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Influence of sex and sex hormones on the oxidation of cholesterol-26-C^{14} by rat liver mitochondria*†

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SUMMARY

The oxidation of cholesterol-26-C^{14}, sodium propionate-1, -2, or -3-C^{14}, and sodium octanoate-1-C^{14} by liver mitochondrial preparations from intact and gonadectomized rats of both sexes, and from intact and gonadectomized rats of both sexes treated with androgens and estrogens, has been studied. Mitochondria from intact female rats and mice consistently oxidized added cholesterol to a greater extent than mitochondria from intact males. There were no significant sex differences in the oxidation of sodium propionate (a possible intermediate in the oxidation of the cholesterol side chain to carbon dioxide). Surgical or chemical castration of male rats enhanced cholesterol oxidation. Androgen treatment of female rats slightly depressed cholesterol oxidation but ovariectomy had no effect. Cholesterol oxidation by preparations of normal male or female rat liver mitochondria was inhibited by sex hormones added *in vitro*. These sex differences in cholesterol oxidation suggest that circulating androgen, rather than estrogen, levels determine the efficiency of cholesterol oxidation. Octanoate oxidation by liver mitochondria was not influenced by prior castration or treatment of male rats with androgens or estrogens. Androgen treatment of female rats slightly inhibited octanoate oxidation while estrogen treatment enhanced octanoate oxidation.

We have repeatedly observed that suitably fortified preparations of liver mitochondria from normal male rats are less active in oxidizing added cholesterol *in vitro* than liver mitochondria from female rats of the same age or weight (1, 2). We have shown (3) that liver mitochondria from male rats that have been castrated either surgically or with estrogens exhibit enhanced cholesterol oxidation activity. This report describes the effects upon cholesterol oxidation by rat liver mitochondria *in vitro* of (*a*) gonadectomy in female rats, and (*b*) estrogen and androgen administration to intact and gonadectomized rats of both sexes. The oxidation of sodium octanoate by these mitochondrial preparations was also studied to provide some indication of nonspecific changes in the oxidative capacity of the mitochondria as a consequence of gonadectomy or hormone administration.

METHODS

Rats of the Wistar strain (150–160 g) were used throughout. Hormone esters (estradiol benzoate, 100 μg/kg/day, or testosterone propionate, 1 mg/160 g/day) were administered intramuscularly in peanut oil for at least 30 days before experiments were begun. Gonadectomized animals were used no earlier than 30 days after the operation. At least 10 rats from each experimental group were killed after 30 days and serum and liver cholesterol levels were determined. Livers

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were homogenized in chloroform–methanol 2:1 and an aliquot of the dried extract was assayed for total cholesterol. Cholesterol was determined using the Mann modification (4) of the ferric chloride method of Zlatkis et al. (5).

Washed liver mitochondria and a boiled particle-free supernatant solution were prepared and incubated with sodium octanoate-1-C^{14} or cholesterol-26-C^{14} as described previously (2, 6). Sex hormones and their esters (30 μmoles) were added to incubations as emulsions of Tween 20 (polyoxyethylene sorbitan monolaurate, Atlas Powder Co., Wilmington, Delaware) in 0.25 M Tris hydrochloride buffer, pH 8.5. An equivalent amount of Tween 20 was added to control incubations. The percentage oxidation of the C^{14}-labeled substrates was calculated as the counts per minute (cpm) of $C^{14}O_2$ (as $BaC^{14}O_3$) evolved during an 18-hr incubation at 37° per 100 cpm of substrate-C^{14}. All results were corrected for variations in amount of mitochondria derived from different experimental groups (determined as mg *N*). Radioactive substrates were purchased from the New England Nuclear Corp., Boston, Massachusetts.

RESULTS

Females. We compared the oxidation of cholesterol-26-C^{14} and octanoate-1-C^{14} by mitochondrial preparations from the livers of four groups of female rats: normal (F), gonadectomized (FG), normal but treated with androgen (FA), and gonadectomized treated with androgen (FGA). The oxidation data are presented in Table 1. In this series of experiments, gonadectomy slightly enhanced cholesterol oxidation by rat liver mitochondria but had no effect on octanoate oxidation. Androgen treatment of either intact or gonadectomized female rats reduced the percentage oxidized of both substrates. For comparison, intact and gonadectomized female rats were also treated with estrogen. After treatment was continued for at least 30 days, a number of rats from each group were sacrificed for serum and liver cholesterol assay and the rest used for oxidation experiments. The oxidation results are presented in Table 2 and the autopsy data in Table 3.

In this latter series of experiments, gonadectomy had no definite effect on the oxidation of cholesterol by female rat liver mitochondria. Estrogen treatment likewise had no effect upon cholesterol oxidation by mitochondria prepared from livers of either the intact or the gonadectomized females. In contrast to this, the liver mitochondria from both the estrogen-treated group and the gonadectomized group oxidized octanoate more readily than the controls.

TABLE 1. PERCENTAGE OF OXIDATION OF CHOLESTEROL-26-C^{14} AND SODIUM OCTANOATE-1-C^{14} BY RAT LIVER MITOCHONDRIA*

Expt. No.	Cholesterol-26-C^{14} Group‡				Octanoate-1-C^{14} Group‡			
	F	FG	FA§	FGA§	F	FG	FA	FGA
1	13.8	Lost	1.2	2.1	41.5	41.2	30.9	29.4
2	2.6	6.7	3.8	3.4	61.5	60.0	Lost	50.0
3	4.7	12.3	1.5	1.4	41.8	40.4	32.5	23.2
4	2.1	5.9	1.4	6.9	31.9	27.4	27.2	24.2
5	7.6	10.3	5.9	3.9	27.1	25.6	11.9	13.6
6	6.8	7.0	1.4	0.8	...	...	...	...
7	10.3	10.9	19.7	1.7	...	...	...	...
Average	6.8	8.8	5.0	2.9	40.8	38.9	25.6	28.1
±S.D.†	3.9	2.4	6.1	1.9	12.6	12.4	8.2	12.1

* Computed as $BaC^{14}O_3$/substrate-C^{14} × 100. All values corrected for equivalent amounts of mitochondria (mg *N*).
† Standard deviation.
‡ F = female; FG = gonadectomized; FA = androgen-treated; FGA = gonadectomized-androgen-treated.
§ 1 mg testosterone propionate/160 g/day.

TABLE 2. PERCENTAGE OF OXIDATION OF CHOLESTEROL-26-C^{14} AND SODIUM OCTANOATE-1-C^{14} BY RAT LIVER MITOCHONDRIA*

Expt. No.	Cholesterol-26-C^{14} Group‡				Octanoate-1-C^{14} Group‡			
	F	FG	FE§	FGE§	F	FG	FE	FGE
1	10.5	1.1	38.9	25.8	14.0	9.3	36.5	27.7
2	48.8	4.7	23.3	29.9	18.9	24.6	26.9	19.3
3	27.9	39.4	29.5	14.8	16.1	22.7	36.4	21.4
4	83.3	6.8	22.5	22.9	53.9	56.6	63.0	57.8
5	8.7	6.5	7.9	30.5	49.1	66.8	89.0	75.6
6	43.7	8.7	61.7	26.0	Lost	54.5	72.0	66.7
7	28.4	52.2	58.1	21.7	...	...	...	...
8	51.4	99.0	30.5	34.0	...	...	...	...
9	14.5	23.3	23.2	16.3	...	...	...	...
10	25.2	21.8	20.5	23.0	...	...	...	...
11	18.4	20.5	25.8	16.0	...	...	...	...
Average	32.8	25.8	31.1	23.7	30.4	39.1	53.9	44.8
±S.D.†	21.3	27.6	15.4	6.0	17.4	21.1	22.3	22.7

* Computed as $BaC^{14}O_3$/substrate-C^{14} × 100. All values corrected for equivalent amounts of mitochondria (mg *N*).
† Standard deviation.
‡ F = female; FG = gonadectomized; FE = estrogen-treated; FGE = gonadectomized-estrogen-treated.
§ 100 μg estradiol benzoate/kg/day.

Gonadectomy generally leads to a slight increase in the serum cholesterol levels of female rats (7–10). This was observed by us in Series I but not in Series II (Table 3). Estrogen treatment also raised serum cholesterol levels, confirming the findings of Moskowitz et al. (7). Coleman et al. (9) had previously observed that gonadectomy had no effect on liver cholesterol levels. The estrogen treatment seemed to elevate liver cholesterol levels in both the intact and gonadectomized females. Our data indicate a slight hypercholesteremia resulting from either gonadectomy or estrogen treatment and a slight hypocholesteremia on androgen treatment. The liver cholesterol level seemed to be affected only by the estrogen treatment. In summary, we conclude that oxidation of cholesterol by female rat liver mitochondria is unaffected by

gonadectomy. However, either androgen or estrogen treatment of intact or gonadectomized rats diminishes cholesterol oxidation to some extent.

Males. In our earlier study (3), we had only compared gonadectomized and estrogen-treated male rats. We, therefore, studied a group of intact and gonadectomized male rats treated with androgen. Animals were sacrificed after 30 days of androgen treatment. Oxidation data are presented in Table 4 and autopsy data in Table 5. It is again evident that castration enhances oxidation of cholesterol, but not of octanoate. The androgen treatment inhibited oxidation of cholesterol. The large rise in liver cholesterol and the lesser, but statistically significant, rise in serum cholesterol (3) that accompanies castration were clearly evident in these experiments.

Effect of Sex Hormones in Vitro. Our previous work (1, 2) had indicated that either the presence of some residual dietary factor or drug in the supernatant fraction from a liver homogenate, or the direct addition of such materials to the incubation mixture, may profoundly affect cholesterol oxidation by isolated liver mitochondria. To ensure that the effects observed following androgen and estrogen administration were not simply due to residual hormone in the liver preparations, we carried out a series of incubations with liver mitochondria from intact male and female rats to which were added 30 μmoles of the sex hormones or some of their hepatic metabolites. As Table 6 shows, direct addition of the hormones to the incubation always depressed cholesterol oxidation. Thus the hormonal effects reported here are considered to be due to biochemical sequellae evoked by the hormones and not to residual amounts of the various steroids.

Further (Control) Experiments. Some further experiments were carried out to substantiate the validity of the experiments already described and to assess the possible contributions of the following factors:

(*a*) *Hormone Vehicle.* To test the effect of the vehicle in which the sex hormones were administered *in vivo*, groups of intact male and female rats were injected with 0.1 ml of peanut oil daily for 30 days. There were no significant differences between the oxidation of cholesterol and octanoate by liver mitochondria from treated and from control (noninjected) rats. Average oxidation (3 experiments) of cholesterol and octanoate by peanut-oil-treated males was 5.8 and 40.6%, respectively, vs 6.4 and 38.0% for controls. Treated females (3 experiments) also oxidized cholesterol (24.0%) and octanoate (80.3%) to the same extent as did the controls (26.9 and 75.4%, respectively).

(*b*) *Mitochondrial Composition.* Little difference was found in the respective nitrogen contents of male and

TABLE 3. RAT AUTOPSY DATA (30 Days)

Group*	No.	Avg. Wt. Gain	Avg. Liver Wt.	Serum Cholesterol†	Liver Cholesterol
		g	*g*	*mg %*	*mg/100 g wet wt*
Series I					
F-I	10	78	9.0	68.8 ± 3.7‡	182.8 ± 4.8‡
FG-I	10	145	11.0	75.8 ± 3.8	187.3 ± 7.0
FA	10	107	10.3	56.8 ± 4.5	180.9 ± 4.7
FGA	10	128	10.5	53.9 ± 2.4	184.6 ± 3.7
Series II					
F-II	11	103	9.1	81.8 ± 5.9	159.2 ± 18.9
FG-II	11	193	11.5	80.3 ± 5.7	150.9 ± 15.3
FE	11	84	9.9	90.8 ± 7.8	177.3 ± 25.2
FGE	11	128	12.1	88.5 ± 6.4	164.1 ± 17.9

* F = female; FG = gonadectomized; A = androgen-treated (1 mg testosterone propionate/160 g/day); E = estrogen-treated (100 μg estradiol benzoate/kg/day).

† *Statistical analysis:* F-I *vs* FA, $p<0.05$; F-I vs FGA, FG-I vs FA, $0.01 > p > 0.001$; FG-I vs FGA, $p<0.001$.

‡ Standard error of the mean.

TABLE 4. PERCENTAGE OF OXIDATION OF CHOLESTEROL-26-C^{14} AND SODIUM OCTANOATE-1-C^{14} BY RAT LIVER MITOCHONDRIA*

Expt. No.	Cholesterol-26-C^{14} Group‡				Octanoate-1-C^{14} Group‡			
	M	MG	MA§	MGA§	M	MG	MA	MGA
1	0.8	13.8	6.4	2.4	18.2	42.7	31.8	24.8
2	3.7	10.8	10.6	11.6	27.7	34.7	17.9	18.2
3	11.9	26.7	4.1	1.8	22.1	9.4	9.5	10.2
4	11.7	26.7	6.0	4.3	Lost	21.5	15.2	16.6
5	7.0	8.2	1.3	0.9	33.0	8.2	24.6	25.3
6	1.4	6.0	3.4	3.0	13.4	7.8	25.2	10.9
7	5.9	8.4	3.0	1.6	...	...	...	...
8	Lost	5.6	7.1	0.7	...	...	...	...
Average	6.1	13.3	5.2	3.3	22.9	20.7	20.7	17.7
±S.D.†	4.2	8.1	2.7	3.1	6.9	13.7	7.4	5.9

* Computed as $BaC^{14}O_3$/substrate-C^{14} × 100. All values corrected for equivalent amounts of mitochondria (mg *N*).

† Standard deviation.

‡ M = male; MG = gonadectomized; A = androgen-treated.

§ 1 mg testosterone propionate/160 g/day.

TABLE 5. RAT AUTOPSY DATA (30 Days)

Group*	No.	Avg. Wt. Gain	Avg. Liver Wt.	Serum Cholesterol†	Liver Cholesterol
		g	*g*	*mg %*	*mg/100 g wet wt*
M	11	230	16.4	59.6 ± 4.0‡	160.2 ± 13.6‡
MG	10	192	13.4	74.9 ± 3.8	187.8 ± 11.5
MA	10	178	14.0	65.3 ± 3.3	173.2 ± 8.2
MGA	11	174	12.9	59.3 ± 3.0	170.5 ± 18.7

* M = male, MG = gonadectomized, A = androgen treatment (1 mg testosterone propionate/160 g/day).

† *Statistical analysis:* M vs MG, $p=0.01$; MG vs MA, $p=0.05$; MG vs MGA, $0.01>p>0.001$.

‡ Standard error of the mean.

female rat liver mitochondria as routinely prepared for these experiments. The RNA contents of the male and female liver mitochondria, measured as ribose (11), were roughly equivalent (0.16 mg RNA/mg protein *N*) indicating no disproportionate contamination by microsomes or nuclei between mitochondrial preparations from the two sexes. In earlier work (1), we had found that the cholesterol content of the mitochondrial preparations (0.10 mg/ml) was the same for both sexes. These findings were confirmed in this series of experiments.

(*c*) *Activity of Microsomes.* In a series of six experiments, mitochondrial and microsomal preparations from male and female rat livers were incubated with cholesterol-26-C^{14}. The average values for percentage cholesterol oxidation were 21.1 and 2.9% for male liver mitochondria and microsomes, respectively, and 32.6 and 2.9% for female liver mitochondria and microsomes.

(*d*) *Species.* In three experiments carried out in Philadelphia with white mice (Swiss Hygienic strain), percentage oxidation of cholesterol-26-C^{14} by male mouse liver mitochondria was 1.5 ± 0.1%; and by female mouse liver mitochondria, 7.6 ± 2.8%. In other experiments carried out at Oxford with a domestic strain of white mice, the average percentage cholesterol oxidation values by male and female liver mitochondria were 9.5 and 13.8%, respectively. There were no significant sex differences in octanoate oxidation (males 29%, females 25%). Thus, the sex-linkage of cholesterol oxidation activity of liver mitochondria is not confined to the Wistar rat.

(*e*) *Propionate Oxidation.* Propionyl coenzyme A has been implicated as an intermediate in the hepatic oxidation to carbon dioxide of the terminal isopropyl group of at least one sterol, 5β-coprostane-3α,7α,12α-triol (12). Male and female rat liver mitochondria were therefore incubated with each of the following substrates: sodium propionate-1-C^{14}, sodium propionate-2-C^{14}, and sodium propionate-3-C^{14}. Table 7 shows the results of these experiments. In addition, no sex difference was found for the oxidation of sodium propionate-2-C^{14} by mouse liver mitochondria.

The sex difference in cholesterol oxidation must, therefore, reflect more than a sex-linked difference in the rate of metabolism of a C_3 or smaller product of sterol catabolism.

TABLE 6. EFFECT OF ADDED HORMONES (30 μmoles) ON PERCENTAGE OXIDATION OF CHOLESTEROL-26-C^{14} BY RAT LIVER MITOCHONDRIA

Compound	No. of Experiments	Average Inhibition Compared to Controls	
		Male	Female
		%	%
Testosterone	4	37	25
Testosterone propionate	4	92	52
Epiandrosterone	3	40	74
Androsterone	3	50	70
Androsterone sulfate	4	53	50
Dehydroepiandrosterone sulfate	3	86	72
Estrone	3	40	57
Estrone sulfate	4	83	86
Estradiol	4	70	16
Estradiol benzoate	4	80	32
Estriol	3	30	52
Etiocholanolone	3	67	89

TABLE 7. OXIDATION OF CHOLESTEROL-26-C^{14} AND OF SODIUM PROPIONATE-1-C^{14},-2-C^{14}, AND -3-C^{14} BY MALE AND FEMALE RAT LIVER MITOCHONDRIA

Substrate	No. of Experiments	Male	Female
		%	%
Cholesterol-26-C^{14}	10	7.7	14.9
Propionate-1-C^{14}	10	72.8	85.1
Propionate-2-C^{14}	4	29.9	31.5
Propionate-3-C^{14}	5	35.6	39.7

DISCUSSION

It is apparent from these data, and from those of other investigators working with enzymic alterations of steroids, that male and female rat livers may contain enzymes that exhibit markedly different levels of activity or degrees of specificity. The distribution of these enzymes differs not only according to sex, but also among the various liver fractions. Forchielli and Dorfman (13) found that liver microsomes contain a Δ^4-5α hydrogenase, while the microsome-free cytoplasm contains a Δ^4-5β hydrogenase. Rubin (14) demonstrated a sex-linked stereospecificity in the reduction of the 3-keto group of C_{19} steroids by rat liver homogenates, male preparations yielding predominantly the 3β,5α-isomer and female preparations giving the 3α,5α-isomer. The reduction of Δ^4-3-ketosteroids is carried out more efficiently by female rat liver homogenates (15, 16) or microsomes (17). It has further been shown that Δ^4,5α-reductase is more abundant in female rat liver microsomes (18), while the Δ^4,5β-reductase is present only in microsomal preparations from male rat livers (15, 18). While castration increases and testosterone treatment decreases the levels of these particular microsomal enzymes in male rat liver (15, 19), ovariectomy or estrogen treatment have little effect (15, 18, 19).

Our own observations on the liver mitochondrial cholesterol oxidation system parallel these properties of the sex-linked liver microsomal steroid reductase and suggest that the efficiencies of these two types of steroid-metabolizing systems are inversely proportional to the circulating androgen levels. It has been suggested that the sex differences observed in the development of ethionine-induced fatty liver (20) are also associated with the presence or absence of androgens.

By contrast, the amounts of steroid-hydroxylating enzymes residing in the microsomal fraction are greater in the male than in the female rat liver (17, 21, 22). Male liver homogenates have also been shown to have greater 3β-hydroxy-dehydrogenase activity (23). These observations suggest that the sex-linked differences are not due merely to differences in the availability of TPNH, which is an essential cofactor for steroid reduction and hydroxylation or in the availability of DPN, which is an essential cofactor for sterol oxidation. The sex-linked metabolism of steroids is the subject of a recent review (24).

Our findings show that castration and estrogen treatment in male rats definitely enhance the cholesterol oxidation activity in liver mitochondria. Neither ovariectomy nor androgen treatment affect this enzyme system in the female rat liver. At the beginning of these studies (3), we found that liver mitochondria from intact female rats consistently oxidized more cholesterol-26-C^{14} to carbon dioxide than did male rat liver preparations. Comparing the data for intact males and females compiled during this series of experiments, we find that the average extent of cholesterol-C^{14} oxidation by females in 63 experiments was 22.9 ± 23.0% and by males in 49 experiments was 8.7 ± 9.1% (standard deviation). The standard errors of the means in these groups were 2.9 and 1.3%, respectively ($p < 0.001$). These data attain greater significance with the finding of a similar sex difference in several experimental strains of mice.

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