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## Lymph Chylomicron Formation during the Inhibition of Protein Synthesis. STUDIES OF CHYLOMICRON APOPROTEINS

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#### Research Article

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Because we had previously demonstrated a markedly increased lymph chylomicron size during the inhibition of protein synthesis, it was first necessary to determine whether chylomicron apoprotein composition normally varied with chylomicron size. Chylomicrons of varying sizes were prepared by differential ultracentrifugation, and their apoprotein composition was determined densitometrically on SDS polyacrylamide gels. No significant difference in apoprotein composition was found normally with varying chylomicron size.

In contrast, however, chylomicrons from ACH-treated animals showed a 50% decrease in a major apoprotein band with  $R_1$  0.67. Other chylomicron apoproteins were not decreased as a result of impaired protein synthesis, suggesting differing rates of synthesis of the various chylomicron apoproteins. In vivo incorporation studies of [<sup>3</sup>H]leucine into the various apoproteins of lymph chylomicrons demonstrated that this apoprotein ( $R_1$  0.67) had the most rapid synthesis rate and suggested that it seemed most affected by impaired intestinal protein synthesis. Immunologic studies indicated that this [...]



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# Lymph Chylomicron Formation during the Inhibition of Protein Synthesis

#### STUDIES OF CHYLOMICRON APOPROTEINS

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ABSTRACT The effect of impaired intestinal protein synthesis on chylomicron apoprotein composition was studied in mesenteric lymph fistula rats. Lymph was obtained from animals with impaired protein synthesis given intraperitoneal acetoxycycloheximide (ACH), a potent inhibitor of protein synthesis. Lymph chylomicrons were then isolated by ultracentrifugation and purified on agarose columns. Purified chylomicrons from control and ACH-treated animals were delipidated, and their apoprotein pattern was examined on sodium dodecyl sulfate (SDS) polyacrylamide gels.

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These studies demonstrate that impaired intestinal protein synthesis is associated with a deficiency in one of the major chylomicron apoproteins and may in part explain the impaired lipid absorption seen during states of impaired protein synthesis.

#### INTRODUCTION

In recent years the intestine has been increasingly appreciated as an active site of lipoprotein synthesis (1-3). When one considers the large quantities of both endogenous and exogenous lipid that can only leave the intestinal cell in combination with specific apoproteins, an impairment in intestinal protein synthesis might be expected to produce a decrease of lipoprotein synthesis and fat absorption. Indeed, the disease a-betalipoproteinemia seems to underscore the importance of specific apolipoprotein synthesis in lipid absorption, in that a defect in  $\beta$ -apoprotein formation is associated with a deranged ability of lipid to leave the intestinal cell either in the form of chylomicrons or very low density lipoproteins (VLDL) (4, 5).<sup>1</sup>

In an attempt to elucidate further the relationship between protein synthesis and lipid absorption, the use of inhibitors of protein synthesis was proposed by Sabesin and Isselbacher (6) and appeared to result in impaired intestinal lipid absorption in the rat. These in-

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<sup>&</sup>lt;sup>1</sup>Abbreviations used in this paper: ACH, acetoxycycloheximide; HDL, high-density lipoprotein; SDS, sodium dodecylsulfate; VLDL, very low-density lipoprotein.

vestigators proposed that protein synthesis, specifically  $\beta$ -lipoprotein, was required for normal fat transport out of the intestine. However, this hypothesis has been questioned by others (7, 8) who found lipid absorption into lymph with impaired protein synthesis to proceed at almost normal rates and attributed decreased absorption to decreases in lymph flow produced by the toxic action of the inhibitor. In an attempt to resolve these conflicting results and to determine whether protein synthesis is required or important for chylomicron formation, we examined the effect of the inhibition of protein synthesis on lymph chylomicron size (9). It was reasoned that if there was a decrease in lipoprotein synthesis during inhibited protein synthesis, this might be reflected in an increased size of chylomicrons in lymph. An increase in chylomicron size would permit the transport of more triglyceride in fewer but larger particles and thereby conserve surface apoproteins and phospholipids. Since there was in fact a dramatic increase in lymph chylomicron size during the inhibition of protein synthesis, it was suggested that protein synthesis is important in the formation and transport of chylomicrons from intestine into lymph (9).

In the present report it is shown that significant changes in the apoprotein composition of lymph chylomicrons also occur during experimental protein deficiency. Thus the present studies together with the previous observations offer direct support for the importance of protein synthesis in chylomicron formation.

#### **METHODS**

Operative technique. Cannulation of the main mesenteric lymphatic duct and duodenum and maintenance of the animals postoperatively have been described previously (9). All animals were studied 16-24 h after surgery. Lipid was administered as a micellar solution of oleic acid and monolein in 20 mM sodium taurocholate solution, pH 7.2 (9). The resultant micellar solutions were completely clear. 5 ml of the above micellar solution was infused intraduodenally over a 20-min interval to control and lymph fistula animals with impaired protein synthesis. Lymph was collected over a 3-h period without anticoagulants and defibrinated before ultracentrifugation.

Inhibition of protein synthesis. Acetoxycycloheximide (ACH) was administered intraperitoneally 1 h before lipid infusion in a 0.9% saline solution at a dose of 0.25 mg/kg. This dose level and schedule was previously shown by us to inhibit L-[<sup>14</sup>C]leucine incorporation into intestinal mucosal protein by at least 80% for at least 3 h (10) and to decrease the absorption of [<sup>14</sup>C]oleic acid-mixed micelles by approximately 33% (9).

Isolation and purification of chylomicrons. Defibrinated lymph, layered beneath 0.15 M NaCl in cellulose nitrate tubes, was centrifuged at  $3 \times 10^{\circ}$  g-min in a Beckman SW39 or SW41 swinging bucket rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). The lipoprotein particles that rose to the top were separated from the subnatant by means of a tube slicer and were designated chylomicrons (11). Further purification of chylomicrons was carried out by either further washing and

| TABLE I                                      |
|--|
| Triglyceride/Protein Ratios of Subfractions; |
| Mesenteric Lymph Chylomicrons                |

| Particle              | Centrifugation   | Sť*        | Triglyceride/<br>Protein<br>Ratio |  |
|-----------------------|------------------|------------|-----------------------------------|--|
|                       | g min            |            |                                   |  |
| Rat lymph             |                  |            |                                   |  |
| Chylomicrons (parent) | $3.0	imes10^6$   | >400       | 204                               |  |
| А                     | $4.1	imes10^4$   | > 5,000    | 297                               |  |
| В                     | $8.2 	imes 10^4$ | ~3,000     | 291                               |  |
| С                     | $5.5	imes10^{5}$ | $\sim 600$ | 228                               |  |
| D                     | $3.0	imes10^6$   | $\sim 400$ | 101                               |  |
| VLDL                  | $1.0	imes10^{8}$ | $\sim$ 75  | 9                                 |  |

\* Subfractions of chylomicrons were prepared from a parent  $S_f > 400$  fraction (Methods) and triglyceride and protein values were determined.  $S_f$  values are based on theoretical predictions (25).

recentrifugation or by agarose column chromatography (12). Disposable columns  $0.7 \times 30.0$  cm were packed with Bio-Gel A-50 m (100-200 mesh) from Bio-Rad Laboratories, Richmond, Calif., and eluted with 0.15 M NaCl. Chylomicrons were recovered in the void volume. Usually one passage through the column was sufficient to remove significant contamination with serum proteins from the chylomicrons (see below). Chylomicron subfractions were prepared from a parent  $S_t > 400$  fraction by differential ultracentrifugation. "Parent" chylomicrons were centrifuged in a SW41 rotor at successively higher speeds (Table I) and the topmost layer of particles was removed from the subnatant with a tube slicer (approximately 0.5 ml). The subnatant was then recentrifuged at the next higher speed and the procedure repeated. As indicated in Table I, the final spin was sufficient to float all remaining lipoprotein particles from the subnatant. Triglyceride was determined on each chylomicron fraction by the enzymatic method of Eggstein and Kreutz (13). Protein was determined by the method of Lowry, Rosebrough, Farr, and Randall (14). Turbidity due to lipid was extracted with diethyl ether after color development was complete (15).

Immunologic studies. Purified chylomicrons were subjected to double diffusion and immunoelectrophoresis in 0.8% agarose in barbital buffer, pH 8.2, against anti-rat sera or anti-rat albumin. Precipitin arcs were allowed to develop up to 1 wk at room temperature and the plates were washed, dried, and stained with amido black (16). They were considered "free" of contamination when there were no detectable nonlipoprotein arcs. While it is possible that small amounts of contaminating serum proteins were tightly adsorbed to the surface of the chylomicrons and were not detected by the above methods, the constancy of apoprotein composition (see below) indicated that a constant degree of purification was achieved by the above procedures.

Polyacrylamide gel electrophoresis. Purified lipoprotein fractions were totally delipidated with ethanol: ether 3:2according to the method of Brown, Levy, and Fredrickson (17). The precipitates were centrifuged for 1 h at 1500 rpm, dried under nitrogen, and dissolved in 0.2 M Tris buffer, pH 7.2, containing 0.1 M decyl sulfate, and incubated at  $37^{\circ}$  for 30 min to completely solubilize all pro-

tein. The solubilized apoproteins were then subjected to polyacrylamide gel electrophoresis either in sodium dodecylsulfate (SDS) or urea. SDS polyacrylamide electrophoresis was carried out in 5.6% polyacrylamide gels and stained for protein with Coomassie blue as described by Fairbanks, Steck, and Wallach (18). Electrophoresis was conducted at 37°C with an initial current of 2 mA/gel for 5 min and 4 mA/gel for the remainder of the run. Polyacrylamide electrophoresis in 6 M urea was carried out in 9% gels at pH 8.3 as described by Davis (19). Samples were applied in sucrose without use of a sample gel.

Turnover studies of individual chylomicron apoproteins. After a constant duodenal micellar lipid infusion had been established, a single dose of 200 µCi [3H]leucine was administered intraperitoneally to lymph fistula rats. Lymph was collected over the indicated time intervals, and the chylomicrons were purified, delipidated, and electrophoresed on SDS polyacrylamide gels. Radioactivity incorporated into various proteins on polyacrylamide gels was determined by slicing a gel into 1-mm slices with a lateral gel slicer. Individual slices were related to specific protein bands by comparison with duplicate gels that were stained and densitometrically scanned. Radioactivity in individual slices was determined by dissolving each slice in NCS (Amersham/Searle Corp., Arlington Heights, Ill.), as described by Ward, Wilson, and Gilliam (20), and counting in a toluene-based scintillation fluid (160 ml Liquiflor per gallon toluene). Since quenching was similar in all samples, no correction was necessary. The efficiency of counting was approximately 25%. Recovery of applied counts was 95%.

Where indicated, individual slices of unfixed, unstained polyacrylamide gel representing specific protein bands were imbedded in warm agar and subjected to double diffusion against the appropriate antisera.

Densitometry. Densitometric scans of Coomassie bluestained gels were carried out at 550 nm with a Gilford linear gel scanner (Gilford Instrument Laboratories, Inc., Oberlin, Ohio). Peak areas were determined directly from the densitometric scan by planimetry (Filotecnica Salmoiraghi S.P.A., Milano, Italy). Each measurement was carried out in duplicate, with error of serial measurements as well as of duplicate gels of the same sample being less than 5%. When varying amounts of protein (delipidated chylomicron apoproteins) were applied to a series of gels, densitometry showed that over the range of applied protein (5-15  $\mu$ g) each band retained its relationship (percent of total) indicating the linearity of the densitometric methods employed (see below).

Preparation of nascent chylomicrons. Chylomicrons were isolated from the intestinal mucosa of rats fed corn oil by stomach tube 2 h before sacrifice. Isolated intestinal epithelial cells were prepared according to the method of Weiser (21). Pooled cells from three animals were suspended in mannitol-EDTA-phosphate buffer (22) and sonicated in a Polytron (Kinematica GMBH, Lucerne, Switzerland) for 20 s (setting #6). Almost all cells were disrupted when checked by phase microscopy. Lipoproteins of  $S_t > 400$  were floated up as previously described, separated by tube slicer from the subnatant, and purified on agarose columns. They were then delipidated as described and used for electrophoretic and immunologic studies.

*Electron microscopy.* Chylomicon subfractions were examined by negative staining with 2% phosphotungstic acid (23) under a Philips Model 200 electron microscope (Philips Electronic Instruments, Mount Vernon, N. Y.).

Materials. Male Sprague-Dawley rats (200-250 g) were purchased from Holtzman Co., Madison, Wis. L-[4,5-<sup>3</sup>H]-

leucine was obtained from New England Nuclear, Boston, Mass. Oleic acid, monolein, and taurocholate were obtained from Calbiochem, San Diego, Calif., and found to be chromatographically pure as described earlier (9). Reagents and enzymes for triglyceride determinations were purchased in kit form from Boehringer Mannheim Corp., New York. Antisera against rat serum and rat albumin, prepared in rabbits, were purchased from Behring Diagnostics, Inc., Woodbury, N. Y., and Nutritional Biochemicals Corp., Cleveland, Ohio. Rat antichylomicron antisera were prepared in rabbits by immunization with purified mesenteric chylomicrons emulsified in complete Freund's adjuvant and injected into the footpads of New Zealand rabbits. Antisera were checked by immunoelectrophoresis and found to contain anti-A and -B activities as described by Ockner, Bloch, and Isselbacher (24). Anti-rat serum high- density lipoprotein (HDL) antiserum was generously supplied by Dr. G. Schonfeld and was found to be monospecific when tested against rat serum. All other chemicals were obtained from standard sources and were purest grades commercially available. ACH was generally supplied by the John L. Smith Memorial for Cancer Research, Chas. Pfizer & Co., Inc., New York.

#### RESULTS

Apoprotein composition of subfractions of control chylomicrons. As indicated above, previous results from this laboratory (9) have demonstrated an increase in lymph chylomicron size after the inhibition of protein synthesis. In order to determine whether these large chylomicrons are deficient in any particular apoprotein, it was first necessary to determine whether the apoprotein composition of mesenteric lymph chylomicrons normally differs as a function of size.

Rat mesenteric lymph chylomicrons of  $S_r > 400$ were purified as described in Methods and then subfractionated by differential ultracentrifugation. The conditions of centrifugation are shown in Table I with the theoretical  $S_r$  values predicted from the Dole and Hamlin normogram (25). Also shown are triglyceride protein ratios for each subfraction indicating that a degree of sizing had been achieved. Further confirmation of varying subfractions within the  $S_r > 400$ class of chylomicrons was provided by direct visualization with negative staining (Fig. 1). While there was some heterogeneity of size, it was evident that significant sizing had been achieved.

The above subfractions were delipidated and subjected to polyacrylamide gel electrophoresis in SDS (Fig. 2) (see Methods). Qualitatively the gel patterns of each subfraction were found to be quite similar. Quantitation of the individual chylomicron aproproteins within each subfraction was obtained by densitometric scanning and planimetric meaurements of SDS polyacrylamide gels (Table II). Since in many chylomicron preparations the band at  $R_t$  0.59 was not clearly separated from the major band at  $R_t$  0.67 and this former band amounted to less than 5% of the total protein, it has been combined with the  $R_t$  0.67 band 5,000 Å



FIGURE 1 Electron microscopy of mesenteric lymph chylomicron subfractions. Samples of the indicated chylomicron subfractions and VLDL were examined after negative staining with 2% phosphotungstic acid (Methods). A and D are subfractions (Table I).

for purposes of calculation (Table II). It can be seen that within the several subgroups of  $S_f > 400$  class, the distribution of the individual chylomicron apoproteins was quite similar. Thus there appeared to be no major differences in chylomicron apoprotein composition as a function of size within the chylomicron class. The reproducibility of densitometric determinations on stained acrylamide gels was verified by less than 5% variation between duplicate gels. The distribution of the apoproteins remained essentially constant despite



FIGURE 2 SDS acrylamide gels of chylomicron subfractions. Chylomicron subfractions (Table I) were delipidated and electrophoresed on SDS acrylamide gels (Methods). O, parent; A-D, subfractions (Table I).

varying amounts of protein applied to the gel  $(5-15 \ \mu g)$ . While the chromogenicity of the various protein bands may vary, the fact that each band retains its distribution (Table III) over the range of applied pro-

TABLE II Distribution of Apoproteins in Mesenteric Chylomicron Subfractions

| Protein Distribution |  |  |   |  |  |  |
|----------------------|--|--|---|--|--|--|
| Parent*              | >5000  | Sf<br>~3000  | ~600  | ~400   |  |  |
|                      |  | % total  |   |  |  |  |
| 6.1                  | 6.6  | 5.7  | 6.1   | 8.0  |  |  |
| 2.8                  | 3.8  | 0.7  | +   | 1.9  |  |  |
| 5.0                  | 6.1  | 5.2  | 3.7   | 3.0  |  |  |
| 13.5                 | 14.2   | 14.5   | 13.7  | 11.0   |  |  |
| 13.5                 | 11.1   | 10.2   | 9.6   | 15.6   |  |  |
| 39.3                 | 38.7   | 40.5   | 43.9  | 42.1   |  |  |
| 22.6                 | 22.4   | 23.7   | 22.9  | 19.4   |  |  |
|                      | Parent*<br>6.1<br>2.8<br>5.0<br>13.5<br>13.5<br>39.3<br>22.6 | Parent* > 5000   6.1 6.6   2.8 3.8   5.0 6.1   13.5 14.2   13.5 11.1   39.3 38.7   22.6 22.4 | Protein DistributiParent*>5000 $\stackrel{Sf}{\sim3000}$ % total6.16.65.72.83.80.75.06.15.213.514.214.513.511.110.239.338.740.522.622.423.7 | Protein Distribution   Parent* >5000 St<br>~3000 ~600 $\frac{\%}{\sim}$ total 6.1 6.6 5.7 6.1   2.8 3.8 0.7 +   5.0 6.1 5.2 3.7   13.5 14.2 14.5 13.7   13.5 11.1 10.2 9.6   39.3 38.7 40.5 43.9   22.6 22.4 23.7 22.9 |  |  |

The distribution of chylomicron apoproteins was determined in the various chylomicron subfractions by planimetric measurements of SDS acrylamide gels shown in Fig. 2. Duplicate gels were run for each subfraction with a variability of less than 5% (Methods). Repeat subfractions of another chylomicron preparation gave similar results.

\* These values included in Table IV as one of control values.

#### TABLE III

Effect of Varying Amounts of Applied Protein on Densitometric Measurement of Rat Chylomicron Apoproteins

|  | Protein Distribution<br>Protein applied to gel |            |       |
|--|--|------------|-------|
| $R_f$  | 5 µg   | 10 µg      | 15 µg |
|  |  | % of total |       |
| 0.1  | 6.7  | 5.1        | 5.1   |
| 0.31   | 4.5  | 3.5        | 3.0   |
| 0.40   | 11.5   | 12.3       | 10.7  |
| 0.53   | 5.5  | 5.1        | 5.0   |
| $\left. \begin{array}{c} 0.59\\ 0.67 \end{array} \right\}$ | 38.3   | 38.4       | 42.0  |
| 0.81   | 32.7   | 35.2       | 33.2  |

Varying amounts of a single chymlomicron sample were applied to SDS gels, and the percentage distribution of proteins was determined densitometrically. Results are the means of two determinations.

tein indicates that the densitometric methods employed reflect the linear relationship of varying amounts of applied protein.

Effect of impaired protein synthesis on lymph chylomicron apoprotein composition. Attempts were next made to determine whether differences in the apoprotein composition occurred in the large chylomicrons formed as a result of the inhibition of protein synthesis.

Lymph chylomicrons were prepared from mesenteric lymph fistula animals treated with ACH in a dose previously shown to inhibit intestinal protein synthesis by greater than 80% for at least 3 h (10). As described in Methods, these chylomicrons were purified, delipidated, and subjected to SDS polyacrylamide gel electrophoresis. A representative densitometric scan from a control and an ACH-treated animal are shown in Fig. 3. It can be seen that in the ACH-treated animal there has been a striking decrease in the chylomicron apoprotein band of  $R_1$  0.67, with other bands being relatively preserved  $(R_t \ 0.09)$  or increased  $(R_t \ 0.52)$ . It is also apparent that the chromatographic characteristics of the individual apoprotein bands appear to have been altered in the ACH-treated animal, in that several of the bands have lost their sharp electrophoretic properties and have become broader. These changes in the apoprotein composition of lymph chylomicrons as a result of the inhibition of protein synthesis were studied quantitatively in a larger number of animals and the results are shown in Table IV. The differential effect of ACH treatment on selective chylomicron apoproteins was verified with approximately a 50% decrease in the  $R_1$  0.67 band and no significant change in  $R_t$  0.09 or the low molecular weight peptides at  $R_1$  0.81. The  $R_1$  0.41 band was variably present

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in different chylomicron samples (see below) and was unaffected by the inhibition of protein synthesis. Since the data in Table IV represent a distribution of the individual apoproteins, there was a proportional increase in the  $R_r$  0.51–0.53 band as a result of the changes seen in the  $R_r$  0.67 band. These data would indicate that there is a differential effect of the inhibition of protein synthesis on the apoproteins of lymph chylomicrons.

One possible explanation for the seemingly differential effects of inhibition of protein synthesis on the chylomicron apoproteins might be differing turnover rates of the individual apoproteins. One could predict that those apoproteins most affected by impaired protein synthesis (i.e.,  $R_r$  0.67) would be those apoproteins with the highest turnover rates.

Rates of chylomicron apoprotein synthesis. In order to test this hypothesis, a single dose of [ ${}^{3}H$ ]leucine (200  $\mu$ Ci) was administered intraperitoneally to lymph fistula rats after a constant micellar fat infusion had been established. Lymph was then collected at hourly intervals, and the chylomicrons purified and subjected to SDS acrylamide electrophoresis as described in Methods. At each time point, duplicate gels were run,



FIGURE 3 Effect of ACH on chylomicron apoproteins. Chylomicrons were prepared from control and ACH treated animals and subjected to SDS acrylamide electrophoresis. The actual densitometric scans of Coomassie blue-stained gels are shown. The relative mobilities  $(R_f)$  are indicated above each peak.

#### TABLE IV

Effect of Protein Synthesis Inhibition (ACH) on the Apoproteins of Lymph Chylomicrons

| Rf           | Control, $n = 5$ | Inhibited, $n = 6$ | Р             |
|--------------|------------------|--------------------|---------------|
|              | % of total (     | $(Mean \pm SE)$    |               |
| 0.09         | $9.4 \pm 1.1$    | $9.8 \pm 1.3$      | NS            |
| 0.31         | 3.0              | +                  |               |
| 0.41         | $8.1 \pm 3.4$    | $10.2 \pm 5.5$     | $\mathbf{NS}$ |
| 0.53         | $7.6 \pm 2.6$    | $21.7 \pm 3.5$     | < 0.01        |
| 0.59<br>0.67 | $50.2 \pm 3.1$   | $27.4 \pm 4.5$     | < 0.01        |
| 0.81         | $25.1 \pm 4.3$   | $28.8 \pm 4.5$     | NS            |

Chylomicrons were prepared from control and ACH-treated animals delipidated and electrophoresed on SDS acrylamide gels. The distribution of apoproteins was determined densitometrically (Methods).

one for staining and densitometry and the other for slicing into 1-mm slices for liquid scintillation counting. Recovery of counts applied to the gels was approximately 95%. Fig. 4 shows the time course of [3H]leucine incorporation of the major apoproteins of lymph chylomicrons in a representative animal. This experiment was repeated with similar results in three animals. It is apparent that the incorporation patterns differ among the individual apoproteins, with certain apoproteins showing little change of labeling with time (i.e.  $R_t$  0.1), while other apoproteins show a rapid acquisition and loss of radioactivity with time (i.e.,  $R_t$  0.67). Of note is that those apoproteins ( $R_t$  0.81) of low molecular weight, i.e., 10,000 (Fig. 5), comprising about 25% of chylomicron protein on the gel, do not label to any significant degree and may not be synthesized de novo by the intestine during lipid absorption. Similarly no labeling of the  $R_t$  0.41 band was observed also indicating that this protein is not newly synthesized during lipid absorption. This protein has an apparent molecular weight of 60,000, is variably present in various chylomicron samples, and may represent small amounts of a contaminating serum protein (i.e., albumin) not detected on immunologic procedures employing whole chylomicrons. The differences in turnover rates of the various chylomicron apoproteins are better appreciated when the data in Fig. 4 are expressed as the total radioactivity incorporated into each apoprotein band (sum of counts under each peak) with time. Since the percentage of each apoprotein remained constant in any given chylomicron preparation (verified densitometrically), the data in Fig. 6 have been corrected for the amount of protein applied to each gel. It is apparent that the most rapid turnover of radioactivity is seen in the  $R_f$  0.67 apopro-



FIGURE 4 Time course of [<sup>a</sup>H]leucine incorporation into lymph chylomicron apoproteins. After constant micellar lipid infusion had been established, a single dose of 200  $\mu$ Ci [<sup>a</sup>H]leucine was given intraperitoneally to a mesenteric lymph fistula rat. Chylomicrons were collected at the indicated times, purified, and electrophoresed on SDS acrylamide gels. The gels were sliced and the radioactivity in each slice determined (Methods). The  $R_t$  of each peak is indicated. Between 1,000-2,000 counts were applied to each gel, and recovery of applied counts was approximately 95%.



FIGURE 5 Molecular weight determination. The indicated proteins of known molecular weight were electrophoresed on 5.6% SDS polyacrylamide gels and their relative mobilities determined densitometrically. Several of the chylomicron apoproteins are inserted at their respective  $R_t$  values.



FIGURE 6 Time course of [<sup>3</sup>H]leucine incorporation into lymph chylomicrons. The data in Fig. 4 have been expressed as the total counts in each protein peak at the indicated times. The figures above each curve indicate the  $R_t$  of each peak.

tein, with significantly slower rates for the other apoproteins shown. It should be pointed out that a constant fat infusion was continued throughout the entire experiment, and therefore the initial radioactivity incorporated into chylomicron proteins was sequentially "diluted" with de novo synthesis of unlabeled chylomicron proteins, reflecting a more rapid isotope dilution in those proteins with the most rapid rates of synthesis (i.e.,  $R_1$  0.67). These data are in agreement with the effects of impaired protein synthesis on the various chylomicron apoproteins shown in Table IV, demonstrating a marked decrease in the  $R_t$  0.67 band. The relative increase in the 0.53 band may largely represent a proportional increase as a result of the changes seen in the  $R_1$  0.67 band, but may also represent a greater availability of this apoprotein during impaired protein synthesis. Incorporation studies into the individual chylomicron apoproteins during inhibition with ACH were attempted; however, the marked impairment of radioactive incorporation and limitations on the amount of protein that could be applied to the gel gave too little radioactivity in each gel slice for analysis.

Immunologic studies. Since impaired protein synthesis was associated with a decrease in one of the major chylomicron apoproteins ( $R_t$  0.67), it was of interest to determine the immunologic identity of this protein. The small amounts of lymph obtained precluded purification of this protein by column chromatography, and therefore immunologic studies were carried out directly from acrylamide gel slices. Attempts to carry out such studies directly from SDS gels were unsuccessful because of nonspecific precipitin lines and inhibition of the immunologic reaction by the SDS

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contained in the gel slice. It was found that these studies could be carried out from slices of acrylamide gels run in urea as described by Kostner and Holasek (26). It was first necessary to locate each of the SDS bands on urea gels. To accomplish this, individual slices from unstained, unfixed SDS gels representing a specific  $R_1$  value were placed directly on top of urea acrylamide gels and re-electrophoresed. The results are shown in Fig. 7, where it can be seen that each slice from SDS representing a given apoprotein band gives distinct bands when re-electrophoresed on urea gels. Significantly, as demonstrated in Fig. 7, each band visible on the SDS gel enters the urea gel with the exception of the  $R_f$  0.1. This band does not enter urea gels, remains at the interface when transplanted, and may represent apo B, since this apoprotein has been shown by others to accumulate at the interface of urea gels (27). With the ability to identify each of the chylomicron apoproteins on urea gels, it was of particular interest to determine the immunologic identity of the rapidly synthesized apoprotein that decreased during protein synthesis inhibition. As described in Methods, delipidated chylomicrons were electrophor-



FIGURE 7 Relationship between SDS and urea acrylamide electrophoresis of rat lymph chylomicrons. Delipidated chylomicrons were electrophoresed on SDS acrylamide gels, sliced, and a given unfixed unstained slice, representing a given Rf, was re-electrophoresed on urea gels (Methods). The position of each SDS band is indicated in the top portion. The position of the individual gel slices ( $R_r$  SDS) on urea gels is shown below. The position of  $R_r$  0.41 photographed poorly; however, its position is indicated above. The  $R_r$  0.10 SDS did not enter the urea gel.



FIGURE 8 Reaction of identity between precipitation lines of protein  $R_f$  0.67 with antibody to rat serum HDL and antibody to mesenteric lymph chylomicrons. Appropriate slices from urea gels were used. A, normal animal; B, ACH-treated animal.

esed on urea acrylamide gels; the appropriate slice from an unfixed, unstained gel was then imbedded in warm agar and subjected to double diffusion. As shown in Fig. 8A, this apoprotein is immunologically related to HDL and gives a reaction of identity with antisera prepared to mesenteric chylomicrons and to rat serum HDL. It was of interest to determine whether this apoprotein from protein synthesis-inhibited animals retained its immunologic identity. As shown in Fig. 8B, this apoprotein from ACH-treated animals retains its immunologic identity with both antisera.

Studies on nascent chylomicrons. The rapid incorporation of radioactivity into the  $R_1$  0.67 bands on SDS acrylamide gels (Fig. 6) derived from mesenteric lymph suggests that this major chylomicron apoprotein is synthesized by the intestine. In view of recent reports (28, 29) that certain apoproteins are transferred from HDL to the lipid-rich particles (i.e., chylomicron and VLDL), it was of interest to determine whether this apoprotein is present in chylomicrons isolated directly from small intestinal mucosal cells (see Methods). When such "nascent" chylomicrons were delipidated and examined by double diffusion utilizing an antibody to rat serum HDL, a line of identity was observed between the delipidated nascent chylomicrons and the  $R_1$  0.67 band as isolated from urea gels. These findings are consistent with the postulate that this apoprotein originates in the intestinal epithelium.

#### DISCUSSION

The importance of protein synthesis during lipid absorption was underscored when it became evident that in a-beta-lipoproteinemia there is a specific defect in lipoprotein synthesis associated with a failure of lipid transport from the intestinal mucosal cell (4, 5). Efforts to produce experimental models of impaired protein synthesis with pharmacologic inhibitors of protein synthesis (i.e. puromycin, ACH) have been difficult because of the toxicity of the drugs employed (7, 8). In an effort to resolve conflicting results for impairment of protein synthesis and its effect on lipid absorption, we previously demonstrated (9) that there was both a decreased absorption of lipid into the lymph of ACHtreated animals and a dramatic increase in lymph chylomicron size associated with impairment of protein synthesis. We proposed that an increase in particle size during conditions of impaired protein synthesis represented an adaptive mechanism to conserve surface components (i.e., apolipoproteins and phospholipids) when these components are limiting. This would permit the transport of more lipid in fewer but larger particles. We have subsequently shown a similar increase in lymph chylomicron size with two other models of protein deficiency, i.e., with dietary protein deficiency and prolonged lymph drainage.<sup>2</sup>

The present studies examine the apoprotein composition of the chylomicrons formed as a result of pharmacologic inhibition of protein synthesis. It is well known that with increasing particle size the ratio of surface components (i.e. protein, phospholipid) to "core" triglyceride decreases, and it was of interest to determine whether the apoprotein composition of chylomicrons normally varies as a function of particle size. As shown (Fig. 2, Table II), there is no significant difference in the distribution of apoproteins with varying particle size as measured densitometrically from SDS polyacrylamide gels. It is of interest that the studies of Eisenberg, Bilheimer, Lindgren, and Levy (30), subjecting subfractions of human serum VLDL to gel filtration, demonstrated varying proportions of apoprotein B to the other apoproteins (Fractions II/I) as a function of size. In view of the recent evidence (29) of the rapid degradation of circulating VLDL, it is not surprising that a heterogeneity of composition exists in the serum VLDL. Lipoproteins from intestinal lymph may well be more homogeneous and vary less in their apoprotein content. The present studies utilizing densitometric methods do not permit any statement concerning the relative amounts of apoprotein B in the various chylomicron subgroups, since this apoprotein does not reliably enter polvacrylamide gels. Studies are currently in progress

<sup>2</sup> Glickman, R. M. Unpublished observations.

utilizing larger quantities of material to examine the content of apoprotein B in subclasses of mesenteric lymph chylomicrons. Certainly, as demonstrated in the present studies, with the possible exception of apo B there are no significant differences in apoprotein content as a function of chylomicron size.

In contrast, the present studies demonstrate that during inhibition of protein synthesis significant changes occur in the apoprotein content of mesenteric lymph chylomicrons, with one of the major apoproteins being reduced approximately 50% (Table IV). This apoprotein has a rapid rate of synthesis (Fig. 6), is present in "nascent" chylomicrons isolated from intestinal epithelial cells, and shares antigenic determinants with HDL (Fig. 8). These data are consistent with the findings of Windmueller, Herbert, and Levy, and Windmueller and Spaeth (27, 31), who used an isolated perfused intestinal preparation. These authors found active synthesis of all the major apoproteins of intestinal VLDL with the exception of the low molecular weight peptides of mol wt ~10,000; these peptides were also not labeled in our studies (Fig. 4,  $R_f$  0.81). It is of interest that these authors also found the incorporation of ['H]lysine to be greater into their Group II apoproteins than into Group I proteins remaining at the origin, including apo B. Since our studies of radioactive incorporation were carried out using SDS acrylamide rather than urea gels, we identified each of the apoproteins present on SDS on urea gels (Fig. 7) to facilitate comparison. The Group II apoproteins referred to by Windmueller et al. comprise the  $R_f$  0.67 and 0.50 bands on SDS, and as in their studies we found these apoproteins to be the most highly labeled. Furthermore we have established that one of the major apoproteins  $(R_t \ 0.67)$  is immunologically related to HDL and is present in chylomicrons isolated directly from intestinal epithelial cells.

We have not been successful in immunologically identifying the Rf 0.1 band directly from gel slices, which on urea gels remains at the interface. Other authors have determined that this material remaining at the interface on urea gels contains apo B, but it is difficult to be certain that other proteins also do not enter the gel (27). The present studies, utilizing SDS gels, may in part avoid this difficulty since approximately 95% of applied counts enter the gel and little radioactivity remains at the surface (Fig. 4). It is probable that the  $R_1$  0.1 band on SDS represents apo B, since almost all protein appears to enter the gel, and all SDS bands, with the exception of this apoprotein, easily enter urea gels (Fig. 7). Furthermore we have shown that apo B prepared from human mesenteric chylomicrons will enter SDS gels with a similar  $R_t$  while remaining at the interface on urea gels.

If, as seems probable, the  $R_t$  0.1 band is apo B, it is of interest that there was no reduction in this apoprotein during the inhibition of protein synthesis. It could be hypothesized that since the present studies examined lymph lipoproteins, those particles that appear in lymph during the inhibition of intestinal protein synthesis represent the minimum requirements for a lipoprotein particle to leave the intestinal cell. In view of the important role of apo B in lipid transfer, the preservation of this apoprotein may be most important, as the present studies would indicate. Rather, pharmacologic inhibition of protein synthesis seems to affect those apoproteins with the most rapid turnover, i.e.,  $R_t$  0.67. As we have shown the turnover of the  $R_t$  0.1 band is significantly slower than  $R_t$  0.67 (Fig. 6) and predictably is less affected by inhibition of protein synthesis. Of additional interest is the  $R_t$ 0.50 band, which also is highly labeled during chylomicron synthesis. This apoprotein is unaffected by inhibition of protein synthesis and actually shows a relative increase after ACH treatment (Table IV, Fig. 3). Since this apoprotein is highly labeled both in our studies and those of Windmueller et al., (27) and is probably synthesized by the intestine, it may be of considerable biological importance in chylomicron formation, as suggested by its relative preservation in chylomicrons formed during the inhibition of intestinal protein synthesis. Further confirmation for the intestinal origin of these apoproteins comes from the work of Mahley et al. (32), who found all the major apoproteins, with the exception of the small molecular weight peptides (V3, V4, V5), to be present in VLDL isolated from intestinal mucosa.

While recent reports have determined the apoprotein composition of serum VLDL in the rat (33, 34), little information is available on the apoproteins of mesenteric chylomicrons. Since significant differences may exist between serum and lymph lipoproteins (35), it is not possible to relate our findings directly to those of others. We have confirmed, however, the presence of the A protein (24) in mesenteric lipoproteins (Fig. 8) and have demonstrated that this apoprotein is a major constituent of lymph chylomicrons.

In view of the many intracellular events required to convert absorbed lipid into a lipoprotein particle capable of leaving the intestinal epithelial cell, it is likely that a basic derangement in cellular function, i.e., impaired protein synthesis, may affect intestinal lipoprotein formation in several ways. Recent studies in puromycin-treated rats have demonstrated decreased intestinal phospholipid synthesis (36) as well as impaired [<sup>14</sup>C]glucosamine incorporation into chylomicrons (37). The present studies demonstrate that defects in apolipoprotein formation may also be seen during impaired intestinal protein synthesis. Further evidence for the effect of impairment of protein synthesis on lipid absorption comes from ultrastructural studies of rat intestine in puromycin-treated animals (38). Ultrastructurally large lipid droplets are present within the intestinal cell and there is a failure of these droplets to enter Golgi vesicles. Indeed, during the inhibition of protein synthesis there is a striking decrease in intracellular organelles. It was suggested that the impaired lipid absorption might be related to impaired synthesis of intracellular membranes. It is possible that the defects in chylomicron apolipoprotein synthesis observed in this study may in part be related to impaired intracellular membrane formation. The precise elucidation of lipoprotein formation within the intestinal cell and the factors controlling synthesis of the various apoproteins is a subject of considerable current interest and must await further study.

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#### REFERENCES

- 1. Windmueller, H. G. 1968. Production of  $\beta$ -lipoprotein by intestine in the rat. J. Biol. Chem. 243: 4878.
- Kessler, J. I., J. Stein, D. Dannacker, and P. Narcessian. 1970. Biosynthesis of low density lipoproteins by cell-free preparations of rat intestinal mucosa. J. Biol. Chem. 245: 5281.
- 3. Ockner, R. K., F. B. Hughes, and K. J. Isselbacher. 1969. Very low density lipoproteins in intestinal lymph: origin, composition and role in lipid transport in the fasting state. J. Clin. Invest. 48: 2079.
- Salt, H. B., O. H. Wolff, J. K. Lloyd, A. S. Fosbrooke, A. H. Cameron, and D. U. Hubble. 1960. On having no beta-lipoprotein. A syndrome comprising a-betalipoproteinemia, acanthocytosis and steatorrhoea. *Lancet*. 2: 325.
- Gotto, A. M., R. I. Levy, K. John, and D. S. Fredrickson. 1971. On the protein defect in abetalipoproteinemia N. Engl. J. Med. 284: 813.
- 6. Sabesin, S. M., and K. J. Isselbacher. 1965. Protein synthesis inhibition: mechanism for the production of impaired fat absorption. *Science* (*Wash. D. C.*). 147: 1149.
- 7. Redgrave, T. G. 1969. Inhibition of protein synthesis and absorption of lipid into thoracic duct lymph of rats. *Proc. Soc. Exp. Biol. Med.* **130**: 776.
- 8. Redgrave, T. G., and D. B. Zilversmit. 1969 Does puromycin block release of chylomicrons from the intestine? *Am. J. Physiol.* 217: 336.
- Glickman, R. M., K. Kirsch, and K. J. Isselbacher. 1972. Fat absorption during inhibition of protein synthesis: studies of lymph chylomicrons. J. Clin. Invest. 51: 356.
- 10. Glickman, R. M., D. H. Alpers, G. Drummev. and K. J. Isselbacher. 1970. Increased lymph alkaline phosphatase after fat feeding: effects of medium chain tri-

glycerides and inhibition of protein synthesis. Biochim. Biophys. Acta. 201: 226.

- 11. Zilversmit, D. B. 1967. Formation and transport of chylomicrons. Fed. Proc. 26: 1599.
- Kalab, M., and W. G. Martin. 1968. Gel filtration of native and modified pig serum lipoproteins. J. Chromatogr. 35: 230.
- Eggstein, M., and F. H. Kreutz. 1966. Eine neue Bestimmung der Neutralfette in Blutserum und Gewebe. I. Mitteilung. Prinzip durch Führung und Besprechung der Methode. Klin. Wochenschr. 44: 262.
- 14. Lowry, O. H., N. J. Rosebrough, A. L. Farr. and R. J. Randall. 1951. Protein measurement with the Folin Phenol Reagent J. Biol. Chem. 193: 265.
- 15. Gustafson, A., P. Alaupovic, and R. H. Furman. 1966. Studies of the composition and structure of serum lipoproteins: separation and characterization of phospholipid-protein residues obtained by partial delipidation of very low density lipoproteins of human serum. *Biochemistry*. 5: 632.
- Oudin, J., and C. A. Williams. 1971. Precipitation analysis by diffusion in gels. Meth. Immunol. Immunochem. 3: 103.
- Brown, W. V., R. I. Levy, and D. S. Fredrickson. 1969. Studies of the proteins in human plasma very low density lipoproteins. J. Biol. Chem. 244: 5687.
- Fairbanks, G., T. L. Steck, and D. F. H. Wallach. 1971. Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. *Biochemistry*. 10: 2606.
- 19. Davis, B. J. 1964. Disc electrophoresis. II. Method and application to human serum proteins. Ann. N. Y. Acad. Sci. 121: 404.
- Ward, S., D. L. Wilson, and J. J. Gilliam. 1970. Methods for fractionation and scintillation counting of radioisotope-labeled polyacrylamide gels. *Anal. Biochem.* 38: 90.
- Weiser, M. M. 1972. Concanavalin A agglutination of intestinal cells from human fetus. Science (Wash. D. C.). 177: 525.
- 22. Rodgers, J. B., E. M. Riley, G. D. Drummey, and K. J. Isselbacher. 1967. Lipid absorption in adrenalectomized rats: the role of altered enzyme activity in the intestinal mucosa *Gastroenterology*. 53: 547.
- Forte, G. M., A. V. Nichols, and R. M. Glaser. 1969. Electron microscopy of human serum lipoproteins using negative staining. *Chem. Phys. Lipids.* 2: 396.
- Ockner, R. K., K. J. Bloch, and K. J. Isselbacher. 1968. Very-low-density lipoprotein in intestinal lymph: evidence for presence of the A protein. Science (Wash. D. C.). 162: 1285.
- 25. Dole, V. P., and J. T. Hamlin, III. 1962. Particulate fat in lymph and blood. *Physiol. Rev.* 42: 674.
- Kostner, G., and A. Holasek. 1972. Characterization and quantitation of apoproteins from human chyle chylomicrons. *Biochemistry*. 11: 1217.
- Windmueller, H. G., P. N. Herbert, and R. I. Levy. 1973. Biosynthesis of lymph and plasma lipoprotein apoproteins by isolated perfused rat liver and intestine. J. Lipid Res. 14: 215.
- Rubenstein, B., and D. Rubenstein. 1972. Interrelationship between rat serum very low density and high density lipoproteins. J. Lipid Res. 13: 317.
- Bilheimer, D. W., S. Eisenberg, and R. I. Levy. 1972. The metabolism of very low density lipoprotein proteins: I. Preliminary in vitro and in vivo observations. Biochim. Biophys. Acta. 260: 212.

- 30. Eisenberg, S., D. Bilheimer, F. Lindgren, and R. I. Levy. 1972. On the apoprotein composition of human plasma very low density lipoprotein subfractions. *Biochim. Biophys. Acta.* 260: 329.
- Windmueller, H. G., and A. E. Spaeth. 1972. Fat transport and lymph and plasma lipoprotein biosynthesis by isolated intestine. J. Lipid Res. 13: 92.
- 32. Mahley, R. W., B. D. Bennett, D. J. Morre, M. E. Gray, W. Thistlethwaite, and V. S. LeQuire. 1971. Lipoproteins associated with Golgi apparatus isolated from epithelial cells from rat small intestine. Lab. Invest. 25: 435.
- 33. Bersot, G. P., W. V. Brown, R. I. Levy, H. G. Windmueller, D. S. Fredrickson, and V. S. Lequire. 1970. Further characterization of the apolipoproteins of rat plasma lipoproteins. *Biochemistry*. 9: 3427.

- 34. Koga, S., L. Bolis, and A. M. Scanu. 1971. Isolation and characterization of subunit polypeptides from apoproteins of rat serum lipoprotein. *Biochim. Biophys. Acta.* 236: 416.
- Herbert, P. N., H. G. Windmueller, and R. I. Levy. 1971. Characterization of rat very low density lipoprotein (VLDL) of intestinal origin. *Circ. Suppl.* 2: 177.
- 36. O'Doherty, P. J. A., I. M. Yousef, and A. Kuksis. 1972. Differential effect of puromycin on triglyceride and phospholipid biosynthesis in isolated mucosal cells. *Fed. Proc.* 31: 701.
- 37. Yousef, I. M., and A. Kuksis. 1972. Release of chylomicrons by isolated cells of rat intestinal mucosa. *Lipids.* 7: 380.
- 38. Friedman, H. I., and R. Cardell, Jr. 1972. Effects of puromycin on the structure of rat intestinal epithelial cells during fat absorption. J. Cell. Biol. 52: 15.