Effects of α-*p*-chlorophenoxyisobutyryl ethyl ester (CPIB) with and without androsterone on cholesterol biosynthesis in rat liver

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SUMMARY α-*p*-Chlorophenoxyisobutyrate (CPIB) or a mixture of CPIB with a small amount of androsterone (Atromid) fed to rats at a level of 0.3% of the diet was found to increase the liver weight by about 25%, and to decrease the cholesterol concentration in liver by about 10% and in plasma by about 30%. No change in rate of growth or of food consumption was observed.

The rate of cholesterol biosynthesis per gram of liver, estimated from the incorporation of acetate-1-C^{14}, was decreased by about 70% in liver slices from rats fed CPIB or Atromid. Conversion of mevalonate-2-C^{14} into cholesterol was decreased only slightly. No decrease in the formation of ketone bodies from acetate-1-C^{14} in liver slices was found to result from CPIB, nor in the synthesis of triglyceride from acetate-1-C^{14}, indicating that the site of the inhibitory action of the drug is between acetyl CoA and mevalonate. CPIB is similar to dietary cholesterol in that it is not inhibitory when added to liver homogenates at concentrations up to about 10^{-3} M.

In intact rats, both CPIB and Atromid significantly decreased the rate of cholesterol synthesis per gram of liver, as measured by the incorporation of acetate-1-C^{14} or of tritium water, but the effect was not as large as in liver slice studies. No decrease was noted in cholesterol synthesis in intestine.

Cholesterol synthesis by the whole liver was decreased less by CPIB or Atromid than when expressed per gram of liver. The decrease in acetate-C^{14} incorporation in liver cholesterol per 100 g of rat body weight was estimated to be about 65% by the liver slice method and about 38% in intact animals; tritium water incorporation gave a mean decrease of 22%. The differences were significant for acetate incorporation but at the borderline for tritium water.

KEY WORDS cholesterol · biosynthesis · α-*p*-chlorophenoxyisobutyrate (CPIB) · Atromid · liver · rat · acetate · intestine · plasma · triglyceride · ketone bodies

Abbreviations: CPIB, α-*p*-chlorophenoxyisobutyrate. Atromid, a mixture of CPIB with a small amount of androsterone. PPO, 2,5-diphenyloxazole. POPOP, 1,4-bis[2-(5-phenyloxazolyl)]-benzene.

In 1962 Thorp and Waring (1) reported that the ethyl ester of α-*p*-chlorophenoxyisobutyric acid (CPIB) was the most active of a series of α-aryloxyisobutyric acid derivatives in decreasing the concentration of cholesterol and other lipids in rat plasma and liver. Thorp (2) also observed that the addition of a small amount of androsterone increased the effect and made it independent of seasonal fluctuation, which led to the suggestion that the effect of CPIB consisted in making orally administered androsterone metabolically active. Oliver (3) reported that the combination (trade name, "Atromid") decreased plasma cholesterol and triglyceride levels in a series of hypercholesterolemic patients. Numerous confirmations of a hypolipidemic effect in humans of both Atromid and CPIB have recently appeared (4), but little has been reported concerning the mechanism of action of either of these preparations.

Since certain other drugs that decrease plasma cholesterol levels have been found to inhibit cholesterol biosynthesis, an investigation of the mechanism of action of Atromid and of CPIB was initiated by a study of their effects on cholesterol biosynthesis in rat liver. Estimates were made of in vitro cholesterol synthesis by incubating rat liver slices with acetate-1-C^{14} and mevalonate-2-C^{14}, and of in vivo synthesis by administering tritium water or acetate-C^{14}, or both.

MATERIALS AND METHODS

Drug Administration

Male Sprague-Dawley rats were fed a stock commercial diet (Purina rat pellets) or the same diet to which 0.3% of ethyl α-*p*-chlorophenoxyisobutyrate (CPIB),[1] or 0.3%

[1] CPIB and Atromid were generously supplied by Ayerst Laboratories, Inc.

TABLE 1 EFFECT OF CPIB AND OF ATROMID ON PLASMA AND LIVER CHOLESTEROL CONCENTRATIONS AND ON LIVER WEIGHT

Expt. No.	Days on Drug	No. of Rats and Group	Body Wt.	Plasma Cholesterol		Liver Cholesterol		Liver Wt/Body Wt		Liver Cholesterol	
				Mean	% Change	Mean	% Change	Mean	% Change	Mean	% Change
			g	*mg/100 ml*		*mg/g*		%		*mg/100 g*	
21	—	6 Control	253	75				4.56			
	10	6 CPIB	239	50	−32			5.65	+24		
	10	6 Atromid	244	51	−32			5.92	+30		
24	6	5 Control	580			2.53		3.29		8.36	
		5 CPIB	607			2.47	−2.4	2.95	−10	7.27	−13
29	—	4 Control	187	65		2.44		3.86		9.91	
	8	3 CPIB	187	45	−31	2.30	−6	4.38	+14	9.87	−1
30	—	4 Control	324	55		2.61		3.28		8.56	
	36	4 CPIB	352	41	−25	2.18	−16	4.30	+31	9.38	+9
31	—	4 Control	317	70		2.76		4.26		10.31	
	6	4 CPIB	305	48	−31	2.06	−25	5.08	+19	10.35	
32	—	5 Control	393			2.16		3.79		8.05	
	7	5 CPIB	369			1.94	−10	4.44	+17	8.47	+5
33	—	5 Control	209	79		2.77		4.08		11.18	
	7	5 Atromid	210	54	−32	2.40	−13.6	5.40	+32	12.92	+16
Mean		Controls		69.9 ± 0.02* (n = 33)		2.48 ± 0.06*		3.93 ± 0.11* (n = 18)		9.37 ± 0.32*	
		Drug-treated		49.0 ± 0.01 (n = 38)		2.22 ± 0.05		4.93 ± 0.12 (n = 17)		9.68 ± 0.39	
Per cent change				−30		−10.5		+25.4		+3.3	
P				<0.001		<0.002		<0.001		>0.1	

* Mean ± SEM.

CPIB and 0.02% androsterone, had been added. The drugs were dissolved in a large volume of reagent grade acetone–ether 1:1 (all solvent ratios v/v), the solution being evenly distributed over the pellets and mixed while the solvents were evaporating. All three diets were fed ad lib.

Labeled Substrates

In the liver slice incubations, acetate-1-C^{14} with a specific activity of 0.1 mc/mmole was added to give a concentration in the incubation mixture of 3 μmoles/ml, or approximately 30 μmoles/g of liver. Mevalonate-2-C^{14} with a specific activity of 0.1 mc/mmole was added to give a concentration in the incubation mixture of 0.05 μmole/ml, or approximately 0.5 μmole/g of liver.

Tritium water with a specific activity of 20 mc/ml was injected intraperitoneally in a dose of 5 mc/100 g body weight. In some experiments it was mixed with sodium acetate-1-C^{14} solution with a specific activity of 0.1 mc/mmole, in others with glycerol-1-C^{14} with a specific activity of 0.1 mc/mmole. Acetate was injected in a dose of 10 μc/100 g and glycerol of 5 μc/100 g body weight.

Liver Slice Incubation

After various periods of drug administration (in most cases 6–13 days) the animals were killed by exsanguination by cardiac puncture while under ether anesthesia. The livers were removed, chilled, and sliced with a Stadie-Riggs microtome. Approximately 1.0 g of slice was added to 10 ml of a previously described modification of the Krebs-Ringer medium (5).

The free acid of CPIB was obtained by saponification of the ester, acidification, and extraction into ether. It was then titrated with 1 N KOH to pH 7.4 and aliquots of the solution were added to the homogenate in certain experiments as described below.

After 1 hr incubation at 37° with gentle shaking, the slices were saponified by heating to 90° in 90% alcohol containing 10% KOH. The unsaponifiable fraction was extracted twice with petroleum ether (3 volumes), and the combined extracts were washed with 50% alcohol, evaporated to dryness, and taken up in a standard volume of ethanol. Aliquots were taken for cholesterol analysis and for isolation of sterol digitonide and C^{14} assay.

At the time of slicing, weighed samples of liver were taken for cholesterol analysis by direct saponification, extraction, and colorimetric analysis. The weight of the liver slices in each flask was most accurately determined from the amount of cholesterol in each flask and the cholesterol content of the liver. Direct weighing of slices before incubation was also done but the weights were not considered to be accurate enough to use in calculating results.

Cholesterol was determined by the method of Abell, Levy, Brodie, and Kendall (6), modified by the use of 2

ml of 0.084% ferric chloride in acetic acid and 3 ml of concentrated sulfuric acid for color development. The solvents were mixed by a standardized procedure since it was observed that the method of mixing could affect the optical density.

Isolation of Cholesterol

An aliquot of the unsaponifiable fraction was evaporated to dryness and taken up in a small volume of alcohol–acetone 1:1, and 1 ml of 1% digitonin was added. After several hours' standing, the digitonide was washed in the centrifuge tube with 95% alcohol, alcohol–ether 2:1, and ether. It was then dissolved in dry pyridine, transferred to a liquid scintillation counting vial, and heated in a stream of nitrogen until the pyridine was evaporated. After addition of a small volume of toluene and evaporation to dryness, repeated several times, to remove the last trace of pyridine, 10 ml of toluene containing 0.5% of PPO and 0.03% of POPOP was added and the C^{14} content was determined in a liquid scintillation counter using a C^{14} standard. The digitonin remained as an insoluble white precipitate adhering to the bottom of the counting vial. Control tests showed that counting rates close to theoretical were obtained from standard samples of cholesterol-C^{14} converted into digitonide and counted by this procedure and that the precipitate of digitonin contained no appreciable amount of activity. Consequently it may be concluded that this procedure is satisfactory providing all the pyridine is removed and each sample is checked for quenching.

Samples that contained both tritium and C^{14} were counted at settings that gave only C^{14} in one channel (efficiency about 38%) and in the other channel gave an efficiency of 37% for tritium and about 18% for C^{14}. Quenching was tested for by adding known amounts of toluene-C^{14} and of toluene-H^3 to all samples and determining the increments in counting rates. Significant quenching was rarely found in cholesterol samples obtained as described above. Counting rates were expressed as disintegrations per minute in the cholesterol isolated from 1 g of liver or from the amount of liver corresponding to 100 g of rat body weight, as indicated.

Purification of Cholesterol

Some samples of cholesterol were purified through the dibromide essentially as described by Fieser (7): after isolation as sterol digitonide and regeneration by the pyridine–ether method, the sterol fraction, if less than 50 mg, was diluted with pure, inert cholesterol to make at least 50 mg, and the specific activity was determined. The sterol was brominated, the dibromide was isolated, and the sterol was regenerated by means of zinc dust; the product was recrystallized from methanol, and its specific activity was determined.

Isolation of Ketone Bodies

To determine the effect of CPIB on ketone body synthesis from acetate, liver slices were incubated with acetate-C^{14} in the usual manner; the incubation medium and liver slices were then acidified with 1 N H_2SO_4, and the the slices ground in a tissue grinder. After centrifugation, the precipitate was reextracted with 0.1 N sulfuric acid in the cold and centrifuged after adding 20 mg of β-hydroxybutyrate as carrier. The combined extracts were treated as described by Van Slyke (8) to convert the ketone bodies to acetone, which was isolated as the Denigès acetone–basic mercuric sulfate compound. The precipitate was centrifuged, washed with water, and counted in a gas flow counter. All samples were at least 20 mg/cm² in thickness so that no self-absorption corrections were necessary. Control experiments demonstrated that acetate-1-C^{14} was not carried down in the precipitate to an appreciable extent.

In Vivo Experiments

Groups of rats fed stock, Atromid, or CPIB diets for different lengths of time were injected intraperitoneally with tritium water and acetate-1-C^{14} in two experiments and with tritium water and glycerol-1-C^{14} in a third. They were killed after 4 hr and cholesterol was isolated from samples of liver and in some cases from the small intestine, as described above, except that the unsaponifiable fraction was treated again with a large volume of alcoholic alkali to ensure the removal of tritium from the hydroxyl group of cholesterol.

RESULTS

Atromid and CPIB administration to rats at a level of 0.3% of the diet resulted in decreases in plasma and liver cholesterol levels (Table 1). The mean decrease in plasma cholesterol was about 29% for CPIB (27 animals) and 32% for Atromid (11 animals) after 6 days or longer of drug administration. The range of decreases in experiments done at different times of year was only 25–40%, in good agreement with the results of Thorp and Waring (1) for the same months.

A small but significant decrease in cholesterol concentration in liver was found. The mean decrease of 10.5% is in good agreement with the results reported by Thorp and Waring (1).

The liver weights of rats treated either with CPIB or with Atromid increased 25% in this study (Table 1). These results are in good agreement with those reported recently by Best and Duncan (9). Since the increase in liver weight was proportionately greater than the decrease in liver cholesterol concentration, there was actually a

TABLE 2 EFFECT OF ATROMID AND OF CPIB ON INCORPORATION OF ACETATE-1-C^{14} INTO CHOLESTEROL IN RAT LIVER SLICES

Expt. No.	Days on Drug	Control Dpm per g Liver × 10^{-3}	Atromid Dpm per g Liver × 10^{-3}	% Inhibition	CPIB Dpm per g Liver × 10^{-3}	% Inhibition
16	13	2.22 ± 0.55*	0.39 ± 0.16*	82.4		
17	6	9.57 ± 3.69	3.08 ± 0.80	67.8		
18	13	1.84 ± 0.13	0.99 ± 0.13	46.2		
19	6	5.90 ± 1.30	0.93 ± 0.11	84.2	1.13 ± 0.41*	80.9
20	13	5.56 ± 1.11	1.27 ± 0.54	77.2	0.82 ± 0.24	84.2
Mean values (6–13 days only)		5.02 ± 1.02	1.33 ± 0.31	71.6	0.98 ± 0.61	82.5
10	45	1.87 ± 0.36	0.16 ± 0.053	85		
14	70	2.96 ± 0.84	0.67 ± 0.27	77.5		

Control (expts. 16–20) vs. Atromid (expts. 16–20) $P < 0.002$
Control (expts. 19–20) vs. CPIB (expts. 19–20) $P < 0.001$

* Mean ± SEM (n = 3).

slight but not significant increase in total cholesterol content of the liver per 100 g body weight.

The dose level of 0.3% of the diet was chosen because the studies of Thorp and Waring (1) had shown this level to have the maximum effect on plasma cholesterol levels without producing any manifestations of toxicity. This dose level was shown not to affect food consumption or weight gain to an appreciable extent. In a separate experiment, 9 rats with a mean weight of 235.4 g were fed 0.3% CPIB for 6 days; the food consumption was 23.26 g per rat day as compared with 22.06 g/day for a group of 9 control rats with a mean weight of 231.3 g. The drug-treated rats gained 7.5 as compared with 8.9 g/day for controls. After 5 days on stock diet, the experiment was repeated with the groups interchanged. The treated rats ate 23.3 and the controls 23.7 g/day; the corresponding weight gains were 4.5 and 4.8 g/day. These results agree with the conclusion of Thorp and Waring (1) and of Best and Duncan (9) that CPIB does not affect the rate of gain in weight.

It is of interest that the liver weights determined at the end of the second experiment were influenced only by the second experiment; the drug-treated rats had a mean liver weight of 4.68% of body weight and the controls of 3.71%, a difference of 26%. The livers of the control group in the second experiment must have been enlarged at the end of the first experiment (in which they were the drug treated group) and consequently their liver weights must have returned to normal during the subsequent 11 days on normal diet.

Synthesis in Liver Slices

The incorporation of acetate-C^{14} into cholesterol per gram of liver slices was considerably less for Atromid-treated rats than for control animals.[2] In a series of five experiments (Table 2) on a total of 30 rats (treated for 6 or 13 days), the drug-treated animals showed a mean decrease of 72% in the incorporation of C^{14} into digitonin-precipitable sterols per gram of liver tissue. As has been previously observed by many investigators, there was a considerable individual variation in controls; however, inhibition was observed in every experiment,

[2] Thorp and Waring (1) report a private communication from Dr. G. S. Boyd (1960), who found an inhibition of 30–50% in incorporation of acetate-1-C^{14} into cholesterol in rat liver slices from rats treated with Atromid.

TABLE 3 LACK OF EFFECT OF ATROMID AND OF CPIB ON INCORPORATION OF MEVALONATE-2-C^{14} INTO CHOLESTEROL IN RAT LIVER SLICES

Expt. No.	Days on Drug	Control Dpm per g Liver × 10^{-3}	Atromid Dpm per g Liver × 10^{-3}	% Inhibition	CPIB Dpm per g Liver × 10^{-3}	% Inhibition
18	13	6.25 ± 0.51*	5.17 ± 0.15*	17.2		
19	6	12.28 ± 1.37	11.47 ± 1.68	6.7	15.35 ± 1.55*	25 (increase)
20	13	14.96 ± 3.94	12.52 ± 3.08	16.2	8.13 ± 1.66	45.4
Mean values		11.16 ± 0.61	9.72 ± 1.53	12.9	11.74 ± 0.61	5 (increase)

P for difference between all controls and all drug-treated animals was >0.10.
* Mean ± SEM (n = 3).

TABLE 4 EFFECTS OF ATROMID AND OF CPIB ON IN VIVO CHOLESTEROL BIOSYNTHESIS IN RAT LIVER FROM ACETATE-1-C^{14} AND FROM TRITIUM WATER

Expt. No.	Days on Drug	No. of Rats and Group	TritiumWater Dpm per g Liver × 10^{-3}	% Decrease	Precursor	Carbon-14 Dpm per g Liver × 10^{-3}	% Decrease
21	10	6 Control	12.37 ± 1.51*		Acetate-C^{14}	5.54 ± 1.73*	
		6 Atromid	6.99 ± 1.64	44		2.31 ± 0.71	58
		6 CPIB	6.85 ± 1.15	45		2.25 ± 0.41	59
24	6	5 Control	16.54 ± 3.71		Acetate-C^{14}	5.60 ± 0.91	
		5 CPIB	10.99 ± 1.68	34		3.85 ± 0.62	31
29	8	4 Control	15.30 ± 2.12		Glycerol-1-C^{14}	0.53 ± 0.24	
		3 CPIB	12.20 ± 3.34	20		0.26 ± 0.06	51
31	6	5 Control	19.66 ± 3.28		Glycerol-1-C^{14}	0.61 ± 0.07	
		5 CPIB	15.62 ± 4.71	21		0.39 ± 0.05	35
33	7	5 Control	18.72 ± 2.62				
		5 Atromid	14.12 ± 2.61	22			
					All acetate results		
Mean		15 Control	14.53 ± 0.46		11 Control	5.56 ± 0.95	
		14 CPIB	9.26 ± 1.14	36	17 Atromid + CPIB	2.74 ± 0.36	51
				$P < 0.001$			$P < 0.01$
					All glycerol results		
					8 Control	0.57 ± 0.12	
					7 CPIB	0.34 ± 0.04	40
							$P < 0.10$

* Mean ± SEM.

the treated animals showing from 46 to 84% decrease of incorporation compared with control animals. No overlap was observed in any one experiment in this series in the values for control and treated animals.

As no significant difference could be found between the 6- and 13-day experiments, the data have been combined for statistical treatment. The mean value for all Atromid-treated animals was significantly less than that for all control animals ($P < 0.002$).

Although most experiments were done on rats that had been treated with the drug for short periods of time, long-term experiments gave very similar results; in two experiments in the 45–70 day range the rats fed Atromid gave 85 and 78% decreases (Table 2).

A comparison of the effects of Atromid and CPIB on acetate-1-C^{14} incorporation is shown in Table 2; the average decrease was 82.5% for CPIB-treated rats compared with 80.8% for Atromid-treated rats in the same experiments. There was no significant difference between the two, and hence no perceptible effect due to the androsterone in Atromid.

The increase in liver weight in drug-treated animals partially offsets the decrease in synthesis; although liver weights were not determined in these experiments, it may be assumed that the livers of the drug-treated animals were 25% larger per 100 g body weight than the controls, corresponding to a decrease in synthesis, per 100 g body weight, of 65%.

When mevalonate-2-C^{14} incorporation was measured in comparison with acetate-C^{14} much less inhibition by either Atromid or CPIB was observed: 13% by Atromid (Table 3) compared with about 70% for acetate in the same experiments (Table 2); CPIB, no significant effect on mevalonate, contrasted with 83% inhibition of acetate incorporation. It may be concluded that the effects on mevalonate conversion to cholesterol were not significant ($P > 0.10$).

In Vivo Results

The rate of cholesterol biosynthesis in intact rats was estimated by measuring the incorporation of tritium from body water and of C^{14} from acetate-1-C^{14} and from glycerol-1-C^{14}. Both methods indicated a decrease in cholesterol biosynthesis per gram of liver tissue in rats treated with either CPIB or Atromid (Table 4).

In two experiments (21 and 24) both labeled substrates were injected into the rats as a single injection. The inhibitory effect of the drugs on the incorporation was similar for both isotopic tracers although the effect on acetate-C^{14} incorporation into liver cholesterol appeared to be slightly greater than for tritium water.

The two methods of estimating cholesterol biosynthesis in intact animals were found to give concordant results in almost every case for individual animals, as indicated by the ratio of C^{14}:H^3 (Table 5). Animals which showed a high rate of acetate incorporation tended to show a high rate of tritium incorporation, which indicates that the large variation in rate of acetate incorporation commonly observed in normal rats is actually due to different rates of synthesis and not to changes in acetate pool

TABLE 5 RATIOS OF C^{14}:H^3 IN THE LIVER CHOLESTEROL OF RATS INJECTED WITH ACETATE-1-C^{14} AND H^3OH

Expt. No.	Control	Atromid	CPIB
21	0.425	0.31	0.33
24	0.32		0.29
Mean of all animals (28) 0.36			
Standard error of the mean 0.026			
Range of all determinations (except 2) 0.22–0.49			

size or other factors concerned with acetate metabolism. This finding is in agreement with previous results (10) and supports the assumption that the relative amount of acetate-C^{14} incorporation into liver cholesterol in an intact animal is a reliable index of the rate of hepatic cholesterol synthesis under these specific experimental conditions. However, it is not clear why the tritium method consistently indicated slightly less inhibitory effect of drug than the acetate-C^{14} method.

To determine if CPIB inhibits the conversion of the sterol intermediates such as desmosterol (24-dehydrocholesterol) into cholesterol, purification through the dibromide was carried out in two in vivo experiments. The decrease in specific activity of liver cholesterol resulting from purification was not significant in drug-treated animals or in controls. Eight samples showed a change in specific activity on purification ranging from -6 to $+13\%$ (mean for 6 drug-treated animals, -0.8%; 2 control animals $+10\%$). Since drugs which inhibit the conversion of desmosterol to cholesterol produce extremely marked decreases in specific activity by this procedure it may be concluded that no significant inhibition between any of the digitonin precipitable sterols and cholesterol is produced by CPIB or Atromid.

Glycerol was much less efficiently converted into cholesterol than was acetate, but there was a measurable incorporation that appeared to be considerably less in the CPIB-treated animals than in controls. However, only two experiments were done with this precursor and the difference is not definitely significant.

Tritium water was injected in a number of experiments and the mean value for all the experiments on male Sprague-Dawley rats given the drug for 6–13 days indicated a decrease of 36%; the effect of the drug on synthesis per gram of liver was significant ($P < 0.001$).

However, the increase in liver weight must be taken into account in considering the effect of the drug on hepatic cholesterol synthesis in the intact animal. The results for both tritium and C^{14} incorporation, calculated in terms of dpm in liver cholesterol per 100 g body weight, are given in Table 6. The results for acetate incorporation showed a decrease of 38% ($P < 0.05$) for the combined results of all experiments, with rats that varied considerably in age and weight. The results from the most recent experiment alone (No. 34) showed almost the same average decrease (39%), with $P < 0.02$. The tritium results showed a smaller decrease, averaging 22%, and the effect was at the borderline of significance, with $P < 0.1$.

Ketone Body Synthesis

No inhibition of ketone body synthesis was found, as shown in Table 7. The drug-treated rats showed a

TABLE 6 EFFECT OF CPIB AND ATROMID ON INCORPORATION OF TRITIUM FROM BODY WATER AND OF C^{14} FROM ACETATE INTO LIVER CHOLESTEROL IN INTACT RATS, PER 100 G BODY WEIGHT

		Liver Cholesterol			
		Tritium		C^{14}	
Expt. No.	No. of Rats and Group	Dpm/100 g Body Weight × 10^{-3}	% Decrease	Dpm/100 g Body Weight × 10^{-3}	% Decrease
21	6 Control	56.78		25.2	
	6 CPIB	40.55	29	13.3	47
	6 Atromid	40.58	28	13.1	48
24	5 Control	55.44		18.5	
	5 CPIB	33.00	40.5	11.4	38
29	4 Control	63.53			
	3 CPIB	51.58	19		
31	4 Control	85.94			
	4 CPIB	83.94	2		
33	5 Control	78.52			
	5 Atromid	76.38	3		
34	9 Control			13.27 ± 1.41*	
	9 CPIB			8.11 ± 1.33	39 ($P < 0.02$)
Mean of all results	Control	67.01 ± 5.20*		18.26 ± 2.77	
	Drug-treated	52.56 ± 6.23	22 ($P < 0.10$)	11.27 ± 1.37	38 ($P < 0.05$)

* Mean ± SEM.

TABLE 7 RATE OF PRODUCTION OF KETONE BODIES BY LIVER SLICES FROM CONTROL AND CPIB-TREATED RATS

Expt. No.	Cpm in Ketone Bodies × 10^{-3}	Effect of Drug	Dpm in Cholesterol × 10^{-3}	Effect of Drug
25 Control	5.39 ± 0.132*		75.15 ± 14.62*	
CPIB	6.50 ± 0.380	20.5% increase ($P < 0.02$)	24.97 ± 6.83	66.8% decrease

* Mean ± SEM (n = 6).

slight increase in the conversion of acetate-1-C^{14} to ketone bodies that was probably significant since $P < 0.02$. Liver slices from the same animals showed a 67% decrease in cholesterol synthesis. Control studies demonstrated that contamination of the acetone–basic mercuric sulfate compound by acetate-C^{14} was less than 2% of the values observed.

Addition of CPIB In Vitro

To determine if CPIB is a direct inhibitor of cholesterol biosynthesis, the effect of adding the drug to normal rat liver slices and homogenates was investigated (Table 8). No significant effect on acetate incorporation was found at concentrations of the drug of 0.6 × 10^{-3} M or below; 15–46% inhibition was found at 2.5 × 10^{-3} M. Incubation of slices or homogenates with CPIB for 30 min before adding acetate-C^{14} did not appear to result in an increase in the inhibitory effect of the drug.

Effect on Cholesterol Synthesis in Intestine

Since the rat intestine is normally the site of as much cholesterol synthesis as the liver (11) it is of interest to determine if CPIB inhibits cholesterol synthesis in intestine. The intestines from one in vivo experiment did not show a significant inhibition of cholesterol biosynthesis per gram of tissue, as indicated by either acetate-C^{14} or tritium incorporation (Table 9). In fact, the rate of synthesis was slightly higher in intestine of drug-treated rats than in controls.

It is of interest that the ratio of C^{14}:H^3 in intestinal cholesterol was almost three times as large as in liver cholesterol from the same animal, in agreement with previous results (10).

TABLE 8 EFFECT OF α-*p*-CHLOROPHENOXYISOBUTYRIC ACID ADDED IN VITRO ON INCORPORATION OF ACETATE-1-C^{14} INTO CHOLESTEROL IN RAT LIVER SLICES AND HOMOGENATES

Expt. No.	Tissue	Time of Preincubation with Drug	Concentration of CPIBA	Dpm per g Liver in Unsaponifiable Fraction	% Inhibition
		min	× 10^{-3} M		
23	Slices	None	0	10,084	
			0.25	12,179	None
			2.5	6,695	33
		30	0	5,928	
			0.25	5,070	14
			2.5	6,574	None
23	Homogenate	None	0	2,873	
			0.25	2,349	18
			2.5	1,922	33
		30	0	2,077	
			0.25	2,049	None
			2.5	1,758	15
27	Homogenate	30	0	5,400	
			0.125	5,660	None
			0.312	4,680	13
			0.625	4,710	13
			2.5	2,900	46

DISCUSSION

The observation that CPIB is as active as the mixture of CPIB and androsterone (Atromid) is relevant to several recent reports (4) that CPIB decreases plasma lipid levels in humans as effectively as Atromid, and makes less likely the suggestion of Thorp and Waring (1) that CPIB acts by potentiating the effect of androsterone.

The liver slice studies indicated a significant inhibitory effect of CPIB on hepatic cholesterol biosynthesis expressed per unit weight of liver. The results on intact animals in the same units are less marked but highly significant. However, the drug also produced an increase in liver weight in rats. When the rate of synthesis is expressed in terms of rat body weight the drug effect was reduced from an inhibition of 72 to one of 65% in liver slice studies, from about 50 to 39% for acetate-C^{14} incorporation in vivo, and from 36 to 22% for tritium water incorporation. The tritium decrease of 22% had $0.05 < P < 0.1$ and cannot therefore be considered highly significant.

TABLE 9 EFFECT OF CPIB ON SYNTHESIS OF CHOLESTEROL IN INTESTINE IN INTACT RATS

	Dpm per g Tissue × 10^{-3}		Ratio C^{14}/H^3	
	Acetate-C^{14}	H^3OH	Intestine	Liver
Control	4.09 ± 0.22*	5.07 ± 0.34	0.806	0.32
CPIB	5.21 ± 0.39	5.56 ± 0.58	0.937	0.29
Change	+28%	+10%		

P for C^{14} data <0.05.
P for H^3 data >0.1.

* Mean ± SEM.

It is puzzling that the over-all results with acetate-C^{14} showed a considerably larger and more significant drug effect per 100 g body weight than those with tritium water. This may be attributed partially to a consistently smaller drug effect on tritium incorporation evident in experiments 21 and 24 (Tables 4–6), in which both isotopic methods were used on the same animals, and partially to the variability in the effect of CPIB at different times of year noted by Thorp and Waring (1). In experiments 31 and 33, which showed no significant inhibition of tritium water incorporation in vivo (Table 6), liver slices were also incubated with acetate-C^{14} and the drug effect was found (Table 4) to be smaller than usual.

In many of these experiments triglyceride synthesis from acetate-C^{14}, tritium water, and glycerol-C^{14} was determined; the results (to be published separately) did not indicate a decrease due to CPIB or Atromid.

Any interpretation of these results in connection with the effect of CPIB in decreasing plasma cholesterol and triglyceride concentrations would be purely speculative, because of the many unknown factors involved in the regulation of plasma lipoprotein levels.

The decrease in cholesterol biosynthesis per unit weight of liver which has been shown to occur both in vivo and in vitro is of interest particularly because it is located between acetate and mevalonate, where the primary physiological control of over-all cholesterol synthesis is thought to be located (12–14). A number of the steps on the pathway from mevalonate to cholesterol are irreversible; consequently inhibition beyond mevalonate (and particularly beyond squalene) is apt to produce abnormal accumulations of intermediates, as has been demonstrated for several drugs that inhibit the conversion of desmosterol (24-dehydrocholesterol) to cholesterol (15, 16). On the other hand the intermediates between acetate and mevalonate have alternative pathways, such as conversion to fatty acids, acetoacetate, or carbon dioxide, so that inhibition before mevalonate would not be expected to result in abnormal accumulation of intermediates.

The lack of effect of CPIB on the conversion of acetate to ketone bodies indicates that there is no inhibition of the formation of acetyl CoA, but does not permit the conclusion that the step affected is the reduction of HMG-CoA to mevalonate. It is still uncertain whether HMG-CoA is an obligatory intermediate in ketone body formation (17–20) and there is recent evidence that it is not an intermediate in mevalonate and cholesterol biosynthesis (21, 22). It has been suggested that an HMG–enzyme complex is the actual precursor of mevalonate (22); if this is the case, the controlling step may be the reduction of HMG–enzyme to mevalonate. In view of the many uncertainties in the intermediates involved in fatty acid, ketone body, and mevalonate biosynthesis, it seems unwarranted to go beyond the statement that administration of CPIB, like cholesterol feeding, decreases the rate of formation of mevalonate from acetate but not the formation of fatty acids or of ketone bodies, and therefore probably acts on one or more steps located on the acetyl CoA to mevalonate pathway beyond the point at which fatty acid and ketone body syntheses branch off.

The failure of α-*p*-chlorophenoxyisobutyric acid to inhibit cholesterol biosynthesis when added to normal liver homogenate in a reasonable concentration also is in contrast to the behavior of a number of other drugs that are inhibitory under these conditions (16), and suggests that the drug acts indirectly.

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