Concurrent inductions of avian hepatic lipogenesis, plasma lipids, and plasma apolipoprotein B by estrogen

Nassrin Dashti, Jim L. Kelley,[1] Rollin H. Thayer, and Joseph A. Ontko[2]

Laboratory of Lipid and Lipoprotein Studies, Oklahoma Medical Research Foundation, Oklahoma City, OK 73104,[3] Department of Animal Science, Oklahoma State University, Stillwater, OK 74074,[4] and Department of Biochemistry and Molecular Biology, College of Medicine, University of Oklahoma, Oklahoma City, OK 73190[5]

Abstract The inductions of hepatic fatty acid synthesis, estrogen-specific plasma proteins, plasma lipids, and apolipoproteins by a single subcutaneous injection of diethylstilbestrol (40 mg/kg body weight) have been examined in immature male turkeys. Estrogen induced the appearance of phosvitin, lipovitellin, and apoVLDL-II in the blood plasma. The highest concentrations of these estrogen-specific plasma proteins were observed 48 hr following hormone administration. Estrogen increased the concentration of triglyceride in the liver, predominantly those molecular species containing 16 carbon fatty acids (triglycerides with 53 and 55 carbon atoms). Liver cholesterol was present predominantly as free cholesterol. Although estrogen did not affect the concentrations of free or esterified cholesterol in the liver, the hormone increased the amount of cholesterol esterified with 20-carbon fatty acids and caused a corresponding decrease in cholesterol esterified with 18 carbon fatty acids. Estrogen treatment elevated the plasma triglycerides 55-fold, tripled the plasma phospholipid, and approximately doubled the plasma cholesterol. The de novo synthesis of fatty acids in the liver in vivo was stimulated by estrogen administration, as exhibited by increased ^3H_2O incorporation into the phospholipids and triglycerides of both liver and plasma. In contrast, hepatic cholesterol synthesis was unaffected. The amounts of newly synthesized trigylceride in the liver and plasma and the specific radioactivities of the plasma triglyceride following 1-hr in vivo labeling periods, 0, 24, 48, and 72 hr after estrogen injection indicate that increased hepatic fatty acid synthesis is a primary and major casuative factor in the development of estrogen-induced hyperglyceridemia in this avian species. The concentration of apolipoprotein B in the plasma increased in parallel with hepatic fatty acid synthesis and the appearance of newly synthesized triglyceride in the plasma, whereas the plasma apolipoprotein A-I level decreased. These observations indicate that in the avian liver estrogen causes a coordination of inductions in the conversion of carbohydrate to triglyceride and in the production of proteins (apolipoprotein B and apoVLDL-II) involved in the assembly of triglyceride-rich lipoprotein particles, leading to hypersecretion of these lipoproteins into the circulation.—**Dashti, N., J. L. Kelley, R. H. Thayer, and J. A. Ontko.** Concurrent inductions of avian hepatic lipogenesis, plasma lipids, and plasma apolipoprotein B by estrogen. *J. Lipid Res.* 1983. **24:** 368–380.

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Estrogen administration in avian species causes a remarkable increase in the concentration of plasma lipids (1, 2), characterized by a marked elevation in triglyceride and also, to a lesser but significant extent, cholesterol in the circulating very low density lipoproteins (VLDL) (3).

Increased incorporation of [^{14}C]acetate into plasma triglycerides (4) and ^{3}H-labeled amino acid into VLDL protein by liver slices (5–7) indicate that the estrogen-induced accumulation of plasma VLDL is caused, at least in part, by increased hepatic production. The observation of increased hepatic concentration of mRNA coding for apoVLDL-II, a low molecular weight apolipoprotein of VLDL, following estrogen treatment (8) provides further evidence for this mechanism.

The turkey has been employed for studies on the pathogenesis of arterial disease since this animal spontaneously develops both hypertension and atherosclerosis (9–13). Consequent death, precipitated by the rupture of aortic aneurysms in these animals, is accelerated by estrogen-induced elevations of the concentrations of blood plasma lipids (14, 15).

Hepatic fatty acids, utilized for the synthesis of VLDL triglyceride, are derived from plasma free fatty acids,

Abbreviations: DES, diethylstilbestrol; PBS, phosphate-buffered saline; VLDL, very low density lipoprotein; LDL, low density lipoprotein; LP-B, lipoprotein B.

[1] Present address: Department of Pathology, University of Texas Health Science Center, San Antonio, TX 78284.

[2] To whom reprint requests should be addressed.

[3] N. Dashti, J. L. Kelley, and J. A. Ontko.

[4] R. H. Thayer.

[5] J. A. Ontko.

fatty acids present in plasma lipoprotein species removed by the liver, and de novo synthesized fatty acids. The relative inductions of hepatic fatty acid synthesis and hyperglyceridemia following estrogen treatment in avian species have not been quantified nor have these responses been examined in parallel with the concentration of plasma apolipoprotein B, the major high molecular weight protein constituent of the triglyceride-rich plasma lipoproteins. In the present study, hepatic de novo fatty acid synthesis in vivo was quantified by the incorporation of ^3H_2O into liver and plasma lipids and the corresponding plasma concentrations of apolipoprotein B were determined by electroimmunoassay, providing a comparison of the temporal patterns of these responses to estrogen treatment.

MATERIALS AND METHODS

Materials

Diethylstilbestrol (DES), silicic acid, digitonin, and ATP were purchased from Sigma Chemical Co. (St. Louis, MO). Glycerol-3-phosphate dehydrogenase was obtained from Boehringer Mannheim (Indianapolis, IN). ^3H_2O was from New England Nuclear (Boston, MA). Indubiose A 45 was from Accurate Chemical and Scientific Corp., (Hicksville, NY). NAD was purchased from PL-Biochemicals (Milwaukee, WI). Glycine was obtained from Eastman Kodak Co. (Rochester, NY). Ultraphore was obtained from BASF AG (Ludwigshafen am Rhein, West Germany). All other chemicals were reagent grade.

Animals

Male Broad Breasted turkeys of the Nicholas strain were used in these experiments 19 days after hatching. The birds weighed 500–600 g at the start of the study and were maintained on regular grower ration. The diet consisted of corn oil, 1.4%; corn (ground yellow), 35.9%; soybean meal, 37.3%; meat and bone scrap, 14.5%; alfalfa meal, 4.5%; dried whey, 2.8%; live yeast culture, 2.5%; DL-methionine, 0.2%; vitamin mix, 0.3%; trace mineral, 0.1%; and NaCl, 0.5%; all by weight. Experimental animals were given a single subcutaneous injection of diethylstilbestrol (DES), dissolved in corn oil at a dose of 40 mg/kg body weight. Control birds were injected with an equal volume of corn oil.

Preparation of liver homogenates

All birds received 20 mCi ^3H_2O intraperitoneally one hr prior to being killed. Birds were decapitated and blood was collected in tubes containing sodium citrate (0.5%). The whole liver was perfused with phosphate-buffered saline (PBS), excised rapidly, and placed in beakers containing cold PBS. After removal of any non-hepatic tissue, the livers were cut into small pieces and washed several times with PBS to remove any further contamination of blood. Each liver was homogenized in a volume of PBS equal to four times the liver weight.

Chemical analyses of triglyceride and total cholesterol of liver and plasma

For determination of triglyceride, an aliquot of plasma was diluted to 1.2 ml with water. For analyses of liver, 1.2 ml of a 20% liver homogenate was used. After addition of 8 ml of methanol and 16 ml of chloroform, the mixture was warmed to 40°C in a water bath for 30 min. The extract was then filtered and washed (16). The lipid fraction was then processed to remove phospholipids, saponified, and extracted to remove fatty acids as previously described (17). The final aqueous phase was analyzed for glycerol (18).

For total cholesterol determination, 3 ml of 8% KOH in ethanol was added to 0.05 ml of plasma and the mixture was saponified at 70°C for 20 min. After addition of 3 ml of water the nonsaponifiable fraction was obtained with two hexane extractions. The combined hexane extracts were evaporated to dryness with N_2, followed by addition of 0.5 ml acetone–ethanol 1:1 (v/v) and one drop of 20% acetic acid in water. After mixing, 0.2 ml of digitonin solution [0.5% in ethanol–water 1:1 (v/v)] was added and the mixture was left at 4°C overnight. The precipitate obtained after 10 min centrifugation at 2000 rpm was washed once with acetone–ether 1:2 (v/v) and centrifuged under the same conditions. The final precipitate was dried in an oven at 110°C for 30 min and dissolved in 0.1 ml of glacial acetic acid and 0.75 ml of $FeCl_3$ solution (0.08% in glacial acetic acid). To enhance this process, the mixture was heated at 60°C in a water bath. The absorbance of the solution was measured at 560 nm 25 min after addition of 0.5 ml of concentrated sulfuric acid (19).

Determination of free cholesterol and molecular species of triglyceride and cholesteryl ester by gas–liquid chromatography

Free cholesterol concentration and the molecular species of triglyceride and cholesteryl ester of liver were determined by the gas–liquid chromatographic method of Kuksis et al. (20) with cholesteryl butyrate as internal standard, after lipid extraction of liver homogenates (above) according to Folch, Lees, and Sloan Stanley (16).

Determination of radioactivity in various lipid fractions in liver homogenates and plasma

One-ml aliquots of liver homogenates and plasma were extracted with 20 ml of chloroform–methanol 2:1. After standing for at least 2 hr at room temperature, the extracts were filtered and adjusted to 25 ml with the same solvent. Complete extraction of lipids from liver and plasma of control and estrogen-treated animals was achieved by this procedure. A 20-ml aliquot of each filtrate was then washed according to the procedure of Folch et al. (16). The washed lipid extract was then adjusted to 25 ml with chloroform–methanol 2:1. Five-ml aliquots of these lipid extracts were evaporated to dryness under nitrogen in counting vials and, after addition of the scintillation fluid Instagel, were counted for determination of radioactivity in the total lipid fraction. For separation of phospholipids, diglycerides plus cholesterol, free fatty acids, triglycerides, and cholesteryl esters, aliquots of the total lipid extracts were placed in conical centrifuge tubes, evaporated to dryness under nitrogen, redissolved in a small volume of petroleum ether (in which the various lipids, above, were completely soluble), and applied to thin-layer chromatography plates coated with Silica Gel G containing the fluorescent agent Ultraphore (21). Although nearly all of the lipids in the extract are transferred onto the chromatography plates in this procedure, quantitative transfer is not necessary at this point, since the total radioactivity in each lipid fraction can be calculated as described below. Lipids were separated with a solvent system of hexane–diethyl ether–glacial acetic acid 80:20:1. By visualization with ultraviolet light, the thin-layer chromatography plates were divided into five regions (phospholipid, diglyceride plus cholesterol, free fatty acid, triglyceride, and cholesteryl ester). Each region was then scraped into a vial containing Instagel for liquid scintillation counting. The radioactivity found in each region was expressed as a percent of the total of the five regions and this percentage was multiplied by the total radioactivity in the lipid extracts of liver homogenates and plasma, obtained as described above, to calculate the total radioactivity in each lipid fraction.

Determination of total fatty acid and digitonin-precipitable sterol radioactivities

Tritium incorporation into the total fatty acids and digitonin-precipitable sterol of plasma and liver homogenates was measured (22) in the following manner. One-ml aliquots of plasma or liver homogenate were placed in culture tubes with Teflon-lined screw caps. To each tube, 1.0 ml of 60% KOH and 2.0 ml of absolute ethanol were added and the samples were saponified (90°C for 2 hr in a water bath). The samples were allowed to cool and were then extracted 3 times with 5 ml of petroleum ether (30°–60°C boiling range). The pooled petroleum ether extracts were backwashed with 7.5 ml of water and processed for digitonin-precipitable sterol radioactivity. The aqueous lower phase from the ether extraction (above) was processed for fatty acids in the saponifiable fraction. The petroleum ether extracts (the nonsaponifiable fractions) were evaporated to dryness under nitrogen at 45°C followed by addition of 10 ml of acetone–absolute ethanol 1:1 (v/v) 0.1 ml of 10% (v/v) acetic acid in water. After mixing, 0.1 ml of unlabeled cholesterol solution (10 mg/ml in acetone) was added, followed by 4 ml of 0.5% digitonin solution in ethanol–water 1:1. The samples were stored at 4°C overnight and then centrifuged at 2000 rpm for 30 min at 4°C. The supernatant was poured off and the precipitate was resuspended in 15 ml of acetone–anhydrous diethyl ether 1:2 and after mixing was centrifuged under similar conditions. The supernatant was removed and the precipitate was resuspended in 15 ml of anhydrous diethyl ether and centrifuged as before. The final precipitate was dissolved in 2 ml of methanol and transferred to the scintillation vial. The methanol was evaporated and, after addition of Instagel, the radioactivity in this sterol fraction was counted. The aqueous phase from the ether extraction (fatty acids in the saponifiable fraction) was acidified (pH 1–2) with 2.5 ml of 5 N HCl and extracted 3 times with 5 ml of petroleum ether. The pooled extracts were placed in culture tubes and backwashed with 7.5 ml of water. The petroleum ether extracts were transferred to counting vials, evaporated to dryness and, after addition of Instagel, the radioactivity in this fatty acid fraction was counted.

Electroimmunoassay of turkey plasma apolipoproteins B and A-I

The electroimmunoassay of Laurell (23) was adopted for the quantification of turkey plasma apolipoproteins B and A-I. Turkey lipoprotein B (LP-B) was prepared from low density lipoprotein (LDL) by concanavalin A-Sepharose affinity chromatography according to the procedure described by McConathy and Alaupovic (24). Immunological and electrophoretic studies showed that the protein moiety of LP-B consisted only of apoB (25). Antiserum to turkey LP-B was produced in rabbits as described previously (26). The antiserum was monospecific for apoB as determined by immunodiffusion (**Fig. 1**) and by electroimmunoassay, and was stored at −20°C in the presence of sodium azide (1 mg/ml) as a preservative. For the apoB electroimmunoassay, the supporting medium contained 2.5 g of agarose (Indubiose A 45) in 100 ml of 0.05 M diethylbarbiturate buffer (pH 8.5) containing 0.01% NaN_3 and 0.01% Thimerosal. The plates were prepared using anti-B

serum, electrophoresed for 3 hr at 10 v/cm, dried, stained, and measured as previously described by Curry, Alaupovic, and Suenram (27). The standard curve was constructed using turkey LP-B, isolated from LDL by concanavalin A-Sepharose affinity chromatography as described above. This curve was based on the protein content determined by the procedure of Lowry et al. (28). The relationships between rocket height and apoB concentration for the standard and for the plasma apoB-containing lipoproteins were very similar, if not identical. In addition, the rocket shapes and heights of intact and partially-delipidized plasma from hypertriglyceridemic estrogenized birds were the same. These observations provide a basis for expressing the plasma apoB concentrations in absolute terms. Electroimmunoassay of turkey plasma apoA-I was performed as described for apoB (above) using monospecific antiserum to apoA-I and turkey lipoprotein A as the primary standard (25).

Preparation of antisera to apoVLDL-II, phosvitin, and lipovitellin

The egg yolk proteins, phosvitin and lipovitellin, were purified and antisera were prepared as described previously (29). ApoVLDL-II was purified from totally delipidized laying turkey plasma VLDL by gel filtration chromatography of apoVLDL by the procedure described by Chan et al. (6). We have observed that turkey apoVLDL-II has a molecular weight and an amino acid composition similar to that reported for chicken apoVLDL-II (6).

Reactions of control and estrogen-treated turkey plasmas to antisera specific for apolipoproteins A-I and B

The monospecificities of the antisera employed for the electroimmunoassay of apolipoproteins A-I and B are shown in Fig. 1. Estrogen treatment caused no gross antigenic differences in either plasma apolipoprotein A-I or B.

Polyacrylamide gel electrophoretic analysis of apoB

The possible heterogeneity of apolipoprotein B was examined by sodium dodecylsulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) (26). Accordingly, the plasma lipoproteins of $d < 1.073$ g/ml were isolated from control and estrogenized turkeys (48 hr after receiving 40 mg of diethylstilbestrol/kg body weight) by centrifugation. They were washed, dialyzed, and partially delipidated; SDS was added and the proteins were electrophoresed on SDS-3.5% polyacrylamide gel. A single band was observed at M_r 360,000 daltons, indicating the absence of the lower molecular weight (240,000 daltons) form of apolipoprotein B in the plasma VLDL and LDL of the control and estrogenized birds.

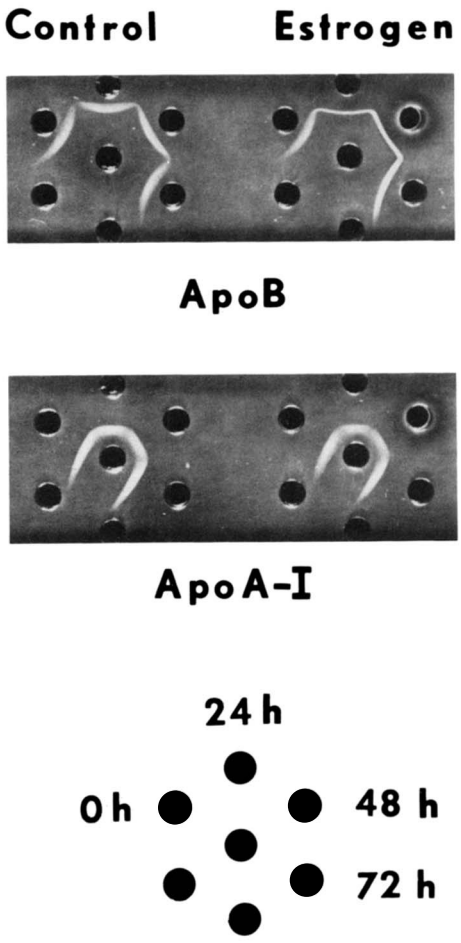

Fig. 1. Immunodiffusion of turkey lipoprotein A-I and lipoprotein B in 1% agarose. Plasma samples from control and estrogen-treated (24, 48, and 72 hr post-treatment) turkeys were placed in the outer wells. The center wells contained anti-lipoprotein B, apoB; anti-lipoprotein A-I, apoA-I.

RESULTS

Induction of estrogen-specific proteins

The appearances of apoVLDL-II, phosvitin, and lipovitellin in blood plasma following the treatment of immature male turkeys with diethylstilbestrol are documented in **Fig. 2.** These proteins were not detected in the plasma of the noninjected male turkeys, but were observed 24, 48, and 72 hr after subcutaneous estrogen administration. These estrogen-induced plasma proteins have not been previously demonstrated in this species. The double precipitin lines against lipovitellin are due to the presence of two forms of lipovitellin, α and β (29, 30). These results (Fig. 2) provide evidence for the effectiveness and specificity of estrogen in this avian system.

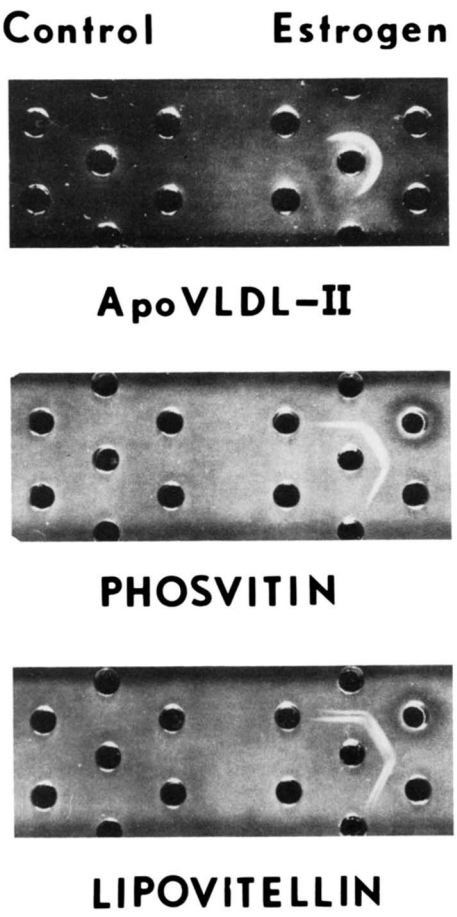

Fig. 2. Time course of induction of plasma apoVLDL-II and egg yolk precursors, phosvitin and lipovitellin; immunodiffusion in 1% agarose. Plasma samples from control and estrogen-treated turkeys were placed in the outer wells (see diagram in Fig. 1). The center wells contained anti-apo-VLDL-II, top; anti-phosvitin, middle; and anti-lipovitellin, bottom.

Although plasma apoVLDL-II, phosvitin, and lipovitellin were not detected in the control turkeys (Fig. 2), in agreement with the polyacrylamide gel electrophoretic analysis of chicken plasma apoVLDL-II by Kudzma, Swaney, and Ellis (31) and the measurement of hepatic apoVLDL-II mRNA in the cockerel by Wiskocil et al. (32), apoVLDL-II and vitellogenin in the plasma of nonestrogenized roosters have been reported (33). The apparent lack of agreement is probably a result of the relative sensitivities of the methods employed. Thus, the radioimmunoassay of Blue and Williams (33) is much more sensitive than immunodiffusion and electrophoresis. The concentrations of apoVLDL-II reported (33) are far below the minimum detectable by the methods employed in the present study. Therefore, these plasma proteins may normally circulate in very low concentrations in avian species in the absence of exogenous estrogen.

Effects of diethylstilbestrol on the liver lipids of young male birds

The liver triglyceride concentration was increased 270% by diethylstilbestrol treatment, whereas the hormone had no effect on the total cholesterol content (**Table 1**). Approximately 90% of the cholesterol was in the free form and this was also unaffected by estrogen treatment (Table 1).

The major triglyceride (65–70% of the total) in both systems contained a total carbon number of 55, consisting mainly of one fatty acid of 16 carbons and two fatty acids of 18 carbons (**Table 2**). In the control system, TG-53 (molecular species containing mainly two fatty acids of 16 carbons and one 18-carbon fatty acid) was the minor species (11% of the total) while TG-57, consisting mainly of three fatty acids of 18 carbons, was intermediate (24% of the total). Diethylstilbestrol treatment decreased the relative percentage of TG-57 and produced a corresponding increase in TG-53. These percentages, however, when multiplied by the corresponding concentrations of triglyceride in the liver (Table 1), while showing no change in the absolute concentration of TG-57 following estrogen treatment, demonstrate a 4-fold increase in TG-55 and a 7-fold increase in TG-53.

The esterified cholesterol molecular species are also shown in Table 2. The cholesterol esterified with fatty acids of 18-carbon chain length was the major form in the livers of both control and DES-treated animals, although significantly less in the latter. The relative abundance of cholesteryl ester with fatty acid of 16 carbons was similar in both systems and constituted 6–8% of the total. The livers of estrogenized birds showed a remarkable increase in cholesterol esterified with 20-carbon fatty acid (32% of the total) relative to the livers from the control birds (2%). The net affect of estrogen treatment was, therefore, a shift from cholesteryl esters containing 18-carbon fatty acids to those containing fatty acids with 20-carbon atoms.

TABLE 1. Triglyceride and cholesterol composition of turkey liver in response to estrogen treatment

Treatment[a]	Triglyceride	Total Cholesterol	Free Cholesterol	Esterified Cholesterol
	mg/g wet wt			
Control	2.65 ± 0.44[b]	2.21 ± 0.16	1.99 ± 0.13	0.21 ± 0.04
Estrogen[c]	9.77 ± 0.97[d]	2.20 ± 0.14	1.94 ± 0.12	0.26 ± 0.03

[a] Livers were removed 24, 48, and 72 hr after injection for analysis. Values are for 48 hr samples, which showed the maximum response.
[b] Values are the mean ± S.E.M. of four birds. The average liver weights were 9.62 g (control) and 12.10 g (estrogen).
[c] The dose of estrogen was 40 mg diethylstilbestrol/kg body wt.
[d] $P < 0.005$.

TABLE 2. Molecular species of triglyceride and esterified cholesterol in turkey liver in response to estrogen treatment

Treatment[a]	Triglyceride			Esterified Cholesterol		
	TG-53[b]	TG-55	TG-57	EC-16[c]	EC-18	EC-20
	% of total			*% of total*		
Control	11.3 ± 0.6[d]	64.8 ± 0.5	24.0 ± 1.1	6.3 ± 2.2	92.0 ± 3.1	1.7 ± 1.7
Estrogen[e]	21.3 ± 1.5[f]	70.3 ± 1.3[f]	8.5 ± 0.2[f]	7.6 ± 0.8	60.0 ± 3.9[f]	32.4 ± 4.6[f]

[a] Livers were analyzed 48 hr after treatment.
[b] TG-53, TG-55, and TG-57 refer to molecular species with total carbon numbers of 53, 55, and 57.
[c] EC-16, EC-18, and EC-20 refer to molecular species with fatty acids containing 16, 18, and 20 carbons.
[d] Values are the mean ± S.E.M. of four birds.
[e] The dose of estrogen was 40 mg diethylstilbestrol/kg body weight.
[f] $P < 0.02$.

Body and liver weights of birds employed for the study of fatty acid synthesis and plasma apolipoproteins in response to estrogen treatment

The effects of diethylstilbestrol on the body and liver weights of young male turkeys are shown in **Table 3.** Acute administration of the hormone did not affect body weight but increased the liver weight, both in absolute terms and, more significantly, in relation to the body weight. The hematocrit was not significantly affected by estrogen treatment, indicating that the plasma volumes were unaltered. The estimated plasma volumes (Table 3) were calculated using 6.0% of the body weight, based on the data of Medway and Kare (34) for birds of the size used. The information shown in Table 3 provides a data base for further calculations on the pool sizes of lipids and lipoproteins.

Concentrations of plasma triglyceride and cholesterol in response to estrogen administration

The plasma triglyceride was elevated 30-fold by estrogen as early as 24 hr after injection (**Table 4**). The maximum hyperglyceridemic effect of estrogen was achieved 48 hr after treatment (55-fold increase). In contrast to the lack of effect of diethylstilbestrol on liver cholesterol (Table 1), the hormone caused an increase in plasma cholesterol (Table 4). A maximum effect of 2.3-fold was observed at 48 hr. An increase in the plasma lipid phosphorus concentration of 203% was observed 48 hr after estrogen injection (data not shown). The concentrations of plasma triglyceride and cholesterol subsequently declined during the third day following hormone treatment.

The relative amounts of triglyceride that accumulated in the liver and plasma in response to estrogen may be, at least approximately, compared from the data in Tables 1, 3, and 4. Thus, 48 hr after estrogen treatment the liver triglyceride was increased from an average of 25 mg to 118 mg per liver (Table 1), whereas the blood plasma of control and estrogen-treated birds contained approximately 7 mg and 376 mg of triglyceride, respectively (Tables 3 and 4). The liver triglyceride content was increased about 5-fold by estrogen, whereas the amount of triglyceride in the plasma was increased 54-fold.

Incorporation of ^3H_2O into phospholipids and triglycerides of liver and plasma

To evaluate the role of fatty acid synthesis in the estrogen-induced accumulation of plasma triglycerides, the effects of diethylstilbestrol on the in vivo syntheses of liver and plasma lipids were measured by incorporation of tritium from ^3H_2O into various lipid fractions. A 1-hr period of tritium incorporation was employed. During this period, the specific activity of ^3H_2O remains constant and hepatic fatty acid synthesis may be measured by analysis of lipid radioactivity in the liver and plasma. A similar procedure has been employed to measure the in vivo rate of hepatic lipogenesis in the rat (35). This procedure assumes no difference in the availability of ^3H_2O at the subcellular sites of lipid synthesis in control and experimental animal groups. In a preliminary experiment it was found that very little secretion

TABLE 3. Body and liver weights and estimated plasma volumes of birds used for the measurements of lipogenesis and plasma lipids and apolipoproteins

Time and Treatment	Body Weight	Liver Weight		Estimated Plasma Volume
	g	*g*	*% of body wt*	*ml*
0-hr Control	523	10.1	1.93	31.4
24-hr Control	559	10.2	1.82	33.5
24-hr Estrogen	539	12.5	2.33	32.3
48-hr Control	594	12.1	2.04	35.6
48-hr Estrogen	575	15.5	2.70	34.5
72-hr Control	593	12.0	2.02	35.6
72-hr Estrogen	591	14.8	2.50	35.5

All values are the mean of three birds in each group. These birds were used for the measurements shown in Tables 4–7 and Figs. 3–6.

TABLE 4. Effects of diethylstilbestrol on turkey plasma triglyceride and cholesterol concentrations

Time after Estrogen Treatment	Plasma Triglyceride		Plasma Total Cholesterol	
	Control	Estrogen[a]	Control	Estrogen[a]
hr	*mg/100 ml*		*mg/100 ml*	
0	11.1 ± 1.0		114.1 ± 11.4	
24	12.9 ± 1.3	388.2 ± 40.0[b]	115.9 ± 4.1	169.9 ± 7.0[c]
48	19.7 ± 3.9	1091.7 ± 457.1[d]	122.5 ± 10.3	278.3 ± 47.4[d]
72	19.8 ± 2.2	88.4 ± 19.2[d]	125.5 ± 5.1	140.4 ± 18.9

[a] Birds were injected subcutaneously at zero time with 40 mg diethylstilbestrol/kg body wt. Values are the mean ± S.E.M. of three birds.
[b] $P < 0.001$.
[c] $P < 0.005$.
[d] $P < 0.05$.

of newly synthesized triglyceride occurs during the first half-hour. This period of time is apparently required for the assembly of VLDL particles, while their secretion proceeds during the second half-hour. Due to mixing of these newly formed particles in the plasma triglyceride pool, little of this newly labeled plasma triglyceride is removed from the circulation during this measurement period. Phospholipids, partial glycerides plus cholesterol, free fatty acids, triglycerides, and cholesteryl esters were separated by thin-layer chromatography of washed chloroform–methanol extracts of liver homogenates and blood plasma.

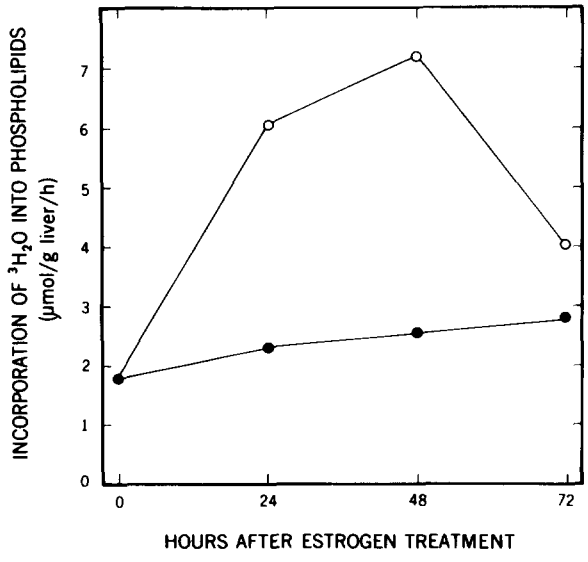

Fig. 3. Effect of estrogen on the incorporation of ^3H_2O into liver phospholipids. Experimental birds were given a single subcutaneous injection of diethylstilbestrol (DES), dissolved in corn oil. The dose was 40 mg/kg body weight. Control birds were injected with an equal volume of corn oil. All turkeys received 20 mCi of ^3H_2O (i.p.) 1 hr prior to being killed. Liver homogenates from 0, 24, 48, and 72 hr control and estrogen-treated birds were analyzed for their content of tritiated phospholipids (see Materials and Methods and Table 3). Each value is the mean of three birds. Control, ●; estrogen-treated, ○.

The incorporation of tritium into liver phospholipids was increased 3-fold in birds injected with estrogen (**Fig. 3**). Diethylstilbestrol also enhanced the appearance of tritiated phospholipids in plasma (**Fig. 4**). The maximum effects of estrogen on liver and plasma phospholipids were at 48-hr post-injection. Greater effects of estrogen were observed on the incorporation of ^3H_2O into liver and plasma triglycerides (**Fig. 5** and **Fig. 6**). The increase in the incorporation of ^3H_2O into liver triglyceride was greatest 48 hr after estrogen administration (Fig. 5). The temporal pattern of the increased de novo hepatic triglyceride synthesis was very similar to the increased plasma triglyceride concentration (Table 4). The most striking effect of diethylstilbestrol was on the appearance of newly synthesized triglyceride in the plasma, which was increased 70-fold (Fig. 6). Diethylstilbestrol had no significant effect on the conversion of ^3H_2O to esterified cholesterol either in the liver or in plasma (data not shown).

Incorporation of ^3H_2O into total lipids and digitonin-precipitable sterols in liver and plasma

The rates of lipogenesis in liver in relation to the amounts of newly synthesized lipid in plasma as a function of time after estrogen treatment are shown in **Table 5.** Lipid synthesis in the liver and its appearance in the circulation were significantly stimulated 24 and 48 hr after estrogen administration. The liver contained far more newly synthesized lipid than plasma at all times. The control plasma contained only 4–6% as much tritiated lipid as found in the liver. In the estrogen-treated birds the relative amounts of newly synthesized lipid in plasma were considerably greater and reached 29–36% of that present in the liver. The total amounts of lipid synthesized were considerably greater than that present in phospholipids plus triglycerides (Figs. 3–6), especially in livers of control birds in which fatty acid synthesis was not stimulated. This is largely accounted for by cho-

lesterol synthesis, reflected by the ^3H_2O incorporation into digitonin-precipitable sterols, which accounted for 23–31% of the hepatic lipogenesis in control birds (Table 5). The rates of hepatic sterol synthesis were not detectably stimulated by estrogen. In contrast, only 5–7% of the tritium present in the plasma lipid of these birds was found in the digitonin-precipitable sterols (data not shown).

Concentrations of plasma apolipoproteins A-I and B in response to estrogen treatment

The concentrations of both apoA-I and apoB in plasma were measured by electroimmunoassay. The apoB concentration in plasma increased from 13.3 mg/dl to 231.0 mg/dl following estrogen treatment, an increase of 17-fold (**Table 6**). The changes in the level of plasma apoB as a function of time following estrogen treatment were closely related to the amounts of newly synthesized and secreted triglyceride in the plasma (Fig. 6). In contrast to the marked increase in plasma apoB, the plasma apoA-I concentrations exhibited a moderate but significant decrease in response to estrogen treatment (Table 6).

Mass ratios of triglyceride to apolipoprotein B in plasma

The ratios of triglyceride to apoB in the plasma in the control animals were 0.83, 0.93, 1.32, and 1.09 at 0, 24, 48, and 72 hr, respectively. In the estrogen-treated birds, the ratios were elevated. The values were 2.01, 4.73, and 1.75 at 24, 48, and 72 hr, respectively.

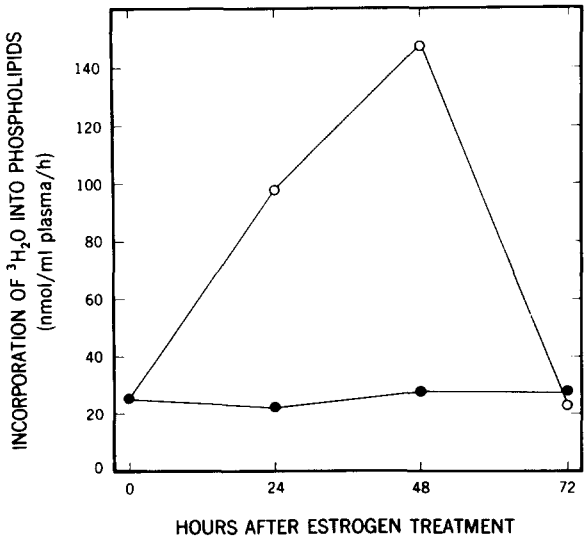

Fig. 4. Effect of estrogen on the incorporation of ^3H_2O into plasma phospholipids. Plasma samples from control and estrogen-treated turkeys (experimental details as described in Fig. 3) were assayed for their content of tritiated phospholipids. Each value is the mean of three birds. Control, ●; estrogen-treated birds, ○.

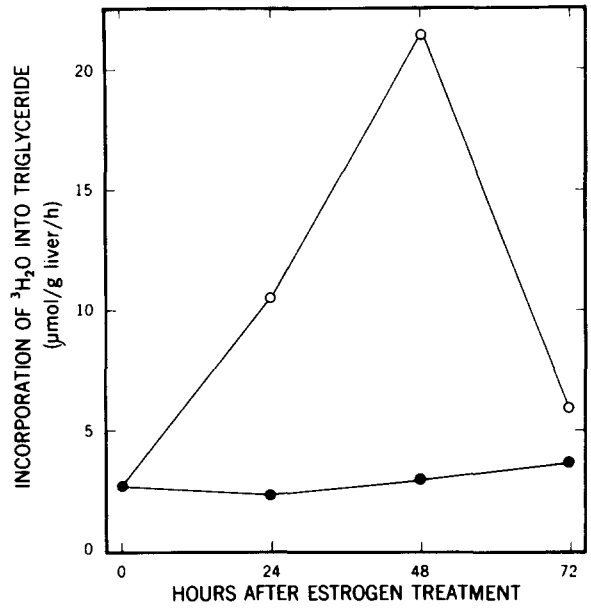

Fig. 5. Action of estrogen on the incorporation of ^3H_2O into liver triglycerides. Liver homogenates from control and estrogen-treated turkeys (for experimental details see Fig. 3) were analyzed for the content of tritiated triglycerides. Each value is the mean of three birds. Control, ●; estrogen-treated birds, ○.

Estrogen, therefore, stimulated the accumulation of triglyceride in the plasma compartment to a greater extent than apolipoprotein B.

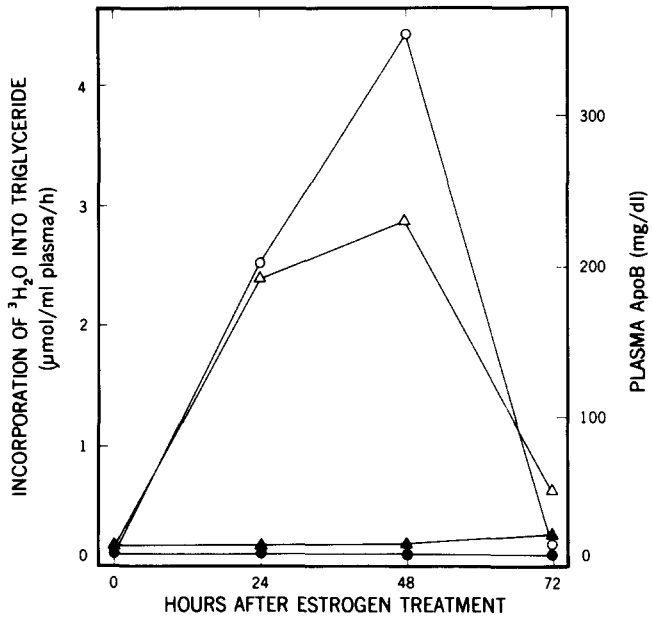

Fig. 6. Concurrent actions of estrogen on the accumulation of newly synthesized triglyceride in the plasma and on the concentration of circulating apolipoprotein B. Plasma samples of control and estrogen-treated turkeys were assayed for both tritiated triglyceride and apoB contents (for experimental details see Fig. 3). Each value is the mean of three birds. Tritiated triglyceride in control plasma, ●; tritiated triglycerides in plasma of estrogen-treated birds, ○; plasma apoB in the control, ▲; plasma apoB in the estrogen-treated birds, △.

TABLE 5. Incorporation of ^3H_2O into total liver lipids, liver sterols, and total plasma lipids

Time and Treatment	Synthesis of Total Liver Lipids: μmol ^3H_2O per g liver	Synthesis of Total Liver Lipids: μmol ^3H_2O per liver	Hepatic Sterol Synthesis[a]: μmol ^3H_2O per g liver	Newly-Synthesized Lipids in Plasma: nmol ^3H_2O/ml	Newly-Synthesized Lipids in Plasma: μmol ^3H_2O in plasma	Total Lipogenesis in Liver and Plasma: μmol ^3H_2O
0-hr Control	9.40 ± 1.30	97.4 ± 22.4	2.14 ± 0.42	133 ± 15	4.18 ± 0.49	101.6 ± 22.4
24-hr Control	8.13 ± 1.04	84.1 ± 15.3	2.17 ± 0.31	128 ± 11	4.30 ± 0.46	88.4 ± 15.7
24-hr Estrogen	20.01 ± 1.38[b]	250.5 ± 17.4[b]	2.09 ± 0.25	2815 ± 253[c]	91.24 ± 9.94[c]	341.7 ± 27.3[b]
48-hr Control	10.51 ± 1.14	127.8 ± 13.8	3.21 ± 0.59	198 ± 15	6.85 ± 0.52	134.7 ± 13.3
48-hr Estrogen	35.69 ± 7.85[d]	576.4 ± 121.8[d]	4.61 ± 1.78	5002 ± 1285[d]	164.69 ± 44.32[e]	741.1 ± 157.7[d]
72-hr Control	11.10 ± 1.81	133.8 ± 21.7	2.91 ± 0.46	220 ± 15	7.70 ± 1.01	141.5 ± 22.0
72-hr Estrogen	13.07 ± 2.81	194.0 ± 42.3	2.31 ± 0.80	246 ± 76	8.67 ± 4.38	202.7 ± 42.9

All values are the mean of three birds ± S.E.M.
[a] Incorporation of ^3H_2O into digitonin-precipitable sterols.
[b] $P < 0.005$.
[c] $P < 0.001$.
[d] $P < 0.05$.
[e] $P < 0.01$.

Estimated quantities of newly synthesized triglyceride in liver and plasma

The quantities of liver and plasma triglyceride produced by the de novo synthesis of fatty acids in the liver, and the subsequent esterification of these fatty acids into triglyceride in this tissue, were estimated (**Table 7**) from the amounts of ^3H_2O incorporated (Figs. 5 and 6, Table 3). These values provide a direct comparison of the absolute amounts of newly synthesized triglyceride in these two compartments at the end of the 1-hr labeling periods. Significant increases were found in both compartments 24 and 48 hr after estrogen treatment. The specific radioactivities of the plasma triglycerides are also shown at the bottom of Table 7, and these data indicate the importance of increased de novo hepatic fatty acid synthesis in the induction of the hyperglyceridemia following estrogen treatment (see Discussion).

Homogeneity of plasma apolipoprotein B in control and estrogen-treated birds

The apoB of plasma lipoproteins d < 1.073 g/ml (combined VLDL and LDL) from both control and estrogenized turkeys (48 hr after 40 mg DES/kg body wt) exhibited only 1 band at M_r 360,000 daltons on SDS–3.5% PAGE.

DISCUSSION

The liver triglyceride concentration was increased 3 to 4-fold by estrogen (Table 1). This increase, together with the predominant increase in molecular species of triglyceride containing 53 and 55 carbons (Table 2), demonstrates an increased amount of fatty acids with 16 carbon atoms, which is the major product of fatty acid synthesis in avian systems (36).

TABLE 6. Effects of estrogen on the concentrations of apolipoproteins A-I and B in turkey plasma

Time after Estrogen Treatment	Plasma Apolipoprotein A-I: Control	Plasma Apolipoprotein A-I: Estrogen[a]	Plasma Apolipoprotein B: Control	Plasma Apolipoprotein B: Estrogen[a]
hr	*mg/100 ml*		*mg/100 ml*	
0	227.1 ± 18.0		13.3 ± 0.2	
24	208.0 ± 11.4	197.0 ± 0.0	13.9 ± 0.9	191.9 ± 11.7[d]
48	267.4 ± 24.6	190.5 ± 20.8[b]	14.9 ± 1.7	231.0 ± 71.4[b]
72	241.7 ± 8.0	153.0 ± 15.4[c]	18.1 ± 1.8	50.4 ± 2.9[d]

[a] Birds were injected subcutaneously at zero time with 40 mg diethylstilbestrol/kg body wt. Values are the mean ± S.E.M. of three birds.
[b] $P < 0.05$.
[c] $P < 0.005$.
[d] $P < 0.001$.

The use of tritiated water, a precursor of known specific activity, provided direct comparison of the rates of fatty acid synthesis as a function of estrogen treatment. Similar amounts of newly synthesized fatty acids were found in liver phospholipids and triglycerides in the control birds (Figs. 3 and 5). The incorporation into liver phospholipid was increased about 3-fold 48 hr after estrogen treatment (Fig. 3) while the incorporation of ^3H_2O into triglyceride increased 7-fold (Fig. 5). This increase in de novo synthesis of triglyceride is consistent with the observed increase in TG-53 and TG-55 molecular species consequent to diethylstilbestrol administration (Table 2). It is clear that in the estrogen-stimulated state, the liver triglyceride accumulation is the result of increased fatty acid synthesis. On the basis of observations in other species, which document the presence of VLDL particles in the tubular network of the endoplasmic reticulum, and on the basis of the observed increase in plasma triglyceride (Table 4) derived from de novo synthesis (Fig. 6), a considerable portion of the estrogen-induced increase in liver triglyceride content (Table 1) probably resides in newly synthesized VLDL particles in intracellular tubules and vesicles in transit toward secretion. Further studies are required, however, to document this.

Total hepatic lipogenesis may be estimated by summation of the ^3H_2O incorporated into both the liver and plasma lipids as shown in Table 5. In the control animals, the amounts of newly synthesized lipid in the plasma were from 4.1–5.4% of the total recovered in liver and plasma (88–142 μmol of ^3H_2O). However, in the estrogen-treated birds that exhibited 3–5 times greater rates of hepatic lipogenesis 24–48 hr after hormone administration, the newly synthesized plasma lipid was 22–27% of the total amount recovered in liver and plasma (342–741 μmol of ^3H_2O). Estrogen, therefore, greatly stimulated the synthesis of lipids in the liver and the secretion of these lipids into the circulation. Considering specifically the newly synthesized triglyceride, the amount found in the plasma of the control birds was only 4.0–5.8% of that recovered in liver and plasma (Table 7). In contrast, 24–48 hr after estrogen treatment, when total triglyceride synthesis increased 8 to 12-fold, 31–38% of the total was present in the plasma. These observations suggest that a massive increase in the secretion of newly synthesized triglyceride occurred.

Estrogen caused the amount of triglyceride in the liver to increase from 25 mg to 118 mg (Table 1). The quantity of triglyceride in the plasma compartment increased under similar experimental conditions from 7 mg to 376 mg (Table 4). It is therefore clear that more triglyceride accumulated in the circulation than in the liver following hormone administration. It is also clear that the rate of secretion of triglyceride into the circulation greatly exceeded its rate of removal from the circulation under these conditions. Although the plasma triglyceride concentration exhibited a 55-fold increase 48 hr after estrogen (Table 4), the specific radioactivity of the plasma triglyceride remained relatively constant. This illustrates that the de novo synthesis of triglyceride by the liver very closely paralleled the hepatic secretion of triglyceride-rich lipoproteins, and clearly demonstrates the importance of accelerated fatty acid synthesis in the induction of hyperglyceridemia by estrogen. Thus, in control birds the specific activity of the plasma triglyceride was between 300–420 nmol of ^3H_2O incorporated/mg of triglyceride throughout the experiment. This value increased to about 650 nmol at 24 hr after estrogen, became 400 nmol at the 48-hr point, and fell to 165 nmol at 72 hr following estrogen. This is exactly as expected if fatty acid synthesis was a dominant causative factor in the estrogen-induced hyperglyceridemia. Thus, as the plasma lipid concentration was on a steep incline at the 24-hr mark, the 1-hr period of synthesis between the 23rd and 24th hr would be greater than the average rate during the first 24 hr, due to an initial lag period after the injection of estrogen. At or near the peak of plasma triglyceride synthesis, the observed rate of fatty acid synthesis would more closely reflect the actual concentration. At 72 hr, when the effects of estrogen on fatty acid synthesis have nearly returned to normal (Fig. 6), but with the plasma triglyceride concentration not yet normalized due to the excessive previous accumulation of triglyceride, the plasma triglyceride specific activity would be less than the control value.

Amounts of triglyceride synthesized from ^3H_2O may

TABLE 7. Estimated quantities of newly synthesized triglyceride in liver and plasma

Time and Treatment	Liver Triglyceride	Plasma Triglyceride[a]	Total Triglyceride
		mg	
0-hr Control	1.07 ± 0.25	0.06 ± 0.01	1.13 ± 0.26
24-hr Control	0.91 ± 0.16	0.05 ± 0.01	0.96 ± 0.17
24-hr Estrogen	5.03 ± 0.34[b]	3.13 ± 0.27[b]	8.16 ± 0.87[c]
48-hr Control	1.46 ± 0.16	0.09 ± 0.01	1.55 ± 0.16
48-hr Estrogen	12.76 ± 2.79[d]	5.83 ± 1.66[d]	18.59 ± 4.43[d]
72-hr Control	1.68 ± 0.22	0.09 ± 0.01	1.75 ± 0.22
72-hr Estrogen	3.23 ± 0.69	0.20 ± 0.10	3.43 ± 0.70

All values are the mean of three birds ± S.E.M.

[a] The specific radioactivities of the plasma triglycerides were 424, 295, 322, and 367 nmol ^3H_2O/mg triglyceride at 0, 24, 48, and 72 hr, respectively, in the control birds and 653, 405, and 165 nmol ^3H_2O/mg triglyceride at 24, 48, and 72 hr, respectively, in the estrogenized birds.

[b] $P < 0.001$.

[c] $P < 0.005$.

[d] $P < 0.05$.

be estimated as done by Brunengraber, Boutry, and Lowenstein (37) based on the formulation developed by Jungas (38). Since this calculation was based on data for fatty acid synthesis in adipose tissue and not liver, it may not strictly reflect the absolute quantity of fatty acid synthesized in liver. However, it should serve as an approximation. Accordingly, 1.15 μmol of acetyl units is generated in the de novo synthesis of fatty acids for each μmole of ^3H_2O incorporated. Thus, employing 17 carbon atoms as the average chain length synthesized, 1 μmol of ^3H_2O incorporated is equivalent to the synthesis of 38.2 μg of triglyceride. The values in Table 7 indicate the approximate quantities of triglyceride synthesized. The values shown for plasma do not reflect the absolute quantities of triglyceride secreted into the circulation during the 1-hr labeling period, owing to the mixing of newly synthesized liver triglyceride with preexisting unlabeled triglyceride in the liver and the uncertain kinetics of entry of ^3H-labeled lipid into the plasma during this 1-hr period. The values are shown primarily to illustrate the approximate rate at which the liver synthesizes fatty acids de novo and the magnitude of the response of this process to the estrogen stimulus.

The hepatic synthesis of cholesterol, as reflected by the incorporation of ^3H_2O into digitonin-precipitable sterols (Table 5), was a significant fraction (23–30%) of the total liver lipid synthesized in normal birds. However, this was not detectably affected by estrogen and therefore became a much smaller fraction of hepatic total lipid synthesis in the estrogen-treated animals. These results are consistent with the lack of effect of estrogen on the liver cholesterol concentration (Table 1). However, the plasma cholesterol level was more than doubled by estrogen (Table 4). It is possible that cholesterol synthesis did increase to some extent, but was not statistically significant due to the sizeable variations. Thus, the highest rate of cholesterol synthesis was observed 48 hr after estrogen treatment (Table 5). During estrogen-induced hyperglyceridemia, when VLDL particles circulate for longer periods due to saturation of removal mechanisms, an increased proportion of particles of intermediate density, which are somewhat enriched with cholesteryl ester, probably also contribute to the observed hypercholesterolemia.

Based on the data in Tables 1, 3, and 4, the relative amount of cholesterol in the liver and plasma may be estimated. Thus, the pool size of cholesterol in the livers of these birds ws 20–30 mg and about 30–40 mg of cholesterol was present in the blood plasma. Estrogen treatment doubled the size of the plasma cholesterol pool but did not alter the liver cholesterol content. The plasma cholesterol pool therefore became 2–3 times greater than that in the liver in the estrogen-treated birds. In relation to the amounts of circulating triglyceride, the control plasma contained 6–10 times more cholesterol than triglyceride; conversely, after estrogen treatment, the plasma triglyceride exceeded the plasma cholesterol by a maximum factor of 4 (48 hr after estrogen).

The concentration of apolipoprotein B in the plasma responded to estrogen almost in parallel with the plasma and liver triglyceride synthesis (Figs. 5 and 6). Since this apolipoprotein is firmly associated with triglyceride-rich lipoprotein particles and remains attached to it, without exchange or transfer during its tenure in the circulation (39), it is reasonable to conclude that the marked increase in plasma apolipoprotein B was a result of increased synthesis and secretion by the liver, as observed to occur in liver slices by Capony and Williams (7). This is entirely consistent with the reported increases in the synthesis of VLDL protein (5, 6) and apoVLDL-II (6, 8) in the livers of birds injected with estrogen. The ratio of triglyceride to apolipoprotein B in plasma was also increased at all periods of measurement following estrogen, and was the highest at 48 hr, the maximum point of triglyceride synthesis (Figs. 5 and 6). The amount of apolipoprotein B per VLDL particle obtained from human plasma is independent of particle size (39).Accordingly, two factors may be responsible for the increased proportion of triglyceride to apolipoprotein B after estrogen treatment in the current study, namely, production of larger VLDL particles by the liver and the existence of larger VLDL particles in the circulation owing to a slower rate of turnover, as observed by Kudzma et al. (4). In contrast, the apolipoprotein A-I was decreased by estrogen (Table 6). This apolipoprotein is a major constituent of HDL and is absent in VLDL turkey plasma (25). The causative factor underlying this decrease in plasma apolipoprotein A-I is not clear. This effect of estrogen on plasma apolipoprotein A-I has also been observed in chicken embryo (29) and in the immature female chicken (31). This alteration may be due to a decrease in the production of apolipoprotein A-I-containing high density lipoproteins under these conditions. Thus, when the rates of synthesis of vitellogenin and VLDL are so markedly increased, the synthesis of other plasma proteins may be decreased. It seems reasonable that when the mRNA species coding for estrogen-responsive plasma proteins are markedly elevated, and when the mRNA coding for other plasma proteins therefore comprise a lower percentage of the total plasma protein mRNA, less synthesis of these other plasma proteins occurs as a result of competition at the level of translation at the ribosome binding sites on the endoplasmic reticulum. Evidence for this type of competition has been provided by Grieninger and Granick (40). It is likely, in addition, that the removal of apoA-I-containing HDL is accelerated

during enhanced net intravascular catabolism of VLDL (41).

The rates of hepatic synthesis and secretion of apolipoprotein B may be estimated from the absolute rates of entry of newly synthesized triglyceride into the circulation (Table 7) and the mass ratios of triglyceride to apolipoprotein B in the circulation at the respective times measured (Tables 4 and 6). Accordingly, in the control animals the rates of apoB synthesis averaged 0.07 mg/hr as compared with 1.55, 1.24, and 0.11 mg/hr at 24, 48, and 72 hr, respectively, in the estrogen-treated birds. These values are approximations, since the assumptions are made. *a*) The secreted triglyceride-bearing lipoproteins contain average amounts of apoB and triglyceride relative to the lipoprotein population in the plasma. This estimate of apoB synthesis is therefore high as a result of the decreased mass ratio of triglyceride to apoB in the plasma caused by lipolytic degradation of VLDL following secretion. *b*) Removal of newly secreted particles is not appreciable during the 1-hr measurement period. The estimate is therefore low as a result of the removal of newly secreted triglyceride from the plasma via lipolysis during the labeling period. These calculations indicate that estrogen stimulated apoB production about 20-fold 24 and 48 hr after hormone administration.

Studies on the synthesis of apolipoprotein B of VLDL by liver cells in primary culture derived from estrogen-treated chickens (42) indicate that a contiguous polypeptide chain of 350,000 daltons is produced. Our observation of a single apolipoprotein B band of M_r 360,000 daltons in the plasma VLDL and LDL of control and estrogenized turkeys is in agreement.

The present findings are consistent with the observations of Kudzma et al. (4) in another avian system, the chicken: that estrogen increases the rate of fatty acid synthesis and that hyperglyceridemia following estrogen administration is characterized by increased production of triglyceride-containing lipoproteins by the liver. In the current study, and as observed by Kudzma et al. (4), it is likely that decreased fractional turnover of triglyceride in the circulation is a secondary factor, resulting from saturation of removal mechanisms. Thus, following the primary metabolic factor, namely overproduction, a decreased fractional removal rate may accentuate the hyperglyceridemia even though net removal is considerably greater than that operative prior to estrogen administration.

The present studies provide a direct measurement of the rates of hepatic fatty acid and cholesterol synthesis in relation to the concentrations of plasma triglyceride, cholesterol, and apolipoproteins A-I and B following estrogen treatment. These studies indicate that increased fatty acid synthesis in the liver is the major process underlying the development of the hyperglyceridemia and that increased formation of apolipoprotein B occurs concurrently with the hypersecretion of triglyceride-rich lipoproteins.

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