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Oxidation of cholesterol-26-C^{14} by rat liver mitochondria: effect of nicotinic acid*

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SUMMARY

The effect of nicotinic acid upon the oxidation of the terminal carbon atoms of cholesterol-26-C^{14} by rat liver mitochondrial preparations has been studied. Addition of nicotinic acid, as the potassium salt, to incubation mixtures containing normal rat liver mitochondria enhances the oxidation of cholesterol-26-C^{14}. Liver mitochondrial preparations from rats administered nicotinic acid in drinking water (12 mg. per day) or diet (0.75 per cent) also oxidize cholesterol-26-C^{14} to a greater extent than do similar preparations from control rats. The mitochondrial preparations from nicotinic acid-fed rats, in the absence of boiled supernatant, exhibit a greater oxidative capacity than do control preparations. This heightened oxidative capacity is further enhanced by addition of the boiled supernatant fraction from these preparations. Addition of boiled supernatant from control preparations has no enhancing effect. Nicotinic acid exerts little effect on the oxidation of sodium pyruvate-2-C^{14} and may have a slightly inhibitory effect upon the oxidation of sodium octanoate-1-C^{14}. No significant differences were observed between serum cholesterol levels of rats ingesting nicotinic acid for 3 weeks and controls.

Since the initial report on the hypocholesterolemic effect of nicotinic acid administered orally to humans (1), a large number of confirmatory publications have appeared (2). Nicotinic acid has been shown to lower serum cholesterol levels in hypercholesterolemic rabbits (3, 4) and dogs (5), although one group of investigators (6) could not confirm the latter result. Nicotinic acid also lessens the degree of aortic atheroma when administered to cholesterol-fed rabbits (3, 4).

The mechanism by which nicotinic acid exerts its hypocholesterolemic effect is not yet known. The feeding of this compound to cholesterol-fed rabbits (4) or rats (7) lowers liver cholesterol levels as compared with cholesterol-fed controls. There is no reduction to normal levels, however. Feeding of nicotinic acid to normal rats (1 to 2 per cent in diet) causes increases in liver fat (8) and liver cholesterol (9) content. Nicotinic acid has also been found to depress cholesterol synthesis from acetate by one group of workers (9) and to enhance it by another (10). Suggestions that the nicotinic acid effect involves the pyridine nucleotides which play an important role in steroid oxidation (11) are weakened by findings that nicotinamide has no hypocholesterolemic effect (10, 12). Hoffer *et al.* (13) have found that beta-pyridylcarbinol is also ineffective, suggesting a specific structural requirement, namely, a free carboxyl group. Nicotinic acid feeding causes a decrease in urinary glycine with increased excretion of nicotinuric acid (10, 14); the latter compound is under test (13). The reduced glycine output might also be due to increased formation of glycocholic acid, but no data are available on this point. Altschul originally suggested that nicotinic acid may act by increasing the *in vivo* oxidation of cholesterol (1).

In the present study we have investigated the effect of nicotinic acid upon the oxidation of the terminal carbon atoms of cholesterol-26-C^{14} by rat liver mitochondrial preparations. In one group of experiments the nicotinic acid (as potassium nicotinate) was added directly to the mitochondrial preparation. In other

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TABLE 1. EFFECT OF ADDITION OF NICOTINIC ACID ON OXIDATION OF CHOLESTEROL-26-C^{14} BY RAT LIVER MITOCHONDRIA

Experiment	Nicotinic Acid †	Percentage of Oxidation of Cholesterol *			
		Males		Females	
		Nicotinic Acid	Control	Nicotinic Acid	Control
	mg.				
1	6	20.5	8.8	12.3	9.2
2	15	9.4	6.0	37.5	31.6
3	3	7.9			
	6	8.2	9.0		
	12	9.2			
4	6	5.6	4.2	6.2	5.4
5	6	2.4	1.9	4.3	4.2
6	6	6.2	2.2	3.4	2.8
7	6	4.5	3.3	6.7	7.1
8	6	5.0	4.4	25.5	22.5

* Computed as $BaC^{14}O_3$/cholesterol-26-C^{14} × 100.
† Added to incubation mixture as potassium salt.

experiments the mitochondrial preparations were obtained from rats which had previously been fed nicotinic acid, either as the sodium salt in the drinking water or as the free acid in the food. Salts of nicotinic acid are as efficacious as the free acid (15).

METHODS

The procedures for obtaining the mitochondrial preparations and supernatant fractions used in these experiments have been described in detail elsewhere (16, 17). It was noted that after the initial low-speed centrifugation the cellular debris from the livers of rats fed nicotinic acid was arranged in layers with sharp boundaries between the various fractions. This phenomenon had not been observed with livers of rats used in other experiments.

The incubation was carried out in stoppered 125 ml. Erlenmeyer flasks containing center wells. The percentage oxidation of cholesterol-26-C^{14}, sodium pyruvate-2-C^{14}, and sodium octanoate-1-C^{14} was calculated from analysis of the $C^{14}O_2$ evolved during the incubation.

The incubation mixture consisted of 1 ml. of the mitochondrial preparation; 1 ml. of a solution containing adenosine triphosphate (25 mg.), diphosphopyridine nucleotide (5 mg.), adenosine monophosphate (8 mg.), reduced glutathione (15 mg.), sodium citrate monohydrate (30 mg.), magnesium nitrate hexahydrate (10 mg.), potassium penicillin G (2,000 units), and streptomycin sulfate (1 mg.); 5 ml. of labeled substrate emulsion in 0.25M tris (hydroxymethyl) aminomethane-HCl, pH 8.5; and boiled supernatant fluid (5 ml.).

The soluble cofactor was prepared by boiling the liver supernatant after centrifugation at 104,000 × *g* for 30 minutes.

The labeled cholesterol was synthesized from 3 β-hydoxy-Δ^5-norcholesten-25-one by the method of Dauben and Bradlow (18). Sodium pyruvate-2-C^{14} and octanoic acid-1-C^{14} were purchased from the Atomic Energy Research Establishment, Amersham, England. The cholesterol suspension was prepared in the manner previously described (17). The acids were dissolved directly in the buffer. All cholesterol determinations were carried out using the method of Trinder (19).

Animals used in the first two series of experiments were maintained on Purina Laboratory Chow. The nicotinic acid diet contained (per 100 g.): Pablum, 70 g.; wheat germ, 6.25 g.; skim milk powder, 20 g.; Vionate, 3 g.; and nicotinic acid, 0.75 g.[1] Sufficient water was added to make a thick dough. The control

TABLE 2. EFFECT OF ADDITION OF NICOTINIC ACID ON OXIDATION OF SODIUM PYRUVATE-2-C^{14} BY RAT LIVER MITOCHONDRIA

Experiment	Nicotinic Acid *	Percentage of Oxidation of Pyruvate			
		Males		Females	
		Nicotinic Acid	Control	Nicotinic Acid	Control
	mg.				
1	6	3.1	1.4	4.4	5.2
2	15	11.0	9.2	24.5	26.8
3	3	9.2			
	6	10.1	3.8	15.3	20.5
	12	8.3			
4	6	2.3	1.9		
5	6	1.3	1.4		
6	6	2.1	1.5		

* Added to incubation mixture as potassium salt.

[1] Pablum: Mead Johnson & Co., Evansville 21, Ind.; wheat germ: Kretschmer Corp., Carrollton, Mich.; skim milk powder: Land O'Lakes Creameries, Inc., Minneapolis, Minn.; Vionate: E. R. Squibb & Sons, New York 22, N. Y.; nicotinic acid: Matheson, Coleman & Bell Division, East Rutherford, N. J.

TABLE 3. AUTOPSY DATA* ON RATS GIVEN NICOTINIC ACID (12 MG./DAY) IN THEIR DRINKING WATER

Sex	Drinking Water	Number of Rats	Final Weight	Weight Change	Liver Weight	Serum Cholesterol
			g.	*g.*	*g.*	*mg./100 ml.*
Male	Nicotinic acid	3	330	+33	10.85	49.3 ± 3.2 †
	Control	5	293	+25	9.99	54.3 ± 5.8
Female	Nicotinic acid	3	251	+22	9.98	48.6 ± 4.7
	Control	5	237	+23	8.88	58 2 ± 4.2

* Averages.
† Standard error of the mean.

rats were fed the same diet minus nicotinic acid. No significant difference in food consumption between the nicotinic acid and control groups was noted. This diet provides 20 per cent protein, 11 per cent fat, and 62 per cent carbohydrate. It is well received by the animals used in these and other experiments.

Experiments using nicotinic acid in the food were carried out with Wistar rats, both sexes, weighing 150 to 160 g. The diet was administered for 3 weeks prior to the experiment. Experiments using sodium nicotinate in the drinking water were carried out with male Wistar rats weighing 260 to 300 g. These animals were maintained on the sodium nicotinate (12 mg. per day) for 3 weeks.

Each experiment was carried out using mitochondria from the same rat. In experiment 3, Table 1, for example, all four incubations (three nicotinic acid, one control) were carried out using aliquots of the same mitochondrial preparation. Preliminary experiments showed that aliquots of the same preparation consistently oxidized cholesterol to the same extent. In one specific example, the range of oxidation with the same mitochondrial preparation varied between 5.7 and 6.3 per cent in a series of six incubations.

RESULTS AND DISCUSSION

Addition of Nicotinic Acid to Normal Mitochondrial Preparations. Experiments were carried out using preparations from both male and female rat livers. The data on oxidation of cholesterol are given in Table 1 and on pyruvate oxidation in Table 2.

In practically all cases the addition of nicotinic acid to the incubation mixture enhanced oxidation of cholesterol-26-C^{14}. Only in experiment 3 (males) and 7 (females) was there not some enhancement of oxidation. As has been noted previously (17), normal female rat liver mitochondria appear to have the greater oxidative capacity for cholesterol-26-C^{14}. The oxidation of pyruvate-2-C^{14} by male rat liver mitochondria is also enhanced by the addition of nicotinic acid, but no such effect is evident in the case of female rat liver preparations. The much greater oxidation of pyruvate by female rat liver mitochondria is noteworthy. This phenomenon, too, has been pointed out before (17).

In one experiment where sodium octanoate-1-C^{14} was the substrate, oxidation was not enhanced by addition of nicotinic acid to the medium (24.3 vs. 24.3

TABLE 4. PER CENT OXIDATION OF CHOLESTEROL-26-C^{14} AND SODIUM PYRUVATE-2-C^{14} BY LIVER MITOCHONDRIA OF RATS GIVEN NICOTINIC ACID (12 MG./DAY) IN THEIR DRINKING WATER

Experiment	Drinking Water	Percentage of Oxidation of Substrates *			
		Males		Females	
		Cholesterol	Pyruvate	Cholesterol	Pyruvate
1	Nicotinic acid	25.8	8.3	lost	lost
	Control	8.8	1.4	5.2	9.2
2	Nicotinic acid	6.6	5.2	19.3	22.1
	Control	6.0	9.2	26.8	31.6
3	Nicotinic acid	22.2	20.3	14.7	14.0
	Control	14.0	14.5	18.6	21.7

* All values corrected for equivalent amounts of mitochondria (mg. N).

TABLE 5. AUTOPSY DATA* ON RATS FED NICOTINIC ACID (0.75% OF DIET)

Sex	Diet	Number of Rats	Final Weight	Weight Change	Liver Weight	Serum Cholesterol
			g.	*g.*	*g.*	*mg./100 ml.*
Male	Nicotinic acid	7	198	48	7.56	60.1 ± 3.4 †
	Control	8	201	49	8.52	62.6 ± 1.9
Female	Nicotinic acid	5	180	24	7.88	63.4 ± 6.9
	Control	7	170	21	6.65	57.1 ± 6.8

* Averages.
† Standard error of the mean.

per cent for the control preparation in the male and 19.0 vs. 25.1 per cent for the control in the female).

Experiments were also carried out in which picolinic acid and pyridine-3-sulfonic acid were added as the sodium salts to various incubation mixtures. The results were variable, but generally a slight enhancement of cholesterol oxidation was observed. Nicotinamide added at levels up to 30 mg. per incubation failed to enhance cholesterol and pyruvate oxidation by normal rat liver mitochondria.

Effect of Nicotinic Acid in Drinking Water. The administration of 12 mg. of nicotinic acid per day in the drinking water was carried out after first establishing that the animals used in these experiments took 2 days to empty a 240 ml. container. We administered fresh water containing sodium nicotinate (0.1 mg. per ml.) daily.

Autopsy data on these animals are presented in Table 3. There was a slight, but nonsignificant, lowering of serum cholesterol levels in both males and females administered nicotinic acid. The results of the oxidation of cholesterol-26-C^{14} and of sodium pyruvate-2-C^{14} are presented in Table 4. There was little doubt about the increased cholesterol oxidation by male liver mitochondria in the nicotinic acid group. Liver mitochondrial preparations from the female control rats oxidized more of both substrates than did the experimental group. The nicotinic acid may not exert a great effect here because of the higher oxidative capacity (as compared with males).

Effect of Nicotinic Acid in Diet. The autopsy data are presented in Table 5. The serum cholesterol differences (higher in male controls and female nicotinic groups) are not significant. Data on the oxidation of various substrates by liver mitochondrial preparations taken from these animals are given in Table 6. Again we find an increase in cholesterol oxidation, slight effect on pyruvate oxidation, and actual reduction of oxidation of octanoate by liver preparations from nicotinic acid-fed rats. On two occasions we added 6 mg. of nicotinic acid (as the potassium salt) to incubation mixtures containing mitochondria from nicotinic acid-fed rats and found a further enhancement of cholesterol oxidation (8.4 vs. 7.1 per cent and 17.7 vs. 14.9 per cent). There was no effect on pyruvate oxidation; if anything, there was a slight reduction (1.5 vs. 1.7 per cent). These results indicate that we had probably not reached the maximum level in the dietary dosage.

In an earlier experiment (17) we had observed appreciable changes in extent of oxidation of choles-

TABLE 6. PER CENT OXIDATION OF CHOLESTEROL-26-C^{14} SODIUM PYRUVATE-2-C^{14} AND SODIUM OCTANOATE-1-C^{14} BY LIVER MITOCHONDRIA FROM RATS FED NICOTINIC ACID (0.75% IN DIET)

Substrate	Experiment	Percentage of Oxidation of Substrates *			
		Males		Females	
		Nicotinic acid	Control	Nicotinic acid	Control
Cholesterol	1	7.1	1.9	34.8	22.5
	2	14.9	2.2	15.0	13.9
	3	17.7	4.4	6.5	2.9
	4	5.7	5.3		
	5	2.1	2.1		
Pyruvate	1	3.1	1.9		
	2	1.7	1.4		
Octanoate	1	13.9	24.3	21.5	25.1
	2	20.5	26.8	30.2	35.6

* All values corrected for equivalent amounts of mitochondria (mg. N).

TABLE 7. EFFECT OF SUBSTITUTION OF SUPERNATANT ON OXIDATION OF CHOLESTEROL-26-C^{14} AND SODIUM OCTANOATE-1-C^{14} BY RAT LIVER MITOCHONDRIA

Mitochondria	Supernatant	Percentage of Oxidation of Substrates *			
		Males		Females	
		Cholesterol	Octanoate	Cholesterol	Octanoate
Nicotinic	None †	13.2	13.9	28.2	27.6
Nicotinic	Nicotinic acid	17.7	13.9	34.8	21.5
Nicotinic	Control	12.7	16.5	27.2	27.1
Control	None	3.6	25.5	15.4	24.9
Control	Nicotinic acid	7.8	25.3	25.0	23.8
Control	Control	4.4	24.3	22.5	25.1

* All values corrected for equivalent amounts of mitochondria (mg. N).

† Equivalent amount of 10% sucrose used.

terol when the boiled supernatant fraction from one dietary group was added to the mitochondria of another. This type of "crossover" experiment was repeated in the present series with results as shown in Table 7. It is evident that the supernatant fraction from the nicotinic acid-fed rat liver preparation has an enhancing property for cholesterol oxidation. In the case of octanoate this supernatant is inhibitory, if anything. The data also suggest that in the absence of "nicotinic" supernatant the "nicotinic" mitochondrial preparation has considerably more oxidative potential than does that from the control rat liver. Thus, a factor within the mitochondria is responsible for most of the heightened oxidative capacity in preparations from livers of nicotinic acid-fed rats, but this effect can be increased by the addition of boiled supernatant from the same preparation.

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