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Mechanism of avian estrogen-induced hypertriglyceridemia: evidence for overproduction of triglyceride

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Abstract Relying on methods other than the determination of turnover rate of triglyceride from the curve of plasma triglyceride radioactivity after administration of labeled precursor, we have confirmed that the endogenous hypertriglyceridemia induced by estrogenization of the chick is accompanied by increased production of triglyceride. Chicks estrogenized with diethylstilbestrol became grossly hypertriglyceridemic and had elevated levels of plasma free fatty acid. Within 5 min of administration of labeled palmitate, estrogenized hypertriglyceridemic birds converted approximately 10 times more plasma free fatty acid to hepatic triglyceride than did controls. In addition, 2 hr after intraperitoneal injection of [^{14}C]acetate or [U-^{14}C]glucose, the specific activity of very low density lipoprotein triglyceride (VLDL-TG) of estrogenized birds reached or exceeded that of the untreated controls, and the rapid enrichment of the vastly expanded plasma VLDL-TG pool with labeled triglyceride further indicated that increased production of triglyceride occurs with estrogenization. Furthermore, [^{14}C]acetate incorporation into VLDL-TG was calculated to be 1.6 and 6.6% of the injected dose in estrogenized birds compared with 0.1 and 0.2% in untreated birds. Increased production of plasma VLDL-TG was confirmed by a kinetic study of VLDL-TG metabolism, employing reinjected, endogenously prepared [^{14}C]triglyceride-labeled VLDL. The fractional turnover rate of VLDL-TG in estrogenized hypertriglyceridemic birds was substantially less than that in untreated controls (0.32 ± 0.03 vs. 0.71 ± 0.03/hr), but the total turnover rate was nearly 50 times greater (244 ± 52 vs. 5 ± 1 mg/hr).

Studies of human endogenous hypertriglyceridemia have provided conflicting explanations of its mechanism, with results of kinetic studies supporting increased production (1–4) being opposed by numerous studies indicating diminished disposal of triglyceride (5–8). Conclusions from these studies have depended on interpretations of curves of plasma triglyceride radioactivity after injection of labeled precursors of triglyceride. Interpretations of such kinetic studies might be more straightforward and less conflicting if one knew the behavior of precursor-produced plasma triglyceride radioactivity that is typical of hypertriglyceridemia due to "overproduction" or "underdisposal." However, determination of overproduction or under disposal for this purpose would require methods other than analysis of plasma triglyceride radioactivity curves, such as experiments with isolated tissue preparations, and has not been achieved to date in human hypertriglyceridemia. Furthermore, spontaneous hypertriglyceridemia does not occur commonly in laboratory animals. Consequently, there are no studies that, for example, describe the kinetics of plasma triglyceride metabolism in hypertriglyceridemia known to be due to overproduction.

However, a suitable model for such studies appears to exist in the hen, which, upon reaching maturity, spontaneously develops endogenous hypertriglyceridemia, a phenomenon that can be produced by administration of estrogen to immature birds (9), and has its qualitative human counterpart in the hypertriglyceridemia of estrogen-treated premenopausal women (10). There are strong indications that this hypertriglyceridemia is due to overproduction of triglyceride: work from this laboratory and elsewhere has demonstrated that liver slices from estrogenized birds synthesize more triglyceride from labeled precursor than similar preparations from untreated birds (9, 11). In addition, estrogen-induced increased synthesis of VLDL apoprotein has recently been described in birds (12). In the first part of this report we describe additional in vivo experiments that confirm that this estrogen-induced hypertriglyceridemia is due to increased production of triglyceride from both lipid and nonlipid precursors.

This model has afforded us the unique opportunity to characterize the kinetics of plasma VLDL-TG metabolism in hypertriglyceridemia known to be due to overproduction. Two kinds of data have been obtained. Kinetics

Abbreviations: DES, diethylstilbestrol; TG, triglyceride; VLDL, very low density lipoprotein.

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derived from disappearance of reinjected, endogenously prepared [^{14}C]triglyceride-labeled VLDL are consonant with overproduction as the primary cause of the hypertriglyceridemia. In hypertriglyceridemic birds, simultaneously derived curves of VLDL-TG radioactivity generated from [^{3}H]palmitate are characterized by descending slopes that are much less than those described by disappearing [^{14}C]triglyceride-labeled VLDL and cannot, therefore, reflect simply disposal of newly synthesized VLDL-TG. Thus, in this situation of endogenous hypertriglyceridemia due to overproduction, precursor–product data generated by labeled fatty acid are interpretable only by complicated kinetic analysis, perhaps along the lines described by Shames et al. (13), rather than by other methods, such as that validated previously by Friedberg et al. (14) in normals and applied by Nestel to the hypertriglyceridemic situation (15).

METHODS

Studies of triglyceride synthesis

A. *In vivo hepatic conversion of plasma free fatty acid to triglyceride.* By sampling the liver shortly after intravenous administration of labeled free fatty acid, at a time before much of the label is secreted as plasma triglyceride, and after most of the labeled substrate has disappeared, one can determine the fraction of administered label converted to triglyceride.

Six New Hampshire Red chicks were studied; their weights were 850–1000 g. Three were estrogenized by four daily doses of diethylstilbestrol, 2.0 mg/day, in sesame oil, and three were given an equivalent volume of sesame oil alone. 24 hr after the last dose, the birds were given 25 μCi of plasma-bound sodium [9,10-^{3}H]palmitate intravenously (0.004 μeq/μCi, New England Nuclear Corp.). 5 to 7 min after injection, the birds were beheaded; blood was collected, and the livers were quickly excised, weighed, and chilled on ice. A weighed portion of liver was extracted by the method of Folch, Lees, and Sloane Stanley (16). Radioactivity of an aliquot of the lipid extract was measured as described below, another aliquot was chromatographed as described below, and a third aliquot was used for quantitation of triglyceride. The percentage of dpm found in triglyceride was used to calculate the fraction of injected label converted to triglyceride. The quantity of plasma free fatty acid converted to triglyceride was calculated from this fraction and the plasma free fatty acid pool. (Fatty acid pool = plasma level of free fatty acid, determined by a modification [17] of the method of Duncombe, multiplied by the plasma volume, determined as described below.)

To ensure that we were sampling the liver prior to the time that it had secreted much of the labeled fatty acid as plasma triglyceride, we extracted 1.0 ml of plasma by the method of Dole (18) and, by methods identical with those used with the livers, calculated the fraction of administered label present as plasma triglyceride.

B. *In vivo lipogenesis.* Eight chicks about 1 wk old were estrogenized with eight daily subcutaneous injections of 0.5 mg of DES (obtained from Sigma Chemical Co., St. Louis, Mo.) in sesame oil. Eight control chicks (same age) were given sesame oil alone. All birds were fed standard Purina starter chow (caloric composition: 75% carbohydrate, 20% protein, 5% fat). The mean weight of estrogenized birds was 70 g, and that of untreated birds was 63 g on the day of study. 24 hr after the eighth injection, the birds were given 50 μCi of sodium [1-^{14}C]acetate intraperitoneally (1 mCi/1.38 mg, New England Nuclear; total sodium acetate, 0.9 mg); they were allowed access to food for the next 2 hr and were then decapitated. Blood was collected in beakers containing EDTA (1.0 mg/ml of blood). The VLDL fraction ($S_f > 20$) of plasma was separated by preparative ultracentrifugation of 1.0 ml of plasma under 4.0 ml of saline (d = 1.006) in a SW 50.1 rotor at 138 × 10^6 *g*-min (temperature held at 25°C). The resultant well-defined top layer (VLDL) was cut away and resuspended in saline.

Lipid extracts of the VLDL were made (16), and aliquots of the extracts were separated into their lipid components by thin-layer chromatography (silica gel G; petroleum ether–diethyl ether–acetic acid 80:20:1). Lipids on the chromatograms were visualized by iodine vapor, and spots corresponding to the triglyceride standard were scraped off and counted in toluene–2,5-diphenyloxazole–1,4-bis-[2-(5-phenyloxazolyl)]-benzene. Counts were corrected for efficiency and expressed as dpm.

Triglyceride was assayed by a modification of the method of Van Handel and Zilversmit (19). In the case of livers, triglyceride was assayed in the lipid extract of Folch et al. (16).

Means and standard errors of means were determined and compared with the *t* test. To compensate for the possibility of unequal variances, the method of paired dependent observations was used after $\log_{10}$ transformation of data (20). (Results, however, are expressed as arithmetic means ± SEM.)

In order to compare the fraction of injected [^{14}C]acetate incorporated into plasma VLDL-TG in the treated and untreated states, the following experiment was done. Four birds, two untreated and two made hypertriglyceridemic by administration of DES, were given 50 μCi of [^{14}C]acetate (0.9 mg of sodium acetate) intravenously. Blood was sampled before and 10, 20, 30, 45, 60, and 120 min after administration of the isotope. VLDL was separated from each specimen by ultracentrifugation (as described above); lipid extracts of specimens of VLDL were made, and triglyceride was separated from each by thin-layer chromatography. The conversion of substrate

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(acetate) to product (VLDL-TG) was estimated by a method previously used to estimate the fraction of injected [1-^{14}C]palmitate converted to plasma triglyceride (14). These calculations have been validated by other investigators using different methods based on a number of theoretical models (8). We have chosen the calculations described because they are largely independent of the previous history of the injected substrate and may be compatible with a number of models.

This type of calculation is valid only if the measurement of K, the fractional turnover rate for plasma triglyceride, is valid. This calculation is invalid if significant amounts of labeled triglyceride are entering the plasma compartment at a time when data are selected for this calculation. Whereas K can be measured with some reliability when plasma triglyceride is normal, the validity of such calculations in hypertriglyceridemia is an uncertainty that may form the basis of the controversy underlying the conflicting results in the literature relating to the mechanism of endogenous hypertriglyceridemia in man. The error would be to underestimate the turnover rate for plasma triglyceride in hypertriglyceridemic states. With respect to the present study, the error would only serve to underestimate the vast difference between triglyceride synthesis from acetate in the normal and estrogenized chick. (Vide infra: the evidence presented in the Results section, that this mathematical analysis of precursor–product VLDL-TG kinetics underestimates fractional turnover rate in the hypertriglyceridemic state.)

In order to be sure that differences in specific activity of acetate-labeled VLDL-TG were not due to differences in the size of the acetate pool, pool size was estimated by assay of hepatic acetyl CoA in six DES-treated and six control birds (21). The liver was used because it is generally considered to be the major source of VLDL and because the liver is the source of DES-induced hyperlipemia (22).

We also evaluated in vivo lipogenesis using glucose, a more physiological nonlipid precursor whose pool size can also be estimated. Four chicks were studied, two DES-treated and two controls. The study protocol was identical with that described above for acetate except that [U-^{14}C]glucose (20 μCi, sp act 10–15 mCi/mmole; New England Nuclear) was injected intraperitoneally. In order to determine what portion of the glucose label appearing in VLDL-TG was incorporated into the glycerol and the fatty acid moieties, the triglyceride was hydrolyzed by heating it in 10% methanolic potassium hydroxide. After acidification with hydrochloric acid, the fatty acids were twice extracted from the water phase with petroleum ether, and the radioactivity in the ether and water phases was measured. The plasma glucose pool was calculated from the plasma glucose concentration (23), total blood volume (8% of body weight [24]), and hematocrit (measured to be 25%).

Kinetics of plasma VLDL-TG

A. *Studies with reinjected, endogenously prepared [^{14}C]triglyceride-labeled VLDL.* Endogenously labeled VLDL-TG was prepared by intravenous injection of 40–50 μCi of [^{14}C]palmitate (in chick serum) into 300–600-g chicks made hypertriglyceridemic by repeated injections of DES. Blood was harvested from the chicks 45 min later (EDTA as anticoagulant). VLDL was separated from the plasma by preparative ultracentrifugation, as described above. After resuspension of VLDL in saline, the triglyceride concentration and radioactivity were determined, and the fraction was refrigerated at 4°C until use. (This fraction is perhaps more precisely designated $S_f >$ 20 lipoproteins, because no attempt was made to separate a "chylomicron" fraction from the VLDL. We know from earlier work that particles resembling chylomicrons physically contribute minutely to total triglyceridemia in estrogenized birds, about 2% at TG levels of 1000 mg/100 ml or greater [9].) The triglyceride concentrations of VLDL so prepared were 20–159 mg/ml, and the radioactivity was 100,000–776,500 cpm/ml. (Radioactivity of VLDL-TG was determined by counting 50 μl of the VLDL directly in phosphor as described below.) Thin-layer chromatography of the lipid-extracted VLDL demonstrated that 90% or more of the radioactivity was in triglyceride.

Prior to use in an experiment, the VLDL was warmed to 38°C. A measured aliquot (0.25–1.0 ml) was then injected via jugular vein into a chick. The amount injected was dictated by the triglyceride concentration of the preparation and by whether the bird was hypertriglyceridemic, in order to alter the steady-state pool size as little as possible. Blood was sampled from the opposite jugular vein before injection and 5, 15, 30, 45, 60, 90, and 120 min thereafter. Plasma triglyceride concentration was measured, and radioactivity of each specimen was determined by counting 50 μl of plasma in 2 parts toluene phosphor and 1 part Triton X-100, as described by Patterson and Greene (25). The amount of aqueous material was kept at 10% of the total volume.

The radioactivity of each specimen was plotted on semilog paper, and the rate of disposal (= fractional turnover rate, K) was determined from the resulting straight line. Turnover rate (mg triglyceride/hr) of VLDL-TG was calculated by multiplying K by the steady-state plasma VLDL-TG pool (= VLDL-TG concentration $\times$ plasma volume). (The plasma volume was estimated by extrapolating the VLDL radioactivity decay curve back to zero time and assuming that the intercept represented total mixing of the injected label. These estimates of plasma volume agreed with those based on body weight [24].)

In an initial experiment, we determined the rate of disposal of a preparation of VLDL-TG immediately after it was made and again after 3 days of refrigerated storage using the same untreated chick as recipient. There was no

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significant change in the rate of disposal and no significant change in turnover rate of VLDL-TG. Consequently, VLDL preparations were commonly used after as long as 3 days of storage. In addition, thin-layer chromatography of lipid extracts of plasma at the 15-, 60-, and 120-min intervals demonstrated that at least 90% of the radioactivity was still in triglyceride (as it was in the injected VLDL). We followed the reinjected label as total triglyceride activity.

For statistical comparison, all studies in the untreated state were considered as one group and all studies done in the estrogenized state as a second group. Data in both groups were again treated by analysis of variance and *t* test.

Chicks used for these experiments, weighing 300–600 g, were raised on standard starter chow. Analysis of fractional turnover rate in untreated birds grouped by weight (300–400 g, 400–500 g, etc.) revealed no differences attributable to weight. Birds were without access to food from the time they were removed from their cages until the end of the experiment (about 2.5 hr). Seven birds were studied before and again after DES treatment (1.0 mg/day), two of them only after 2 days, one of them after 2 days and again after 6 days of treatment, and four of them only after 6 days of treatment. 10 additional birds were studied only before treatment. 11 birds were studied only after large-dose estrogenization (total of 17.5 mg of DES administered over 4 days), in order to provide data for very high plasma triglyceride levels. In all, there were 37 experiments, 18 before and 19 after estrogenization.

B. *Studies with [^{3}H]palmitate and [^{14}C]triglyceride-labeled VLDL injected simultaneously.* To determine whether turnover rate of VLDL-TG derived from the radioactivity curve of plasma VLDL-TG after administration of labeled fatty acid precursor is the same as that derived from the disappearance of reinjected labeled VLDL, four estrogenized hypertriglyceridemic and two untreated birds were studied as follows.

Birds were injected simultaneously with labeled VLDL (prepared as described earlier) and 50 μCi of sodium [^{3}H]palmitate (specific activity and preparation the same as described earlier). Blood was sampled at intervals thereafter for 4 hr. Plasma ^{14}C radioactivity was followed by counting plasma directly (see above). Radioactivity of plasma VLDL [^{3}H]triglyceride was determined in chromatographically separated lipid extracts of VLDL prepared ultracentrifugally. Curves of total ^{14}C-labeled TG and [^{3}H]triglyceride-labeled VLDL were drawn on semilog paper, and turnover rates were calculated, in the case of ^{3}H-labeled VLDL, from the late portion of the curve, subsequent to the peak radioactivity.

We used ultracentrifugally separated VLDL, rather than extracts of whole plasma, to follow [^{3}H]triglyceride activity because one could not otherwise be sure how much of the newly synthesized palmitate-labeled triglyceride was in VLDL as opposed to lipoproteins of higher density. Using extracts of whole plasma to follow total ^{3}H-labeled TG activity resulted in curves that had the same shapes as those found with ^{3}H-labeled VLDL alone. That kinetics of VLDL-TG and whole plasma triglyceride appear to be the same has also been documented in humans (26).

RESULTS

Studies of triglyceride synthesis

A. *In vivo hepatic conversion of plasma free fatty acid to triglyceride.* The results are summarized in **Table 1.** Estrogenized birds were substantially hypertriglyceridemic and had larger plasma free fatty acid pools resulting from elevated levels of plasma free fatty acid, a phenomenon that has been described before in estrogenized birds (9, 11). Also noted was increased triglyceride content in liver (an average of 1394 mg in estrogenized birds compared with 291 mg/liver in untreated birds).

In the three untreated birds, 8%, 13%, and 18% of the injected labeled palmitate was found as hepatic triglyceride at 5 to 7 min; in the three estrogenized birds, the percentages were 18, 24, and 36. (Less than 1% of the label was present in plasma triglyceride at this time, indicating that sampling of liver was indeed accomplished before much secretion of triglyceride had occurred and, therefore, before substantial disposal of label as plasma triglyceride could have occurred.)

On the average, the pool size of plasma free fatty acid was increased fivefold and the fractional conversion of labeled precursor to hepatic triglyceride was increased twofold in estrogenized birds. If one assumes that the (fractional) turnover rate of plasma free fatty acids was not altered by treatment, these observations indicate that 10 times more free fatty acid was converted to triglyceride in the liver of estrogenized animals.

B. *In vivo lipogenesis from acetate and glucose.* The DES-treated birds had a plasma concentration of VLDL-TG 84 times that of the untreated birds (2604 $\pm$ 417 vs. 31 $\pm$ 9 mg/100 ml, means $\pm$ SEM). The amount of [^{14}C]acetate found in the VLDL-TG of treated birds exceeded that in VLDL-TG of untreated birds by a factor of 86 (1,176,000 vs. 13,680 dpm/ml of plasma), so that the specific activity of the VLDL-TG of the treated group was of the same magnitude as that of the untreated group (53,400 vs. 41,340 dpm/mg). These data are summarized in **Table 2.** The amount of acetyl CoA in livers of six treated birds was the same as that in livers of six untreated birds (45.5 and 43.0 nmoles/g, respectively); hence, the increased acetate labeling of VLDL-TG in estrogenized birds was not due to a smaller pool of acetate. Table 2

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TABLE 1. Conversion of labeled palmitate to triglyceride by livers of estrogenized and untreated birds

	Estrogenized Birds			Untreated Birds		
	No. 1	No. 2	No. 3	No. 1	No. 2	No. 3
Plasma triglyceride (mg/100 ml)	7320	3710	4040	56	118	89
Plasma FFA pool (μeq)	143.8	74.6	41.6	12.8	18.3	14.3
% FFA converted to liver triglyceride	24	18	36	8	18	13

again demonstrates that the liver, as well as the plasma, of estrogenized birds became triglyceride-laden.

In the experiment designed to compare the total amount of [^{14}C]acetate incorporated into VLDL-TG in the treated and untreated birds, the treated birds had plasma VLDL-TG concentrations of 960 and 1520 mg/100 ml whereas the untreated birds had 39 and 29 mg/100 ml. From the calculations described in Methods, 3,608,000 and 882,389 cpm of the injected 55 × 10^6 cpm (6.6 and 1.6%) were incorporated into VLDL-TG over 2 hr in the estrogenized birds; 50,500 and 123,587 cpm (0.1 and 0.2%) were incorporated into the VLDL-TG in the untreated birds (**Table 3**). Curves of VLDL-TG radioactivity from the untreated and the estrogenized birds having the larger acetate incorporations are compared in **Fig. 1.** The disappearance of triglyceride synthesized from acetate in the estrogenized birds appears to be zero. If, as seems most likely from comparison of labeled precursor VLDL-TG kinetic behavior and kinetic behavior of reinjected [^{14}C]triglyceride-labeled VLDL to be presented below, there is actually disposal of the acetate-labeled VLDL-TG in estrogenized birds during the interval observed, then the calculated amounts of acetate incorporated are minimum estimates. Even so, these amounts greatly exceed those calculated for control birds.

These data indicate that equal specific activity of plasma VLDL-TG in estrogenized birds could have been achieved only by increased triglyceride synthesis from a precursor acetate pool. The fraction of labeled acetate converted to triglyceride in the untreated state was insufficient to achieve the specific activities observed in the estrogenized state.

The results of the [^{14}C]glucose experiments were similar to those described above in all respects. Plasma content of VLDL-TG increased markedly in estrogenized birds (5025 and 4230 compared with 138 and 157 mg/100 ml in untreated birds). An even greater increase in the quantity of glucose label in the VLDL-TG was found, such that the specific activity of the VLDL-TG of the estrogenized birds exceeded that of the untreated controls (1518 and 1764 compared with 900 and 668 dpm/mg of triglyceride). Plasma glucose pools were the same in both sets of birds (20.2 mg in the estrogenized and 18.7 mg in the untreated birds). These data are listed in **Table 4.** Hydrolysis of the VLDL-TG demonstrated that the increased glucose labeling of VLDL-TG of estrogenized birds occurred in both the glycerol and the fatty acid moieties. The pro-

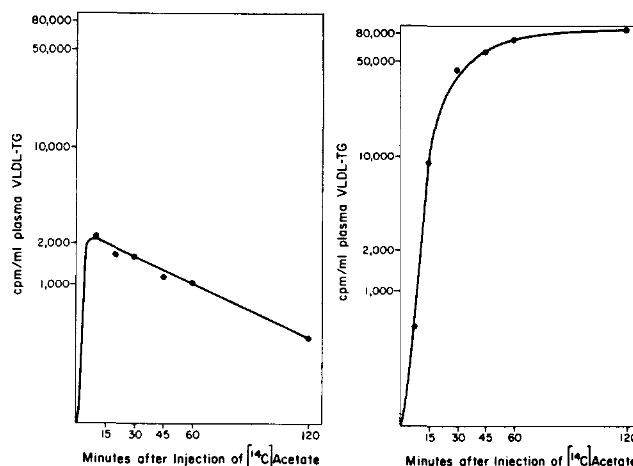

Fig. 1. *Left,* radioactivity of plasma VLDL-TG in unestrogenized bird after i.v. injection of [^{14}C]acetate. VLDL-TG concentration, 157 mg/100 ml. *Right,* radioactivity of plasma VLDL-TG in estrogenized bird after i.v. injection of [^{14}C]acetate. VLDL-TG concentration, 5025 mg/100 ml.

TABLE 2. Summary of experiments done with [^{14}C]acetate[a]

	Plasma VLDL-TG	Specific Activity of VLDL-TG	Liver Triglyceride Content
	mg/100 ml	*dpm/mg*	*mg*
Untreated (n = 8)	31 ± 9[b]	41,340 ± 12,898	27.2 ± 4.1
Estrogenized (n = 8)	2,604 ± 417[c]	53,400 ± 10,077	161.2 ± 42.3[c]

[a] 50 μCi of sodium [^{14}C]acetate (0.9 mg) given intraperitoneally; birds killed 2 hr later.
[b] Data are means ± SEM.
[c] Differs from observation in untreated group ($P < 0.01$).

TABLE 3. Comparison of amount of [^{14}C]acetate incorporated into VLDL-TG by untreated and estrogenized birds[a]

	VLDL-TG	^{14}C Incorporated into VLDL in 2 hr	% of Administered ^{14}C Incorporated
	mg/100 ml	*cpm*	
Estrogenized			
Bird no. 1	960	3,608,000	6.6
Bird no. 2	1520	882,000	1.6
Untreated			
Bird no. 1	39	50,500	0.1
Bird no. 2	29	123,587	0.2

[a] Fed birds given 55 × 10^6 cpm of [^{14}C]acetate intravenously. Blood was sampled at intervals after injection through 120 min.

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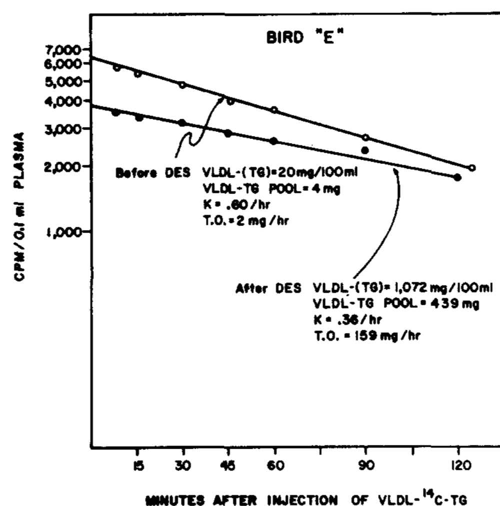

Fig. 2. Rate of disposal (fractional turnover rate, K) of reinjected [^{14}C]triglyceride-labeled VLDL (VLDL-^{14}C-TG) before and after diethylstilbestrol (DES) estrogenization in bird E. Also shown: concentrations of VLDL-TG, VLDL-TG pool size, and turnover rates (T.O.).

portion of label in each moiety was not changed by estrogenization, 81% to 87% appearing in the fatty acids in both untreated and estrogenized birds.

Although we have no explanation for the apparently higher VLDL-TG specific activity in estrogenized birds when glucose was used as precursor rather than acetate, the results support the same conclusion as that prompted by the acetate experiments: that VLDL-TG synthesis from nonlipid precursors is increased.

TABLE 4. Summary of experiments done with [^{14}C]glucose[a]

Group	Plasma VLDL-TG	Specific Activity of VLDL-TG
	mg/100 ml	*dpm/mg*
Estrogenized		
Bird no. 1	5025	1518
Bird no. 2	4230	1764
Untreated		
Bird no. 1	138	900
Bird no. 2	157	668

[a] 20 μCi of [U-^{14}C]glucose (10–15 mCi/mmole) given intraperitoneally at zero time; birds were killed 2 hr later.

Kinetics of plasma VLDL-TG

A. *Studies with reinjected, endogenously prepared ^{14}C-labeled VLDL-TG.* The steady-state VLDL-TG pool was seldom significantly disturbed in estrogenized hypertriglyceridemic birds by the quantity of [^{14}C]triglyceride-labeled VLDL administered (less than 10% of the VLDL-TG pool in 13 birds and 13% to 68% in the other 6). However, because it was often difficult to avoid disturbing the steady state in untreated birds, the quantity of [^{14}C]triglyceride-labeled VLDL administered (although as small as consistent with requirements for scintillation counting) represented a load in most instances. However, the kinetics observed with labeled palmitate (no disturbance of steady state) were identical with those observed with ^{14}C-labeled VLDL in two untreated birds, each of which received a different load of ^{14}C-labeled

TABLE 5. Plasma levels, pool sizes, fractional turnover rates, and turnover rates of VLDL-TG in untreated birds

Bird	Plasma VLDL-TG Concentration	VLDL-TG Pool Size	Amount of VLDL-^{14}C-TG[a] Injected	K VLDL-^{14}C-TG[a]	Turnover of VLDL-TG
	mg/100 ml	*mg*	*mg*		*mg/hr*
A	8	1	82	0.76	1
B	11	3	7	0.86	3
C	14	5	15	0.82	4
D	20	4	29	0.71	3
E	20	4	54	0.60	2
F	20	5	159	0.50	3
G	21	6	26	0.80	5
H	24	7	21	0.75	5
I	25	8	20	0.73	6
J	29	8	36	0.74	6
K	29	8	23	0.66	5
L	32	6	120	0.47	3
M	33	5	64	1.01	5
N	36	10	93	0.56	6
O	50	13	20	0.83	10
P	51	9	26	0.73	7
Q	55	10	64	0.70	7
R	62	16	20	0.78	12
Mean ± SEM	30 ± 4	7 ± 1		0.72 ± 0.03	5 ± 1

[a] [^{14}C]Triglyceride-labeled VLDL.

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VLDL (see below, Table 8). Hence, the transient disturbance of the steady state and the use of a variable quantity of VLDL-TG from bird to bird appear not to have been factors in the kinetics observed. Furthermore, though the very large loads of VLDL-TG used in a few untreated birds might have produced the relatively low fractional transport rates that accompanied them, the weak correlation between all the fractional rates (including those of Table 8) and injected loads ($r = -0.52$) suggests that the former were not critically influenced by the latter. For these reasons, although it is possible that our observed mean transport rates in untreated birds are lower than they might have been without the experiments done with very large injected loads, we consider the reported rates to be reasonable approximations. The identity of kinetics observed with labeled palmitate and [^{14}C]triglyceride-labeled VLDL also makes it unlikely that the VLDL-TG kinetics were influenced by the use of early secretory ^{14}C-labeled VLDL that was potentially nonuniformly labeled (since the labeled VLDL was harvested 45 min after injection of precursor, shortly before peak labeling) or by the use of "estrogenized" [^{14}C]triglyceride-labeled VLDL in control birds.

Fig. 2 shows representative curves of disappearance of reinjected [^{14}C]triglyceride-labeled VLDL, before and after estrogenization, obtained in one bird (designated "E" here and in the tables that follow).

Tables 5 and **6** present the plasma levels of VLDL-TG, VLDL-TG pool sizes, fractional turnover rates, and total turnover rates in untreated and estrogenized birds, respectively. Birds are ranked according to increasing levels of VLDL-TG. Comparison of the data in each group reveals obvious differences: the plasma level of VLDL-TG (and, consequently, the plasma VLDL-TG pool) is substantially greater in the estrogenized birds (2078 ± 396 compared with 30 ± 4 mg/100 ml in untreated birds, means ± SEM), and the fractional turnover rate of VLDL-TG is substantially less (0.32 ± 0.03 compared with 0.71 ± 0.03/hr). Nevertheless, the estrogenized birds have a much greater total turnover rate of VLDL-TG (233 ± 52 compared with 5 ± 1 mg/hr). All differences are significant at the level of $P < 0.01$.

The changes in these parameters (VLDL-TG level, fractional turnover, and turnover rates of VLDL-TG) induced by estrogen are epitomized in the seven birds studied both before and after treatment, whose data are presented in **Table 7.** In every case, estrogenization was accompanied by a rise in plasma VLDL-TG concentration. Although the rise in VLDL-TG concentration was accompanied by a decrease in fractional turnover rate of the triglyceride, the decrease was so small in comparison with the magnitude of increase of VLDL-TG that the net effect was a marked increase in total turnover rate.

B. *Studies with [^{3}H]palmitate and [^{14}C]triglyceride-labeled VLDL injected simultaneously.* **Fig. 3** shows curves of plasma ^{3}H-labeled VLDL and ^{14}C-labeled VLDL radioactivity of one of the four estrogenized hypertriglyceridemic birds. The curves are representative of

TABLE 6. Plasma levels, pool sizes, fractional turnover rates, and turnover rates of VLDL-TG in estrogenized birds

Bird	Plasma VLDL-TG Concentration	VLDL-TG Pool Size	Amount of VLDL-^{14}C-TG[a] Injected	K VLDL-^{14}C-TG[a]	Turnover of VLDL-TG
	mg/100 ml	*mg*	*mg*		*mg/hr*
P	311	59	26	0.36	21
J	323	154	25	0.67	104
H	523	136	93	0.28	39
A	841	143	62	0.40	57
K	958	383	25	0.52	201
E	1014	213	54	0.54	115
S	1030	443	20	0.20	89
E	1071	439	41	0.36	159
L	1276	822	13	0.17	140
T	1279	307	41	0.23	71
U	1283	526	20	0.32	166
V	2036	794	20	0.25	196
W	2604	1172	7	0.19	223
X	2973	1100	30	0.45	498
Y	3055	1436	20	0.21	297
Z	3200	3488	100	0.24	837
AA	4404	4187	13	0.06	251
BB	4431	1728	30	0.28	486
CC	6870	1934	20	0.36	692
Mean ± SEM	2078 ± 396	1024 ± 262		0.32 ± 0.03	244 ± 52

[a] [^{14}C]Triglyceride-labeled VLDL.

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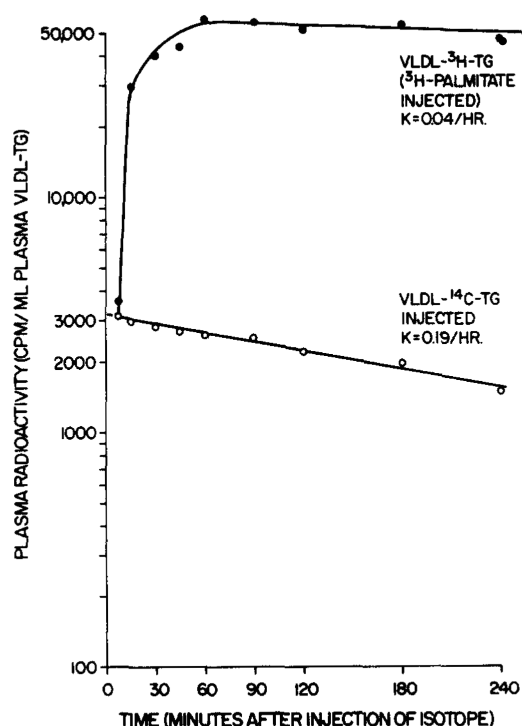

Fig. 3. Radioactivity curves of ^{14}C-labeled TG (VLDL-^{14}C-TG) and [^{3}H]triglyceride-labeled VLDL (VLDL-^{3}H-TG) after simultaneous injection of ^{14}C-labeled VLDL and [^{3}H]palmitate. Estrogenized, hypertriglyceridemic bird. Calculation of fractional turnover rates (*K*) is described in Methods.

those obtained in this group, demonstrating that fractional turnover rate (*K*) derived from the descending portion of the ^{3}H-labeled VLDL curve (0.04/hr) is much smaller than that derived from the ^{14}C-labeled VLDL curve (0.19/hr). On the other hand, no such difference exists in the untreated bird whose level of triglyceride is normal (**Fig. 4**): the late (postpeak) portion of the ^{3}H-labeled VLDL curve and the slope of the ^{14}C-labeled VLDL curve are essentially identical. Additional differences between the ^{3}H-labeled VLDL curves of estrogenized and untreated birds are the level of peak plasma radioactivity and the time elapsing before this peak is reached (Figs. 3 and 4). The peak is lower and prompt in untreated birds (15 min) and much higher and delayed in estrogenized birds (60–90 min). Summarizing the results of experiments done in the four estrogenized and two untreated birds, **Table 8** emphasizes that the late portion of the palmitate-generated VLDL-TG radioactivity curve cannot be assumed to represent only removal of labeled TG from the plasma in hypertriglyceridemic birds.

DISCUSSION

Consideration of the mechanism of endogenous hypertriglyceridemia devolves to considering the relative roles of increased synthesis and diminished disposal of plasma triglyceride.

The estrogenized hypertriglyceridemic chick afforded an opportunity to evaluate the kinetics of endogenous plasma triglyceride from the standpoint of a priori knowledge that the hypertriglyceridemia is related to increased production of triglyceride. Rather than having to decide from triglyceride radioactivity curves whether the curves indicated overproduction or underdisposal, we could ascribe whatever curves resulted to overproduction because of the several consistent lines of evidence that the liver of the estrogenized hypertriglyceridemic bird synthesizes triglyceride at a supernormal rate. Earlier work from this laboratory (9) and the work of Hawkins and Heald (11) showed that liver slices from estrogenized birds synthesize more triglyceride than control slices, from both acetate and free fatty acid precursors. Confirming these findings are the results of the present in vivo studies. First, there is the observation of 10-fold increased incorporation of plasma free fatty acids into triglyceride by livers of estrogenized birds within 5 min of injection of labeled palmitate, the in vivo

TABLE 7. Plasma levels, fractional turnover rates, and turnover rates of VLDL-TG in birds studied before and after estrogenization

	VLDL-TG Level			Fraction Turnover Rate (*K*) of VLDL-^{14}C-TG			Turnover rate of VLDL-TG		
Bird	Before	After DES[a]	After DES[b]	Before	After DES[a]	After DES[b]	Before	After DES[a]	After DES[b]
	mg/100 ml plasma						*mg/hr*		
A	8	841		0.76	0.40		1	57	
E	20	1014	1071	0.60	0.54	0.36	2	115	159
H	24		523	0.75		0.28	5		39
J	29		323	0.74		0.67	6		104
K	29		958	0.66		0.52	5		201
L	32		1279	0.47		0.23	3		71
P	51	311		0.73	0.36		7	21	

[a] 1.0 mg/day for 2 days.
[b] 1.0 mg/day for 6 days.

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TABLE 8. Comparison of fractional turnover rate (K) and turnover rate of VLDL-TG determined simultaneously from reinjected [^{14}C]triglyceride-labeled VLDL[a] and [^{3}H]triglyceride-labeled VLDL[b] generated from [^{3}H]palmitate[c]

	K ^{14}C-TG	Amount of VLDL-^{14}C-TG Injected	K ^{3}H-TG	Turnover Rate of VLDL-TG Derived from VLDL-^{14}C-TG/ VLDL-^{3}H-TG
	fraction/hr	*mg*	*fraction/hr*	*mg/hr*
Untreated	0.98	20	0.98	15/15
	0.50	7	0.48	6/6
Estrogenized	0.19	20	0.04	291/46
	0.19	7	0.04	272/57
	0.17	13	0.04	129/30
	0.06	13	0.00	215/0

[a] VLDL-^{14}C-TG.
[b] VLDL-^{3}H-TG.
[c] Fractional turnover rate of VLDL-^{3}H-TG calculated from late portion of VLDL-^{3}H-TG curve.

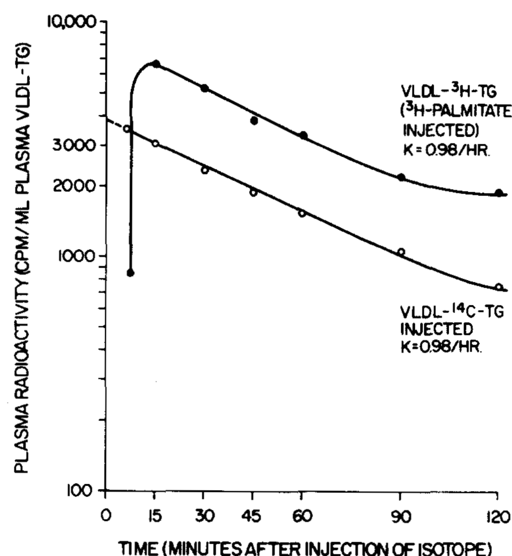

Fig. 4. Same experiment as in Fig. 3, except bird was unestrogenized and had normal plasma triglyceride level.

counterpart of earlier in vitro observations (11). Then, there are (*1*) the observation that the estrogen-induced 84-fold rise in plasma VLDL-TG content is accompanied by an 86-fold increase in incorporation of [^{14}C]acetate into the same triglyceride and (*2*) the similar observation when [^{14}C]glucose is the triglyceride precursor. If synthesis of triglyceride and its secretion into plasma had not been increased by estrogen, i.e., if labeled precursor were being secreted as triglyceride at similar rates in estrogenized and control birds, in which case the increased VLDL-TG levels would be due to diminished disposal alone, then one would have expected the specific activity of the triglyceride to be much lower in the estrogenized birds, because the newly synthesized labeled molecules would have been diluted by the large number of unlabeled molecules in the vastly expanded, stagnant plasma triglyceride pool. Increased synthesis from a precursor acetate pool is further supported by the observation that estrogenized birds diverted from 8- to 66-fold more [^{14}C]acetate to synthesis of VLDL-TG than did untreated birds. (For reasons discussed in the Methods section, these numbers are only estimates and must be regarded as minima.) Although these precursor–product experiments yield neither information about metabolism of precursors by tissues other than liver nor quantitative information on estrogen-induced alterations of hepatic pathways of precursor and TG metabolism beyond augmentation of those pertinent to synthesis of plasma TG, because of the overwhelming quantitative differences observed between estrogenized and untreated birds lack of such data does not detract from the conclusion that estrogen-induced hypertriglyceridemia results from increased hepatic production of trigylceride from both lipid precursor (plasma free fatty acid) and nonlipid precursor (acetate, glucose) as a result of augmented de novo lipogenesis.

When the kinetics of plasma VLDL-TG are examined in the estrogenized bird by the method of reinjecting endogenously labeled native [^{14}C]triglyceride-labeled VLDL, the results are entirely consistent with overproduction. Although the fractional turnover rate of plasma VLDL-TG is greater in the untreated bird than in the estrogenized bird, the total turnover rate is overwhelmingly greater in the latter. For example (Table 7), five birds treated with six doses of estrogen had 11–40-fold increases in VLDL-TG concentration but only 1.1–2.6-fold decreases in fractional turnover rate; consequently, on the average, a 34-fold increase in turnover rate resulted. When all results in the estrogenized state are compared with those in the control, estrogenization is associated with a 50-fold increase in turnover rate. Viewed kinetically, then, from the standpoint of disappearance of reinjected VLDL-TG, the hypertriglyceridemia results primarily from increased production of VLDL-TG.

It is clear that the kinetics of plasma VLDL-TG as described by TG radioactivity curves generated from labeled fatty acid precursor cannot be analyzed precisely (in this model of hypertriglyceridemia, at least) by a mathematical approach that derives fractional turnover rate of TG solely from the descending slope of TG radioactivity. Applying, for instance, the mathematical scheme of Friedberg et al. (14), we find turnover rates of VLDL-TG ranging from 0 to 57 mg/hr in estrogenized birds, rates that are, at best, but 20% of those derived simultaneously from reinjected [^{14}C]triglyceride-labeled VLDL (Table 8). Such is not the case, however, in the unestrogenized state when TG concentrations are normal; in this situation, turnover rates derived from [^{3}H]palmitate precursor and the model of Friedberg et al. (14) are identical with those

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derived simultaneously from reinjected ^{14}C-labeled VLDL (Table 8). (In fact, the model of Friedberg et al. was validated only in nonhypertriglyceridemic humans, and attention was directed to this point in the original report [14].)

Thus, though the Friedberg mathematical analysis of palmitate-generated VLDL-TG radioactivity approximates the true flux of VLDL-TG in nonhypertriglyceridemic birds (as it does in nonhypertriglyceridemic humans), it cannot be regarded as applicable when VLDL-TG levels have been elevated by estrogen. The reason for this lies in the lack of parallelism of the late portion of the palmitate-generated ^{3}H-labeled VLDL radioactivity curve with the curve of reinjected ^{14}C-labeled VLDL in hypertriglyceridemic birds. Quite clearly, the flattened (if not flat) late portion, characteristic of estrogen-induced hypertriglyceridemia, represents more than simply removal of VLDL-TG from plasma; otherwise, it would parallel the curve of disappearance of ^{14}C-labeled VLDL. For instance, such a curve might be produced by slow, continuous secretion of newly synthesized labeled VLDL-TG into the circulation beyond the time when peak levels have been reached. That is, the rate constant from liver to plasma (k_1) could be small enough in comparison with the rate constant out of plasma (k_2) that measurements of k_2 by graphic methods could be unreliable (14). There are two reasons to consider this possibility to be the case. First, in hypertriglyceridemic birds, the early (prepeak) portion of the plasma VLDL-TG radioactivity curve is also distinctive, differing from normal in the longer time required for the peak to be reached (slow "up-slope"), suggesting that the rate of secretion of labeled triglyceride is slower. Second, as the present experiments reaffirm, avian estrogen-induced hypertriglyceridemia is accompanied by fatty liver. The increased hepatic triglyceride pool might be expected to retard secretion of newly synthesized labeled triglyceride molecules, diluted by the crowd of preexisting unlabeled molecules.

Though our experimental subjects were birds who received estrogen, it is possible to suggest applications of the present observations to human endogenous hypertriglyceridemia. The most obvious suggestion is that the avian model may be fundamentally similar to human estrogen-induced hypertriglyceridemia, which may also be due to increased production of triglyceride (27). This possibility requires further investigation. Beyond this is the suggestion, implicit in our discussion of precursor-derived VLDL-TG kinetic behavior, that flux of plasma TG calculated from the rate of disappearance of fatty acid-labeled TG be viewed as a possible underestimate in hypertriglyceridemia. Some have used this method in human hypertriglyceridemia (15); others have suggested caution in interpretation of these data (13). The caution seems even more appropriate in light of our findings. Whether use of labeled glycerol as VLDL-TG precursor would avoid the complexities of kinetic analysis that accompany use of labeled fatty acid in such studies as these is a matter for future investigation. As they stand, our studies allow us to conclude that avian estrogen-induced hypertriglyceridemia is characterized by large increases in production of VLDL-TG.

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