Sex differences in plasma cholesterol-esterifying activity in rats

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ABSTRACT Esterification of free cholesterol was investigated after incubation at 37°C of plasma from immature and adult rats of both sexes kept on stock, fat-free, or cholesterol-supplemented diets. According to measurements of the decrease in free cholesterol, plasma from the fat-deficient rats showed the highest cholesterol-esterifying activity. Esterification was higher in the mature female rats than in the mature males on stock or cholesterol-containing diets, although no sex differences were observed in the sexually immature young or in the fat-free animals.

There were no sex differences in the fatty acid composition of the plasma sterol esters, phospholipids, and triglycerides in the immature animals, but arachidonic acid increased at the expense of linoleic acid in the sterol ester fraction in the adult female (not, however, in the adult male). In the phospholipid fraction the higher ratio of palmitic to stearic acids in the male was confirmed. There was an increase in linoleic acid in all three plasma lipid fractions of the mature male after cholesterol feeding. It is suggested that cholesterol may inhibit the conversion of linoleate to arachidonate.

During the incubation of plasma, there was little change in the distribution of fatty acids except for a decrease in palmitoleate, and increases in C_{20} tri- and tetraenoic acids, in the sterol esters of mature female rats on the stock ration and the fat-free diet. These C_{20} acids decreased concomitantly in the phospholipid fraction, as the transesterification reaction mechanism proposed by earlier workers would predict.

KEY WORDS cholesterol-esterifying enzyme · acyltransferase · plasma · rat · sex differences · diet · fat-free · cholesterol-containing · · cholesteryl esters · fatty acid composition

RECENTLY (1) we have observed that, in general, the lower plasma cholesterol levels in male rats are associated with a lower concentration of plasma arachidonate. Administration of estrogen to castrated females on a fat-free diet results in an increase in plasma cholesterol levels and a concomitant increase in the plasma arachidonate level. During essential fatty acid depletion, rapid loss of unsaturated fatty acids from plasma of castrated females is prevented by administration of estrogen. In view of these facts, we undertook an investigation of the effect of gonadal hormones on some aspects of the metabolism of plasma cholesteryl esters.

As early as 1935, Sperry (2) observed that there was an increase in esterified cholesterol in serum incubated at 37°C, which indicated the presence of a cholesterol-esterifying enzyme. The difference in fatty acid composition between plasma and liver cholesteryl esters has suggested that the site and mechanism of the formation of cholesteryl esters might be different for plasma and liver (3). Polyunsaturated esters of cholesterol predominate in plasma; liver contains mainly the monounsaturated and saturated esters (4).

Phospholipid has been suggested as the source of fatty acids for cholesterol esterification in incubated plasma, since LeBreton and Pantaléon observed (5, 6) that the level of phospholipid decreases as cholesterol esterification increases. This suggestion has been supported by Glomset and his associates (7, 8). The concentration of lecithin decreased significantly during incubation in vitro, and both cholesterol and esterified fatty acid in the cholesteryl ester fraction increased to the same degree. The existence of a plasma lecithin:cholesterol acyltransferase which acts specifically to transfer highly unsaturated fatty acid from the β-position of lecithin to the cholesterol molecule has been postulated. More recently Glomset, Janssen, Kennedy, and Dobbins (9) suggested that the transferase acts on all high density lipoprotein molecules, but has a particular affinity for those of relatively high lecithin content. Rowen and Martin (10, 11) have confirmed the theory that an ap-

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

parent conversion of lecithin to lysolecithin accompanies cholesterol esterification in the incubated plasma.

In the study reported here, we investigated not only the effect of sex hormone activity on enzymatic (esterase) activity in the rat, but also the influence of age (sexual maturity or immaturity) and of diet. In addition to measuring possible inhibition or activation of the enzyme systems, we analyzed the fatty acids of various plasma lipid fractions for changes that might result from enzyme action.

MATERIALS AND METHODS

Male and female albino rats of the former USC strain were selected at random and divided into the following groups:

Group I. Animals 6 wk of age fed from weaning on a stock ration (Purina pellets).

Group II. Animals 6 months of age fed from weaning on a stock ration (Purina pellets).

Group III. Like Group II, but placed for 1 month thereafter on a cholesterol-containing ration consisting of 24% casein, 15% cottonseed oil, 1% cholesterol, 0.25% bile salts, 4% salt mix, 4% celluflour, vitamins, and sucrose.

Group IV. Animals placed for 18 wk from weaning on a fat-free diet consisting of 24% casein, 4% salt mix, 4% celluflour, vitamins, and sucrose.

Animals were killed by removal of blood from the heart under Nembutal anesthesia. Heparinized blood from three animals (six animals in Group I) was pooled to yield about 15 ml of plasma after centrifugation at 0°C. Total lipids were immediately extracted from 5 ml of plasma with dimethoxymethane–methanol 4:1. The same amount of plasma was incubated at 37°C in a Dubnoff shaker for 5 hr, and the lipids were extracted similarly. Alcohol–acetone 1:1 was used to extract lipids for cholesterol analysis from separate aliquots of plasma taken before and after incubation. Cholesterol was determined by a modified Sperry-Schoenheimer method as reported by Nieft and Deuel (12). The difference in the amount of free cholesterol in the total cholesterol before and after incubation was considered to indicate enzyme activity.

Total lipid was fractionated by thin-layer chromatography with petroleum ether–anhydrous ether–acetic acid 83:16:1. Methyl esters of fatty acids derived from sterol esters, phospholipids, and triglycerides were prepared by methanolysis with methanol–benzene–HCl 15:4:1. The methyl esters were purified by additional thin-layer chromatography, which also measured completeness of transmethylation. The fatty acid methyl esters were analyzed on a gas–liquid chromatograph (Barber-Colman Model 20 with ionization detector, radium source). The stationary phase was 15% ethylene glycol succinate polyester on 80–100 mesh Gas-Chrom P at 190°C and the argon inlet pressure was 20 psi. 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 frequently by means of standard fatty acid mixtures provided by NIH.

To minimize oxidation, we kept samples under a nitrogen atmosphere whenever feasible, and when necessary, stored them at −15°C.

RESULTS AND DISCUSSION

The extent of cholesterol esterification during the 5 hr incubation of rat plasma is presented in Table 1. The

TABLE 1 EFFECT OF INCUBATION ON PLASMA CHOLESTEROL LEVELS

Group	Diet and Age	Sex and Number of Samples	Cholesterol				
			Total (T)	% Free (F)		$-\Delta\ \%F$	$-\Delta \frac{\%F}{T} \times 100$
				Before	After		
			mg/100 ml				
I	Stock 6 wk	M 3	54.3 ± 4.1*	33.6	19.7	13.9 ± 3.0	25.5
		F 5	60.0 ± 10.1	35.1	21.7	13.4 ± 3.7	22.3
II	Stock 6 months	M 7	59.2 ± 5.0	34.7	27.0	7.7 ± 2.6	13.0
						$P<0.01$	
		F 6	61.8 ± 9.3	35.6	24.0	11.6 ± 2.6	18.8
III	Stock 6 months; cholesterol 1 month	M 4	74.9 ± 5.9	30.4	25.6	4.8 ± 0.2	6.3
						$P<0.005$	
		F 4	78.1 ± 12.8	32.7	21.5	11.2 ± 3.3	14.3
IV	Fat-free 18 wk	M 4	42.8 ± 5.2	33.1	14.6	18.5 ± 2.1	43.2
		F 7	67.3 ± 4.8	36.0	15.2	20.8 ± 3.1	30.9

* SD.

TABLE 2 MAJOR FATTY ACIDS OF PLASMA STEROL ESTERS (% OF TOTAL)

Diet and Age	Sex and Number of Samples	16:0	16:1	18:0	18:1	18:1	20:3	20:4
Stock 6 wk	M 5	13.2 ± 2.4*	4.8 ± 1.2	2.5 ± 0.7	11.8 ± 1.6	18.6 ± 5.0		42.7 ± 7.5
	F 6	13.5 ± 2.4	5.0 ± 0.4	2.8 ± 0.6	10.0 ± 1.6	20.0 ± 2.9		43.3 ± 6.9
Stock 6 months	M 9	12.0 ± 0.6	5.9 ± 1.0	3.8 ± 0.9	10.9 ± 1.7	16.2 ± 3.1		44.4 ± 3.1
		$P<0.001$			$P<0.01$	$P<0.005$		$P<0.001$
	F 6	9.8 ± 1.0	4.6 ± 0.7	3.3 ± 0.6	8.0 ± 0.6	11.3 ± 0.8		57.1 ± 1.0
Stock 6 months; cholesterol 1 month	M 6	11.2 ± 1.0	7.0 ± 0.7	3.1 ± 0.6	19.1 ± 2.5	25.4 ± 3.8		29.7 ± 4.0
						$P<0.025$		
	F 6	10.7 ± 3.0	7.7 ± 1.0	3.0 ± 1.0	22.7 ± 7.2	18.7 ± 4.6		30.6 ± 5.0
Fat-free 18 wk	M 7	14.3 ± 1.2	14.5 ± 2.1	4.1 ± 1.7	32.1 ± 3.0	5.3 ± 1.5	19.9 ± 3.4	3.4 ± 1.0
		$P<0.05$	$P<0.001$		$P<0.001$	$P<0.25$	$P<0.01$	
	F 5	11.6 ± 2.7	29.2 ± 4.3	4.5 ± 1.3	18.7 ± 1.6	8.5 ± 2.3	14.1 ± 2.2	3.8 ± 1.4

* SD.

greatest decrease in free cholesterol occurred in fat-deficient rats (Group IV), which indicates that plasma from these animals exhibited the highest cholesterol-esterifying activity, particularly when the activity is related to the total cholesterol content. This agrees with the observation of Sugano and Portman (13), who have also reported an increased plasma cholesterol esterification in essential fatty acid-deficient rats. They ascribed this effect to an increased enzyme concentration.

The young male and female animals (Group I) showed the next highest decrease in free cholesterol, and thereafter the mature females (Group II). Sex differences in the extent of cholesterol esterification in vitro were not apparent in the young animals or in the fat-deficient rats (Group IV). However, sex differences can be observed in the 6-month old animals fed either the stock laboratory ration or the experimental, cholesterol-containing diet (Groups II and III).

In Table 2 are reported the major fatty acids in the plasma sterol esters. Here again the immature animals do not exhibit any sex differences. As the animal grows older and matures sexually, an increase in arachidonic acid at the expense of linoleic acid is observed in the female, but not in the male. Ostwald, Bouchard, Shannon, Miljanich, and Lyman (14) have proposed the theory that estradiol facilitates the conversion of linoleic acid to arachidonic acid. They also suggest that a significant amount of cholesterol is esterified in plasma in addition to that in the liver.

The cholesterol-fed females (Group III) showed a lower linoleate concentration than the similarly fed male rats (18.7 vs. 25.4%). Arachidonate levels are lower here than in Group II. The possibility exists that excess cholesterol may interfere with the conversion of linoleate to arachidonate.

A different situation exists in the fat-deficient rats, for both their arachidonate and linoleate levels are quite low and are accompanied by high levels of 5,8,11-eicosatrienoic, oleic, and palmitoleic acids. The essential fatty acid-deficient female rats have twice as high a percentage of palmitoleate as the males, whereas the reverse is true of oleate. The fat-deficient female rats also have higher linoleate and lower 5,8,11-eicosatrienoate levels than the males.

Table 3 presents fatty acid composition of plasma phospholids. As previously observed for the sterol fatty acids (Table 2), no sex differences are apparent in the immature animals. In the older animals on all diets studied (Group II, III, IV), the males have higher palmitic acid content than the corresponding females, and in Groups II and III, a lower stearic acid content. This relationship could reflect a pathway of lecithin synthesis in the female different from that in the male (15).

In the 6-month old animals, females have higher arachidonate levels and slightly, but not significantly, lower linoleate levels than the males. On the cholesterol diet, in the phospholipids as in the sterol esters, males have a significantly higher linoleate content than the females.

In essential fatty acid deficiency, in the phospholipid fraction, the levels of both arachidonate and linoleate are very low in both sexes, and 5,8,11-eicosatrienoic acid appears in significant amounts, although much higher in the female than in the male. This group (both sexes) is also characterized by increased oleic acid levels, in contrast to the other experimental groups.

Fatty acids in plasma triglycerides are shown in Table 4. Several sex differences are apparent in the young animals (Group I). The concentration of 16:0 and 18:0 is higher and that of 18:2 lower in the female rats than in the male rats. These differences are not maintained in the older rats but are significantly accentuated in rats on the cholesterol diet. In addition, in

TABLE 3 MAJOR FATTY ACIDS OF PLASMA PHOSPHOLIPIDS (% OF TOTAL)

Diet and Age	Sex and Number of Samples	16:0	16:1	18:0	18:1	18:2	20:3	20:4	16:0/18:0
Stock 6 wk	M 6	26.4 ± 3.8*	7.1 ± 2.3	18.4 ± 2.8	16.1 ± 3.8	12.3 ± 4.1		14.7 ± 2.0	1.43
	F 6	24.2 ± 3.8	7.8 ± 1.1	20.0 ± 2.4	14.0 ± 1.6	13.4 ± 2.6		12.8 ± 1.8	1.21
Stock 6 months	M 9	22.1 ± 1.5 $P<0.005$	10.1 ± 2.5	17.9 ± 1.3 $P<0.005$	15.7 ± 2.1	12.8 ± 2.8		12.6 ± 3.1 $P<0.01$	1.23
	F 8	19.4 ± 1.5	7.6 ± 2.1	21.5 ± 2.3	15.9 ± 2.7	10.2 ± 3.1		16.6 ± 2.2	0.90
Stock 6 months; cholesterol 1 month	M 6	24.6 ± 1.8 $P<0.05$	5.9 ± 2.0	16.6 ± 0.8 $P<0.001$	15.3 ± 1.0	18.3 ± 1.5 $P<0.01$		13.8 ± 1.8	1.48
	F 6	21.1 ± 2.9	6.7 ± 2.0	22.6 ± 2.9	14.0 ± 1.0	13.2 ± 3.3		14.9 ± 2.9	0.93
Fat-free 18 wk	M 7	27.8 ± 1.6 $P<0.001$	10.6 ± 2.3	18.0 ± 3.4	20.3 ± 5.4	4.7 ± 2.2	7.0 ± 2.8 $P<0.001$	tr.	1.54
	F 5	20.7 ± 0.4	10.0 ± 1.0	19.7 ± 3.3	22.0 ± 1.1	2.8 ± 0.9	17.8 ± 3.0	tr.	1.05

* SD.

TABLE 4 MAJOR FATTY ACIDS OF PLASMA TRIGLYCERIDES (% OF TOTAL)

Diet and Age	Sex and Number of Samples	16:0	16:1	18:0	18:2	18:2	20:3	20:4
Stock 6 wk	M 6	21.8 ± 3.9* $P<0.025$	6.8 ± 1.2	6.4 ± 1.7 $P<0.025$	26.7 ± 2.1	24.8 ± 3.7 $P<0.001$		5.9 ± 1.8
	F 6	27.4 ± 3.1	8.1 ± 1.6	8.4 ± 0.6	25.4 ± 1.3	16.9 ± 1.4		5.2 ± 1.7
Stock 6 months	M 9	22.4 ± 3.0	8.8 ± 1.4	7.1 ± 1.1	25.4 ± 1.8 $P<0.005$	23.6 ± 2.4		4.2 ± 1.3
	F 9	22.9 ± 2.5	7.3 ± 1.7	6.0 ± 1.6	27.6 ± 2.3	22.2 ± 2.8		5.4 ± 2.2
Stock 6 months; cholesterol 1 month	M 6	19.8 ± 1.3 $P<0.025$	5.4 ± 0.9 $P<0.001$	3.8 ± 0.9 $P<0.005$	26.2 ± 2.0	37.7 ± 3.3 $P<0.001$		2.2 ± 0.6
	F 6	23.9 ± 3.1	10.1 ± 1.8	7.5 ± 2.4	25.3 ± 2.0	24.1 ± 5.2		1.7 ± 3.1
Fat-free 18 wk	M 8	26.7 ± 2.5	11.4 ± 1.1 $P<0.001$	4.8 ± 0.6	45.7 ± 2.9 $P<0.05$	4.1 ± 1.9	2.6 ± 0.8	
	F 4	24.4 ± 0.9	17.4 ± 2.5	4.8 ± 0.9	41.7 ± 3.0	3.0 ± 1.1	2.1 ± 0.6	

* SD.

rats on the latter diet, the level of palmitoleic acid is significantly higher in the females than in the males. An accumulation of linoleic acid in all three lipid fractions in plasma of the male seems to be a characteristic response to cholesterol feeding.

In the fat-deficient animal, there is a disappearance of the 20:4 acid, an extreme decrease in linoleate accompanied by a very large increase in oleate (more pronounced in the male), and an increase in palmitoleate (more pronounced in the female).

After the 5 hr incubation of plasma, during which some free cholesterol was esterified (Table 1), the composition of fatty acids observed before incubation was, in general, maintained. There were, however, some significant changes which are shown in Table 5. First, all changes occurred exclusively in fatty acids of the female animals; second, changes occurred only in the sterol esters and phospholipid fractions; third, changes were observed in the older animals on stock rations and on the fat-free diet only; and fourth, only three fatty acids (16:1, 20:3, and 20:4) were affected by incubation. The level of arachidonate was quite high in the sterol ester fraction of female plasma, and esterification increased it further. Similarly, in fat-deficient females, it was the unsaturated 20:3 that increased. In both cases, the change was accompanied by a significant decrease in palmitoleic acid. Changes in the sterol ester 20:3 and 20:4 fatty acids were accompanied by a corresponding inverse change in the phospholipid fraction. No changes were observed in the immature animals, which suggests the influence of gonadal hormones in effecting these changes. Similarly, no changes were observed in sterol esters and phospho-

TABLE 5 MAJOR CHANGES IN PERCENTAGE COMPOSITION OF FATTY ACIDS IN PLASMA STEROL ESTERS AND PHOSPHOLIPIDS OF FEMALE RATS RESULTING FROM INCUBATION FOR 5 HR AT 37°C

Fraction	Diet and Age	16:1		20:3		20:4	
		Before	After	Before	After	Before	After
Sterol esters	Stock 6 months	4.6	3.6			57.1	62.4
		$P<0.01$				$P<0.01$	
	Fat-free 18 wk	29.2	25.6	14.1	19.3		
		$P<0.05$		$P<0.01$			
Phospholipids	Stock 6 months					16.6	13.8
						$P<0.05$	
	Fat-free 18 wk			17.8	14.7		
				$P<0.1$			

lipids of cholesterol-fed females, or in the triglyceride fraction of any group.

Lyman, Shannon, Ostwald, and Miljanich (15) have shown previously that plasma lecithins from female rats contain higher levels of arachidonate than those obtained from male rats. More recently (16), these investigators reported that during the development of essential fatty acid deficiency, female rats and estrogen-treated castrated male rats maintained higher proportions of arachidonate in plasma phospholipid and plasma cholesteryl ester than intact males or testosterone-treated castrates.

These observations confirm the presence of lecithin: cholesterol acyltransferase in rat plasma. The transesterification process seems to be influenced by gonadal hormones (either depressed by androgens or enhanced by estrogens).

Changes in fatty acid composition suggest that in the fat-free animals there is a conversion of 16:1 to 18:1 to 20:3, while in the nondeficient animals 18:2 is converted to 20:4. An excess of cholesterol appears to inhibit this latter synthesis. This has been previously observed in rat liver by Morin, Bernick, Mead, and Alfin-Slater (17), and in both rat liver and plasma by Klein (18).

Thus, the higher ratio of palmitic to stearic acids in the male, the stimulation of the conversion, on incubation, of linoleic to arachidonic acids in plasma from the female, and the presence of a larger concentration of unsaturated fatty acids in the female confirm that the fatty acid composition of plasma lipid fractions is a sex characteristic of rats.

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