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Oxidation of cholesterol by rat liver mitochondria: effect of dietary fat *

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SUMMARY

Rats were maintained on normal diets or on diets containing 20 per cent of corn oil (unsaturated, iodine number 127) or commercial shortening (saturated, iodine number 73) for 40 days. The liver mitochondria of the rats fed saturated fat oxidized cholesterol-26-C^{14} to $C^{14}O_2$ to a much greater extent than did liver mitochondria from rats fed unsaturated fat. In general, liver mitochondria from control rats also oxidized more cholesterol-26-C^{14} than did mitochondria from rats fed unsaturated fat, but this difference was not nearly as consistent (7 of 12 experiments). These results were the same with rats of either sex. Oxidation of sodium pyruvate-2-C^{14} did not vary with diet. The results could not be attributed to differences in liver lipid or cholesterol content. It has been shown that the homologous combination of liver mitochondria and boiled supernatant from the livers of the unsaturated-fat-fed rats greatly impairs cholesterol oxidation. Addition of boiled supernatant from the other dietary groups to liver mitochondria from the rats fed unsaturated fat restores the cholesterol oxidation to normal levels as does substitution of a 10 per cent sucrose solution for the boiled supernatant. Addition of the boiled liver supernatant from the animals fed unsaturated fat to liver mitochondria from the normal or saturated-fat-fed rats has no appreciable inhibitory effect.

The concept that cholesterol is the metabolic precursor of the bile acids has been amply reviewed in the last few years (1, 2, 3). The oxidation of the terminal isopropyl group of the cholesterol side chain has been observed in rat liver slices (4) and in suitably fortified preparations of mouse (5, 6) and rat (7) mitochondria. We have now studied the influence of dietary fats upon the ability of such preparations to oxidize cholesterol and form bile acids. A preliminary report of some of our findings has been given elsewhere (8). Our interest was aroused by several reports that the administration of highly unsaturated fat to humans (9, 10, 11) results in a lowering of serum cholesterol levels and increased fecal excretion of bile acids. In rats (12), increased biliary excretion of bile acids is observed when the rats are fed unsaturated fat.

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METHODS

Wistar rats weighing between 150 and 180 g. were fed rabbit chow [1] which had been thoroughly mixed with 20 per cent fat—either commercial shortening, which is relatively saturated (iodine number 73), or corn oil, which is unsaturated (iodine number 127). The chow was mixed with the added fat until all the fat had been absorbed. These diets will henceforth be designated as "unsaturated" and "saturated." Control animals were maintained on untreated rabbit chow. The rabbit chow used in these experiments contained 2.2 per cent fat (48-hour ether-alcohol extraction), which had an iodine number of 115. It had previously been reported (13) that Purina chow contained 2 per cent fat, iodine number 121, and that 70 per cent of the fatty acids were unsaturated, consisting mostly of linoleic acid. More recently the analysis has been repeated and the new findings are in substantial agreement with the earlier results.[2]

[1] Ralston-Purina Co., St. Louis, Mo.
[2] Personal communication, J. D. Evans.

After 40 days the rats were killed and mitochondrial preparations were made from the livers (7). The livers were removed and placed in 10 per cent aqueous sucrose (w/v). The livers were minced, then homogenized in cold aqueous sucrose (3 volumes of sucrose per volume of minced liver), using a loose-fitting glass Potter-Elvehjem homogenizer. The homogenate was centrifuged at 600 × *g* for 10 minutes to remove nuclei, unbroken cells, and cellular debris. The supernatant suspension was then centrifuged in a Spinco Model L preparative centrifuge for 12 minutes at 8500 × *g* to separate mitochondria. The supernatant layer from the second centrifugation was used to prepare the soluble cofactor. The mitochondria were resuspended in 10 per cent sucrose and recentrifuged at 8500 × *g* for 12 minutes to remove adhering microsomes. The supernatant layer from this centrifugation was discarded. Before use, the mitochondria were resuspended in enough 10 per cent aqueous sucrose to give 1 ml. of suspension per incubation. Generally, the mitochondria obtained from one liver were sufficient for four incubations. The soluble cofactor was prepared by boiling the supernatant from the initial 8500 × *g* centrifugation. After the boiled solution was freed of denatured protein (filtration or low-speed centrifugation), it was used directly.

Cholesterol-26-C^{14} was synthesized from 3β-hydroxy-Δ^5-norcholesten-25-one by the method of Dauben and Bradlow (14). Solutions of cholesterol were prepared by dissolving cholesterol and Tween 20 (10:1) in a small amount of methanol and, after removal of the methanol in a stream of nitrogen at 40°-50°C, dissolving in the desired buffer. Sodium pyruvate-2-C^{14} was purchased from the Atomic Energy Research Establishment, Amersham, England, and was dissolved directly in the buffer.

Incubations were carried out in stoppered 125 ml. Erlenmeyer flasks containing center wells. The incubation mixture consisted of 1 ml. of the mitochondrial preparation: [3] 1 ml. of a solution containing ATP (25 mg.), DPN (5 mg.), AMP (8 mg.), reduced glutathione (15 mg.), sodium citrate monohydrate (30 mg.), magnesium nitrate hexahydrate (10 mg.), potassium penicillin G (2000 U) and streptomycin sulfate (1 mg.); 5 ml. of labeled substrate in 0.25 M tris (hydroxymethyl) aminomethane.HCl, pH 8.5; and 5 ml. of boiled supernatant. The center well contained 2.0 ml. of 2.5 N sodium hydroxide. The flasks were shaken at 37°C in a water bath for 18 hours. In each experiment cited below, incubations of mitochondria from animals in all three dietary groups were carried out simultaneously. After the incubation period, 2.5 ml. of 25 per cent aqueous trichloroacetic acid was added and the flasks were shaken for 3 hours on a rotary shaker to displace $C^{14}O_2$ from the suspension. The contents of the center well were added to 2.5 ml. of 2 N ammonium chloride and 1 ml. of 1.5 M barium chloride. The resulting barium carbonate precipitate was filtered, weighed, and counted in a Packard Tri-Carb liquid scintillation counter. The barium carbonate was counted as a thixotropic gel using Thixcin [4] as the suspending agent. The method is a modification of the procedure of Funt and Hetherington (15).

For determination of liver lipids, liver samples were thoroughly extracted with petroleum ether, and the extracts were dried and diluted in a volumetric flask. Aliquots were taken for gravimetric determination of total lipid and colorimetric determination of cholesterol. All cholesterol determinations were carried out by the method of Trinder (16). Nitrogen levels of all mitochondrial preparations were determined colorimetrically using Nessler's reagent.

RESULTS AND DISCUSSION

Preliminary experiments were carried out with pooled livers, one male and one female. In view of the findings that male and female rats accumulate liver lipid at different rates (17) and that sex differences exist in the amounts of certain rat-liver enzymes (18), subsequent experiments were carried out with livers from either male or female rats alone. The results (Table 1) show that, regardless of sex, in every case the oxidation of the terminal carbon atoms of cholesterol was greater in liver mitochondrial preparations from rats fed large amounts of saturated fats than in preparations from rats fed equivalent quantities of unsaturated fat. In general, the oxidation of cholesterol by liver mitochondrial preparations from rats on the normal diet was greater than that by mitochondrial preparations from rats fed the unsaturated fat. However, these differences were not as consistent as were those between the oxidative power of the preparations from rats fed saturated and unsaturated fats. The normal rabbit chow contained about 2 per cent of fat with an iodine number of 115. Rats ingesting this diet would be consuming considerably less unsaturated fat than those on the corn oil augmented diet. The variability of the oxidative ability of the preparations most likely reflects differences in activation of this

[3] Abbreviations: ATP, adenosine 5'-triphosphate; DPN, diphosphopyridine nucleotide; AMP, adenosine 5-monophosphate; Tris, tris (hydroxymethyl) aminomethane.HCl; THC, 3α, 7α, 12α-trihydroxycoprostane.

[4] Baker Castor Oil Co., New York, N. Y.

TABLE 1. OXIDATION OF CHOLESTEROL-26-C^{14} BY LIVER MITOCHONDRIA OF RATS *

Sex	Experiment Number	Percentage of Oxidation †		
		Diet		
		Normal	Unsaturated	Saturated
Mixed	1	15.6	1.7	9.6
	2	15.2	17.4	17.6
	3	21.4	3.0	15.5
Females	1	5.3	6.6	15.2
	2	4.2	3.3	12.0
	3	21.0	1.9	10.4
	4	1.3	22.4	31.8
	5	11.7	2.9	4.5
Males	1	7.2	4.9	7.9
	2	15.1	13.9	23.7
	3	15.0	3.0	20.3
	4	11.1	2.8	11.2

* All values corrected for equivalent amounts of mitochondria (mg. N).
† Computed as $BaC^{14}O^3$/cholesterol-26-C^{14}.

TABLE 2. OXIDATION OF PYRUVATE-2-C^{14} BY LIVER MITOCHONDRIA OF RATS AT pH 8.5 *

Sex	Diet	Percentage of Oxidation †	
		Experiment 1	Experiment 2
M	normal	0.6	2.0
M	unsaturated	0.4	0.7
M	saturated	0.9	0.6
F	normal	14.4	3.6
F	unsaturated	6.4	4.1
F	saturated	7.5	3.9

* All values corrected for equivalent amounts of mitochondria (mg. N).
† Computed as $BaC^{14}O_3$/sodium pyruvate-2-C^{14}.

mitochondrial oxidase system. The system has been shown to be sensitive to many minor variations in preparation or technique (7). The consistency of our findings in the face of this sensitivity is worth noting.

When incubations were conducted with boiled mitochondria for periods up to 22 hours, there was no oxidation of cholesterol and no production of radioactive carbon dioxide. This finding, plus the presence of two antibiotics in all incubations, would appear to exclude bacterial contamination as the source of cholesterol oxidase activity. In one time-course experiment several preparations from the same liver were incubated for 2, 4, 6, and 18 hours. A gradual oxidation with time is noted for all three preparations (Fig. 1).

Oxidation of Other Substrates. The effect of these diets on mitochondrial oxidation of substrates other than cholesterol was also of interest, and several experiments were carried out in which the substrate was sodium pyruvate-2-C^{14}. In general, there was little effect of diet, as can be seen from Table 2. The limited extent of pyruvate oxidation as compared with cholesterol oxidation may be largely attributable to the relatively high pH (8.5) at which these incubations were carried out. Relative thiamine deficiency of the mitochondria due to extraction by the aqueous sucrose solution may also contribute to this discrepancy. In one series in which the substrate was 3α, 7α, 12α-trihydroxycoprostane-26-C^{14} (19), the extent of oxidation was 42.8, 46.0, and 75.4 per cent for the normal, unsaturated, and saturated mitochondrial preparations, respectively.

Analysis of Liver Lipids. The finding of differences in oxidation rates associated with dietary differences raises the question of the amount of fat being deposited in the liver and its possible effect on the mitochondria. Some autopsy data are given in Table 3. Several other studies have been concerned with the liver weights, liver lipids, and serum cholesterol levels

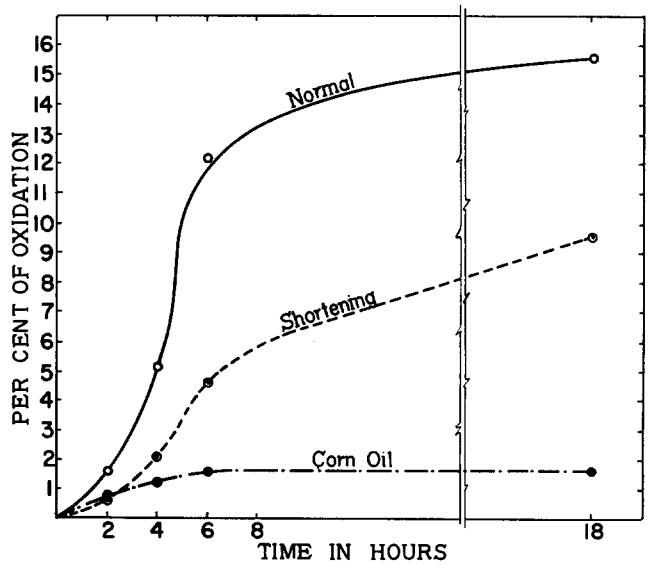

FIG. 1. Time-course of oxidation of cholesterol-26-C^{14} by liver mitochondria.

TABLE 3. AUTOPSY DATA ON RATS FED SATURATED AND UNSATURATED FAT
(Average of 12 Rats per Group)

Sex	Diet	Avg. Body Wt.	Avg. Liver Wet Wt.	Liver Wt. as Percentage of Body Wt.	Serum Cholesterol
		gm.	*gm.*		*mg./100 ml.*
M	normal	258	9.00	3.45	104.4 ± 9.9 *
M	unsaturated	259	8.58	3.31	77.2 ± 23.1
M	saturated	230	7.11	3.09	110.0 ± 5.9
F	normal	206	7.21	3.50	78.9 ± 20.5
F	unsaturated	200	6.78	3.39	82.4 ± 10.7
F	saturated	192	6.83	3.56	110.0 ± 16.0

* Standard deviation.

of rats maintained on diets containing saturated and unsaturated fat (20, 21, 22). These investigators have also found lower serum cholesterol levels in animals fed unsaturated fat, and larger livers in the males. Our data also show that the males had larger livers; however, we found that the ratio of liver weight to body weight was greater in the females. Analysis of the liver lipid and cholesterol content shows slightly more fat in the female livers than in the males, and in both sexes more liver lipid in the groups on the unsaturated fat diets. Our data are presented in Table 4.

The lower fat content of the livers of the rats fed saturated fat might possibly affect the oxidative ability of the mitochondrial preparations if the amount of lipid in the mitochondria reflected the over-all differences in liver lipid.

The amounts of lipid found in the livers of animals fed saturated and unsaturated diets were closer to the normal than has been reported by the other workers cited. However, in these other studies the fats compared were cottonseed oil and either lard or coconut oil, both of which differ in composition and extent of saturation from the saturated fat we have used. One experiment, in which corn oil was compared with margarine, among other fats, showed essentially similar levels of liver fat (23). All the other work was done with formula diets.

The question remained whether the differences in liver lipid were reflected in the mitochondrial lipid content. Previous analyses of liver fractions have shown most of the cholesterol to be present in a fraction sedimenting at 105,000 × *g* (24). The mitochondria contained about 0.2 mg. of cholesterol per 4 ml. (25). In these experiments the mitochondria were sedimented by centrifugation at 24,000 × *g* for 10 minutes. Our own analyses of liver mitochondria (sedimented at 8500 × *g* for 12 minutes) showed that about 0.10 mg. of cholesterol per ml. was present in the mitochondria taken from livers of rats of both sexes on all three diets. The mitochondrial lipid content was similar in all groups of rats.

Rat mitochondria have been shown to contain almost no esterified cholesterol (25). To test possible effects of transesterification, the incubation mixtures were subjected to extraction with ether and petroleum ether and, after addition of carrier cholesterol, the free cholesterol was precipitated as the digitonide. The supernatant fluid was saponified, carrier cholesterol was added, and digitonin precipitation was repeated. All of the radioactivity was present in the first (free cholesterol) digitonide.

To ascertain the possible effects of the liver lipids on cholesterol oxidation, pooled livers from rats (one male and one female) fed each of the three diets were extracted with ether-alcohol (1:3). The extracts were dried over anhydrous sodium sulfate and all the solvent removed at reduced pressure. Weighed amounts of the extracted liver fat from all three preparations were emulsified according to the method used for the labeled substrates, using Tween 20 and Tris buffer. Solutions of the fat from each preparation (1 mg.) were added to normal male and female rat liver mitochondrial preparations. The extent of inhibition of oxidation of cholesterol-26-C^{14} by each type of liver fat was of the same order of magnitude (Table 5). In experiments using sodium pyruvate-2-C^{14} as the substrate, we again observed similar inhibitory effects of all three fat preparations in the male series, while in the females there was no inhibition when fat from normal livers was added. These data are also presented in Table 5.

"Crossover" Experiments. In another effort to eluci-

date the source of the observed differences in cholesterol oxidation, we added supernatant fractions of one preparation to the mitochondrial preparation of another. These "crossover" experiments were carried out with both cholesterol-26-C^{14} and sodium pyruvate-2-C^{14} as substrates. In all, six such experiments were carried out with cholesterol and two with pyruvate. The results for cholesterol were in general agreement in all cases, regardless of sex. They showed that the addition of "foreign" supernatant or of 10 per cent aqueous sucrose to liver mitochondria from controls or saturated fat-fed animals resulted in roughly the same extent of oxidation as did the addition of homologous supernatant fractions. There was considerable enhancement of oxidation by liver mitochondria from animals fed the unsaturated fat when either aqueous sucrose or the supernatant fractions from livers of the control rats or saturated-fat-fed rats were substituted for the homologous supernatant fraction. A representation of the average effects may be found in Table 6. In view of the variability of the oxidative power of individual preparations, any differences of less than 100 per cent may be considered within the range of variability. Whether the oxidative activity of the mitochondria alone or of a homologous combination of mitochondrial preparation and supernatant be taken as the reference, it is apparent that the combination of mitochondria from the livers of animals fed unsaturated fat and the homologous supernatant has far less oxidative activity than the mitochondria alone. The results of adding the supernatant from the livers of animals fed unsaturated fat to liver mitochondria prepared from normal or saturated-fat-fed animals indicate that the "unsaturated" supernatant exerts no noxious effect per se. The cause of the observed inhibition in the homologous unsaturated preparation is under investigation. The data from the crossover experiments with sodium pyruvate-2-C^{14} are given in Table 7. Very little difference is seen between the various pyruvate incubations.

TABLE 4. LIVER LIPIDS OF RATS FED SATURATED AND UNSATURATED FAT
(Average of 5 Rats per Group)

Sex	Diet	Percentage of Liver Lipid	Percentage of Liver Cholesterol
M	normal	3.5 ± 0.95 *	0.38 ± 0.05 *
M	unsaturated	4.0 ± 1.65	0.46 ± 0.18
M	saturated	3.7 ± 0.80	0.34 ± 0.06
F	normal	3.7 ± 1.07	0.35 ± 0.15
F	unsaturated	4.8 ± 1.36	0.45 ± 0.10
F	saturated	4.2 ± 0.19	0.44 ± 0.12

* Standard deviation.

GENERAL COMMENTS

Fredrickson (26, 27) has demonstrated that cholesterol may be oxidized by mouse liver preparations to products resembling, but not identical with, deoxycholic and cholic acids. The acidic fractions obtained from experiments in which cholesterol-4-C^{14} had been incubated with rat liver mitochondrial preparations from all dietary groups were subjected to paper

TABLE 5. INHIBITION OF OXIDATION OF CHOLESTEROL-26-C^{14} AND PYRUVATE-2-C^{14} BY LIVER FAT FROM RATS ON VARIOUS DIETS

Sex	Added Fat	Cholesterol-26-C^{14}		Pyruvate-2-C^{14}	
		Percentage of Oxidation *	Percentage of Inhibition †	Percentage of Oxidation *	Percentage of Inhibition †
M	normal	1.8	54	4.5	25
M	unsaturated	2.0	50	4.2	30
M	saturated	1.4	64	4.2	30
M	——	3.9	——	6.0	——
F	normal	1.5	69	3.2	0
F	unsaturated	1.8	63	2.4	25
F	saturated	1.9	61	2.1	14
F	——	4.8	——	2.8	——

* All values corrected for equivalent amounts of mitochondria (mg. N).
† Inhibition based on control.

TABLE 6. EFFECT OF DIFFERENT SUPERNATANT FRACTIONS ON OXIDATION OF CHOLESTEROL-26-C^{14} BY RAT LIVER MITOCHONDRIA (Average of 6 Experiments)

Mitochondria	Supernatant	Effect on Oxidation	
		Homologous = 1 †	No Supernatant = 1 ‡
normal	normal	1.0	1.4 ± 0.3
normal	unsaturated	1.0 ± 0.1 §	1.5 ± 0.4
normal	saturated	1.3 ± 0.1	1.8 ± 0.4
normal	——*	0.7 ± 0.1	1.0
unsaturated	normal	4.2 ± 1.7	1.3 ± 1.0
unsaturated	unsaturated	1.0	0.3 ± 0.2
unsaturated	saturated	2.3 ± 1.1	0.7 ± 0.3
unsaturated	——	3.2 ± 2.1	1.0
saturated	normal	1.1 ± 0.3	1.1 ± 0.2
saturated	unsaturated	1.0 ± 0.1	1.1 ± 0.1
saturated	saturated	1.0	1.0 ± 0.1
saturated	——	1.0 ± 0.3	1.0

* 10% sucrose used instead of boiled supernatant.
† Ratio of oxidative ability if oxidation by homologous mitochondria and supernatant = 1.
‡ Ratio if mitochondria plus sucrose = 1.
§ Standard error.

TABLE 7. EFFECT OF DIFFERENT SUPERNATANT FRACTIONS ON OXIDATION OF SODIUM PYRUVATE-2-C^{14} AT pH 8.5 BY RAT LIVER MITOCHONDRIA

Mitochondria	Supernatant	Percentage of Oxidation	
		Experiment 1 (Males)	Experiment 2 (Females)
normal	normal	0.6	3.6
normal	unsaturated	0.4	1.8
normal	saturated	0.6	3.6
normal	——	0.4	3.0
unsaturated	normal	0.5	4.2
unsaturated	unsaturated	0.4	4.1
unsaturated	saturated	0.4	6.2
unsaturated	——	0.3	1.8
saturated	normal	0.8	2.3
saturated	unsaturated	0.9	3.8
saturated	saturated	1.0	3.9
saturated	——	0.9	2.4

chromatography in two different solvent systems (28, 29). In all cases the bulk of the radioactivity (assayed by scanning) was found at an R_f approximating that of a known sample of cholic acid detected with antimony trichloride (30).

Since the early reports that human serum cholesterol levels could be lowered by ingestion of unsaturated fat (31, 32), the effect of this fat on cholesterol metabolism has been the subject of considerable speculation. Data suggesting that the mechanism of serum cholesterol lowering involves increased fecal excretion of bile acids have been cited (9 to 12). Apparently the increased excretion of bile acid and lowering of serum cholesterol levels observed during intake of unsaturated fat take place despite increased cholesterol biosynthesis (33, 34, 35) and, as these studies show, decreased cholesterol oxidation. Our animals showed a relative hypocholesterolemia when fed unsaturated fat despite increased hepatic synthesis (inferred from references 33, 34, 35) and decreased hepatic oxidation. Unsaturated fat has also been shown to enhance intestinal absorption of cholesterol (12). It has been found that decreased reabsorption of bile acid results in a decrease of plasma cholesterol levels (36). Reduced bile acid production may have a similar effect. Perhaps the increased oxidation of cholesterol observed on the saturated-fat diet may result in elevated levels of circulating bile acids or bile salts which may, in turn, have a hypercholesterolemic effect such as is observed when bile acids or salts are fed to rats (37, 38) or rabbits (39). These speculations must await experimental testing.

Our data are sustained to some extent by the findings of Wilson and Siperstein (40), that rats fed saturated fat (lard) over long periods of time, when given a single tracer dose of cholesterol-4-C^{14}, excrete more radioactivity as bile acid than do rats maintained on corn oil or fat-free diets for similar lengths of time.

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