Effect of the Ethyl Ester and Sodium Salt of α-*p*-Chlorophenoxyisobutyric Acid on Cholesterol Oxidation by Rat Liver Mitochondria[1,2] (34152)

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Ethyl α-*p*-chlorophenoxyisobutyrate (CPIB) has been shown to inhibit cholesterol synthesis from acetate (1) and mevalonate (2) and to interfere with lipoprotein synthesis or release from the liver or both (3). We have investigated the effect of CPIB upon the oxidation of 26-^{14}C-cholesterol to $^{14}CO_2$ by rat liver mitochondrial preparations in an effort to further clarify the mode of action of this compound.

Since the ethyl ester is probably hydrolyzed in the course of metabolism of this drug, we have also tested the effect of sodium *p*-cholorophenoxyisobutyrate (NaCPIB) in this system.

Moyer *et al.* (4) found that addition of choline chloride to a cholesterol-rich, choline-free diet resulted in reduction in liver size and lipid content, presumably due to increased formation and release of liver lipoprotein. In order to ascertain if the hepatomegaly and increased liver triglyceride observed in rats fed CPIB (5) was due to a deficiency in lipoprotein components, we added 0.5% choline chloride to some diets.

Methods. Male, Wistar rats (150–190 g) were maintained for 3 weeks on a synthetic test diet containing mixed cereal (70%), skim milk powder (22%), wheat germ (7%) and vitamin mix (1%). The CPIB or the sodium salt (NaCPIB) were added (0.3%) at the expense of the cereal, as was choline chloride (0.5%). The rats were killed by decapitation. The serum cholesterol (6), triglyceride (7), and phospholipid (8) levels were determined. A portion of liver was homogenized in chloroform–methanol 2:1, and the liver cholesterol, triglyceride, and phospholipid levels were determined using aliquots of the lipid extract. The mitochondrial oxidation was carried out according to published methods (9, 10).

The incubation mixture consisted of 1 ml of a rat liver mitochondrial preparation fortified with 1 ml of a solution containing ATP (25 mg); NAD (5 mg); AMP (8 mg); reduced glutathione (15 mg); Na-citrate·H_2O (30 mg); $Mg(NO_3)_2 \cdot 6H_2O$ (10 mg); potassium penicillin G (2000 units); and streptomycin sulfate, (1 mg); 5 ml of a solution labeled substrate in 0.25 *M* tris(hydroxymethyl)aminomethane·HCl, pH 8.5, and 5 ml of boiled supernatant obtained during the preparation of the mitochondrial suspension. The reaction vessel was a stoppered 125-ml Erlenmeyer flask containing a center well in which there was 2.0 ml of 2.5 *N* NaOH. The flasks were shaken for 18 hr at 37°. The $^{14}CO_2$ evolved during the reaction was trapped in the base, from which it was precipitated as $Ba^{14}CO_3$, and assayed for radioactivity by liquid scintillation spectrometry. Acid phosphatase was determined by the methd of Bessey *et al.* (11), and β-glucuronidase by the method of Fishman (12). Labeled substrates were purchased from New England Nuclear Co., Boston, Mass. The sodium salt and ethyl ester of α-*p*-chlorophenoxyisobutyrate acid were generously provided by Dr. J. Noble, Ayerst Co., New York.

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TABLE I. Serum and Liver Lipids of Rats Fed 0.3% CPIB, NaCPIB or CPIB + 0.5% Choline Chloride for 3 Weeks.

	Group			
	CPIB	NaCPIB	CPIB + choline	Control
Expt. 1				
No. of animals	6/6	5/6	6/6	6/6
Wt gain (g)	86	88	80	112
Liver wt (g)	11.6 ± 0.6[e]	13.8 ± 0.7[e,f]	12.6 ± 0.6	11.3 ± 0.5
Liver (% of body wt)	4.43 ± 0.20[a]	5.27 ± 0.09[b]	4.96 ± 0.21[b]	3.95 ± 0.06
Cholesterol serum lipids (mg/100 ml)	39.0 ± 5.5	23.5 ± 9.5	50.2 ±10.3	33.0 ± 5.0
liver lipids (g/100 g)	117 ±10	98 ± 5[a]	99 ± 6	126 ±12
plus liver pool (mg)	16.64	15.37	16.30	17.07
Triglyceride serum lipids (mg/100 ml)	51.6 ± 3.6[b]	54.5 ± 8.8[c]	48.3 ± 8.5[d]	91.3 ± 7.7
liver lipids (g/100 g)	161 ±50	205 ±41	98 ±16	210 ±75
plus liver pool (mg)	22.74	32.57	16.03	34.29
Expt. 2				
No. of animals	5/6	6/6	4/6	6/6
Wt gain (g)	85	61	77	106
Liver wt (g)	12.3 ± 0.9[c]	14.3 ± 0.9[b]	13.4 ± 0.3[b]	9.7 ± 0.1
Liver (% of body wt)	4.46 ± 0.19[d]	5.65 ± 0.21[b]	4.95 ± 0.08[b]	3.24 ± 0.25
Cholesterol serum lipids (mg/100 ml)	33.0 ± 5.5	26.3 ± 3.6	33.5 ± 2.7	26.5 ± 4.2
liver lipids (g/100 g)	176 ±16	166 ± 9	210 ±17	165 ±42
plus liver pool (mg)	24.38	25.74	30.86	18.39
Triglyceride serum lipids (mg/100 ml)	37.2 ± 6.1	33.9 ± 4.1	39.8 ± 4.2	34.5 ± 3.8
liver lipids (g/100 g)	577 ±59[a]	507 ±94	464 ±70	394 ±33
plus liver pool (mg)	71.05	75.07	65.42	41.31

[e] Standard error.

[f] Significance compared to control:

[a] $p < .05$.

[b] $p < .001$.

[c] $p < .02$.

[d] $p < .01$.

Results and Discussion. In one series of experiments the influence of CPIB, NaCPIB, and CPIB plus 0.5% choline chloride upon serum and liver lipids of rats was determined and the influence of the ethyl ester and sodium salt of α-*p*-chlorophenoxyisobutyric acid upon cholesterol oxidation by rat liver mitochondria was determined. The results of the lipid analyses are presented in Table I.

The hepatomegaly resulting from CPIB administration which Best and Duncan (5) reported was observed in both experiments. The liver size of the groups fed the sodium salt of α-*p*-chlorophenoxyisobutyric acid was larger than that of the groups fed CPIB alone ($p < .01$ both experiments) or CPIB plus choline chloride ($p < .02$ in expt. 2). Ad-

TABLE II. Oxidation of Cholesterol-26-^{14}C to $^{14}CO_2$ by Mitochondrial Preparations from Rats Fed 0.3% CPIB or NaCPIB for 3 Weeks.

		% Oxidation[a]	
Group	No.		Corrected for mg of N
Expt. 1			
A (CPIB)	6	11.1 ± 1.0[b]	8.4 ± 0.5
B (NaCPIB)	5	9.8 ± 0.7	5.4 ± 0.4
N (control)	6	8.8 ± 2.2	—
Expt. 2			
A	5	15.7 ± 2.5	10.2 ± 1.1
B	6	12.7 ± 0.8	7.4 ± 0.4
N	6	9.4 ± 1.5	—

[a] Calculated as (cholesterol-26-^{14}C/$Ba^{14}CO_3$) × 100.

[b] Standard error.

TABLE III. Serum and Liver Lipids of Rats Fed 0.3% CPIB for 3 Weeks.

	Expt. 3		Expt. 4		Expt. 5	
	Normal	CPIB	Normal	CPIB	Normal	CPIB
No. of animals	9	10	5	5	6	6
Wt gain (g)	93	84	93	54	23	41
Liver wt (g)	10.0 ± 0.4[e]	13.4 ± 0.4[a]	11.4 ± 0.5	12.4 ± 0.9	8.6 ± 0.3	11.0 ± 0.7[b]
Liver (% of body wt)	3.35	4.48	3.67	4.43	3.71	4.40
Liver cholesterol (mg/100 g)	270 ± 18	194 ± 24[c]	328 ± 10	289 ± 14	167 ±10	166 ± 6
triglyceride (mg/100 g)	238 ± 18	357 ± 32[b]	222 ± 42	336 ± 70	67 ± 6	101 ± 4[a]
Serum cholesterol (mg/100 ml)	26.3 ± 1.8	24.1 ± 1.6	52.4 ± 3.9	40.0 ± 3.4[c]	37.6 ± 2.3	40.4 ± 1.8
triglyceride (mg/100 ml)	39.9 ± 2.4	39.1 ± 2.4	127.6 ± 26.0	40.8 ± 7.8[d]	67.3 ± 9.3	62.0 ± 6.4
phospholipid (mg/100 ml)	126.8 ± 10.8	141.4 ± 8.9	96.0 ± 12.3	88.5 ± 10.6	102.5 ± 7.9	95.0 ±13.0
Cholesterol in serum (mg)	2.45	2.16	4.89	3.36	2.63	3.03
in liver (mg)	28.08	26.00	37.39	35.84	14.35	18.29
serum plus liver pool (mg)	30.53	28.16	42.28	39.20	16.98	21.32
Triglyceride in serum (mg)	3.71	3.51	11.91	3.43	2.62	3.03
in liver (mg)	24.75	47.84	25.31	41.66	5.76	11.11
serum plus liver pool (mg)	28.46	51.35	37.22	45.09	8.38	14.04

[a] $p < .001$.
[b] $p < .01$.
[c] $p < .05$.
[d] $p < .02$.
[e] Standard error.

dition of choline chloride to the CPIB did not reduce the liver size nor did it affect serum or liver lipids. In neither experiment did cholesterol levels differ among groups. Serum triglycerides were significantly elevated in the normal rats in the first, but not the second experiment. This may be due to the fact that smaller rats were used in this experiment (175 vs 190 g rats in Expt. 2). The liver triglycerides, however, were lower throughout in Expt. 1. In this experiment the average liver weights of the CPIB and normal groups were almost the same and no elevation in liver triglycerides was observed in the CPIB group. The second experiment showed the expected high liver triglyceride levels. The feeding pattern of rats seems to affect liver size and triglyceride levels (13) and this may have had some influence on our findings.

The extent of oxidation of 26-^{14}C-cholesterol to $^{14}CO_2$ by suitably fortified preparations of rat liver mitochondria showed a greater degree of cholesterol oxidation by liver mitochondria from rats fed the test diet, but the level of oxidation was comparable to the controls when corrected for milligrams of nitrogen. The results are presented in Table II. It is evident that the gross level of oxidation is not due to liver size alone, since the livers of group B were significantly larger than those of group A.

Earlier work (9, 14) has shown that omission of the boiled supernatant cofactor (cytosol) from the incubation mixture reduced the extent of cholesterol oxidation. The nature of the cytosol has not been elucidated but it is thought to be a soluble protein of low molecular weight. In view of the relative abundance of protein in the liver mitochondria of CPIB-fed rats we proposed to carry out a number of incubations in which sucrose was substituted for the cytosol of both normal and CPIB-fed rats.

In three of the experiments we carried out complete lipid analysis of serum and liver and these are listed in Table III. These results show again the general pattern of higher liver triglyceride and usually lower liver cholesterol in the CPIB-fed group. The only difference in serum lipids was in Expt. 4 in which the normal rats exhibited significantly higher triglycerides.

TABLE IV. Oxidation of Cholesterol-26-^{14}C to $^{14}CO_2$ by Mitochondrial Preparations from Rats Fed 0.3% CPIB for 3 Weeks: Effect of Cytosol.

	No.	Cytosol	% Oxidation[a]	Corr. for mg of N
Expt. 3A				
CPIB	5	+	70.0 ± 5.7[b]	42.0 ± 3.4
Control	5	+	43.1 ± 7.2	
Expt. 3B				
CPIB	5	+	40.8 ± 0.6	22.5 ± 0.3
Control	4	+	34.0 ± 2.0	
Expt. 4				
CPIB	5	+	11.3 ± 2.8	6.8 ± 1.7
Control	5	+	3.6 ± 0.1	
CPIB	5	—	10.2 ± 2.1	6.1 ± 1.3
Control	5	—	1.4 ± 0.3	
Expt. 5				
CPIB	6	+	17.0 ± 3.7	12.3 ± 2.7
Control	6	+	9.7 ± 2.9	
CPIB	6	—	13.1 ± 4.0	9.5 ± 2.8
Control	6	—	4.2 ± 1.9	
Expt. 6				
CPIB	6	+	20.3 ± 2.8	10.1 ± 1.8
Control	4	+	13.0 ± 0.6	
CPIB	6	—	14.7 ± 2.4	8.5 ± 1.4
Control	6	—	2.4 ± 0.5	

[a] Calculated as (cholesterol-26-^{14}C/Ba$^{14}CO_3$) × 100.
[b] Standard error.

The results of a further series of cholesterol oxidation experiments are presented in Table IV. It is clear from these data that the mitochondria prepared from the livers of CPIB-fed rats do not require as much cytosol as do control preparations. These findings suggest that the cytosol factor may be some nonspecific protein whose presence at certain concentrations is required for maximum oxidation. In Expt. 4 (Table IV) another set of incubations was carried out in which CPIB cytosol was incubated with normal mitochondria and vice versa. The results were: CPIB mitochondria plus normal cytosol (5 incubations) 13.1 ± 1.9% oxidation (7.8 ± 1.2% corrected); normal mitochondria plus

TABLE V. Oxidation of 26-^{14}C-Cholesterol and 1-^{14}C-Octanoate to $^{14}CO_2$ by Mitochondrial Preparations from Rats and Mice Fed 0.3% CPIB for 3 Weeks.

		% Oxidation[a]			
		Cholesterol		Octanoate	
	No.		Corrected for mg of N		Corrected for mg of N
Rats					
CPIB	4	8.5 ± 2.2[b]	6.6 ± 1.7	79.1 ± 3.0	63.2 ± 2.4
Control	4	5.6 ± 1.1		84.0 ± 5.7	
Mice					
CPIB	2[c]	6.0	7.8	73.0	93.5
Control	2	3.3		80.2	

[a] Calculated as (substrate-^{14}C/$Ba^{14}CO_3$) × 100.

[b] Standard error.

[c] Two pools of mitochondria, 2 livers each.

CPIB cytosol 3.7 ± 0.9% oxidation. The levels of oxidation compare well with those obtained using homologous mitochondria and cytosol.

Duncan *et al.* (13) reported that if rats fed CPIB are fasted for 12–24 hr before they are killed, liver size and liver triglyceride levels revert to normal. Accordingly, we fasted CPIB-fed and control rats (6/group) for 18 hr before carrying out the experiment. The liver weights of the control and test animals were 8.6 g (3.17% of body wt) and 9.6 g (3.8% of body wt), respectively. Liver triglyceride levels were 90.0 and 73.2 mg/100 g for the two groups. The fast did not affect cholesterol oxidation, however. Mitochondrial preparations from the control rats oxidized 9.7% of cholesterol-26-^{14}C to $^{14}CO_2$ when all cofactors were present, and 4.2% in the absence of cytosol. Corresponding levels of oxidation by mitochondria from CPIB-treated rats were 17.0% (12.3% corrected for mg of N) and 13.1% (9.4% corrected for mg of N), respectively.

We also decided to test the effect of CPIB on cholesterol oxidation by mouse liver mitochondria. Accordingly two groups of rats and two of mice were maintained on normal and test diets for 3 weeks. At the end of this period the normal rats had gained 98 g and their livers weighed 10.6 g (3.80% of body wt) and the CPIB-fed rats had gained 84 g and their livers weighed 12.2 g (4.60% of body wt). In contrast the average weight gain for the mice was the same for both groups, 2 g. The livers of the normal mice were larger (2.2 g; 6.87% of body wt) than those of the test group (2.0 g; 6.25% of body wt). Mitochondrial oxidation of 26-^{14}C-cholesterol and 1-^{14}C-octanoate was tested in all four groups. The results are detailed in Table V. In mice, as well as rats, the administration of CPIB results in increased mitochondrial oxidation of cholesterol. Fatty acid oxidation (as octanoate) is unaffected in either species.

Over the course of these experiments we conducted 42 oxidations with mitochondria from CPIB-fed rats, the average oxidation of cholesterol was 24.1 ± 3.2% (14.9 ± 1.8% corrected for mg of N). The average extent of oxidation for 40 expts. with normal mitochondria was 15.3 ± 2.3% ($p<.05$). In the absence of cytosol (17 expts.) average oxidation of cholesterol by normal rat liver mitochondria was 2.7 ± 0.8% as compared with 12.8 ± 1.7% for liver mitochondrial preparations from CPIB-fed rats (7.9 ± 1.2% corrected for mg of N) ($p<.001$ for gross oxidation; $p<.01$ for net oxidation).

Paget (15) and Svoboda *et al.* (16) reported that the liver of rats fed CPIB showed a striking increase in "microbodies," possibly containing oxidizing enzymes. Hess *et al.* (17) examined some aspects of the enzyme profile of livers from normal and CPIB-fed

TABLE VI. Acid Phosphatase and β-Glucuronidase Activities (μmoles/min) in Liver Fraction of Rats Fed 0.3% CPIB for 3 Weeks.

Fraction	Expt. 2			Expt. 5	
	CPIB	NaCPIB	Normal	CPIB	Normal
			Acid phosphatase[a]		
Cellular debris	53.4 ± 8.4[b]	46.9 ± 4.7	52.5 ± 4.3	69.3 ± 3.7	54.6 ± 0.8
Mitochondria	111.6 ± 11.0	89.4 ± 9.4	125.0 ±11.2	132.4 ± 9.2	120.2 ± 1.5
Microsomes	57.6 ± 10.2	41.2 ±12.5	71.2 ±18.1	43.7 ± 2.6	36.5 ± 6.7
Supernatant	60.0 ± 5.7	52.8 ± 6.4	57.0 ± 6.0	66.7 ± 1.5	63.1 ± 4.7
Whole homogenate	89.8 ± 6.3	70.6 ± 4.8	92.2 ± 8.3	58.9 ± 2.4	75.6 ± 1.6
			β-glucuronidase[c]		
Cellular debris	184.2 ± 35.7	231.2 ± 12.2	261.2 ± 36.6	824 ± 157	597 ± 114
Mitochondria	419.1 ± 96.4	506.2 ± 48.0	693.1 ± 61.3	1935 ± 603	1436 ± 309
Microsomes	307.2 ±194.9	1124.4 ±464.3	811.0 ±222.8	1667 ± 865	506 ± 325
Supernatant	128.7 ± 20.2	121.8 ± 6.5	143.2 ± 24.3	457 ± 93	313 ± 42
Whole homogenate	281.7 ± 49.7	354.2 ± 17.6	467.1 ± 76.1	1351 ± 472	1687 ±1038

[a] Hydrolysis of *p*-nitrophenylphosphate.

[b] Standard error.

[c] Hydrolysis of phenolphthalein glucuronide.

rats and found the mitochondrial and supernatant fractions of CPIB-treated rats to contain considerably more catalase. There were no differences in urate oxidase or in cytochrome oxidase in these fractions. To test for a possible alteration in lysosomal activity, we examined the acid phosphatase and β-glucuronidase content of the various liver fractions. The results are presented in Table VI. The data do not suggest that increased lysosomal activity may be the cause of the increased oxidation of cholesterol observed in mitochondrial preparations from rats fed ethyl *p*-chlorophenoxyisobutyrate.

Summary. Mitochondrial preparations from livers of rats fed 0.3% ethyl α-*p*-chlorophenoxyisobutyrate (CPIB) show an increased capacity to oxidize cholesterol-26-^{14}C to $^{14}CO_2$. When oxidation is calculated per milligram of mitochondrial nitrogen, oxidation of cholesterol by mitochondria from CPIB-fed rats is the same as that observed in normal rat liver mitochondria. In the absence of the boiled supernatant cofactor (cytosol) the mitochondria from CPIB-treated rats show a significantly higher capacity for cholesterol oxidation than do normal mitochondrial preparations. Mitochondria prepared from livers of rats fed sodium α-*p*-chlorophenoxyisobutyrate also oxidize more cholesterol than do control preparations. Fasting of the rats for 18 hr prior to mitochondrial preparation does not affect the extent of oxidation either in the presence or absence of cytosol. Mitochondrial preparations from CPIB-fed mice also oxidize more cholesterol than do controls. Studies of mitochondrial acid phosphatase and β-glucuronidase activity suggest that the increased oxidation of cholesterol is not due to lysosomal activity. Feeding of either the ethyl ester or sodium salt of α-*p*-chlorophenoxyisobutyric acid causes increase in liver size and usually an increase in liver triglyceride levels, confirming results of others.

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