Hepatic acetoacetyl-CoA deacylase activity in rats fed ethyl chlorophenoxyisobutyrate (CPIB)

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ABSTRACT Intact or sonicated mitochondria from the livers of rats fed a diet containing 0.2% ethyl chlorophenoxyisobutyrate (CPIB) for 3 wk showed acetoacetyl-CoA deacylase activity enhanced 26 and 39%, respectively, over that shown by comparable fractions from rats fed the same diet without CPIB. The corresponding supernatant fractions did not differ in activity. The enhanced activity of mitochondrial acetoacetyl-CoA deacylase in the livers of the CPIB-treated rats could effectively decrease the amount of acetoacetyl CoA available within the cell for synthetic processes.

SUPPLEMENTARY KEY WORDS cholesterol biosynthesis · acetoacetate · HMG-CoA

In 1965 Avoy, Swyryd, and Gould (1) showed that the incorporation of acetate-^{14}C into cholesterol by liver slices from CPIB-treated rats was depressed, whereas the incorporation of mevalonate-^{14}C into cholesterol was unchanged when compared to liver from normal rats. They concluded that the inhibition of cholesterol biosynthesis after the administration of CPIB was probably due to decreased HMG-CoA reductase activity, which mediates the reduction of HMG-CoA to mevalonic acid. However, these authors emphasized the possibility that other enzymes involved in the conversion of acetyl CoA to HMG-CoA could also be affected by CPIB.

Evidence presented in this paper shows that one of the enzymes influencing the metabolic sequence from acetyl CoA to HMG-CoA, namely acetoacetyl CoA deacylase, is sensitive to CPIB administration. This sequence of reactions, which is common to both acetoacetate formation and the early stages of cholesterol biosynthesis, is shown in Fig. 1. Since acetoacetyl CoA is a substrate and acetoacetate a product in both the HMG-CoA and acetoacetyl CoA pathways for the production of acetoacetate, it was necessary to inhibit the HMG-CoA pathway by preincubating the liver preparations with iodoacetamide (2–4). It was not possible to evaluate acetoacetate production via the HMG-CoA pathway adequately, since no satisfactory method has been devised to completely inhibit acetoacetyl CoA deacylase.

METHODS AND MATERIALS

Male rats of the Wistar strain were obtained from Albino Farms, Red Bank, N.J. All rats weighed between 200 and 500 g. "CPIB-treated" animals were allowed free access to ground Purina rat chow containing 0.2% CPIB. To prepare the diet we dissolved the CPIB in diethyl ether and sprayed the solution on the ground rat chow. After evaporation of the ether the food was mixed thoroughly. Normal animals received the same diet without added CPIB.

At the end of 3 wk normal and CPIB-treated rats were killed by cervical dislocation and thoroughly bled, and the livers were removed and chilled. The livers were minced and then homogenized in 3 volumes of 0.25 M sucrose by means of a loose-fitting Potter–Elvehjem homogenizer for less than 30 sec. Mitochondria were isolated and washed as described previously (5) and then diluted with 0.25 M sucrose to a final protein concentration of 7–15 mg/ml. An aliquot of this solution, containing intact mitochondria, was then diluted with an equal volume of 1.0 M Tris buffer, pH 7.9, and subjected to 3 min of sonic disruption with the 9 mm probe of a Bronwill Biosonik III sonicator (Bronwill Scientific

Abbreviations: CPIB, ethyl ester of chlorophenoxyisobutyrate; HMG-CoA, the coenzyme A ester of β-hydroxy-β-methylglutaric acid; D.P.S., digitonin precipitable sterols.

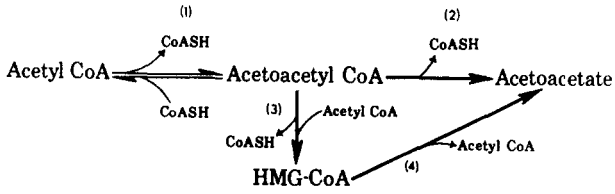

Fig. 1. Reactions associated with the two major pathways for the production of acetoacetate. The enzymes catalyzing the various reactions are: (*1*) β-ketothiolase, (*2*) acetoacetyl-CoA deacylase, (*3*) HMG-CoA condensing enzyme, (*4*) HMG-CoA cleavage enzyme. Enzymes *1*, *3*, and *4* are completely inhibited by preincubation with iodoacetamide (refs. 2–4).

Div., Will Scientific, Inc., Rochester, N. Y.) operating at half the maximum intensity.

The 10,000 *g* supernatant obtained during the initial isolation of the mitochondria was again centrifuged at 10,000 *g* for 10 min, and this supernatant was carefully decanted. This process was repeated, and the supernatant obtained from this final centrifugation was used in the studies of supernatant acetoacetyl-CoA deacylase activity. Further fractionation of the 10,000 *g* supernatant at 105,000 *g* for 1 hr in a Beckman L-2 preparative ultracentrifuge revealed that approximately 95% of the deacylase activity was in the supernatant and 5% was in the microsomal fraction. Determination of the mitochondrial marker, glutamic dehydrogenase, in the 10,000-*g* supernatant fractions revealed almost no enzyme present and thereby excluded the possibility that supernatant acetoacetyl-CoA deacylase activity was due to liberation from damaged mitochondria.

Assay Procedure

The assay procedure used in these studies has been described previously in detail (2). Briefly, it was as follows. Triplicate aliquots of approximately 1 mg of enzyme were preincubated at room temperature for 10 min with 10^{-3} M iodoacetamide (final concentration) in the presence of 100 μmoles of Tris buffer (pH 7.9) and 5 μmoles of $MgCl_2$. Approximately 0.2 μmole of purified acetoacetyl CoA (6) was then added, and the mixture was incubated at 37°C; final volume was 2.0 ml. If iodoacetamide was purposely omitted, final volume was also 2.0 ml. After the reaction had been stopped by the addition of 25% trichloroacetic acid, the amount of acetoacetyl CoA disappearing was determined enzymatically (7). Results were averaged, and after subtraction of the amount of acetoacetyl-CoA that disappeared nonenzymatically, acetoacetyl-CoA deacylase activity per mg of protein was determined. Nonenzymatic deacylation varied between 23 and 45 mμmoles, the lower values being observed in 10-min incubations and the higher values in 20-min incubations.

Free acetoacetate was determined enzymatically with β-hydroxybutyric dehydrogenase according to the method of Mellanby and Williamson (8) and agreed closely with the amount of acetoacetyl CoA that disappeared during an incubation.

Reagents

Acetoacetyl CoA was synthesized from diketene and reduced coenzyme A (9). Coenzyme A was obtained from P-L Laboratories, Milwaukee, Wisconsin. β-Hydroxyacyl-CoA dehydrogenase, β-hydroxybutyric dehydrogenase, reduced diphosphopyridine nucleotide, ADP, and AMP were purchased from Boehringer Mannheim Corp., New York. DL-carnitine was purchased from Calbiochem, Los Angeles, Calif. Reduced glutathione was purchased from Mann Research Laboratories, Inc., New York.

Methods

Acetoacetyl CoA was purified according to the method of Sauer and Erfle (6) on an ECTEOLA-cellulose column and was assayed spectrophotometrically with β-hydroxyacyl-CoA dehydrogenase, according to the method of Decker (7). The purified acetoacetyl CoA has previously been shown to contain only acetoacetyl CoA and is not contaminated with acetoacetyl-glutathione (2). Protein was determined by the method of Lowry, Rosebrough, Farr, and Randall (10). Blood acetoacetate was determined with β-hydroxybutyric dehydrogenase according to the method of Mellanby and Williamson (8).

The incorporation of acetate-1-^{14}C into digitonin-precipitable sterols (D.P.S.) was determined in aliquots from livers in which deacylase activity was also measured. Liver slices were prepared and incubated; the sterols were extracted and precipitated with digitonin, and their radioactivity was determined as previously described (11).

RESULTS

Exclusion of Other Possible Pathways

It has previously been shown in this laboratory (2) that preincubation of intact mitochondria with 10^{-3} M iodoacetamide inhibits both β-ketothiolase and HMG-CoA condensing enzyme (reactions 1 and 3 in Fig. 1). To be certain that iodoacetamide pretreatment of hepatic enzymes from CPIB-treated rats was equally efficacious in inhibiting these two enzymes the following experiments were performed. Either acetyl CoA or coenzyme A, in the amount of 100 mμmoles, was added to incubations utilizing both untreated and iodoacetamide-treated intact and sonicated mitochondria and 10,000 *g* super-

natant from CPIB-fed rats. The amount of acetoacetyl CoA disappearing was compared to the disappearance of acetoacetyl CoA utilizing the same source of enzyme without added acetyl CoA or coenzyme A. These data are given in Table 1 and show that the addition of either acetyl CoA or coenzyme A to iodoacetamide-treated enzyme fails to increase the disappearance of acetoacetyl CoA, whereas similar additions to the same enzyme preparation which has not been preincubated with iodoacetamide stimulate the disappearance of acetoacetyl CoA. It has previously been shown in this laboratory (2) that in those experiments where free coenzyme A is added to iodoacetamide-treated mitochondria all the added CoASH does not react with the excess iodoacetamide. It must be emphasized that we measured the disappearance of acetoacetyl CoA in untreated enzyme preparations only to demonstrate the presence of both β-ketothiolase and HMG-CoA condensing enzyme activities. Although these studies show qualitatively that β-ketothiolase and HMG-CoA condensing enzyme are present in the untreated enzymes, no quantitative information about these two enzymes can be inferred from these studies since, under these circumstances, the above two enzymes and acetoacetyl-CoA deacylase are all effectively utilizing acetoacetyl CoA simultaneously.

Since acetoacetyl CoA disappearance after preincubation of the enzyme with iodoacetamide could not be stimulated by either acetyl CoA or coenzyme A over control values without such additions, it can be concluded that both β-ketothiolase and HMG-CoA condensing enzyme have been inhibited. Further, since the acetoacetate formed during incubations, as determined enzymatically with β-hydroxybutyric dehydrogenase, closely approximated acetoacetyl CoA disappearance, this assay procedure seemed to be an accurate reflection of acetoacetyl CoA deacylase activity. This is consistent with studies previously done in this laboratory with intact mitochondria from fed and fasted rats (2).

To be sure that acetoacetyl CoA was giving rise to acetoacetate directly by deacylation and not through the intermediacy of other reactions which could conceivably be involved in acetoacetate production, we preformed a series of experiments to exclude this possibility. If other pathways were responsible for acetoacetyl CoA disappearance then, theoretically, addition of the substrates involved in these reactions should stimulate the disappearance of acetoacetyl CoA. Addition of the following substances did not stimulate acetoacetyl CoA disappearance: succinate, ADP and inorganic phosphate, AMP and inorganic pyrophosphate, DL-carnitine, and reduced glutathione. Thus, the possibilities that a transferase reaction, a reversal-of-a-kinase type reaction, the formation of acetoacetyl-carnitine with subsequent conversion to free acetoacetate, and the nonenzymatic formation of acetoacetyl-glutathione with the subsequent enzymatic hydrolysis to acetoacetate were excluded as sources of the results reported in this work. Both mitochondrial and supernatant fractions were evaluated in the above manner. These results are similar to those reported by Drummond and Stern (3), who used a partially purified enzyme preparation from normal ox liver.

TABLE 1 EFFECT OF ADDED ACETYL-CoA AND COENZYME A ON ACETOACETYL CoA DISAPPEARANCE FROM UNTREATED AND IODOACETAMIDE-TREATED MITOCHONDRIA*

Enzyme Obtained from CPIB-Treated Rat Liver	Acetoacetyl CoA Disappearing	
	Iodoacetamide-Treated	Untreated
	mμmoles/mg protein/20 min	
Expt. 1		
Intact mitochondria	47.6	120
+ CoASH	40.7	172
+ AcCoA	12.2	173
Expt. 2		
Sonicated mitochondria	167	357
+ CoASH	151	396
+ AcCoA	151	382
	mμmoles/mg protein/10 min	
Expt. 3		
Supernatant	35.2	39.3
+ CoASH	27.8	55.4
+ AcCoA	27.8	55.5

* Where indicated, 100 mμmoles of either acetyl CoA or reduced coenzyme A were added after the addition of acetoacetyl CoA.

Acetoacetyl-CoA Deacylase Activity in Liver Fractions from CPIB-Treated Rats

The data shown in Table 2 reflect hepatic acetoacetyl-CoA deacylase activity in intact and sonicated mitochondria and in 10,000 *g* supernatant from normal rats and rats fed 0.2% CPIB in their diet for 3 wk. The deacylase activity in both the intact and sonically disrupted mitochondria is greater in the preparations from CPIB-treated rats than in similar preparations from normal rats. However, the deacylase activities in the 10,000 *g* supernatant from these two groups of animals are almost identical. There is a 26% increase in activity of the deacylase per mg mitochondrial protein in intact mitochondria and 39% in sonically disrupted mitochondria. When one considers that the livers of the CPIB-treated rats were 18% larger (Table 3) than the livers of normal rats, this increase becomes even more significant. In fact, if one assumes that mitochondria constitute 5% of the liver weight (12) and, further, that the 18% increase in liver weight is accompanied by a similar increase in deacylase protein, then the amount of acetoacetate that could be produced via deacylation by sonicated mitochondria from the whole liver would be 2.63 μmoles/min in the normal and 4.33 μmoles/min in the CPIB-treated animals. This calculation suggests

TABLE 2 ACETOACETYL-CoA DEACYLASE ACTIVITY IN IODOACETAMIDE-TREATED LIVER FRACTIONS FROM NORMAL AND CPIB-FED RATS

Enzyme Preparation	Acetoacetyl CoA Disappearing	
	mμmoles/mg protein/20 min	*mμmoles/mg protein/10 min*
Mitochondria		
1. Intact		
Normal (26)	40.6 ± 1.6	
CPIB (38)	51.1 ± 1.5	
2. Sonicated		
Normal (19)	94.4 ± 2.8	
CPIB (31)	131 ± 4.7	
10,000 g Supernatant		
Normal (10)		28.7 ± 1.0
CPIB (7)		30.6 ± 2.3

Means ± SEM. Number of rats in parentheses.

TABLE 3 EFFECT OF CPIB DIET ON LIVER AND RAT WEIGHTS

	Rat Weight	Liver Weight	Weight gained in 18 hr Preceding Experiments
	g	*g*	*g*
Normal (27)	369 ± 15.1	11.2 ± 0.5	7.7 ± 0.9
CPIB (38)	344 ± 10.7	13.2 ± 0.5	6.6 ± 0.7

Means ± SEM (n in parentheses).

an increase of 65% in the capacity of this pathway to produce acetoacetate after CPIB therapy. The amount of acetoacetate that could be produced by the supernatant deacylase pathway amounts to 1.3 μmoles/min in the normal liver and 1.61 μmoles/min in the CPIB-treated liver.

The data shown in Table 3 indicate that the normal and CPIB rats were approximately the same size at the time the studies were done, that each group of animals gained approximately the same amount of weight in the 18 hr preceding the experiment, and that the livers of CPIB-treated rats were larger than the untreated animals.

To evaluate further the stimulating effect of CPIB on acetoacetyl-CoA deacylase activity, we added CPIB in a final concentration of 2.5×10^{-2} M directly to incubations utilizing mitochondria from normal rat liver. There was no stimulatory effect of the CPIB over control values obtained without added drug. This finding is consistent with the observation of Avoy et al. (1) that CPIB does not inhibit cholesterol synthesis when added directly to liver homogenates at concentrations of 10^{-3} M or less.

Inhibition of Cholesterol Synthesis from Acetate in Liver Slices from CPIB-Treated Rats

In Table 4, if the specific activity of the digitonin precipitable sterols (D.P.S.) from CPIB-treated liver slices is compared with the D.P.S. specific activity of liver slices from normal rats, it can be seen that the indicated synthesis of cholesterol from acetate was inhibited 66%. The percentage diminution in D.P.S. synthesis varied from 19 to 87%. For the sake of comparison, acetoacetyl-CoA deacylase activities in sonically disrupted mitochondria are shown for each of these preparations in this table, and the percentage increase in activity of the deacylase is shown. The increase in deacylase activity varied from 28 to 135%, with a mean of 69%. The close agreement of the percentile decrease in D.P.S. specific activity and the percentile increase in deacylase activity is probably fortuitous, but the inverse relationship of the two activities is of interest.

DISCUSSION

Avoy et al. found (1) that cholesterol synthesis from acetate was diminished in CPIB-treated rat liver but that the synthesis of cholesterol from mevalonate was not impaired. They felt that the site of inhibition was, therefore, somewhere between acetate and mevalonate in the cholesterol synthetic sequence and postulated that the site of inhibition was at the HMG-CoA reductase step. Azarnoff, Tucker, and Barr showed (13) that the incorporation of mevalonic acid-2-^{14}C into cholesterol by liver homogenates from CPIB-treated rats was lower than normal. These authors, on the basis of diminished $^{14}CO_2$ liberation by liver homogenates from CPIB-treated rats when mevalonic acid-1-^{14}C was used as substrate, placed the site of inhibition between mevalonic acid and isopentenyl pyrophosphate. Thus, it is entirely possible that there are several sites of action of CPIB in diminishing cholesterol biosynthesis.

As noted under Results, we have validated the contention that our assay measures only acetoacetyl-CoA deacylase activity. The administration of 0.2% CPIB in the diet is associated with a 26 and 39% increase in deacylase activity in intact and sonicated mitochondria, respectively. However, there is no increase in deacylase activity in the supernatant. This observation is consistent with the studies by Avoy et al. (1) showing that ketone body production by liver slices from CPIB-treated rats was 20% higher than control values. However, this increase is not reflected in the blood, since we found that blood acetoacetate, determined enzymatically, was not elevated in CPIB-treated rats when they were killed. Why this is so we do not know.

Since the addition of CPIB to tissue preparations from normal animals failed to stimulate deacylase activity, the effect of CPIB on this enzyme cannot be a direct effect. Similarly, the enhanced deacylase activity cannot be accounted for by a fasting effect (2) since the CPIB animals gained weight and did eat in the 18 hr preceding

TABLE 4 EFFECT OF CPIB THERAPY ON RAT LIVER CHOLESTEROL SYNTHESIS AND MITOCHONDRIAL DEACYLASE ACTIVITY*

	Weight of Rats	Weight of Livers	Digitonin-Precipitable Sterols	% Decrease	Acetoacetyl-CoA Deacylase Activity in Sonicated Mitochondria	% Increase
		g	*dpm/mg*		*mμmoles/mg protein/20 min*	
Expt. 1						
Control	390	11	484	—	80.3	—
CPIB	400	15	64	86	188	135
CPIB	410	16	162	66	172	114
Expt. 2						
Control	372	8.4	370	—	94.4	—
CPIB	390	16	298	19	133.5	41
CPIB	370	14	126	66	121.4	28
Expt. 3						
Control	384	12	385	—	74.6	—
CPIB	364	12	158	59	115.2	55
CPIB	316	10	140	64	134.2	81
Expt. 4						
Control	361	11	515	—	104.9	—
CPIB	319	14	112	78	172.5	65
CPIB	360	16	65	87	140.1	34
			Average	66		

* Synthesis of digitonin-precipitable sterols determined by incubation of liver slices with 2.7 μc of acetate-1-^{14}C.

an experiment. It is also apparent that the increased activity of the deacylase may be even greater than the values we present since the livers of CPIB-treated rats are 18% larger than livers from control rats. Hepatomegaly with CPIB administration has been reported by others (13).

The data presented in Table 4 indicate that D.P.S. synthesis from acetate was inhibited 19–87% in liver slices from CPIB-treated rats, in agreement with the findings of others (1). In these same animals acetoacetyl-CoA deacylase activity is increased 28–135%.

Cholesterol synthesis from acetate is inhibited in liver slices from CPIB-treated rats. Further, there is an associated rise in acetoacetyl-CoA deacylase activity in the mitochondria from these same rats. Whether these two observations are related as cause and effect is a moot point. It can be postulated that increased deacylase activity could remove acetoacetyl CoA so that it is no longer available for synthetic processes which include cholesterol synthesis. However, it is also possible that with a decrease in HMG-CoA reductase activity, there is a compensatory, or a noncompensatory, simultaneous increase in acetoacetyl-CoA deacylase activity. It is also possible that the data of Avoy et al. (1) could be explained on the basis of increased acetoacetyl-CoA deacylase activity and that there is no block in the conversion of HMG-CoA to mevalonate. One other fact should be borne in mind, namely, that cholesterol synthesis occurs in the microsomes and the increased deacylase activity is seen in mitochondria. However, until the source of acetoacetyl CoA for microsomal cholesterol synthesis is known this neither adds to nor detracts from the hypothesis that mitochondrial acetoacetyl-CoA deacylase could control cholesterol synthesis.

Recently Williamson, Bates, and Krebs (14) have reported that the acetoacetyl-CoA deacylase pathway is not physiologically significant in the formation of acetoacetate. Although their assay differed from that cited in this paper in several respects (such as lack of preincubation with iodoacetamide, a greater concentration of iodoacetamide, and the use of unpurified acetoacetyl CoA as substrate), the acetoacetyl-CoA deacylase activity these authors observed, rather than being insignificant, could be quite important physiologically. According to their data a liver from a normal rat could produce 3.3 μmoles of acetoacetate per minute via deacylation. Although this value is probably excessive, because of the use of impure acetoacetyl CoA and the consequent measurement of acetoacetyl-glutathione hydrolase in addition to acetoacetyl-CoA deacylase, it gives some idea of the potential magnitude for acetoacetate production via this pathway.

Thus, it may be concluded that cholesterol synthesis from acetate is diminished in livers from CPIB-treated rats and that there is an increase in acetoacetyl-CoA deacylase activity in mitochondria from the same rats.

These observations are consistent with each other, but it is impossible to state that a cause and effect relationship exists. If there is such a relationship, then CPIB is an example of an agent whose mechanism of action in decreasing cholesterol biosynthesis is potentiation of an enzyme rather than inhibition of one.

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References

1. Avoy, D. R., E. A. Swyryd, and R. G. Gould. 1965. *J. Lipid Res.* **6:** 369.
2. Burch, R. E., and D. Triantafillou. 1968. *Biochemistry.* **7:** 1009.
3. Drummond, G. I., and J. R. Stern. 1960. *J. Biol. Chem.* **235:** 318.
4. Stewart, P. R., and H. Rudney. 1966. *J. Biol. Chem.* **241:** 1212.
5. Burch, R. E., H. Rudney, and J. J. Irias. 1964. *J. Biol. Chem.* **239:** 4111.
6. Sauer, F., and J. D. Erfle. 1966. *J. Biol. Chem.* **241:** 30.
7. Decker, K. 1963. *In* Methods of Enzymatic Analysis. H. U. Bergmeyer, editor. Academic Press, Inc., New York. 425–428.
8. Mellanby, J., and D. H. Williamson. 1963. *In* Methods of Enzymatic Analysis. H. U. Bergmeyer, editor. Academic Press, Inc., New York. 454–458.
9. Wieland, T., and L. Rueff. 1953. *Angew. Chem. Int. Ed. Engl.* **65:** 186.
10. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. *J. Biol. Chem.* **193:** 265.
11. Curran, G. L., and R. L. Costello. 1956. *J. Exp. Med.* **103:** 49.
12. Schneider, W. C., A. Claude, and G. H. Hogeboom. 1948. *J. Biol. Chem.* **172:** 451.
13. Azarnoff, D. L., D. R. Tucker, and G. A. Barr. 1965. *Metabolism.* **14:** 959.
14. Williamson, D. H., M. W. Bates, and H. A. Krebs. 1968. *Biochem. J.* **108:** 353.