Stimulative and Inhibitive Effects of Estrogen on Cholesterol Synthesis in Rats

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Synopsis

Effect of estradiol on hepatic cholesterol synthesis, acetate and mevalonate incorporation into 3β -hydroxysterols *in vitro* and acetate incorporation *in vivo*, was examined in relation to the effect of this steroid on plasma and liver cholesterol levels in male rats. A 3-day treatment with estradiol, daily 0.3 mg/rat, caused a decrease in plasma cholesterol level with a concomitant increase in liver cholesterol level and reduced the rate of hepatic cholesterol synthesis from both acetate and mevalonate. Statistical analysis revealed a) significant positive correlations between plasma cholesterol level and the rate of cholesterol synthesis in the liver, and between acetate incorporation and mevalonate incorporation, and b) significant negative correlations between plasma cholesterol level and liver cholesterol level, and between liver cholesterol level and the rate of cholesterol synthesis in the liver. By contrast, a 3-week treatment with estradiol (0.3 mg/rat, twice a week) produced a marked hypercholesterolemia and a significant stimulation of cholesterol synthesis from acetate both *in vitro* and *in vivo*, but not from mevalonate. Liver cholesterol levels after chronic treatment were in normal range. These data suggest that the inhibition of hepatic cholesterol synthesis after the acute treatment with estradiol is indirectly caused by the cholesterol accumulated in the liver as the result of cholesterol shifting action of this hormone and the stimulation after the chronic treatment is the effect of estradiol upon cholesterol synthesis to induce hypercholesterolemia.

It has been well established that estrogen decreases plasma cholesterol level (Boyd and McGuire, 1956; Boyd, 1961; Uchida and Miyake, 1961; Merola *et al.*, 1968) and inhibits cholesterol synthesis (Boyd and McGuire, 1956; Merola and Arnold, 1964; Merola *et al.*, 1968; Mukherjee and Bhowmik, 1968) in rats. The inhibition of cholesterol synthesis, however, does not reconcile with the findings that plasma cholesterol level and hepatic activity to synthesize cholesterol are higher in female rats than in males (Fillios, 1957; Kritchevsky *et al.*, 1961) and that estradiol or other naturally occurring estrogens produced a marked hypercholesterolemia after a long term treatment such as 3 weeks

(Uchida *et al.*, 1967 and 1969).

In the present experiments, the activity of the liver to synthesize cholesterol, i.e. the *in vivo* incorporation of acetate- $1-^{14}\text{C}$ and mevalonate- $2-^{14}\text{C}$ into digitonin precipitable sterols by liver slices and *in vivo* incorporation of acetate- $1-^{14}\text{C}$ into plasma and liver 3β -hydroxysterols, was examined in the rats treated with estradiol for 3 days or for 3 weeks in order to elucidate the relations between plasma and liver cholesterol levels and hepatic activity to synthesize cholesterol.

Materials and Methods

Animals, estrogen treatment and autopsy

Wistar strain male rats initially weighing around

Received for publication February 22, 1973.

200 g were maintained in an air-conditioned room ($25 \pm 1^\circ\text{C}$, 50–60% humidity) with free access to chow diet and water. A 0.3 mg per rat of estradiol in 0.2 ml aqueous suspending vehicle consisting of sodium chloride (0.9%), polysorbate-80 (0.4%), carboxymethylcellulose (0.5%) and benzylalcohol (0.9%), was injected subcutaneously either once daily for 3 consecutive days or twice a week for 3 weeks. Blood samples were withdrawn by cardiac puncture under sodium methylhexabital anesthesia (125 mg/kg i.p.). The livers were then removed, washed in cold saline, and placed in ice-chilled Krebs-Ringer phosphate buffer (pH 7.4).

Cholesterol determination

Plasma and liver cholesterol extracted with ethanol by reflux were determined by the method reported previously (Uchida and Miyake, 1961) after fractionation to free and ester forms by thin-layer chromatography in the solvent system of petroleum ether-ether-acetic acid (90:15:3) described by Kremer *et al.* (1969).

Determination of *in vitro* cholesterol synthesis

A 0.3 mg each of liver slices in about 0.5 mm thickness was incubated in 3 ml Krebs-Ringer phosphate buffer (pH 7.4) containing 2 μCi (20 μmoles) of sodium acetate-1- ^{14}C or 0.4 μCi (2 μmoles) of sodium mevalonate-2- ^{14}C at 37°C for 3 hr with air as gas phase. The incubation mixture was added with 5 ml ethanol and 1 ml of 10 N potassium hydroxide and left overnight for saponification. Unsaponified materials were extracted with petroleum ether and

digitonin-precipitable sterols were isolated by the procedures of Sobel *et al.* (1953). Dried digitonides were then dissolved in 1 ml of 1% phosphoric acid in dioxane as described by Kabara (1957) and made up to 10 ml with dioxane. An aliquot of this solution was served for cholesterol determination and for the counting of radioactivity. Sodium acetate-1- ^{14}C and N, N'-dibenzylethylenediamine DL-mevalonate-2- ^{14}C were purchased from Daiichi Pure Chemicals Co. Ltd. (Tokyo).

Determination of *in vivo* cholesterol synthesis

Rats were intraperitoneally injected with sodium acetate-1- ^{14}C (5 μCi /25 μmoles per 100 g body weight) and sacrificed by bleeding under sodium methylhexabital anesthesia 1 and 3.5 hr after the injection. A portion of the liver was homogenized in the same volume of saline. The homogenate and plasma were extracted with 20 volumes of chloroform-ethanol (2:1) twice. The extracts were combined and purified by the method of Folch *et al.* (1957). The subsequent procedures were the same as *in vitro* experiments.

Results

Plasma and liver cholesterol levels

Effect of estradiol on plasma and liver cholesterol levels is shown in Table 1. A 3-day treatment with estradiol decreased plasma cholesterol level without affecting the free/

Table 1. Effect of estradiol on plasma and liver cholesterol levels in male rats

	Control	Estradiol	
		3 Days ^{a)}	3 Weeks ^{b)}
No. of rats	6	5	5
Plasma cholesterol level (mg/100 ml)			
Free	11 $\pm$ 1.9 ^{c)}	8 $\pm$ 0.7 ^{c)}	21 $\pm$ 2.8 ^{*c)}
Ester	42 $\pm$ 3.7	23 $\pm$ 2.6*	81 $\pm$ 5.2*
Total (Free + Ester)	54 $\pm$ 5.4	31 $\pm$ 2.9*	102 $\pm$ 7.9*
Free/Total ratio (%)	21 $\pm$ 1.6	25 $\pm$ 2.1	20 $\pm$ 1.3
Liver cholesterol level (mg/g wet weight)			
Free	2.1 $\pm$ 0.03	2.3 $\pm$ 0.05	2.2 $\pm$ 0.09
Ester	0.2 $\pm$ 0.01	0.7 $\pm$ 0.13*	0.3 $\pm$ 0.05
Total (Free + Ester)	2.3 $\pm$ 0.04	3.0 $\pm$ 0.13*	2.5 $\pm$ 0.13
Free/Total ratio (%)	92 $\pm$ 0.4	78 $\pm$ 3.5*	90 $\pm$ 1.4

a) A 0.3 mg/rat of estradiol was injected subcutaneously for 3 days.

b) A 0.3 mg/rat of estradiol was injected subcutaneously twice a week for 3 weeks.

c) Mean $\pm$ S.E.

* Statistically significant against the control ($p < 0.05$).

total cholesterol ratio but caused an accumulation of cholesterol in the liver, mainly in the esterified form. On the other hand, a 3-week treatment increased plasma cholesterol level almost twice, but the liver cholesterol level and the free/total cholesterol ratios in both plasma and liver in the treated rats were in normal range.

Figure 1 shows the correlation between

Fig. 1. Correlation between plasma and liver cholesterol levels in rats given 0.3 mg/rat of estradiol for 3 days. Regression line; $Y = -0.028X + 4.945$ ($r = -0.753$, $p < 0.01$).

plasma and liver cholesterol levels in rats given estradiol for 3 days and in control rats. The data were collected from the present (Table 1) and previous experiments (Uchida *et al.*, 1970). The increase of liver cholesterol level after the acute treatment with estradiol inversely correlated with the decrease of plasma cholesterol level ($p < 0.01$).

In vitro cholesterol synthesis

As shown in Table 2, the acute treatment with estradiol decreased cholesterol synthesis. Acetate incorporation, expressed in terms of 3 hr incorporation, was reduced by 49% and mevalonate incorporation was by 33%. On the contrary, the treatment for 3 weeks resulted in a marked stimulation of cholesterol synthesis from acetate, but not from mevalonate. Acetate incorporation after the chronic treatment was about 2.5-fold of the control but the rate of mevalonate incorporation was in the control range.

Table 3 gives the correlation coefficients between cholesterol levels and cholesterol synthesis activities after the 3-day treatment with estradiol. Statistically significant correlations were found between plasma total cholesterol

Table 2. Effect of estradiol on the incorporation of acetate-1- 14 C and mevalonate-2- 14 C into digitonin precipitable sterols by liver slices

	Control	Estradiol	
		3 Days ^{a)}	3 Weeks ^{b)}
No. of rats	6	5	5
Acetate-1- 14 C incorporation			
3 Hrs. incorporation (10^4 dpm/g tissue)	4.19 ± 0.49^c	$2.15 \pm 0.40^{*c)}$ (-49%)	$11.58 \pm 0.92^{*c)}$ (+176%)
Specific activity (10^4 dpm/mg cholesterol)	1.50 ± 0.24	$0.59 \pm 0.14^*$	$3.62 \pm 0.35^*$
Mevalonate-2- 14 C incorporation			
3 Hrs. incorporation (10^4 dpm/g tissue)	26.44 ± 1.61	$17.59 \pm 2.56^*$ (-33%)	25.88 ± 2.15 (-2%)
Specific activity (10^4 dpm/mg cholesterol)	9.30 ± 0.67	$4.75 \pm 0.77^*$	8.05 ± 0.63

a) A 0.3 mg/rat of estradiol was injected subcutaneously for 3 days.

b) A 0.3 mg/rat of estradiol was injected subcutaneously twice a week for 3 weeks.

c) Mean $\pm$ S.E.

* Statistically significant against the control ($p < 0.05$).

() Percent changes against the control.

Table 3. Correlation coefficients between plasma cholesterol, liver cholesterol, acetate incorporation and mevalonate incorporation in rats given estradiol for 3 days and in control rats

	Plasma Cho- lesterol	Liver Cho- lesterol	Acetate Incorpo- ration	Acetate Specific activity	Mevalonate Incorpo- ration	Mevalonate Specific activity
Plasma Cholesterol		-0.780**	0.746**	0.726*	0.660*	0.760**
Liver Cholesterol	n = 11		-0.839**	-0.869**	-0.579	-0.822**
Acetate Incorporation ^{a)}	n = 11	n = 11		0.986**	0.703*	0.889**
Acetate Specific activity ^{b)}	n = 11	n = 11	n = 11		0.653*	0.874**
Mevalonate Incorporation ^{a)}	n = 11	n = 11	n = 11	n = 11		0.923**
Mevalonate Specific activity ^{b)}	n = 11	n = 11	n = 11	n = 11	n = 11	

a) Acetate or mevalonate incorporation, dpm/g tissue/3 hr.

b) Acetate or mevalonate specific activity, dpm/mg cholesterol.

* Statistically significant ($p < 0.01$).** Statistically significant ($p < 0.05$).Table 4. Effect of estradiol on *in vivo* acetate-1-¹⁴C incorporation into digitonin precipitable sterols in plasma and liver of male rats

	1 hr		Acetate-1- ¹⁴ C incorporation ^{a)}		Mean	
	dpm/ml	Sp act	dpm/ml	Sp act	dpm/ml	Sp act
i) Plasma						
Control	366 ^{c)}	584 ^{c)}	430 ^{c)}	636 ^{c)}	391 ± 36.5 ^{d)}	605 ± 99.9 ^{d)}
Estradiol ^{b)}	918	809	784	737	864 ± 98.2*	780 ± 72.5
ii) Liver						
Control	4,648 ^{c)}	3,383 ^{c)}	3,915 ^{c)}	3,807 ^{c)}	4,282 ± 599.0 ^{d)}	3,595 ± 462.6 ^{d)}
Estradiol ^{b)}	6,187	4,243	4,056	2,808	5,121 ± 677.7	3,526 ± 491.6

a) 5 μ Ci/25 μ mole per 100 g body weight of sodium acetate-1-¹⁴C was injected intraperitoneally.

b) A 0.3 mg/rat of estradiol was injected subcutaneously twice a week for 3 weeks.

c) Mean value in 3 rats.

d) Mean $\pm$ S.E. in 6 rats.* Statistically significant against the control ($p < 0.05$).

level and cholesterol synthesis from both acetate and mevalonate, and between acetate incorporation and mevalonate incorporation. Significant inverse correlations were also found between plasma total cholesterol level and liver total cholesterol level, and between liver cholesterol level and the rate of cholesterol synthesis.

In vivo cholesterol synthesis

As shown in Table 4, there was an about 2-fold increase in the *in vivo* acetate incorpora-

tion into plasma cholesterol after the chronic treatment with estradiol. The specific activity (dpm/mg cholesterol) in the plasma also increased but statistically insignificant ($p > 0.05$). The incorporation into liver cholesterol was not significantly altered but showed a trend of increase in the treated rats.

Discussion

The present experiments demonstrated that

the effect of estrogen on hepatic cholesterol synthesis is biphasic, an acute treatment decreases cholesterol synthesis but a chronic treatment significantly increases it, resembling to the biphasic effect of this steroid on rat plasma cholesterol levels (Uchida *et al.*, 1967 and 1969).

A 3-day treatment with estrogen significantly decreased cholesterol synthesis from both acetate and mevalonate (Table 2) in agreement with the previous findings that liver slices or homogenates prepared from rats treated with estrogen show lower activities in cholesterol synthesis than that from the controls (Boyd and McGuire, 1956; Merola and Arnold, 1964; Merola *et al.*, 1968; Mukherjee and Bhose, 1968). In contrast to the 3-day treatment, a 3-week treatment with estradiol significantly stimulated cholesterol synthesis both *in vitro* (Table 2) and *in vivo* (Table 4). This stimulation appears to occur at the site before mevalonate formation since acetate incorporation was stimulated but mevalonate incorporation was not (Table 2). Fillios (1957) and Kritchevsky *et al.* (1961) have shown that the female rat synthesizes cholesterol to a greater extent than the male does, suggesting that estrogen acts favorably in stimulating cholesterol synthesis. Noble and Boucek (1957) have shown that estrogen treatment for 3 weeks increases cholesterol synthesis, but Mukherjee and Bhose (1968) have claimed that estradiol causes a marked inhibition of cholesterol synthesis, where the longer the period of treatment the larger inhibition occurs. Our present data rather coincide with the observation of Noble and Boucek (1957) but not with that of Mukherjee and Bhose (1968). This disagreement may be partly due to the difference in the mode of estrogen administration. The treatment with estradiol at intervals of 3 or 4 days produces a marked hypercholesterolemia by 3 weeks (Uchida *et al.*, 1967 and 1969) while the daily treatment only restores plasma cholesterol level to the control range after 60 days (Uchida *et al.*, 1963), suggesting that hypercholesterolemic

effect of estradiol manifests more rapidly in the rats given at intervals than in the rats given daily. Moreover, hypercholesterolemia was found to manifest more easily in the rats given a smaller dose of estrogen than in the rats given a higher dose (Uchida *et al.*, 1967). We injected 0.3 mg/rat of estrogen (about 150 μ g/100 g body weight) at intervals of 3 to 4 days and Noble and Boucek (1957) daily injected 50 μ g/rat of estrogen, while Mukerjee and Bhose (1968) daily injected a dose of 100 μ g/100 g body weight.

The hypocholesterolemic effect of estrogen is explained by the shifting of cholesterol from blood to the liver (Uchida, 1966; Merola *et al.*, 1968), since acute treatment with estrogen decreases cholesterol level in plasma and concomitantly increases in the liver (Table 1). The statistically significant correlation between plasma and liver cholesterol levels (Fig. 1 and Table 3) further supports the occurrence of cholesterol shifting.

On the other hand, it is well known that cholesterol, when fed an excess, resulted in a marked inhibition of cholesterol synthesis by negative feedback inhibition (Gould, 1959). Sites of inhibition are shown as before mevalonate (Siperstein, 1960; Gould and Swyryd, 1966), between mevalonate and farnesyl phosphate, and after farnesyl phosphate (Gould and Swyryd, 1966). In order to confirm the inhibition, we fed a diet supplemented with 2% cholesterol to rats for 4 weeks and determined *in vitro* acetate incorporation by liver slices. Plasma and liver cholesterol levels in these treated rats increased by 16 and 31%, respectively, and acetate incorporation into cholesterol decreased to about one twentieth of the paired controls.

The inhibition of cholesterol synthesis by estrogen (Table 2), as compared with that by cholesterol feeding, is presumed to be indirectly caused by the cholesterol accumulated in the liver as the result of cholesterol shifting action of this steroid. However, it can not be excluded the possibility that estrogen directly inhibits cholesterol synthesis, since estrogen is

reported to reduce the rate of cholesterol synthesis when added into the incubation flasks (Humber *et al.*, 1962; Carroll and Pritham, 1966). This inhibition, however, would take a minor part in either hypocholesterolemic or cholesterol synthesis inhibiting action of estrogen *in vivo*, because testosterone is also reported to exert a potent inhibition on cholesterol synthesis in that *in vitro* system (Carroll and Pritham, 1966), but testosterone when administered by a similar regime as estrogen produces no decrease in plasma cholesterol level in rats (Uchida and Miyake, 1961; Uchida, 1966).

We have previously reported that estrogen treatment increases chenodeoxycholic acid formation in rats (Uchida *et al.*, 1970). This increase in chenodeoxycholic acid formation occurs in association with the increase in liver cholesterol level, and the increased hyodeoxycholic acid converted from chenodeoxycholic acid acts to reduce the elevated liver cholesterol level to the normal range by preventing the accumulation of cholesterol in the liver (Uchida *et al.*, 1970). Therefore, the initial elevation in the liver cholesterol level during the estrogen treatment may be explained as the result of cholesterol shifting from blood to the liver, and the subsequent normalization appears to be due to the increase in chenodeoxycholic acid formation. This may account for the fact that the cholesterol level in the liver is kept in a normal range in spite of hepatic cholesterol synthesis being stimulated in the rats treated with estrogen for 3 weeks.

Acknowledgment

The authors wish to thank Dr. N. Yoshitani of Shionogi Research Laboratory for his help in the statistical analysis of the present data.

References

- Boyd, G. S. and W. G. McGuire (1956). *Biochem. J.* **62**, 19p.
- Boyd, G. S. (1961). *Fed. Proc. Supplement* No. 7, 152.
- Carroll, J. J. and G. H. Pritham (1966). *Biochim. Biophys. Acta* **115**, 320.
- Fillios, L. C. (1957). *Endocrinology* **60**, 22.
- Folch, J., M. Lees and G. H. S. Stanley (1957). *J. Biol. Chem.* **226**, 497.
- Gould, R. G. (1959). In *Hormones and Atherosclerosis*, G. Pincus (ed), Academic Press Inc., New York, p. 75.
- Gould, R. G. and E. A. Swyryd (1966). *J. Lipid Res.* **7**, 698.
- Humber, L. G., M. Kraml and J. Dubuc (1962). *Biochem. Pharmacol.* **11**, 755.
- Kabara, J. J. (1957). *J. Lab. Clin. Med.* **50**, 146.
- Kremmer, T., E. Ferenczy and E. Posch (1969). *Chromatographia* **2**, 142.
- Kritchevsky, K., E. Staple, J. L. Rabinowitz and M. W. Whitehouse (1961). *Am. J. Physiol.* **200**, 519.
- Merola, A. J. and A. Arnold (1964). *Science* **144**, 301.
- Merola, A. J., R. R. Dill and A. Arnold (1968). *Arch. Biochem. Biophys.* **123**, 378.
- Mukherjee, S. and A. Bhose (1968). *Biochim. Biophys. Acta* **164**, 357.
- Noble, N. L. and R. J. Boucek (1957). *Circulation Res.* **5**, 573.
- Siperstein, M. D. (1960). *J. Am. Clin. Nutr.* **8**, 645.
- Sobel, A. E., M. Goldberg and S. R. Slater (1953). *Anal. Chem.* **25**, 629.
- Uchida, K. and T. Miyake (1961). *Ann. Rept. Shionogi Res. Lab.* **11**, 119.
- Uchida, K., M. Kadowaki and T. Miyake (1963). *Folia Endocrinol. Japon.* **38**, 1094.
- Uchida, K. (1966). *Ph. D. Thesis*, Tokyo University.
- Uchida, K., M. Kadowaki, K. Miyata and T. Miyake (1967). *Folia Endocrinol. Japon.* **42**, 1159.
- Uchida, K., M. Kadowaki, K. Miyata and T. Miyake (1969). *Endocrinol. Japon.* **16**, 211.
- Uchida, K., Y. Nomura, M. Kadowaki, K. Miyata and T. Miyake (1970). *Ibid.* **17**, 107.