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STUDIES ON THE HYPOGLYCAEMIC COMPOUND CYCLOPROPANECARBOXYLIC ACID

EFFECTS ON FATTY ACID OXIDATION *IN VITRO*

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Abstract—The effects of the species specific hypoglycaemic agent cyclopropanecarboxylic acid and its carnitine ester on certain synthetic and oxidative mitochondrial processes in rat and guinea-pig liver were examined. No major species differences were found in the following responses. Cyclopropanecarboxylate (0·2 mM) inhibited oxygen uptake and $^{14}CO_2$ evolution from [1-^{14}C]acetate and [1-^{14}C]palmitate but not from [1-^{14}C]laurate, [1-^{14}C]octanoate or [1-^{14}C]butyrate. Palmitoyl carnitine utilization was not significantly affected but palmitoyl CoA levels were decreased. The oxidation of tricarboxylic acid cycle intermediates was not significantly inhibited. Acetoacetate synthesis from fatty acids was inhibited possibly at the stage of HMG CoA formation. Cyclopropanecarboxyl carnitine had little effect on fatty acid oxidation and ketogenesis. Possible mechanisms for the inhibitory effects of cyclopropanecarboxylate are discussed.

THE EFFECTS of cyclopropanecarboxylic acid, an orally active hypoglycaemic agent, have been investigated by Stewart.[1] He found marked species differences in the hypoglycaemic response, guinea-pigs being most sensitive and rats virtually unresponsive, but ketosis resulted in all species. The possible link between hypoglycaemic effect and fatty acid metabolism led Duncombe and Rising[2] to examine the relation of cyclopropanecarboxylate to lipid metabolism. It was found that the cyclopropyl ring was incorporated into long-chain fatty acids *in vitro* in tissues from both guinea-pigs and rats, but the results did not explain the hypoglycaemic effect or its variation with species.

Since the oxidation of fatty acids provides an alternative glucose-sparing source of energy for the cell it was thought that any effects of cyclopropanecarboxylate on β-oxidation might help to explain the hypoglycaemic effect and species differences. We have therefore extended the work on lipid metabolism to an investigation of the actions of cyclopropanecarboxylate and some of its derivatives on mitochondrial fatty acid oxidation and ketone body synthesis in tissues from guinea-pigs and rats, as representing sensitive and insensitive species.

MATERIALS AND METHODS

Reagents. Cyclopropanecarboxylic acid was synthesized by the method of McCloskey and Coleman,[3] and ^{14}C-labelled cyclopropanecarboxylic acid was synthesized as described by Duncombe and Rising.[2] Both were used as their sodium salts in all experiments. Palmitoyl carnitine was prepared according to Bremer.[4] Cyclopropanecarboxyl carnitine was synthesized from DL-carnitine hydrochloride and the

acid chloride by essentially the same method as for palmitoyl carnitine. The yield was about 60 per cent when based on the amount of DL-carnitine used; the product was soluble in both ethanol and water and had a melting point of 120°. Suspensions of ^{14}C-labelled long chain fatty acids were prepared by adding an aqueous suspension of the inactive acid sodium salt to a minimal ethereal solution of the labelled acid and shaking until the ether had evaporated. Before use this was immersed in an ultrasonic water bath for several minutes to produce a very finely divided suspension, homogeneous enough to give counting rates of ± 3 per cent.

[1-^{14}C]Acetyl CoA was purchased from Boehringer, Mannheim, and all other labelled materials were obtained from the Radiochemical Centre, Amersham, Bucks.

Tissue preparations. Rat and guinea-pig livers were homogenized in 0·25 M sucrose–0·1 mM EDTA and mitochondria prepared according to the method of Johnson and Lardy.[5] Mitochondria were finally suspended in 0·25 M sucrose at a concentration of 10–20 mg protein/ml as determined by the method of Lowry *et al.*[6] Electron microscopic examination showed these preparations to be largely devoid of broken mitochondria and nonmitochondrial particles.

Incubations. When oxygen uptake was to be measured, mitochondria were incubated in Warburg flasks at 37° in a gas phase of air with a shaking rate of 1 stroke/sec. Each flask contained potassium phosphate buffer, pH 7·0 (20 mM); $MgCl_2$ (0·014 M); KCl (0·1 M); ATP (1·0 mM); AMP (1·0 mM); sodium succinate (1·0 mM); mitochondrial suspension (1 ml) plus substrate solution or water to a total volume of 2·5 ml. Sodium succinate was replaced by 0·5 mM fumarate when acetoacetate was to be determined since it has been shown by Martin and De Luca[7] that succinate reduces acetoacetate conversion to β-hydroxybutyrate. The centre well contained 0·2 ml of 10% KOH and the side arm 0·3 ml of either 3·4 M citric acid or 1·4 M trichloroacetic acid. The reaction was started by the addition of the mitochondrial suspension. At the end of the incubation period (30–40 min) the side arms were tipped, shaking being continued for a further 15 min before the final manometric reading was recorded and the KOH removed. $^{14}CO_2$ was determined by the method of Duncombe and Rising.[8] When either the rate of $^{14}CO_2$ evolution or total $^{14}CO_2$ production solely was to be determined, incubations were carried out in modified 25 ml conical flasks. These flasks contained a centre well and were fitted with a rubber serum cap, thus allowing the KOH in the well to be removed and replaced at suitable intervals by means of a hypodermic syringe.

Extraction of cyclopropanecarboxylic acid derivatives. Incubations were terminated with conc. HCl (0·2 ml) and the flask contents extracted with butanol. The extracts were run on silica gel TLC in two different solvents: (1) $CHCl_3$–MeOH–H_2O, 70:30:5 and (2) ethanol–H_2O–NH_4OH, 96:28:4, the position of the radioactive bands being located by autoradiography. The relative percentages of the metabolites were estimated by scraping off the silica gel and counting it in Bray's[9] scintillator containing 4 per cent finely divided silica.

Assay procedures. Palmitoyl CoA synthesis was assayed by the method of Van Tol *et al.*[10] This procedure was also adapted for use with whole, unsonicated mitochondria, when not less than 75 per cent of the total radioactivity was recovered in $^{14}CO_2$, [^{14}C]-palmitate and its CoA and carnitine esters. Acetoacetic acid was determined by the method of Wadkins and Lehninger[11] and β-hydroxybutyrate by the method of Williamson and Mellanby.[12] To measure the incorporation of ^{14}C into the carboxyl

group of acetoacetic acid 0·4 ml of aniline citrate solution (3·75 g of aniline-HCl plus 6 ml of 62·5% citric acid) was tipped from the side arm of the Warburg flask and the evolved $^{14}CO_2$ collected in the normal way.[13,14] Radioactivity was determined by counting in a Packard Tri-Carb liquid scintillation spectrometer, followed by internal standardization and calculation of absolute radioactivities.

Statistical analysis. The P values for the significance of the difference between means were calculated, where applicable, by the Student's *t*-test using a computer programme.

RESULTS

The effects of cyclopropanecarboxylate and its carnitine ester on fatty acid oxidation by rat and guinea-pig liver mitochondria. The results shown in Tables 1 and 2 and in Fig. 1 demonstrate typical rates of oxidation of palmitic acid and its carnitine ester

TABLE 1. THE EFFECT OF CYCLOPROPANECARBOXYLATE AND CYCLOPROPANECARBOXYL CARNITINE ON THE RATES OF OXIDATION OF PALMITATE AND PALMITOYL CARNITINE IN RAT LIVER MITOCHONDRIA

Substrate	Addition		% Inhibition of total oxygen uptake	P
Palmitate	Cyclopropanecarboxylate	0·2 mM	38 (32–50)	0·001
		1·0 mM	33 (30–36)	—
		2·0 mM	44 (42–48)	0·001
	Cyclopropanecarboxylate plus DL-carnitine 1 mM	1·0 mM	+ 14	—
	Cyclopropanecarboxyl carnitine	0·2 mM	25 (23–27)	—
		1·0 mM	+ 7	0·1
		2·0 mM	+ 25	0·001
Palmitoyl carnitine	Cyclopropanecarboxylate	0·2 mM	10 (8–20)	0·05
		2·0 mM	17 (10–24)	0·01
	Cyclopropanecarboxyl carnitine	0·2 mM	17 (13–20)	—
		2·0 mM	+ 4	N.S.

Experimental details were as given in the Methods section. Palmitic acid and Palmitoyl carnitine were at a final concentration of 1 mM. The results are the means of the percentage inhibition in two to five experiments of the total oxygen uptake, with the ranges given in parenthesis. Triplicate determinations were carried out in all experiments. The control rates of oxygen uptake were (mμg atoms/min/mg of protein): palmitate, 20–30; palmitoyl carnitine 25–35.

and the effects thereon of cyclopropanecarboxylate and cyclopropanecarboxyl carnitine. In general the oxidation rates were linear for the duration of the incubation period. At concentrations of between 0·2 mM and 2·0 mM cyclopropanecarboxylate inhibited oxygen uptake by liver mitochondria utilizing palmitate by up to 44 per cent in rats and 35 per cent in guinea-pigs. In rat liver mitochondria cyclopropanecarboxyl carnitine at concentrations of 1 mM and 2 mM did not inhibit palmitate oxidation but stimulated oxygen uptake. However, when the concentration was decreased to 0·2 mM, palmitate oxidation was inhibited. In guinea-pig liver mitochondria at a carnitine ester concentration of 2·0 mM there was again inhibition of oxidation. In liver mitochondria from both species cyclopropanecarboxylate (2·0 mM) had a small inhibitory

TABLE 2. THE EFFECT OF CYCLOPROPANECARBOXYLATE AND CYCLOPROPANECARBOXYL CARNITINE ON THE RATES OF OXIDATION OF PALMITATE AND PALMITOYL CARNITINE IN GUINEA-PIG LIVER MITOCHONDRIA

Substrate	Addition		% Inhibition of total oxygen uptake	P
Palmitate	Cyclopropanecarboxylate	0·2 mM	17 (13–25)	0·01
		2·0 mM	35 (31–39)	—
	Cyclopropanecarboxyl carnitine	2·0 mM	7 (2–13)	0·1
Palmitoyl carnitine	Cyclopropanecarboxylate	0·2 mM	10 (7–21)	0·05
		2·0 mM	15 (11–19)	—
	Cyclopropanecarboxyl carnitine	2·0 mM	0	—

Experimental details were as given in the Methods section. Palmitic acid and Palmitoyl carnitine were at a final concentration of 1 mM. The results are the means of the percentage inhibition in two to four experiments of the total oxygen uptake, with the ranges given in parenthesis. Triplicate determinations were carried out in all experiments. The control rates of oxygen uptake were (mμg atoms/min/mg of protein): palmitate, 18–25; palmitoyl carnitine 25–30.

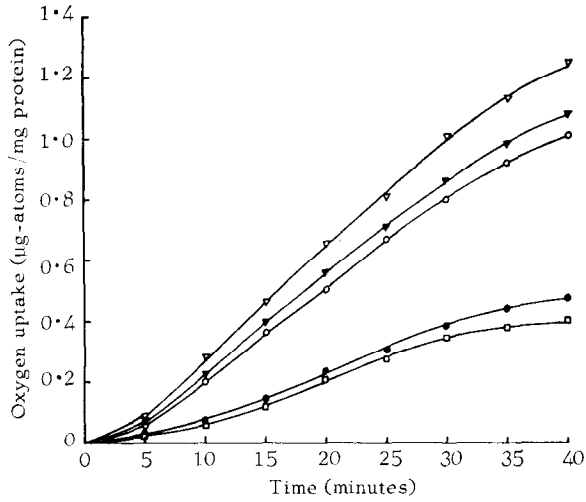

FIG. 1. Rates of oxidation of palmitate and palmitoyl carnitine in rat liver mitochondria incubated with cyclopropanecarboxylate. General conditions of incubation were as described in the Methods section, with a mitochondrial protein concentration of 19 mg/ml. □, endogenous; ○, palmitate (1 mM); ●, palmitate (1 mM) plus cyclopropanecarboxylate (2 mM); ▽, palmitoyl carnitine (1 mM); ▼, palmitoyl carnitine (1 mM) plus cyclopropanecarboxylate (2 mM).

effect (about 5 per cent) on endogenous respiration, and its carnitine ester (2·0 mM) increased the endogenous oxidation rate by approximately 15 per cent.

In further experiments, palmitoyl carnitine was used as the oxidizable substrate. The role of carnitine in the stimulation of fatty acid oxidation has been attributed to its ability to transfer long chain fatty acyl groups across the mitochondrial permeability barrier as their carnitine esters.[4,15] Since fatty acyl CoA formed in the external mitochondrial compartment does not penetrate this barrier, but first must be transacylated

to the carnitine ester for translocation to the site of β-oxidation, the use of palmitoyl carnitine might provide some information about the actual site of inhibition.

In both rat and guinea-pig mitochondria utilizing palmitoyl carnitine rather than the free acid, the degree of inhibition of oxidation caused by cyclopropanecarboxylate was reduced to about 10 per cent, suggesting that fatty acid activation was being inhibited in some way. Again the effect of cyclopropanecarboxyl carnitine seemed to depend on its concentration (Table 1).

Tables 3 and 4 and Fig. 2 show the effects of cyclopropanecarboxylate and its carnitine ester on $^{14}CO_2$ evolution from 1-^{14}C-labelled fatty acids. When [1-^{14}C]laurate

TABLE 3. THE EFFECTS OF CYCLOPROPANECARBOXYLATE AND CYCLOPROPANECARBOXYL CARNITINE ON $^{14}CO_2$ EVOLUTION AND ACETOACETATE SYNTHESIS FROM ^{14}C-LABELLED FATTY ACIDS IN RAT LIVER MITOCHONDRIA

Substrate	Addition	$^{14}CO_2$ evolved	Acetoacetate concentration
[1-^{14}C]lauric acid	None (control)	53 ± 6	42 ± 4
	Cyclopropanecarboxylate	106 (103–109)*	68 (64–73)†
	Cyclopropanecarboxyl carnitine	99 (95–104)	94 (93–95)
	Cyclopropanecarboxylate plus DL-carnitine	93 (85–100)	129 (125–133)
[1-^{14}C]octanoic acid	None (control)	39 ± 2	70 ± 0
	Cyclopropanecarboxylate	124 (122–124)	67 (63–70)
[1-^{14}C]palmitic acid	None (control)	9·8 ± 2·4	18 ± 3
	Cyclopropanecarboxylate	17 (10–37)†	53 (45–58)†
	Cyclopropanecarboxyl carnitine	88 (86–90)	41 (40–43)
[1-^{14}C]palmitoyl carnitine	None (control)	22·3 ± 1·2	40 ± 13
	Cyclopropanecarboxylate	77 (75–79)	105 (96–114)

Experimental details were as given in the Methods section, with 1·4 M trichloroacetic acid in the side arm. All fatty acid substrates were at a concentration of 1 mM except lauric acid (0·15 mM), which has been shown by Enser[20] to be surface-active and to inhibit its own oxidation at concentrations above 0·2 mM. $^{14}CO_2$ evolution and acetoacetate concentration are expressed as the mean values of the percentage of control of two to four experiments, with the ranges given in parenthesis. Control values (means ± S.E.M.) are expressed as the percentage of initial substrate radioactivity for $^{14}CO_2$ evolution and as nmoles/mg protein for acetoacetate concentration. All additives were at a final concentration of 0·2 mM. Significance of the difference: * P is not significant; † $P \leq 0{\cdot}05$.

was the substrate, cyclopropanecarboxylate tended to increase the amount of $^{14}CO_2$ evolved in both rat and guinea-pig mitochondria, although the difference was not significant in rats. Carnitine and cyclopropanecarboxyl carnitine had little or no effect in rat mitochondria but in guinea-pig preparations the carnitine ester generally increased $^{14}CO_2$ production, thus demonstrating a distinct difference between the two species.

When [1-^{14}C]palmitic acid was the substrate, on the other hand, the hypoglycaemic agent at a concentration of 0·2 mM greatly inhibited oxidation by mitochondria, the $^{14}CO_2$ released being reduced by 83 per cent in rats and by 75 per cent in guinea-pigs. That the inhibition of $^{14}CO_2$ evolution was greater than the inhibition of the measured

TABLE 4. THE EFFECTS OF CYCLOPROPANECARBOXYLATE AND CYCLOPROPANECARBOXYL CARNITINE ON $^{14}CO_2$ EVOLUTION AND ACETOACETATE SYNTHESIS FROM ^{14}C-LABELLED FATTY ACIDS IN GUINEA-PIG LIVER MITOCHONDRIA

Substrate	Addition	$^{14}CO_2$ evolved	Acetoacetate concentration
[1-^{14}C]lauric acid	None (control)	47 ± 5	54 ± 6
	Cyclopropanecarboxylate	126 (115–133)*	50 (44–60)*
[1-^{14}C]octanoic acid	None (control)	31 ± 3	104 ± 9
	Cyclopropanecarboxylate	120 (115–125)	86 (82–90)
[1-^{14}C]palmitic acid	None (control)	4·9 ± 2·4	6 ± 2
	Cyclopropanecarboxylate	25 (15–30)*	40 (35–45)
	Cyclopropanecarboxyl carnitine	54 (48–59)	60 (45–75)
Palmitoyl carnitine	None (control)	—	16 ± 3
	Cyclopropanecarboxylate	—	68 (65–71)

Significance of the difference: * $P \leq 0{\cdot}05$. Experimental details were as given in the Methods section. All other conditions were the same as for Table 3, except that two or three experiments were carried out.

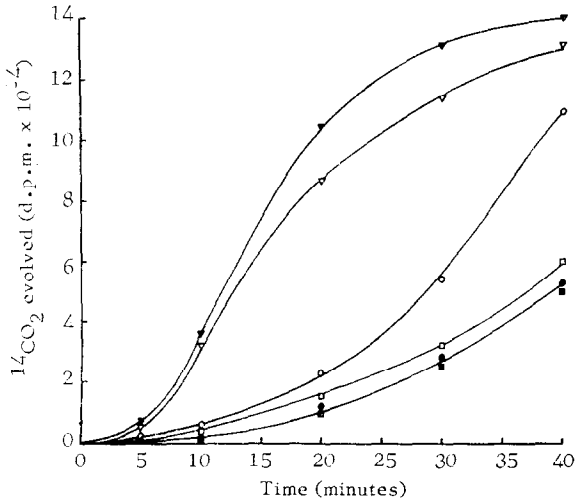

FIG. 2. The effects of added cofactors and cyclopropanecarboxylate on $^{14}CO_2$ evolution from [1-^{14}C]palmitate in rat liver mitochondria. General conditions of incubation and $^{14}CO_2$ measurement were as described in the Methods section. All samples contained [1-^{14}C]palmitate (1 mM, 0·2 μC/μmole) with, in addition: ○, control; ●, cyclopropanecarboxylate (0·2 mM); ▽, DL-carnitine (2 mM); ▼, DL-carnitine (2 mM) plus cyclopropanecarboxylate (0·2 mM); □, CoA (0·2 mM); ■, CoA (0·2 mM) plus cyclopropanecarboxylate (0·2 mM).

oxygen uptake was due to the presence in the incubation medium of succinate as sparker. Much less inhibition was caused by the carnitine ester of cyclopropanecarboxylic acid. The inclusion of CoA in the incubation medium decreased [1-^{14}C]-palmitate utilization as was reported by Ontko and Jackson.[16] Evolution of $^{14}CO_2$ from octanoic acid, like that from lauric acid, was not influenced by cyclopropanecarboxylate. It therefore seemed likely that the activation and not the β-oxidation of long chain fatty acids was being inhibited.

That the effects of cyclopropanecarboxylate on palmitate oxidation were not influenced by the presence of succinate in low concentration was concluded from the results of additional experiments using [1-^{14}C]palmitate as substrate with succinate either at its normal concentration of 1·0 mM or at 0·1 mM. The following observations were made. (1) Palmitate successfully competed with succinate for mitochondrial entry and suppressed succinate oxidation. (2) $^{14}CO_2$ evolution in the presence of cyclopropanecarboxylate was independent of the succinate concentration, whereas $^{14}CO_2$ evolution in control samples was reduced when using the lower sparker concentration.

The effect of cyclopropanecarboxylate on the oxidation of tricarboxylic acid cycle intermediates. When ^{14}C-carboxyl labelled long chain fatty acids are oxidized in the mitochondria, the $^{14}CO_2$ is ultimately derived from the oxidation of an acetyl group *via* the tricarboxylic acid cycle. To investigate whether the inhibition of fatty acid oxidation was a result of decreased oxidation of tricarboxylic acid cycle intermediates, a number of ^{14}C-labelled intermediates were used as oxidative substrates in the rat liver mitochondrial preparations. The results are given in Table 5. In one experiment (not reported in detail), using succinate as substrate with varying concentrations of AMP, rates of respiration were not affected by the absence from the incubation medium of an ADP generating system. Cyclopropanecarboxylate had little effect on oxygen uptake or $^{14}CO_2$ evolution when either acetyl CoA, citrate or succinate were the substrates but significantly inhibited $^{14}CO_2$ evolution from [1-^{14}C]pyruvate and greatly inhibited the utilization of acetate. These results would indicate that the cyclopropanecarboxylate inhibition of palmitate oxidation was at a stage prior to the tricarboxylic acid cycle. The depression of both acetate and [1-^{14}C]pyruvate oxidation was probably due to the inhibition of acetyl CoA formation before entry into the tricarboxylic acid cycle.

The effect of cyclopropanecarboxylate on ketone body synthesis in rat and guinea-pig liver mitochondria. The synthesis of acetoacetic acid from octanoate, laurate and palmitate was inhibited by cyclopropanecarboxylate in both species (Tables 3 and 4). However, there was generally a greater inhibitory effect in guinea-pigs than in rats. This difference is in accordance with the results reported for $^{14}CO_2$ evolution, since when ketone body synthesis is being inhibited more acetyl CoA will be available for tricarboxylic acid cycle oxidation. Again the effects of carnitine and cyclopropanecarboxyl carnitine varied according to both the species and the acid employed. In two experiments (not reported in detail) there was no major effect on β-hydroxybutyrate formation.

To find the site of inhibition we observed the effects of cyclopropanecarboxylate on the more immediate precursors of ketone bodies—butyric acid and acetyl CoA—both of which were labelled in the carboxyl position. Both substrates would contribute to the [1-^{14}C]acetyl CoA pool and therefore both would be incorporated into acetoacetate *via* the HMG CoA* pathway[17,18] or be oxidized *via* the tricarboxylic acid cycle. Since acetoacetate is not oxidized in liver,[19] any label incorporated into the ketone body would not be lost and therefore could be determined. The results from Tables 6 and 7 show that cyclopropanecarboxylate generally increased oxygen uptake and inhibited both acetoacetate formation and the incorporation of label into the carboxyl

* Abbreviation used: HMG CoA=β-Hydrox-β.methylglutaryl CoA.

TABLE 5. $^{14}CO_2$ EVOLUTION AND OXYGEN UPTAKE WITH ^{14}C-LABELLED TRICARBOXYLIC ACID CYCLE INTERMEDIATES IN RAT LIVER MITOCHONDRIA

Substrate	Addition	O_2 uptake	% difference from control	$^{14}CO_2$ evolved	% difference from control
[1-^{14}C]succinate (1·0 mM)	None (control)	12·3 ± 0·5	—	9·31 ± 0·01	—
(1·88 × 10^5 dis./min)	Cyclopropanecarboxylate (1·0 mM)	11·1 ± 0·6	9·8	8·92 ± 0·01	− 4·2
Acetate (1·0 mM)	None (control)	5·5 ± 0·3	—		
	Cyclopropanecarboxylate (1·0 mM)	1·5 ± 0·3	73·2		
Citrate (1·0 mM)	None (control)	10·7 ± 0·2	—		
	Cyclopropanecarboxylate (1·0 mM)	10·1 ± 0·3	4·8		
[1-^{14}C]citrate (0·1 mM)	None (control)			4·59 ± 0·10	—
(0·74 × 10^5 dis./min)	Cyclopropanecarboxylate (1·0 mM)			4·19 ± 0·07	− 8·7
+ 1 mM acetate					
[1-^{14}C]acetate (0·1 mM)	None (control)			49·80 ± 0·83	—
(4·24 × 10^6 dis/min)					
+ 1 mM citrate	Cyclopropanecarboxylate (1·0 mM)			3·42 ± 0·04	− 93·1
[1-^{14}C]acetate (1·0 mM)	None (control)			30·76 ± 0·90	—
(1·37 × 10^7 dis./min)					
+ 0·1 mM succinate	Cyclopropanecarboxylate (0·2 mM)			6·85 ± 2·03	− 77·7
	CoA (0·05 mM)			32·70 ± 1·60	+ 6·3
	CoA (0·05 mM) + Cyclopropanecarboxylate (0·2 mM)			5·58 ± 0·11	− 81·9
[1-^{14}C]acetyl CoA (0·2 mM)	None (control)			0·71 ± 0·03	—
(3·27 × 10^6 dis./min)	Cyclopropanecarboxylate (0·2 mM)			0·71 ± 0·01	0·0
+ 0·1 mM succinate					
[1-^{14}C]pyruvate (5·0 mM)	None (control)			1·19 ± 0·08	—
(2·31 × 10^5 dis./min)	Cyclopropanecarboxylate (0·2 mM)			0·80 ± 0·12	− 32·8
+ 1 mM fumerate					

Experimental details were as given in the Methods section. Oxygen uptake (mμg. atoms/min/mg protein), and $^{14}CO_2$ evolved (total dis./min × 10^{-4}) are expressed as the means ± S.E.M. for each treatment in a single mitochondrial experiment.

TABLE 6. THE EFFECTS OF CYCLOPROPANECARBOXYLATE ON OXIDATION AND KETOGENESIS FROM [1-^{14}C]BUTYRATE AND [1-^{14}C]ACETYL CoA IN RAT LIVER MITOCHONDRIA

Substrate	Determination*	Addition		% difference from control	P
		None	Cyclopropane-carboxylate		
[1-^{14}C]butyric acid	A	26·6 ± 0·7	28·8 ± 0·9	+ 8·3	0·02
(5·35 × 10^4 dis./min)	B	2·65 ± 0·04	3·44 ± 0·03	+ 33·6	0·01
	C	65 ± 2	42 ± 2	− 35·4	0·001
	D	0·87 ± 0·14	0·57 ± 0·08	− 34·5	—
Butyrate + [1-^{14}C]acetyl CoA	A	28·9 ± 2·6	24·9 ± 1·0	− 13·8	—
(0·3 × 10^{-6} M)	B	1·17 ± 0·02	0·40 ± 0	− 65·8	—
	C	95 ± 6	80 ± 4	− 15·8	N.S.
Butyrate + [1-^{14}C]acetyl CoA	B	6·01 ± 0·21	3·01 ± 0·11	− 49·9	0·001
(1·6 × 10^{-6} M)	D	0·50 ± 0·05	0·22 ± 0·01	− 56·0	—
[1-^{14}C]acetyl CoA	B	2·28 ± 0·10	1·14 ± 0·04	− 50·0	—
(0·5 × 10^{-6} M)					

Experimental details were as given in the Methods section. Each sample contained either [1-^{14}C]butyric acid (1 mM) or [1-^{14}C]acetyl CoA (45·5 mc/m-mole), or both. Cyclopropanecarboxylate was at a concentration of 0·2 mM. Each result is the mean ± S.E.M. of two to four experiments.

* Determinations: A = oxygen uptake; mμg. atoms/min/mg protein, B = $^{14}CO_2$ evolution: total dis./min × 10^{-4}, C = acetoacetate synthesized: n-moles/mg. protein, D = ^{14}C incorporated into the carboxyl group of acetoacetate; total dis./min × 10^{-4}.

TABLE 7. THE EFFECTS OF CYCLOPROPANECARBOXYLATE ON OXIDATION AND KETOGENESIS FROM [1-^{14}C]BUTYRATE AND [1-^{14}C]ACETYL CoA IN GUINEA-PIG LIVER MITOCHONDRIA

Substrate	Determinations*	Addition: None	Addition: Cyclopropanecarboxylate	% difference from control	P
[1-^{14}C]butyric acid	A	17·1 ± 0·6	18·8 ± 0·4	+ 9·9	0·02
(5·48 × 10^4 dis./min)	B	2·27 ± 0·15	2·66 ± 0·04	+ 17·2	0·05
	C	66 ± 7	18 ± 5	− 72·7	0·01
	D	0·82 ± 0·09	0·27 ± 0·03	− 67·1	0·001
[1-^{14}C]acetyl CoA	B	5·26 ± 0·41	5·42 ± 0·10	+ 3·0	N.S.
(3·0 × 10^5 dis./min) + butyrate	D	0·38 ± 0·04	0·13 ± 0·00	− 65·8	—

Experimental details were as given in the Methods section. Each sample contained either [1-^{14}C]butyric acid (1 mM) or [1-^{14}C]acetyl CoA (1·2 × 10^{-6} M) plus butyrate (1 mM). Cyclopropanecarboxylate was at a concentration of 0·2 mM. Each result is the mean ± S.E.M. of two to four experiments.

* Determinations A = oxygen uptake; mμg. atoms/min/mg protein, B = $^{14}CO_2$ evolution: total dis./min × 10^{-4}, C = acetoacetate synthesized: nmoles/mg protein, D = ^{14}C incorporated into the carboxyl group of acetoacetate; total dis./min × 10^{-4}.

TABLE 8. EFFECTS OF CYCLOPROPANECARBOXYLATE ON $^{14}CO_2$ EVOLUTION AND PALMITIC ACID ESTER FORMATION IN RAT LIVER WHOLE AND SONICATED MITOCHONDRIA

Addition	Mitochondria	No. of expts.	$^{14}CO_2$	Palmitate	Palmitoyl CoA	Palmitoyl carnitine
None (control)	Whole	4	6·7	96·9	1·6	1·5
Cyclopropanecarboxylate			1·4*	98·6*	0·5*	0·9†
CoA	Whole	2	10·6	71·6	27·9	0·5
Cyclopropanecarboxylate + CoA			6·2	78·9	19·7	1·4
Carnitine	Whole	1	39·0	83·4	10·3	6·3
Cyclopropanecarboxylate + carnitine			45·2	90·5	5·5	4·0
Carnitine + CoA	Whole	1	35·5	88·5	6·4	5·1
Carnitine + CoA + cyclopropanecarboxylate			38·5	91·1	5·8	3·1
None (control)	Sonicated	3		63·8	35·8	0·4
Cyclopropanecarboxylate				71·0	28·8†	0·2

Significance of the difference: * $P \leq 0{\cdot}01$. † $0{\cdot}1 \geq P \geq 0{\cdot}05$.

For whole mitochondria the normal incubation medium was used plus 0·5 mM [1-^{14}C]palmitate; incubation time 30 min. For sonicated mitochondria the basic medium of Van Tol *et al.*[10] excluding carnitine but containing CoA was used; incubation time 15 min. The concentrations of additional substrates were: cyclopropanecarboxylate 0·2 mM; DL-carnitine 2·0 mM; CoA 0·2 mM. $^{14}CO_2$ evolution is expressed as the percentage of the initial palmitate radioactivity. [^{14}C]palmitate and its CoA and carnitine esters are expressed as mean percentages of the total radioactivity recovered in the three compounds. Determinations in each experiment were carried out in duplicate.

group of acetoacetate, these last two effects being greater in guinea-pig. When [1-^{14}C]-acetyl CoA (0·5–1·6 $\times$ 10^{-6} M) was used as the labelled substrate, there was inhibition of $^{14}CO_2$ production in rats but not in guinea-pigs. This inhibition in rat liver mitochondria, however, was not found when using [1-^{14}C]acetyl CoA at a concentration of 2×10^{-4} M (Table 5). These are further examples of concentration and species differences. A more detailed interpretation of these results is complicated by the probability that acetyl CoA undergoes recycling and does not enter a common pool. However, the observed increase in $^{14}CO_2$ evolution might be explained by the cyclopropanecarboxylate inhibition of HMG CoA synthase (EC 4.1.3.5), thereby directing more [1-^{14}C]acetyl CoA into the tricarboxylic acid cycle and less into acetoacetate synthesis.

The effect of cyclopropanecarboxylate on palmitoyl CoA synthesis in rat liver mitochondria. The results shown in Table 8 suggest that the effect of cyclopropanecarboxylate on palmitate utilization was due to the inhibition of palmitoyl CoA synthesis. The addition of CoA to the medium largely overcame the inhibition of $^{14}CO_2$ evolution as would be expected on the basis of this hypothesis. When carnitine or both carnitine and CoA were present, cyclopropanecarboxylate had little effect, the major observation being a stimulation of $^{14}CO_2$ evolution. As can be seen from Fig. 2, this increase only appeared after the first 10 min and was probably due to a later effect of cyclopropanecarboxylate, possibly on the transport of acetyl CoA derivatives out of the mitochondria. The results using sonicated mitochondria supported those reported for the intact organelle.

The metabolism of cyclopropanecarboxylate in rat and guinea-pig liver mitochondria. The results from TLC and autoradiography showed that there were always two and sometimes four labelled metabolites of [1-^{14}C]cyclopropanecarboxylate (0·2 mM). In rat liver mitochondria one metabolite (about 3 per cent of the total radioactivity) co-chromatographed in both solvent systems with [^{14}C]cyclopropanecarboxyl carnitine. When DL-carnitine (0·2 mM) was added to the mitochondrial incubation medium, this percentage increased to 12 per cent. Another metabolite, consistently observed from both rat and guinea-pig preparation, remained at or close to the origin. By analogy with the separation of long chain fatty acid esters in these solvent systems, it is possible that this metabolite (1–3 per cent of the total radioactivity) was the CoA ester of cyclopropanecarboxylic acid.

DISCUSSION

We have shown that cyclopropanecarboxylate inhibits the oxidation of long chain fatty acids and the synthesis of acetoacetate in both rat and guinea-pig liver mitochondria. Senior *et al.*,[21] while investigating the hypoglycaemic activities of a number of short chain fatty acids, reported a lower inhibitory effect of cyclopropanecarboxylate on palmitate oxidation in rat liver mitochondria than was found by us, but their incubation medium contained DL-carnitine. Our results for the inhibition of short chain fatty acid oxidation and acetoacetate synthesis are in agreement with those reported by them.

In unpublished experiments we found by using [1-^{14}C]cyclopropanecarboxylate in guinea-pigs *in vivo* that 120 nmoles of the hypoglycaemic agent were incorporated/g wet wt. of liver 2 hr after a subcutaneous injection of the minimum hypoglycaemic dose (31 mg/kg). This is of the same order as that found for *in vitro* incubations—42 $\pm$ 8 nmoles/g wet wt. of liver/2 hr when cyclopropanecarboxylate was at a

concentration of 0·1 mM. It is thus likely that any effect found *in vitro* when using the lowest inhibitory concentration, 0·2 mM, is relevant to events in the intact animal after a hypoglycaemic dose.

Cyclopropanecarboxylic acid was found to inhibit both oxygen uptake and CO_2 evolution in liver mitochondria of rat and guinea-pig, with palmitate as substrate. Since there was no major effect on these two parameters when using short chain fatty acids and since the degree of inhibition of tricarboxylic acid cycle intermediates was small, this suggests that cyclopropanecarboxylate inhibits a stage prior to β-oxidation; either activation or transport of the long chain fatty acids.

As cyclopropanecarboxylate had little effect on either laurate or octanoate oxidation but inhibited palmitate oxidation it appears that ATP-dependent long chain fatty acyl-CoA synthetase (EC 6.2.1.3) is a possible site of inhibition. (The GTP-dependent enzyme is inhibited by phosphate,[22] which was present in all determinations.) This inhibition could be due either to competition by cyclopropanecarboxylate for CoA or to a direct effect on the enzyme. Both possibilities would explain the decreased synthesis of palmitoyl CoA that we observed. The former is supported by the fact that added CoA reduces the inhibitory effect of cyclopropanecarboxylate on palmitate oxidation (Table 8).

There is now direct evidence for the existence in the mitochondria of two ATP-dependent palmitoyl-CoA synthesizing enzymes, one located in the outer membrane and the other in the inner membrane-matrix compartment.[24,25] In the absence of carnitine and at high concentrations of long-chain fatty acids the latter is responsible for activation, whereas in the presence of carnitine at low fatty acid concentrations the former is predominant.[24] In our experiments the addition of 1 mM DL-carnitine reversed the inhibition of palmitate oxidation by cyclopropanecarboxylate. This reversal can be explained if the inhibitor affects the inner enzyme but not the outer one which is functional in the presence of exogenous carnitine.

When palmitoyl carnitine was used as substrate, cyclopropanecarboxylate only inhibited oxygen consumption by about 10 per cent, even at a concentration of 2 mM. It is thus apparent that the cyclopropanecarboxylate-induced inhibition of palmitate oxidation results from an action at some stage prior to palmitoyl carnitine. This could be at the level of palmitoyl-CoA-carnitine *O*-acyltransferase (EC 2.3.1.–), which is rate-limiting in fatty acid oxidation,[26] possibly due to competition for carnitine by cyclopropanecarboxylate. We have shown that a major metabolite of the latter is its carnitine ester. A similar type of inhibition was reported by Entman and Bressler[27] for hypoglycin or its metabolites, compounds which also contain a cyclopropyl ring.

Cyclopropanecarboxylate also inhibited $^{14}CO_2$ evolution from two other labelled substrates requiring CoA for their oxidation—pyruvate and acetate. In our experiments $^{14}CO_2$ evolution from [1-^{14}C]pyruvate (and thus intramitochondrial acetyl CoA formation) was inhibited to a lesser degree than from [1-^{14}C]acetate, whereas the effect on $^{14}CO_2$ evolution from [1-^{14}C]acetyl CoA depended markedly on the concentration of the thioester (50 per cent inhibition at 0·5 μM; no inhibition at 0·2 mM). These results suggest that cyclopropanecarboxylate has a greater inhibitory effect on extra-mitochondrial acetyl CoA formation than on its formation intramitochondrially from either pyruvate or acetylcarnitine.

Since we found a major metabolite of cyclopropanecarboxylate to be its carnitine ester it is possible that the hypoglycaemic agent requires activation before mitochondrial

entry. If this is the case, then when cyclopropanecarboxylate is incubated with mitochondria in the presence of pyruvate there is sufficient endogenous CoA and carnitine for its activation and transport, resulting in some inhibition of intramitochondrial pyruvate decarboxylation. With exogenous acetyl CoA at a low concentration, cyclopropanecarboxylate competes successfully for available carnitine, resulting in appreciable inhibition of transport of C_2 units, whereas at a high level of acetyl CoA, competition and inhibition is lessened and the carnitine is preferentially utilized in the transport of the more natural substrate. The mechanism of cyclopropanecarboxylate inhibition of acetate oxidation is probably analogous to the inhibition of palmitate oxidation discussed above.

The synthesis of acetoacetate in liver *in vitro* was significantly inhibited from all acid precursors except octanoate. This inhibition is not merely due to decreased rates of fatty acid oxidation, since acids whose oxidation was not effected by cyclopropanecarboxylate also showed decreased conversion to ketone bodies. It is therefore likely that cyclopropanecarboxylate has a direct inhibitory effect on acetoacetate production. It is of interest that production of acetoacetate, like fatty acid oxidation, is associated with CoA. The increased levels of acetoacetate found *in vivo*[1] probably arise from inhibition of the peripheral utilization of ketone bodies.[28]

Whatever the immediate agent concerned, the general effects on fatty acid oxidation that we have found could in principle explain the hypoglycaemia produced in sensitive species by cyclopropanecarboxylate. However, the hope that species differences in hypoglycaemic response might be related to quantitative differences in fatty acid oxidation cannot be sustained, since the greatest inhibition of oxidation was found in mitochondria of an almost unresponsive species, rat. It seems unlikely, therefore, that the inhibition of long chain fatty acid oxidation is the primary stimulus for increased glucose utilization in any species. The reported species differences are, however, often influenced by the presence of carnitine, and it is possible that endogenous carnitine levels may control the hypoglycaemic activity of cyclopropanecarboxylate in different species.

It is evident that the main reason for the hypoglycaemic effect in guinea-pig, and an explanation why rats do not respond, must be sought elsewhere. In the following paper[29] we report on the effects of cyclopropanecarboxylate on carbohydrate metabolism.

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