Biochem. J. (1978) **174**, 45–51
Printed in Great Britain

Reversal by Triton WR-1339 of Ethynyloestradiol-Induced Hepatic Cholesterol Esterification

By ROGER A. DAVIS,* RADENE SHOWALTER and FRED KERN, JR.
Division of Gastroenterology, Department of Medicine, University of Colorado Medical School, Denver, CO 80220, *U.S.A.*

(*Received* 18 *October* 1977)

Rats treated with ethynyloestradiol have marked hypolipidaemia: serum cholesterol is decreased to 5%, triacylglycerol to 10% and phospholipid to 70% of control concentrations. Loss of serum cholesterol follows an exponential decay, with a half-life of 1.13 ± 0.09 days. After 4 days of treatment, serum cholesterol concentrations remain relatively constant (ranging from 1 to 20mg/100ml) for at least 30 days. There is a concomitant 20-fold decrease in the $d < 1.21$ fraction of serum proteins and a similar decrease in serum apolipoproteins as measured by sodium dodecyl sulphate/10%-polyacrylamide-gel electrophoresis. The activity of hepatic microsomal acyl-CoA–cholesterol *O*-acetyltransferase (EC 2.3.1.26) was significantly increased by ethynyloestradiol treatment ($P < 0.05$). This activation caused hepatic cholesteryl esters containing mainly $C_{18:1}$ fatty acids to increase linearly as serum cholesterol concentrations decreased ($r = 0.9675$, $P < 0.001$). Triton WR-1339, a non-ionic detergent that inhibits lipoprotein catabolism, was used to estimate hepatic lipid secretion by measuring the increment in serum lipids after its administration. At 15h after Triton WR-1339 administration, serum cholesterol concentrations were increased equally in both control and ethynyloestradiol-treated rats. In contrast, the increment of serum triacylglycerol of treated rats was 40% of that found in control rats, indicating that ethynyloestradiol inhibits hepatic triacylglycerol secretion. Triton WR-1339 inhibited the oestrogen activation of hepatic microsomal acyl-CoA–cholesterol *O*-acyltransferase and restored hepatic cholesteryl ester concentrations to normal values. These data suggest that ethynyloestradiol and its pharmacological 'antagonist' Triton WR-1339 alter hepatic triacylglycerol secretion via a mechanism associated with changes in hepatic cholesterol esterification.

Rats treated with ethynyloestradiol (17α-ethynyloestra-1,3,5-triene-3,17-β-diol) have diverse alterations of hepatic function, including decreased bile flow (Simon & Arias, 1973) and decreased bile-acid synthesis (Davis & Kern, 1976). In addition, several microsomal (MacKinnen & Simon, 1975) and surface-membrane enzyme activities are altered (Simon & Arias, 1973). In a previous study (Davis *et al.*, 1978) we found that in ethynyloestradiol-treated rats the decreased bile flow caused by impaired activity of $(Na^+ + K^+)$-dependent ATPase (ATP phosphohydrolase, EC 3.6.1.3) could be correlated with structural alterations in the surface membrane. The increased microviscosity, as measured by electron-paramagnetic-resonance probes, was found to be due in part to an accumulation of membrane-associated cholesterol esters caused by ethynyloestradiol treatment (Davis *et al.*, 1978). Since cholesteryl esters are crystalline at physiological temperatures (Katz *et al.*, 1976) and have limited interaction with membrane phospholipids (Davis & Sinensky, 1977), their accumulation in membranes may indirectly alter membrane enzyme function. Furthermore, loss of membrane-accumulated cholesteryl esters after administration of Triton WR-1339 to ethynyloestradiol-treated rats was associated with restoration of bile flow and $(Na^+ + K^+)$-dependent ATPase activity (Davis *et al.*, 1978). Triton WR-1339 also restored bile-acid synthesis to normal (Davis & Kern, 1977). We hypothesized that bile-acid synthesis was returned to normal values by increasing the availability of free cholesterol [a substrate for bile-acid synthesis (Balasubramaniam *et al.*, 1975; Ogura *et al.*, 1971)] from the metabolically inactive form of esterified cholesterol.

In the present study we report the effects of ethynyloestradiol and Triton WR-1339 on hepatic and serum cholesterol concentrations. Our results show that ethynyloestradiol decreases and Triton WR-1339

Abbreviations used: VLD lipoprotein, very-low-density lipoprotein ($d < 1.0063$); LD lipoprotein, low-density lipoprotein ($d < 1.063$); ATPase, adenosine triphosphatase.

* To whom reprint requests should be addressed at the following address: Department of Medicine M-013, Division of Metabolic Disease, University of California San Diego Medical School, La Jolla, CA 92093, U.S.A.

increases hepatic lipid secretion by either increasing (ethynyloestradiol) or decreasing (Triton WR-1339) the activity of hepatic microsomal acyl-CoA–cholesterol *O*-acyltransferase (EC 2.3.1.26). The results support the concept that free cholesterol appears to be an obligatory component for the secretion of hepatic triacylglycerol (as VLD lipoprotein) (Goh & Heimberg, 1977). Thus by decreasing (ethynyloestradiol) or increasing (Triton WR-1339) the availability of hepatic free cholestrol through changes in cholesterol esterification, ethynyloestradiol and its pharmacological 'antagonist' Triton WR-1339 may subsequently alter hepatic lipid secretion.

A preliminary report of this work has been published (Davis *et al.*, 1977).

Experimental

Animals

Male Sprague–Dawley rats (180–220g) (Charles–River, Wilmington, MA, U.S.A.) were housed in wire-mesh cages in a well-ventilated room with 12h light cycle (07:00–19:00h) for 1 week before use.

Ethynyloestradiol (Wyeth Laboratories, Fort Washington, PA, U.S.A.) was administered subcutaneously (5mg/kg body wt. per day) as a propylene glycol solution (1mg/ml). Control rats received propylene glycol alone. After 4 days of either treatment, Triton WR-1339 (Rohm and Haas, Philadelphia, PA, U.S.A.) was administered intraperitoneally (22.5mg/100g body wt.) as a solution (62.5mg/ml) in 0.9% NaCl. Animals were starved for 24h before death.

Assay of cholesterol and cholesteryl esters

Daily serum samples, obtained for cholesterol analysis via the tail vein, were saponified and extracted as described (Davis & Kern, 1976). At the times indicated animals were killed by exsanguination and the liver was removed immediately after death and homogenized with a Potter–Elvehjem homogenizer with a Teflon pestle. [1α,2α(n)-^{3}H]cholesterol and cholesteryl [1-^{14}C]oleate (Amersham/Searle, Arlington Heights, IL, U.S.A.) were each added as internal standards to both the serum and liver homogenates. Cholesterol and cholesteryl esters were extracted from the serum and liver homogenates with 20vol. of chloroform/methanol (2:1, v/v) as described by Davis *et al.* (1977). Recovery of free cholesterol averaged 90% and the recovery of cholesteryl oleate averaged 80%. The combined chloroform extracts (serum or liver) were evaporated to dryness and the residues were dissolved in 20ml of hexane/diethyl ether (19:1, v/v). The free cholesterol and cholesteryl esters were separated on a Florisil column (1cm×24cm) as described by Davis *et al.* (1977). The recovery of cholesteryl [1-^{14}C]oleate and [1α,2α(n)-^{3}H]cholesterol from the column averaged greater than 90%.

The t.l.c. system used throughout this study [hexane/diethyl ether/acetic acid (85:15:1, by vol.)] monitored the purity of each of the column fractions. Triacylglycerol did not contaminate the cholesteryl ester fraction and the free cholesterol and cholesteryl ester fractions did not overlap.

The amount of cholesterol obtained after saponification and extraction of the cholesteryl ester fraction was quantified by g.l.c. with a glass column (1.8m×2mm) filled with 3% SP-2250 on 100–120 supelcon AN-DMCS (Supelco, Bellefonte, PA, U.S.A.) with 3β-coprostanol as a standard (Davis *et al.*, 1977). The free cholesterol fractions from the column were similarly quantified.

Non-esterified fatty acids, which were esterified to the cholesteryl esters, were obtained from the aqueous solutions of the saponification reactions after acidification to pH1 at 0°C with 6M-HCl and extraction with 3×10ml of diethyl ether. Recovery of [1-^{14}C]oleic acid (from the added cholesteryl [1-^{14}C]oleate) averaged 85%. Nonadecanoic acid was added as an internal standard. The combined diethyl ether extracts were evaporated to dryness and methylated with ethereal diazomethane. The distribution of fatty acids was determined by g.l.c. on 10% diethyl glycol succinate (Applied Science, State College, PA, U.S.A.) at 140°C, by using peak-area ratios based on nonadecanoic acid.

Assay of hepatic microsomal acyl-CoA–cholesteryl O-acyltransferase

The assay for the microsomal liver enzyme that esterifies cholesterol was performed essentially as described by Goodman *et al.* (1964). Livers from control and ethynyloestradiol-treated rats (4 days of treatment) were removed and perfused as described above and were homogenized with a Potter–Elvehjem homogenizer with a loose-fitting pestle in 2.5ml of 0.1M-potassium phosphate buffer (pH7.4)/g of liver. The homogenates were centrifuged (109000*g* for 1h at 4°C) in a Beckman L2-65B ultracentrifuge in a type 40 rotor. The final 'washed' microsomal preparation was diluted to 5ml with buffer and protein concentrations were determined by the method of Lowry *et al.* (1951) with bovine serum albumin as the standard.

Each incubation mixture (2.5ml) contained 0.5mg of microsomal protein, 3mg of bovine serum albumin (deficient in non-esterified fatty acid), 0.1M-potassium phosphate buffer, pH7.4, 6μmol of ATP, 0.3μmol of CoA, 20μmol of [4-^{14}C]cholesterol (0.04μCi) and 75μmol of oleic acid and 50μl of acetone as a lipid solvent. Incubations were carried out at 37°C

in test tubes open to the atmosphere in a metabolic shaker for 1 h, after which mixtures (5 ml) of chloroform/methanol (1:1, v/v) were added to the tubes. The contents were extracted with 20 vol. of the chloroform/methanol and the chloroform layer was drawn off and evaporated to dryness under reduced pressure. The residue was dissolved in benzene and free cholesterol and cholesteryl esters were separated on 0.25 mm-thick silica-gel G t.l.c. plates as described above.

The silica gel containing the free cholesterol and the cholesteryl esters was scraped into glass vials and the radioactivity was counted in PCS scintillation mixture (Amersham/Searle). Recovery of [^{14}C] cholesterol was 85–95%. No significant radioactivity was detected in areas of the t.l.c. plate other than those corresponding to cholesterol and cholesteryl esters. Production of cholesteryl esters was linear with time and no esterification occurred when boiled microsomal fractions were used. Glucose 6-phosphatase, a microsomal enzyme marker, was assayed as described by de Duve *et al.* (1955).

Lipoprotein isolation and characterization

Serum obtained from control and ethynyloestradiol-treated rats (4 days of treatment) was adjusted with NaBr to a density of 1.24 g/ml and separated into a single $d < 1.21$ fraction and washed once as described by Havel *et al.* (1955) by centrifugation in a 40.3 rotor at 114000*g* for 44 h at 10°C. Electrophoresis of the $d < 1.21$ lipoprotein was performed on sodium dodecyl sulphate/10% polyacrylamide gels essentially as described by Maizel (1971).

Assay of radioactivity

^{14}C and ^{3}H radioactivity was determined by scintillation counting in a Packard 2425 spectrometer with automatic external standard for correction of quenching. Results are reported as d.p.m. ^{3}H was counted at an average efficiency of 50% and ^{14}C counted at an average efficiency of 78%.

Statistical analysis

Statistical differences were analysed by using Student's *t* test. All values are reported as the mean ± 1 S.E.M. *P* values (calculated from double-tailed tables) equal to or less than 0.05 were considered statistically significant. Non-linear regression was performed by computer analysis.

Results

Effects of ethynyloestradiol on serum concentrations

Treatment with ethynyloestradiol caused a rapid and severe hypocholesterolaemia (Fig. 1). The decrease in serum cholesterol concentrations is described by an exponential function giving a half-life of 1.13 ± 0.09 days (mean ± S.E.M. for four individual rats). Serum cholesterol concentrations reached a minimal value after 4 days of ethynyloestradiol treatment and remained relatively constant (in the range 1–20 mg/100 ml) for as long as 30 days. Similar values for the total decrease and the rate of cholesterol loss from serum have been reported for rats treated with 4-aminopyrazolo[3,4]pyrimidine, a drug that inhibits hepatic lipoprotein secretion (Shiff *et al.*, 1971). Clearly, although this dosage of ethynyloestradiol causes severe hypocholesterolaemia, rats can tolerate it for a substantial period. Since after 4 days of ethynyloestradiol treatment a new 'steady state' of serum cholesterol content is obtained, additional experiments were performed after this time point.

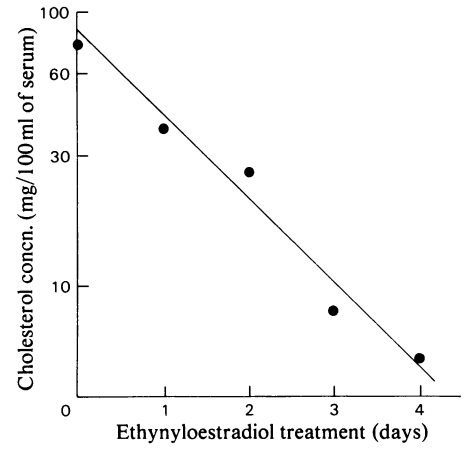

Fig. 1. *Time course of serum cholesterol concentrations during ethynyloestradiol treatment*

Rats were given ethynyloestradiol (5 mg/kg body wt. per day) subcutaneously and bled daily at 16:00 h. Total serum cholesterol was determined in duplicate by g.l.c. by using Supelco SP-2250 100-mesh column support at 285°C. Coprostanol was used as an internal standard. The values represent one typical experiment. The line represents the best-fit line determined by non-linear least-squares analysis of a single exponential function.

In addition to decreasing serum cholesterol from 60 ± 6 to 3 ± 1 mg/100 ml ($P < 0.001$) ethynyloestradiol treatment also decreases the concentration of both serum triacylglycerol from 78 ± 4 to 8 ± 6 mg/100 ml ($P < 0.001$) and phospholipid from 132 ± 9 to 78 ± 3 mg/100 ml ($P < 0.001$) ($n = 6$ in each group). Isolation of a total lipoprotein fraction ($d < 1.21$) of serum showed a similar 20-fold decrease in protein concentrations [69 ± 8 mg/100 ml for controls compared with 3.3 ± 0.6 mg/100 ml for ethynyloestradiol-

treated rats ($n = 3$, $P < 0.001$)]. Analysis of the $d < 1.21$ lipoprotein by sodium dodecylsulphate/10% polyacrylamide discontinuous gel electrophoresis showed a similar 20-fold decrease in all Coomassie Blue-stained bands, indicating that ethynyloestradiol treatment lowers the concentration of all serum apolipoproteins.

Effect of ethynyloestradiol on hepatic cholesterol concentrations

After 5 days of treatment, hepatic cholesteryl ester concentrations were doubled from 0.5 ± 0.2 to 1.10 ± 0.08 mg/g of liver ($n = 6$, $P < 0.001$). In contrast, free cholesterol concentrations remained unchanged [2.8 ± 0.7 mg/g of liver for the control compared with 2.1 ± 0.2 mg of liver for ethynyloestradiol-treated rats ($n = 6$, P was non-significant)]. The fatty acid compositions of the hepatic cholesteryl esters were examined and are shown in Fig. 2. The results, expressed as percentage composition by weight, show a 15% increase in cholesteryl esters containing $C_{18:1}$ and $C_{18:2}$ fatty acids and a decrease in those containing $C_{16:0}$ and $C_{18:0}$ fatty acids. Cholesteryl esters containing $C_{18:1}$ fatty acids accounted for most of the hepatic cholesteryl esters both before and after ethynyloestradiol treatment. Intracellular cholesterol esterification produces mainly cholesteryl esters containing $C_{18:1}$ fatty acids (Goodman, 1965).

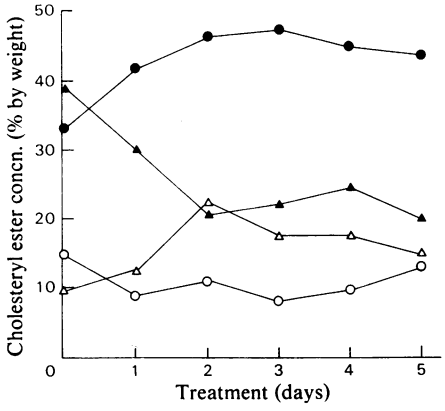

Fig. 2. *Fatty acid distribution in hepatic cholesteryl esters during ethynyloestradiol treatment*

The fatty acid composition of the hepatic cholesteryl esters (calculated as percentage by weight) was determined by g.l.c. analysis of their corresponding methyl esters by using 10% diethylene glycol succinate (100-mesh) column support. Values represent the mean for duplicate determinations in two animals for each time period. Symbols: ●, $C_{18:1}$ fatty acids; ▲, $C_{16:0}$ fatty acids; △, $C_{18:2}$ fatty acids; ○, $C_{18:0}$ fatty acids.

We next examined the influence of ethynyloestradiol on hepatic cholesterol esterification. The activity of hepatic microsomal acyl-CoA–cholesterol *O*-acyltransferase increased after 4 days of ethynyloestradiol treatment from a control value of 6.7 ± 0.4% for [^{14}C]cholesterol esterified/h per mg of microsomal protein to a value of 8.2 ± 0.4% in treated rats ($n = 6$, $P < 0.05$). The value obtained in ethynyloestradiol-treated rats is probably an underestimation, since the microsomal fraction from these rats contains nearly double the amount of free cholesterol (Davis & Sinensky, 1977), which probably results in a greater dilution of exogenous labelled substrate. To ensure that the microsomal membrane preparations from livers of control and ethynyloestradiol-treated rats were of equal purity, the enrichment (isolated microsomal fraction/crude homogenate ratio) of the specific activity of glucose 6-phosphatase, a specific microsomal membrane enzyme (de Duve *et al.*, 1955) was compared, but no significant difference was found (4.7 ± 0.8, control; 4.9 ± 0.7, treated; $n = 6$ in each group).

Relationship between hepatic cholesteryl ester and serum cholesterol concentrations

A significant linear correlation was found between serum cholesterol concentrations and the mass of total hepatic cholesterol esters observed during 6 consecutive days of ethynyloestradiol treatment (Fig. 3; $r = 0.9675$, $P < 0.001$). Furthermore, by calculating

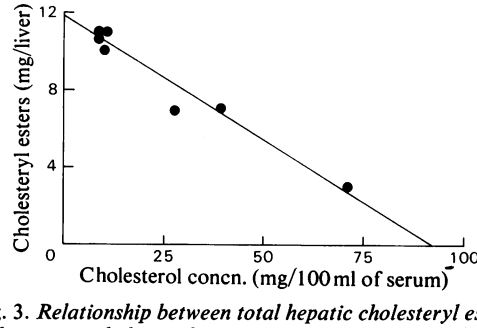

Fig. 3. *Relationship between total hepatic cholesteryl esters and serum cholesterol concentrations during ethynyloestradiol treatment*

Rats were treated with ethynyloestradiol daily for 7 days. Each day two rats were bled and livers were taken at 16:00h. Hepatic cholesteryl esters were isolated by extraction and column chromatography. Serum cholesterol concentrations and total hepatic cholesteryl esters were determined by g.l.c. Each point represents the mean for two determinations. A linear negative correlation exists between serum cholesterol concentrations and the total mass of hepatic cholesteryl esters; $y = -0.126x + 11.74$, $r = 0.9675$, $P < 0.001$.

the average loss of cholesterol from the serum after 4 days of treatment [3.2mg/animal (assuming 6.7ml of blood/100g body wt.; Ormond & Rivera-Valez, 1965)], it can be seen that the increase in hepatic cholesteryl esters after 4 days (7.8mg of cholesteryl ester/animal) can easily account for all of the cholesterol that is lost from the blood. Thus the rate and amount of cholesterol lost from serum is closely related to the rate and amount of the increase of hepatic cholesteryl esters. The additional 4.6mg (range 7.8–3.2mg) of hepatic cholesteryl esters may be derived from cholesterol that would have been catabolized to bile acids in the absence of ethynyloestradiol treatment, which inhibits bile-acid synthesis (Davis & Kern, 1976).

Reversal of ethynyloestradiol-induced hypolipidaemia by Triton WR-1339

Triton WR-1339 inhibits lipoprotein lipolysis and causes an accumulation of serum lipids secreted by the liver (Byers *et al.*, 1963). The increment in serum lipid concentrations after Triton WR-1339 treatment gives an estimate of hepatic lipid secretion.

In both control and ethynyloestradiol-treated rats serum cholesterol increased linearly, reaching maximal values 15h after Triton WR-1339 administration. We chose this time point for subsequent determinations. Triton WR-1339 increased serum cholesterol concentrations to nearly equal values in both control and ethynyloestradiol-treated rats (Table 1). The percentage of serum cholesterol in the form of cholesteryl ester decreased in both groups after Triton WR-1339 treatment from 78±8 to 22±3% in the controls, and from 55±14 to 17±2% in ethynyloestradiol-treated rats. Thus free cholesterol accounts for most of the increment in serum cholesterol after Triton WR-1339 treatment, consistent with the observation that Triton WR-1339 inhibits serum cholesterol esterification (Klauda & Zilversmit, 1974). The increment in serum triacylglycerol after Triton WR-1339 treatment in control rats was 3 times that in ethynyloestradiol-treated rats ($P<0.005$; Table 1), suggesting that ethynyloestradiol treatment inhibits triacylglycerol (VLD lipoprotein) secretion.

It also appears that Triton WR-1339 augments hepatic triacylglycerol (VLD lipoprotein) secretion by ethynyloestradiol-treated rats. Before Triton WR-1339 administration, control serum triacylglycerol concentrations were 10 times those of ethynyloestradiol-treated rats, whereas after Triton WR-1339 administration this difference was decreased to only 3-fold. These data show that Triton WR-1339 produced a greater relative increase in serum triacylglycerol concentrations in ethynyloestradiol-treated rats compared with control rats.

Table 1. *Effect of Triton WR-1339 on serum lipid concentrations in control and ethynyloestradiol-treated rats*

Control ($n=6$) and ethynyloestradiol-treated ($n=6$) (5mg/kg body wt. per day for 4 days) rats were injected with Triton WR-1339 (22.5mg/100g body wt.) and 15h later serum was obtained and the lipid concentrations (mg/100ml of serum) were determined.

	Lipid concn. (mg/100ml of serum)	
Lipid	Control+Triton	Ethynyloestradiol treatment+Triton
Cholesterol	141± 36	127±29
Triacylglycerol	1270±153	429±97*
Phospholipid	188± 36	105±20
Triacylglycerol	1192±109†	421±95*†

* Significant difference at $P<0.005$.

† Values represent the change in concentration after administration of Triton WR-1339 compared with values without the detergent.

Reversal of ethynyloestradiol-induced hepatic cholesteryl ester accumulation by Triton WR-1339

Triton WR-1339 did not significantly alter hepatic free cholesterol concentrations. In contrast, Triton WR-1339 greatly decreased hepatic cholesteryl ester concentrations to 0.16±0.05mg/g of liver for control rats treated with Triton WR-1339 and to 0.6±0.3mg/g of liver for ethynyloestradiol-treated rats further treated with Triton WR-1339 ($n=6$, $P<0.001$). Thus Triton WR-1339 totally restored hepatic cholesteryl ester concentrations of ethynyloestradiol-treated rats to values found in untreated control rats. Since the increment serum cholesterol after Triton WR-1339 administration was found in the form of free cholesterol, Triton WR-1339 increases serum cholesterol through a process involving hepatic cholesteryl ester hydrolysis and its secretion as free cholesterol. These data suggest that the greater increment of free cholesterol caused by Triton WR-1339 administration to ethynyloestradiol-treated rats compared with control rats (Table 1) is due to a larger reserve of sequestered hepatic cholesteryl esters that subsequently become mobilized by their hydrolysis.

Effect of Triton WR-1339 on hepatic microsomal acyl-CoA–cholesterol O-acyltransferase activity

Microsomal fractions of three ethynyloestradiol-treated rats were combined and Triton WR-1339 was added to these fractions as indicated in Fig. 4. Triton WR-1339 inhibited hepatic microsomal acyl-CoA–cholesterol *O*-acyltransferase by a relation-

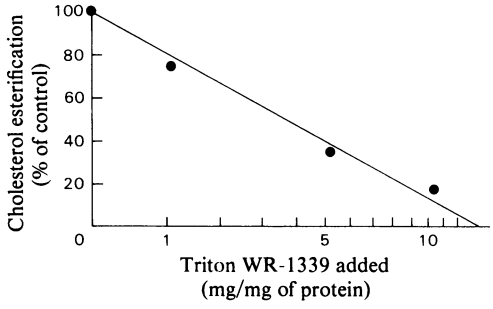

Fig. 4. *Inhibition of microsomal acyl-CoA–cholesterol O-acyltransferase in ethynyloestradiol-treated rats*
Microsomal fractions from three rats treated with ethynyloestradiol (5mg/kg body wt. per day) for 5 days were prepared and combined. Triton WR-1339 was added to each incubation as indicated. Cholesterol esterification was assayed by measuring the amount of [4-^{14}C]cholesterol (20μmol, 0.04μCi) esterified in 1h. Each point represents the mean for triplicate determinations. The S.D. for each determination is less than 5%.

ship depending on the logarithm of the dose administered. When administered to ethynyloestradiol-treated rats, Triton WR-1339 decreased the hepatic microsomal enzyme activity to control values [6.2±0.4% of cholesterol esterified/h per mg of microsomal protein ($n = 3$)].

Discussion

The results show that ethynyloestradiol and its pharmacological 'antagonist' Triton WR-1339 alter reciprocally hepatic and serum cholesterol concentrations as well as causing correlative changes in the activity of hepatic microsomal acyl-CoA–cholesterol *O*-transferase. Thus ethynyloestradiol treatment increases hepatic microsomal acyl-CoA–cholesterol *O*-transferase activity and increases hepatic cholesteryl ester concentrations, whereas hepatic triacylglycerol and cholesterol secretion are decreased. Conversely, Triton WR-1339 decreases hepatic microsomal acyl-CoA–cholesterol transferase activity and restores both serum and hepatic concentrations toward normal. The correlation between hepatic esterification and hepatic triacylglycerol secretion that exists under the influence of these pharmacological agents does not necessitate the presence of a cause and effect relationship. However, since oestrogens, but not other steroid hormones, activate hepatic microsomal acyl-CoA–cholesterol *O*-transferase when added to microsomal preparations from untreated rats (Schweppe & Jungmann, 1969) it is likely that this is a primary oestrogen effect. If increased hepatic cholesterol esterification is the primary effector through which ethynyloestradiol inhibits cholesterol and triacylglycerol secretion, then inhibition of this process should restore hepatic lipid secretion. This is, indeed, what was observed in experiments with Triton WR-1339. It is clear that Triton WR-1339 inhibits hepatic cholesterol esterification both directly *in vitro* (Fig. 4) and *in vivo*. As a result, both serum cholesterol and hepatic cholesteryl ester concentrations are restored to normal values. In addition to altering hepatic triacylglycerol (VLD lipoprotein) secretion these drugs inhibit (ethynyloestradiol) or restore to normal (Triton WR-1339 administration to ethynyloestradiol-treated rats) the rate of bile-acid synthesis (Davis & Kern, 1977). Since non-esterified, but not esterified, cholesterol plays an essential role in VLD lipoprotein secretion (Goh & Heimberg, 1976, 1977) and bile-acid synthesis (Ogura *et al.*, 1971; Balasubramaniam *et al.*, 1975), changes in the rate of hepatic cholesterol esterification may subsequently alter the availability of free cholesterol necessary for these processes.

Triacylglycerol is hydrophobic and therefore cannot be secreted as a single entity. Instead, hepatic triacylglycerol is secreted as a complex VLD lipoprotein particle, which contains a polar shell of specific polypeptides, phospholipids and free cholesterol that emulsifies an inner liquid core consisting of mainly triacylglycerol and a small amount of cholesteryl esters (Deckelbaum *et al.*, 1977). Hepatic triacylglycerol secretion is therefore coupled to the secretion of each of the polar 'shell' components of VLD lipoprotein. The essential role of free cholesterol in maintaining a stable phospholipid structure containing triacylglycerol has been demonstrated in model membranes (Davis & Sinensky, 1977). Under normal physiological conditions, a constant supply of free cholesterol for hepatic triacylglycerol secretion is maintained by a co-ordinate activation of β-hydroxy-β-methylglutaryl-CoA reductase, the regulatory enzyme of cholesterol biosynthesis (Goh & Heimberg, 1976, 1977).

It is well established that in pharmacological doses oestrogens cause hypolipidaemia and increased hepatic cholesterol esters in rats (Fewster *et al.*, 1967; Aftergood *et al.*, 1968). The results of the present study confirm the earlier findings and show that ethynyloestradiol treatment similarly decreases both serum cholesterol and triacylglycerol concentrations as well as apolipoproteins to values that are 5–10% of control values. The two ways in which serum lipid concentrations are altered are through changes in the rate of secretion and/or the rate of catabolism of lipoproteins. The increment of serum triacylglycerol subsequent to Triton WR-1339 administration to ethynyloestradiol-treated rats was only 30% of that of controls, suggesting that ethynyloestradiol inhibits hepatic triacylglycerol (VLD lipoprotein) secretion.

In a previous study the half-life of ^{125}I-labelled LD lipoprotein was decreased in oestrogen-treated rats, suggesting that oestrogens increase lipoprotein catabolism (Hay *et al.*, 1971). This apparent discrepancy from our conclusion that ethynyloestradiol inhibits hepatic triacylglycerol (VLD lipoprotein) secretion is readily reconciled. It now appears that fibroblasts contain high-affinity cell-surface receptors that bind, internalize and degrade LD lipoproteins (Brown & Goldstein, 1976). In the presence of low concentrations of LD lipoprotein, the number of receptors is increased so that more LD lipoprotein can be bound, internalized and degraded to increase cellular cholesterol concentrations. In the hypocholesterolaemic state, low serum cholesterol concentrations would trigger the normal cellular response to increase the number of LD lipoprotein receptors. Therefore increased degradation of ^{125}I-labelled LD lipoprotein of oestrogen-treated rats would be secondary to the initial event that caused the hypocholesterolaemia (decreased hepatic cholesterol and VLD lipoprotein secretion).

The hypothesis that the availability of free cholesterol regulates hepatic triacylglycerol secretion and bile-acid synthesis remains to be proved. Although results of the present study show that increased hepatic cholesterol esterification is closely associated with the inhibition of VLD lipoprotein secretion it is also possible that ethynyloestradiol and/or Triton WR-1339 may have other effects. Furthermore, the finding that ethynyloestradiol treatment increases microsomal free cholesterol concentrations argues against the hypothesis. However, recently we found that this increased concentration of microsomal free cholesterol is necessary to maintain membrane structure owing to a 10-fold increase in hydrophobic cholesteryl esters (Davis & Sinensky, 1977). It thus appears important to understand what portion of membrane cholesterol is available for purposes other than maintaining membrane structure. In the adrenal gland, steroidogenesis is regulated by the availability of free cholesterol produced by corticotropin-mediated activation of cholesteryl ester hydrolysis (Mahafee *et al.*, 1974). It therefore seems possible that a similar mechanism may exist in regulating hepatic cholesterol metabolism.

This work was supported by Grant AM-12626 from the National Institutes of Health. The excellent technical assistance of Ms. Renee LeComte and Mr. William Lutz is gratefully acknowledged. We thank Dr. Paul Roheim, in whose laboratory the serum lipoprotein studies were carried out. Merck, Sharp and Dohme Inc. supported the serum lipoprotein studies.

References

Aftergood, L., Hernandez, H. J. & Alfin-Slater, R. B. (1968) *J. Lipid Res.* **9**, 447–452

Balasubramaniam, S., Mitropoulis, K. A. & Myant, N. B. (1975) *Biochim. Biophys. Acta* **398**, 1720–1727

Brown, M. S. & Goldstein, J. L. (1976) *Science* **191**, 150–154

Byers, S., Friedman, M. & Sugiyama, T. (1963) *Am. J. Physiol.* **204**, 1100–1102

Davis, R. A. & Kern, F., Jr. (1976) *Gastroenterology* **70**, 1130–1135

Davis, R. A. & Kern, F., Jr. (1977) *Gastroenterology* **72**, 1045

Davis, R. A. & Sinensky, M. (1977) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **37**, 2290

Davis, R. A., Showalter, R. B. & Kern, F., Jr. (1977) in *Bile Acid Metabolism in Health and Disease* (Paumgartner, G. & Stiehl, A., eds.), pp. 25–33, MTP Press, London

Davis, R. A., Kern, F., Showalter, R., Sutherland, E., Sinensky, M. & Simon, F. (1978) *Proc. Natl. Acad. Sci. U.S.A.* in the press

Deckelbaum, R. J., Tall, A. R. & Small, D. M. (1977) *J. Lipid Res.* **18**, 164–168

de Duve, C., Pressman, B. C., Gianetto, R., Wattiaux, R. & Applemans, F. (1955) *Biochem. J.* **60**, 604–617

Fewster, M. E., Pirrie, R. E. & Turner, D. A. (1967) *Endocrinology* **80**, 263–271

Goh, E. H. & Heimberg, M. (1976) *FEBS Lett.* **63**, 209–210

Goh, E. H. & Heimberg, M. (1977) *J. Biol. Chem.* **252**, 2822–2826

Goodman, De W. S. (1965) *Physiol. Rev.* **45**, 747–839

Goodman, De W. S., Deykin, D. & Shiratori, T. (1964) *J. Biol. Chem.* **239**, 1335–1345

Havel, R. J., Eder, H. A. & Bragdon, J. H. (1955) *J. Clin. Invest.* **35**, 1345–1353

Hay, R. V., Pottenger, L. A., Teingold, A. L., Getz, G. S. & Wissler, R. W. (1971) *Biochem. Biophys. Res. Commun.* **44**, 1471–1477

Katz, S. S., Shipley, G. G. & Small, D. M. (1976) *J. Clin. Invest.* **58**, 200–211

Klauda, H. C. & Zilversmit, D. B. (1974) *J. Lipid Res.* **15**, 593–601

Lowry, O. H., Rosebrough, N. J, Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275

MacKinnen, M. & Simon, F. R. (1975) *Biochem. Pharmacol.* **24**, 748–749

Mahafee, D., Reitz, R. C. & Ney, R. L. (1974) *J. Biol. Chem.* **249**, 227–233

Maizel, J. V., Jr. (1971) *Methods Virol.* **5**, 179–245

Ogura, M., Shiga, J. & Yamasuki, K. (1971) *J. Biochem. (Tokyo)* **70**, 967–972

Ormond, A. P. Jr. & Rivera-Valez, J. M. (1965) *Proc. Soc. Exp. Biol. Med.* **118**, 600–602

Schweppe, J. S. & Jungmann, R. A. (1969) *Proc. Soc. Exp. Biol. Med.* **131**, 868–870

Shiff, T. S., Roheim, P. S. & Eder, H. A. (1971) *J. Lipid Res.* **12**, 596–603

Simon, F. R. & Arias, I. M. (1973) *J. Clin. Invest.* **52**, 765–775