Effect of large doses of the oral contraceptive Enovid on cholesterol metabolism in the rat

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ABSTRACT Short-term effects of the oral contraceptive drug, Enovid, a mixture of estrogenic and progesterone compounds, have been determined in experiments on male and female rats. Oral administration of large doses for 7 days resulted in marked decreases of cholesteryl esters in plasma accompanied by only slight elevations of hepatic cholesterol content. Cholesteryl esters were also much lower in adrenals and ovaries, organs which are usually responsible for steroid hormone biosynthesis. At the same time, cholesterol-esterifying activity in plasma was substantially increased.

Enovid administration was shown also to affect the fatty acid composition of sterol esters remaining in plasma, adrenals, and ovaries. The concentration of linoleate and arachidonate was significantly decreased in plasma sterol esters, whereas the concentrations of arachidonate and docosatetraenoate in adrenals and of docosatetraenoate in ovaries were significantly lowered.

All of the changes reported were more pronounced in the female than in the male rat. It is hypothesized that the decreased levels of cholesteryl ester found in the organs investigated, together with the increase in plasma cholesterol-esterifying activity, are probably associated either with changes in cholesterol biosynthesis and(or) transport or with an increase in cholesterol transformation to other steroids and excretion. These possibilities are now under investigation.

KEY WORDS Enovid · oral contraceptive drugs · cholesteryl esters · fatty acid composition · plasma cholesterol-esterifying activity · adrenal steroids

THE ADMINISTRATION OF ESTROGENIC SUBSTANCES to humans has resulted in the depression of serum cholesterol levels (1–3). In the rat the results of treatment with sex hormones are somewhat less well defined. In some cases it has been reported (4–6) that plasma cholesterol levels increase after the administration of estrogen, whereas in others, results similar to those in humans have been obtained (7). The effects are apparently related to the dose level, route of administration, age of animal, length of treatment, and nutritional status of the animal.

The changes in lipid metabolism that occur during pregnancy are probably related to the changes caused by hormone administration. DeAlvarez, Goodel, and Zighelboim (8) have reported that serum cholesterol levels rise progressively in pregnancy; this elevation is accompanied by characteristic changes in fatty acid composition of lipids. The fatty acid composition of various tissues is also affected by administration of sex hormones. A decreased deposition of total unsaturated fatty acids in the adipose tissue of the ox has been attributed to diethylstilbestrol treatment (9). Fewster, Pirrie, and Turner (10) reported that the percentage of cholesteryl oleate increased in the plasma cholesteryl ester fractions of estrogen-treated rats. Oliver and Boyd (11) observed in humans that the ratio of linoleate : oleate : palmitate in the cholesteryl ester fraction was markedly influenced by estrogen administration, changing from 46:25:15 to 30:20:22. This decrease in relative linoleate concentration led the authors to suggest that cholesteryl linoleate may be more readily metabolized than the other fatty acid esters of cholesterol.

An increased proportion of plasma cholesteryl arachidonate in female rats and estrogen-injected, castrated male rats was reported by Lyman, Shannon, Ostwald, and Miljanich (12). Aftergood and Alfin-Slater (13) have related estrogenic deficiency to decreased concentration of unsaturated fatty acid in plasma and liver during essential fatty acid deficiency.

Since many women are now using antifertility agents consisting of mixtures of progesterone-type hormones with

Fatty acids are designated by number of carbon atoms : number of double bonds.

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estrogens, it seemed desirable to measure some of the metabolic effects of these steroids. A series of experiments was undertaken to determine the effect of one of these, Enovid E (consisting of a mixture of 2.5 mg of progesterone [norethynodrel] and 0.1 mg of estrogen [mestranol] manufactured by G. D. Searle & Co., Chicago, Ill.), on some aspects of lipid metabolism in the adult male and female rat. The dose level used (1 mg of the mixture daily) is unphysiological for the rat. This level of hormone is approximately 40 times higher than the minimal estrogenizing dose in rats and about 3 times the minimal progestational dose. These high levels of hormone were used to emphasize and hasten the appearance of any effects which might result from the use of smaller doses over a longer period of time.

MATERIALS AND METHODS

A group of male and female rats of our colony (the former USC strain), 5–6 months old, were kept on a stock diet (Purina pellets) and given over a 7 day period either 1 mg of Enovid E in 0.5 ml of sesame oil or 0.5 ml of plain sesame oil by intubation daily. Food consumption was measured. Some animals given Enovid were then placed on plain sesame oil for a 7 day "recovery period."

At the end of the experimental periods, the animals were killed by removal of blood from the heart under nembutal anesthesia. Heparinized blood was centrifuged and total lipids were extracted from plasma with dimethoxymethane–methanol 4:1. Free and total cholesterol were determined on alcohol–acetone extracts of plasma by a modified Sperry–Schoenheimer method (14). Total lipid was fractionated by thin-layer chromatography in petroleum ether–anhydrous ether–acetic acid 83:16:1. Methyl esters of fatty acids derived from sterol esters, phospholipids, triglycerides, and free fatty acids were prepared by methanolysis with methanol–benzene–concd. HCl 15:4:1. The methyl esters were purified by additional thin-layer chromatography which also measured completeness of transmethylation. These esters were analyzed on a gas–liquid chromatograph (Varian Aerograph model 1200, hydrogen flame detector; packing 14.5% Hi-Eff 2BP on 100–120 mesh Gas-Chrom P; column temperature 190°C; carrier gas, nitrogen, flow rate 33.0 ml/min). Chromatographic peaks were identified either by comparison of retention times with those of standards or from a graph representing the relationship between log retention time and the number of carbon atoms. The accuracy of recovery of fatty acids from a mixture was checked by means of a standard fatty acid mixture provided by NIH.

As soon as the blood had been drawn, the liver was excised, trimmed, and frozen. Cholesterol and total lipid determinations were carried out on petroleum ether extracts, while fatty acid determinations were performed on dimethoxymethane–methanol extracts. Adrenals and ovaries were likewise trimmed immediately and extracted with dimethoxymethane–methanol. The cholesterol-esterifying activity in plasma was investigated by a previously described procedure (15).

RESULTS AND DISCUSSION

Table 1 shows the growth of male and female rats over the experimental period as well as food consumption of the female rats. It may be noted that in 7 days the experimental males lost about 5% of their weight, while the females lost 8%. The control female rats consumed 16.5 g of diet per day whereas the Enovid-treated animals ate an average of approximately 7 g/day. This loss of appetite is probably a reflection of the unphysiological level of hormone administration. Fewster et al. (10), who injected 1.7 mg of estradiol benzoate per day into rats, did not report their observations on growth and food consumption, but Lyman et al. (12) mentioned that rats receiving 30 μg of estradiol benzoate per week showed a gain in weight and food consumption identical to those for the controls. Reduced growth rates in animals fed estrogen–progesterone mixtures have been noted by Holmes and Mandl (16). Pincus (17) suggested that a limited inhibition of growth-promoting stimulation (perhaps by pituitary somatotropin) takes place when rats are fed large doses of Enovid.

Table 2 presents plasma and liver cholesterol levels, liver weights, and total liver lipid levels. Enovid administration results in a marked decrease in the level of total cholesterol in plasma in both female and male animals. Although both the esterified and the free portion of cholesterol are lowered, the esterified cholesterol is decreased more than is the unesterified cholesterol.

In the liver, total lipids are essentially unchanged but cholesterol values are significantly affected. Enovid administration results in an increase of total liver cholesterol with a concomitant lowering of the proportion of free cholesterol. This could be due either to an influx of

TABLE 1 BODY WEIGHT AND FOOD CONSUMPTION OF CONTROL AND ENOVID-TREATED RATS

Group	Starting Weight	Change in Body Weight over 7 Day Experimental Period	Food Consumption
	g	*g*	*g/day*
F (8)	205	−4	16.5
FE (32)	214	−17	7.1
M (4)	300	+6	n.d.
ME (8)	305	−16	n.d.

Numbers in parentheses indicate number of rats. F, normal female; FE, Enovid-treated female; M, normal male; ME, Enovid-treated male; n.d., not determined.

esterified cholesterol from other sources or to an increase in cholesterol synthesis, followed by a stimulation of the esterification process.

When the administration of Enovid to female rats was discontinued for 7 days, both plasma and liver cholesterol levels returned to their control values (Table 2).

It has been already established (18) that the turnover of cholesteryl esters in mammalian tissues depends on whether the acyl moieties are saturated or unsaturated. Since esterified cholesterol appears to be primarily involved in Enovid-induced metabolic changes, the fatty acid composition of various lipid fractions was determined.

Major fatty acids of plasma lipid fractions are presented in Table 3. Significant changes in the sterol ester fractions are apparent. The most striking change is the decrease in arachidonate content of both male and female treated animals, as compared with the untreated animals—from 27.4 to 9.4% in the treated male, and from 41.4 to 8.9% in the treated female. Enovid administration also causes decreased linoleate concentrations in both male and female animals. These changes are compensated for by a significant increase in oleate, stearate, and palmitoleate. The phospholipid fatty acids seem to be affected by hormone treatment but no statistically significant differences are apparent. As far as triglycerides are concerned, the most marked changes occur in the female rats, where lower palmitoleic, stearic, and arachidonic acid levels and higher oleic and linoleic acid levels result from the hormone treatment.

Since no changes were observed in the composition of free fatty acids in plasma and in the fatty acids of the

TABLE 2 Plasma and Liver Cholesterol Levels, Liver Weights, and Total Lipids in Control and Enovid-Treated Rats

Group	Plasma Cholesterol			Liver			
						Cholesterol	
	Total	Ester	Free	Weight	Lipids	Total	% Free
	mg/100 ml			*g*	*mg/g*	*mg/g*	
F	49.9 ± 9.3	30.5 ± 5.3	19.4 ± 3.7	7.51 ± 0.79	40.6 ± 5.7	2.24 ± 0.22	89.0
	$P < 0.001$					$P < 0.001$	
FE	10.5 ± 3.7	4.5 ± 3.3	6.0 ± 2.5	8.51 ± 0.83	36.4 ± 4.2	3.02 ± 0.33	75.5
FE + Recovery*	59.9 ± 11.0	41.6 ± 9.9	18.3 ± 4.7	8.28 ± 0.73	41.6 ± 5.2	2.05 ± 0.20	95.6
M	50.9 ± 3.3	34.8 ± 4.2	16.1 ± 3.7	11.04 ± 1.05	35.0 ± 5.2	1.89 ± 0.20	82.0
	$P < 0.001$					$P < 0.001$	
ME	16.9 ± 2.8	9.1 ± 3.2	7.8 ± 1.6	11.37 ± 1.43	38.4 ± 7.7	2.88 ± 0.36	59.5

Values are means ± SD (n given in Table 1).
* Rats sacrificed 7 days after the last dose of Enovid.

TABLE 3 Percentages of Major Fatty Acids of Plasma Lipid Fractions in Control and Enovid-Treated Male and Female Rats

	16:0	16:1	18:0	18:1	18:2	20:4
Sterol esters			%			
M (4)	16.3 ± 1.4*	10.3 ± 1.8	5.5 ± 1.6	14.9 ± 1.3	15.2 ± 2.9	27.4 ± 2.7
		$P < 0.05$	$P < 0.025$	$P < 0.001$		$P < 0.001$
ME (7)	17.8 ± 2.9	13.4 ± 1.9	9.3 ± 2.2	25.0 ± 1.6	11.0 ± 3.1	9.4 ± 1.7
F (7)	11.3 ± 2.3	6.6 ± 1.9	4.4 ± 1.3	9.7 ± 2.3	16.6 ± 1.9	41.4 ± 6.1
	$P < 0.01$	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$
FE (9)	18.0 ± 4.6	12.4 ± 2.0	8.8 ± 1.5	21.8 ± 1.5	10.8 ± 1.3	8.9 ± 2.2
Phospholipids						
M	21.3 ± 3.1	10.3 ± 3.6	16.3 ± 1.6	20.7 ± 2.8	12.3 ± 3.7	7.0 ± 5.4
ME	21.8 ± 5.2	11.4 ± 3.3	14.5 ± 3.2	18.1 ± 1.5	9.7 ± 1.9	2.4 ± 1.7
F	16.5 ± 2.0	7.9 ± 2.7	17.1 ± 4.3	14.9 ± 2.7	9.7 ± 1.9	10.1 ± 4.5
FE	18.3 ± 2.2	8.9 ± 2.5	13.6 ± 1.6	17.8 ± 4.2	10.2 ± 2.0	6.4 ± 2.5
Triglycerides						
M	20.5 ± 2.0	6.8 ± 1.5	8.4 ± 2.7	29.9 ± 1.8	25.7 ± 2.4	2.3 ± 0.5
			$P < 0.005$		$P < 0.025$	
ME	23.2 ± 1.5	6.1 ± 2.1	4.0 ± 0.9	30.5 ± 3.4	28.7 ± 1.9	1.8 ± 0.5
F	22.2 ± 0.9	15.8 ± 3.3	9.5 ± 1.9	23.8 ± 2.6	16.0 ± 4.2	4.6 ± 0.9
		$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$
FE	23.3 ± 2.2	4.9 ± 0.7	3.9 ± 1.4	30.1 ± 4.0	24.5 ± 2.1	2.4 ± 0.5

Values are means ± SD (n in parentheses). Abbreviations as in Table 1.

TABLE 4 EFFECT OF ENOVID ADMINISTRATION ON PLASMA CHOLESTEROL-ESTERIFYING ACTIVITY IN FEMALE RATS

Group	Cholesterol			% Arachidonate in Cholesteryl Ester Fraction	
	Total	% Free at 0 hr	Decrease in % Free after 4 hr*	0 hr	After 4 hr
	mg/100 ml				
F (7)	61.0 ± 9.6	35.8 ± 3.7	10.5 ± 3.9	44.7 ± 5.8	51.8 ± 5.7
			$P < 0.01$		
FE (5)	14.2 ± 5.2	50.4 ± 12.7	23.0 ± 4.9	9.5 ± 2.6	13.5 ± 4.3

Values are means ± SD (n in parentheses). Abbreviations as in Table 1.
* Incubation at 38°C.

various lipid fractions in liver, these results are not included here.

It has been shown previously (15) that the activity of a cholesterol-esterifying enzyme in plasma (a lecithin: cholesterol acyltransferase) is influenced by gonadal hormones; female hormones enhance the esterification process. In Table 4 it can be seen that Enovid treatment similarly enhances cholesterol esterification in plasma. Whereas approximately one-third of the available free cholesterol becomes esterified during the incubation of control plasma for 4 hr at 38°C, approximately 50% of the free cholesterol is esterified in the case of the hormone-treated animals. The concentration of arachidonate in the cholesteryl esters is also significantly increased during the process. In the control animals the percentage increase of arachidonic acid is approximately 16%; in the experimental rats a 42% increase is observed. These changes may reflect the increased requirement for esterified rather than for free cholesterol in the hormone-treated animal. However, as was shown in Table 2, the proportion of free cholesterol in plasma actually increases. Apparently the rate of removal of the esterified cholesterol is sufficiently rapid to depress the proportion of it in plasma despite an increased rate of esterification.

Cholesterol levels in adrenals of control and Enovid-treated rats are shown in Table 5. Although the actual weight of the organs is not affected by the administration of hormones, the cholesterol levels decrease by 78% in females and by 54% in the male rats. In addition, as shown for the female rats, the change seems to be primarily a decrease in the esterified portion of cholesterol, which results in an increase in the proportion of the free cholesterol.

Although the adrenals are able to synthesize cholesterol (19), most adrenal cholesterol is derived from plasma (20). Moore and Williams (21), on the basis of studies on the cholesteryl esters of the adrenal glands of the rabbit, put forward the theory that the gland incorporates plasma cholesteryl esters in a nonselective fashion but that subsequently linoleate is utilized for synthesis of C_{20} and C_{22} polyunsaturated fatty acids.

TABLE 5 CHOLESTEROL LEVELS IN ADRENALS AND OVARIES OF CONTROL AND ENOVID-TREATED RATS

	Weight	Cholesterol			
		Total	Free	% Free	Total
	mg/rat	*mg/g*	*mg/g*		*mg/rat*
Adrenals					
F (11)	59.6 ± 14.2	33.0 ± 7.2	2.4	9.1	2.187
FE (7)	58.3 ± 13.7	7.0 ± 3.3	1.5	29.7	0.419
M (6)	50.6*	25.9*	n.d.	n.d.	1.311
ME (10)	51.0*	11.9*	n.d.	n.d.	0.607
Ovaries					
F (5)	57.6 ± 11.8	6.5 ± 0.2	1.8	27.0	0.376
FE (6)	34.8 ± 8.0	5.0 ± 1.2	1.5	31.0	0.167

Values are means ± SD (n in parentheses). Abbreviations as in Table 1.
* Pooled samples.

In the ovaries (Table 5), there is only a small decrease in cholesterol content per g of tissue; however, since the weight of the organs of the treated rat is rather markedly lowered, a substantial decrease of total ovarian cholesterol results. Herbst (22) has recently reported significant variations in ovarian cholesterol content due to administration of gonadotropins and anterior pituitary hormones. Luteinizing hormone, in particular, caused a depletion of cholesteryl esters. These data confirm the theory that cholesterol is the intermediary in progesterone synthesis.

Table 6 shows the major fatty acids of the cholesteryl esters isolated from adrenals and ovaries of control and Enovid-treated females. It is apparent that in these organs the subsequent disposition of cholesteryl esters depends on the type and amount of the fatty acid with which cholesterol is esterified. Thus, in the adrenal the most significant decrease after hormone treatment is found in the 20:4 and 22:4 acids, while in the ovaries, where initially the 20:4 acid is rather low, it is the 22:4 fatty acid that decreases.

Sinclair (23) has suggested that essential fatty acid esters of cholesterol may be preferentially converted to steroid hormones. The 22:4 acid which arises from linoleic acid has been identified by several workers (24, 25)

TABLE 6 PERCENTAGES OF MAJOR FATTY ACIDS IN ADRENAL AND OVARIAN STEROL ESTERS FROM CONTROL AND ENOVID-TREATED FEMALE RATS

	16:0	16:1	18:0	18:1	18:2	20:4	22:4	22:5	22:6
					%				
Adrenals									
F (6)	11.1 ± 1.0	4.8 ± 1.4	4.2 ± 0.9	16.7 ± 3.0	6.6 ± 1.0	15.9 ± 1.9	25.4 ± 3.3	2.0 ± 0.8	3.6 ± 0.9
	$P < 0.01$	$P < 0.001$	$P < 0.001$	$P < 0.005$		$P < 0.001$	$P < 0.001$		
FE (7)	14.4 ± 1.9	10.7 ± 1.3	8.2 ± 1.9	22.2 ± 2.7	7.2 ± 0.9	8.2 ± 1.3	11.0 ± 4.5	2.9 ± 1.5	1.7 ± 1.0
Ovaries									
F (5)	17.8 ± 5.7	10.7 ± 1.6	9.3 ± 1.8	18.1 ± 2.0	4.0 ± 1.9	4.3 ± 0.6	11.0 ± 1.1	2.7 ± 0.8	3.6 ± 2.4
							$P < 0.001$		
FE (6)	18.0 ± 3.8	11.4 ± 2.2	10.5 ± 0.7	20.2 ± 2.8	4.3 ± 1.5	4.3 ± 0.8	7.0 ± 1.3	2.9 ± 1.5	4.6 ± 1.7

Values are means ± SD (n in parentheses). Abbreviations as in Table 1.

as the 7,10,13,16-docosatetraenoic acid, and has been named "adrenic" acid by Chang and Sweeley (26).

Steroid hormones are synthesized in both adrenals and ovaries. Krum, Morris, and Bennett (27) indicate that cholesterol is an obligatory precursor in the biosynthetic pathway of adrenocortical steroids, and Riley (28) believes that sterol esters rather than free sterol are the starting materials for steroid biosynthesis. The results presented here support these reports. However, they do not answer the question of whether the high doses of Enovid act directly on adrenals and ovaries or indirectly through the pituitary gland. The fact remains that a rather large amount of cholesterol, mainly as a highly unsaturated ester, undergoes transformation in, or removal from, the ovaries, adrenals, and plasma. Simultaneously there is a stimulation of esterification in plasma, which preferentially supplied the unsaturated esters.

The effects of Enovid administration appear to be more pronounced in the female rats. The depression of the plasma cholesterol level averages 80% in the female as compared with 67% in the male (Table 2); the depression in relative percentage of cholesteryl arachidonate is 78% in the female and 58% in the male; and the decrease in total percentage of major unsaturated fatty acids in plasma cholesterol esters is from 74% to 54% in the female and from 68% to 59% in the male (Table 3). Only in the liver cholesterol levels is there a greater increase in the male rat, and this increase is the only compensation observed so far for the lowered cholesterol levels in plasma, ovaries, and adrenals. The increase in liver cholesterol levels may reflect either increases in cholesterol biosynthesis or decreases in cholesterol excretion. These questions are being investigated.

In summary, studies of the administration of large doses of Enovid have been carried out in short-term experiments on rats. This level of Enovid affects some aspects of lipid metabolism in the rat. The effects are more pronounced in the female, possibly because of the supplementation of endogenous hormones by the Enovid. The effects observed consist primarily of a marked removal of cholesteryl esters from plasma and from those organs concerned with the manufacturing of steroid hormones, e.g. adrenal and ovaries. The dependence of the response of cholesteryl esters on the type of the fatty acid contained in the molecule has been established.

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