. S. Fredrickson. 1966. *J. Clin. Invest.* **45:** 63.
27. Haft, D. E., P. S. Roheim, A. White, and H. A. Eder. 1962. *J. Clin. Invest.* **41:** 842.

28. Roheim, P. S., D. E. Haft, L. I. Gidez, A. White, and H. A. Eder. 1963. *J. Clin. Invest.* **42:** 1277.
29. Merrill, J. M., O. Roels, and W. Lacy. 1966. *Federation Proc.* **25:** 210.
30. Wilcox, H. G., M. Fried, and M. Heimberg. 1965. *Biochim. Biophys. Acta.* **106:** 598.
31. Cohen, N. R. 1966. *Biol. Rev.* **41:** 503.
32. Kenney, F. T. 1962. *J. Biol. Chem.* **237:** 1610.
33. Feigelson, P., and O. Greengard. 1962. *J. Biol. Chem.* **237:** 3714.
34. Schimke, R. T., E. W. Sweeney, and C. M. Berlin. 1965. *J. Biol. Chem.* **240:** 322.
35. Kruh, J., and H. Borsook. 1956. *J. Biol. Chem.* **220:** 905.
36. Gribble, T. J., and H. C. Schwartz. 1965. *Biochim. Biophys. Acta.* **103:** 333.
37. Hammel, C. L., and S. P. Bessman. 1964. *J. Biol. Chem.* **239:** 2228.
38. Bruns, G. P., and I. M. London. 1965. *Biochem. Biophys. Res. Commun.* **18:** 236.
39. Grayzel, A. I., P. Hörchner, and I. M. London. 1966. *Proc. Natl. Acad. Sci. U. S.* **55:** 650.
40. Drysdale, J. W., and H. N. Munro. 1966. *J. Biol. Chem.* **241:** 3630.