Biochem. J. (1968) **110**, 511
Printed in Great Britain

Biochemical Effects of the Hypoglycaemic Compound Pent-4-enoic Acid and Related Non-hypoglycaemic Fatty Acids

FATTY ACID OXIDATION

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(*Received* 17 *May* 1968)

1. The effects of the hypoglycaemic compound, pent-4-enoic acid, and of four structurally related non-hypoglycaemic compounds (pentanoic acid, pent-2-enoic acid, cyclopropanecarboxylic acid and cyclobutanecarboxylic acid), on the oxidation of saturated fatty acids by rat liver mitochondria were determined. 2. The formation of $^{14}CO_2$ from [1-^{14}C]palmitate was strongly inhibited by 0·01 mM-pent-4-enoic acid. 3. The inhibition of oxygen uptake was less than that of $^{14}CO_2$ formation, presumably because fumarate was used as a sparker. 4. The oxidation of [1-^{14}C]-butyrate, -octanoate or -laurate was not strongly inhibited by 0·01 mM-pent-4-enoic acid. 5. The other four non-hypoglycaemic compounds did not inhibit the oxidation of any saturated fatty acid when tested at 0·01 mM concentration, though they all inhibited strongly at 10 mM. 6. The oxidation of [1-^{14}C]-myristate and -stearate, but not of [1-^{14}C]decanoate, was strongly inhibited by 0·01 mM-pent-4-enoic acid. 7. The oxidation of [1-^{14}C]palmitate was about 50% carnitine-dependent under the experimental conditions used. 8. The percentage inhibition of [1-^{14}C]palmitate oxidation by pent-4-enoic acid was the same whether carnitine was present or not. 9. Acetoacetate formation from saturated fatty acids was inhibited by 0·1 mM-cyclopropanecarboxylic acid to a greater extent than their oxidation. 10. The other compounds tested inhibited acetoacetate formation from saturated fatty acids proportionately to the inhibition of oxidation. 11. Possible mechanisms for the inhibition of long-chain fatty acid oxidation by pent-4-enoic acid are discussed. 12. There was a correlation between the ability to inhibit long-chain fatty acid oxidation and hypoglycaemic activity in this series of compounds.

Holt, Holt & Böhm (1966) showed that methylenecyclopropylacetic acid, a metabolite of hypoglycin, inhibited the oxidation of long-chain fatty acids, but not of short-chain or medium-chain fatty acids, in rat liver mitochondria. Here we report that the hypoglycaemic compound pent-4-enoic acid (Anderson *et al.* 1958) also inhibits long-chain fatty acid oxidation in mitochondria and that structurally similar yet non-hypoglycaemic short-chain fatty acid compounds are not inhibitory. A preliminary account of some of this work has appeared (Senior & Sherratt, 1967).

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METHODS

Chemicals. DL-Carnitine was obtained from Koch–Light Laboratories Ltd., Colnbrook, Bucks. Decanoic acid, lauric acid, myristic acid, palmitic acid and stearic acid were obtained from Fluka A.-G., Buchs, Switzerland. Hyamine (1 M solution in methanol) was obtained from Nuclear Enterprises Ltd., Chicago, Ill., U.S.A. The 1-^{14}C-labelled saturated fatty acids were obtained from The Radiochemical Centre, Amersham, Bucks. All other chemicals used were described in the preceding paper (Senior & Sherratt, 1968*b*).

Miscellaneous. The animals and equipment used were described in the preceding paper (Senior & Sherratt, 1968*b*).

Measurement of fatty acid oxidation. 1-^{14}C-labelled fatty acids were incubated with mitochondria in Warburg flasks. The incubation medium contained DL-carnitine (1 mM), fumarate (0·4 mM) as sparker, ATP (2 mM), hexokinase (15 units), glucose (40 mM), sucrose (48 mM), KCl (33·6 mM), $MgCl_2$ (12·8 mM), glycylglycine (16 mM), AMP (1 mM),

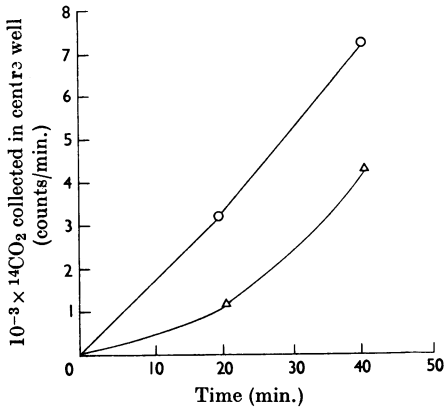

Fig. 1. Retention of $^{14}CO_2$ by the medium during the oxidation of [1-^{14}C]butyrate by rat liver mitochondria. The $^{14}CO_2$ collected in the centre well of the Warburg vessels is shown: ○, collected after addition of 0·2ml. of 3·7N-trichloroacetic acid from the side arm and 10min. further shaking of vessels; △, collected directly without tipping. The O_2 uptake was linear during the incubations. Experimental details are given in the Methods section.

potassium phosphate (18mM) and rat liver mitochondria (4–8mg. of protein) in a final volume 2·50ml., pH7·0. Flasks were incubated at 30°, the shaking rate was 100 strokes/min. and the gas phase was air. The O_2 uptake and acetoacetate production were measured as described in the preceding paper (Senior & Sherratt, 1968*b*). Incubation was for 40min. in all experiments.

Solutions (10mM) of the potassium salts of fatty acids were prepared by neutralizing their solutions at pH12 to pH7·0 with HCl. With acids of chain length greater than 10 carbon atoms suspensions formed, which were easily pipetted. Fatty acid salts (specific radioactivity 0·03–0·1 μc/μmole) were added to the incubation mixture to give final concentrations of 1mM (butyrate, octanoate, decanoate, palmitate and stearate) or 0·16mM (laurate and myristate). The concentrations of the long-chain fatty acids quoted were apparent only, since the acids were added as suspensions. Laurate and myristate were very surface-active, and strongly inhibited their own oxidation at concentrations higher than 0·2mM (Enser, 1964; Ahmed & Scholefield, 1961).

$^{14}CO_2$ was absorbed in either Hyamine or KOH. At first Hyamine was used; 0·25ml. of 1M-Hyamine in methanol was put in the centre well as CO_2 absorbent. Control experiments showed that no filter paper was necessary with the rates of oxidation measured. At the end of incubation 0·10ml. was withdrawn from the centre well with a blood pipette and added to 5·0ml. of scintillation fluid containing 5·0g. of 2,5-diphenyloxazole and 0·3g. of 2,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene in 1l. of toluene. The samples were counted with a counting efficiency of 70% determined from internal standards. This technique was soon discontinued because Hyamine is a viscous substance that solidifies if it absorbs too much CO_2, and it caused inexplicable oscillations of the fluid levels in the manometers when shaking was stopped to take readings.

A technique modified from that of Herberg (1960) was then used, in which 0·25ml. of 2N-KOH was put in the centre well. Again, with the rates of oxidation used filter papers were not necessary. At the end of incubation 0·10ml. was withdrawn from the centre wells and added to 5·0ml. of scintillation fluid consisting of 104g. of naphthalene, 6·5g. of 2,5-diphenyloxazole and 130mg. of 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene in 500ml. of toluene, 500ml. of dioxan and 300ml. of methanol. Counting efficiency was 66%. Similar results were obtained with both methods of CO_2 absorption.

Incubation media containing phosphate buffer retain CO_2 (Umbreit, Burris & Stauffer, 1964). Therefore, to measure total CO_2 production, 0·20ml. of 3·7N-trichloroacetic acid coloured with acid fuchsin was put in the side arms of the Warburg flasks, which were tipped at the end of the incubation period. The acid fuchsin revealed cases of accidental tipping and this dye did not interfere with acetoacetate estimations since it was adsorbed by denatured protein. The incubation was continued for a further 10min. to release retained CO_2. Fig. 1 illustrates the importance of liberation of retained CO_2. This elementary precaution has been overlooked by some workers, and values should be accepted only with reservation where the inhibition of CO_2 evolution is presented as a percentage in such cases. The evolution of CO_2 was not linear (Fig. 1), so the observed percentage inhibition depends on whether or not an acid tip is used. Occasionally some $^{14}CO_2$ was liberated during incubation even when mitochondria were omitted, presumably due to small amounts of contaminants in the radioactive fatty acids. When required the necessary control experiments were done.

RESULTS

Both short-chain and long-chain fatty acids were readily oxidized in this reaction system with rates of oxygen uptake of 60–90mμg.atoms/min./mg. of protein with octanoate and laurate, 40–60 with butyrate and 40–50 with palmitate. The rates were nearly linear for 40min. in the presence of fumarate. If fumarate was omitted the rate of oxygen uptake soon decreased. With palmitate as substrate P/O ratios 1·5–1·7 were obtained (determined as described by Senior & Sherratt, 1968*b*).

Effects of pent-4-enoic acid and related compounds on $^{14}CO_2$ production from 1-^{14}C-labelled fatty acids. In control experiments of 40min. duration 3–8% of the labelled carbon added was recovered as $^{14}CO_2$. Close agreement was usually obtained between replicates.

Pent-4-enoic acid and the control fatty acids at concentrations of 1·0 and 10mM inhibited $^{14}CO_2$ production from all 1-^{14}C-labelled fatty acids used (Table 1). Pent-4-enoic acid at concentrations of 0·01 and 0·1mM strongly inhibited palmitate oxidation. None of the control fatty acids used had such powerful inhibitory effects at these lower concentrations, and pent-4-enoic acid did not strongly inhibit the oxidation of 1-^{14}C-labelled fatty acids with chain lengths of 12 carbon atoms

Table 1. *Effect of pent-4-enoic acid and related compounds on $^{14}CO_2$ production from 1-^{14}C-labelled saturated fatty acids by rat liver mitochondria*

The results are given as means of the percentage inhibition in two to six experiments of total $^{14}CO_2$ production during 40min. and the ranges are given in parentheses. The experimental conditions are given in the Methods section. In control experiments 3–8% of the labelled carbon added was recovered as $^{14}CO_2$.

Compound	Substrate	Inhibition of CO_2 production (%)				
	Concn. of compound ...	1 μM	10 μM	0·1 mM	1·0 mM	10 mM
Pent-4-enoic acid	Butyrate	0	6 (0–16)	36 (24–42)	80 (75–82)	92 (92–93)
	Octanoate	0	0	12·5 (0–25)	56 (51–59)	82 (77–84)
	Laurate	3 (0–9)	2 (0–7)	10 (9–11)	88 (87–91)	95 (93–99)
	Palmitate	7 (5–10)	82 (78–85)	90 (87–91)	90 (89–92)	91 (88–92)
Pentanoic acid	Butyrate	0	8 (6–10)	9 (9, 9)	40 (40, 40)	62 (56–68)
	Octanoate	6 (4–8)	9 (8–9)	9 (9, 9)	14 (11–17)	85 (55–95)
	Laurate	2 (2, 2)	3 (0–6)	34 (33–36)	92 (85–99)	96 (94–98)
	Palmitate	2 (0–4)	11 (9–16)	43 (42–43)	69 (62–74)	62 (57–64)
Pent-2-enoic acid	Butyrate	0	0	17 (13–21)	23 (23–24)	47 (40–62)
	Octanoate	2 (0–4)	3 (0–5)	3 (0–5)	18 (17–19)	85 (83–86)
	Laurate	3 (0–6)	7 (0–17)	13 (0–35)	91 (89–93)	95 (95, 95)
	Palmitate	4 (0–7)	10 (5–16)	54 (49–59)	72 (67–76)	64 (64, 64)
Cyclopropanecarboxylic acid	Butyrate	0	8 (6–11)	16 (15–17)	51 (51–52)	63 (63–65)
	Octanoate	9 (9, 9)	9·5 (2–18)	38 (27–52)	46 (41–51)	70 (65–75)
	Laurate	0	6 (4–7)	4 (0–13)	90 (88–92)	93 (92–93)
	Palmitate	0	7 (0–17)	51 (50–53)	65 (54–73)	54 (49–61)
Cyclobutanecarboxylic acid	Butyrate	11 (6–16)	12 (10–14)	3 (0–7)	48 (46–49)	73 (73, 73)
	Octanoate	0	11 (0–23)	26 (24–28)	50 (48–51)	72 (67–76)
	Laurate	10 (0–21)	0	9 (9, 9)	66 (23–90)	95 (95–96)
	Palmitate	14 (12–20)	8 (0–15)	36 (30–43)	68 (60–77)	64 (64, 64)

or less (Table 1). The inhibition of [1-^{14}C]palmitate oxidation by different concentrations of pent-4-enoic acid is shown in Fig. 2, indicating a threshold effect.

A second series of experiments was undertaken with the homologous series of 1-^{14}C-labelled fatty acids, octanoate, decanoate, laurate, myristate, palmitate and stearate. Surprisingly there were some quantitative differences between this series and our earlier experiments. Inhibitions caused by pent-4-enoic acid, though still marked, were less than in the first series (Fig. 2). In the interval between these two series of experiments the management of our animal-house was changed, so that there was a more rigorous control of the environment and diet of the rats supplied. These differences are relevant in view of reported effects of dietary factors in the toxicity of hypoglycin and pent-4-enoic acid (Senior, 1967). Table 2 shows a profile of the inhibition of $^{14}CO_2$ production from 1-^{14}C-labelled fatty acids of different chain lengths.

Effects of pent-4-enoic acid and related compounds on oxygen uptake with fatty acids as substrates. The effects of these compounds on the initial rate of oxygen uptake are shown in Table 3. Often inhibition of oxygen uptake increased progressively during an experiment. Again the greatest inhibition found was that of palmitate oxidation by low concentrations of pent-4-enoic acid. Inhibition of oxygen uptake, however, was always less than inhibition of $^{14}CO_2$ production and this was probably due to the use of fumarate as sparker.

Effect of serum albumin on palmitate oxidation. Most workers who have studied the mitochondrial oxidation of palmitate manometrically (which requires high concentrations of palmitate) have used palmitate combined with serum albumin (Bjorntorp, Ells & Bradford, 1964; Holt *et al.* 1966). The palmitate–albumin complex is less surface-active than free palmitate yet allows oxidation of the palmitate. We were reluctant to use albumin in case it formed complexes with pent-4-enoic acid. Van den Bergh (1966) showed that albumin was not necessary for manometric measurements of mitochondrial oxidation of long-chain fatty acids added as ethanolic solutions. It appeared better to avoid the use of ethanol, so we therefore introduced the use of neutral suspensions of long-chain fatty acids. Two control experiments were performed, however, with the palmitate–albumin complex as substrate to ascertain whether any differences were apparent. The rates of $^{14}CO_2$ production and oxygen uptake

and the extent of the inhibition of palmitate oxidation by pent-4-enoic acid were similar to those with free palmitate (Table 4). These results also suggest that albumin does not strongly bind pent-4-enoic acid.

Effects of carnitine on fatty acid oxidation. DL-Carnitine had no effects on the oxidation of butyrate and of octanoate (Table 5). The production of $^{14}CO_2$ from [1-^{14}C]laurate or from [1-^{14}C]-palmitate was approximately halved when 1·0mM-DL-carnitine was omitted (Table 5). However, the percentage inhibition of palmitate oxidation by pent-4-enoic acid was the same whether carnitine was present or not (Table 6).

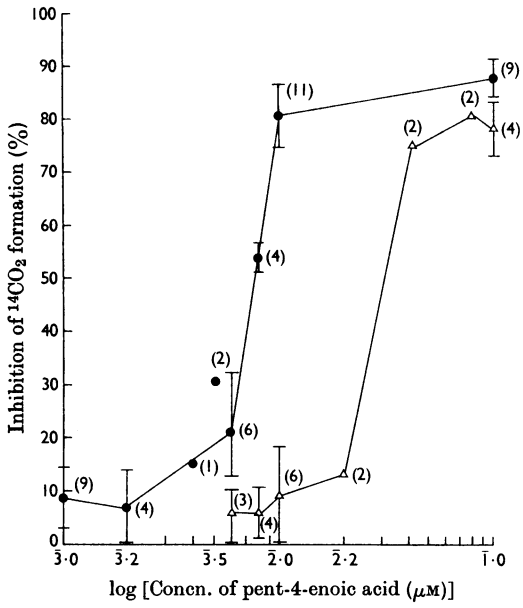

Fig. 2. Effect of concentration of pent-4-enoic acid on the percentage inhibition of $^{14}CO_2$ formation from [1-^{14}C]-palmitate in two series of experiments: ●, first series; △, second series (see the text). The results are means and the bars indicate the standard deviations where applicable with the numbers of observations in parentheses. Experimental details are given in the Methods section.

Effects of pent-4-enoic acid and related compounds on fumarate oxidation. It was necessary to find the effects of these compounds on the oxidation of 0·4mM-fumarate, since this was used in all experiments with fatty acids. The inhibition of fumarate oxidation by these fatty acids (Table 7) may contribute to the inhibition of the oxidation of butyrate, octanoate, laurate and palmitate at 1·0 and 10mM concentrations of inhibitors (Table 3).

Effects of pent-4-enoic acid and related compounds on acetoacetate formation from fatty acids. Acetoacetate was formed during the mitochondrial oxidation of butyrate, octanoate and laurate, but very little was formed from palmitate with our experimental conditions (Table 8). The only inhibitory compound tested that decreased acetoacetate formation to a greater extent than $^{14}CO_2$ production and oxygen uptake was cyclopropanecarboxylic acid. The other compounds inhibited oxidation and acetoacetate formation to the same extent and this parallelism was remarkably constant at different concentrations of the inhibitors (Table 3).

Comparison of the inhibition of the oxidation of long-chain fatty acids by pent-4-enoic acid and by methylenecyclopropylacetic acid. Holt *et al.* (1966) used 1-^{14}C-labelled fatty acids at concentrations of 0·4286mM at pH 7·4 and 25°. Albumin was included in their incubation medium, but hexokinase + glucose, carnitine and a tricarboxylic acid-cycle sparker were not. They reported that methylenecyclopropylacetic acid had the following effects on palmitate oxidation: at 0·6μM it had no effect, at 60μM it inhibited $^{14}CO_2$ production, oxygen uptake and acetoacetate production by 63, 77 and 55% respectively and at 6mM it inhibited by 96, 84 and 94% respectively. These authors also reported wide

Table 2. *Effect of pent-4-enoic acid on $^{14}CO_2$ production from 1-^{14}C-labelled saturated fatty acids of different chain lengths by rat liver mitochondria*

The results are given as means of the percentage inhibition in four to six experiments of total $^{14}CO_2$ production during 40min. and the ranges are given in parentheses. The experimental conditions are given in the Methods section.

	Inhibition of CO_2 production (%)				
Concn. of pent-4-enoic acid ...	1·0μM	10μM	0·1mM	1·0mM	10mM
Saturated fatty acid					
Decanoate (10:0)	0	19 (12–21)	21 (17–25)	38 (34–42)	44 (36–52)
Laurate (12:0)	10 (0–20)	4 (0–8)	9 (4–14)	41·5 (37–45)	88·5 (86–90)
Myristate (14:0)	15 (7–22)	23 (17–26)	57·5 (46–64)	78·5 (76–80)	89 (88–91)
Palmitate (16:0)	5 (4–6)	9 (4–21)	81 (78–83)	80 (78–84)	90 (76–94)
Stearate (18:0)	16 (13–19)	25 (18–31)	65·5 (57–70)	77 (65–89)	85 (83–89)

Table 3. *Effect of pent-4-enoic acid and related compounds on oxygen uptake by rat liver mitochondria with saturated fatty acids as substrate*

The results are given as means of the percentage inhibition in two to six experiments of the initial rate of O_2 uptake and the ranges are given in parentheses, determined from the same experiments described in Table 1. The experimental conditions are given in the Methods section. The 100% rates of O_2 uptake were (mμg.atoms/min./mg. of protein): butyrate, 40–60; octanoate and laurate, 60–90; palmitate, 40–50.

		Inhibition of initial rate of O_2 uptake (%)				
Concn. of compound ...		1·0 μM	10 μM	0·1 mM	1·0 mM	10 mM
Compound	Substrate					
Pent-4-enoic acid	Butyrate	0	0	0	8 (0–16)	21 (13–29)
	Octanoate	2 (0–4)	6 (0–12)	5 (2–8)	20·5 (20–21)	44 (31–51)
	Laurate	8 (0–16)	11 (6–19)	13 (5–21)	56 (50–62)	87 (84–90)
	Palmitate	4·5 (0–9)	45 (21–61)	56 (54–58)	58 (47–69)	69 (68–70)
Pentanoic acid	Butyrate	0	0	19 (18–24)	21 (17–25)	21 (17–26)
	Octanoate	2 (0–4)	6 (4–7)	7 (7, 7)	14 (13–14)	53 (35–71)
	Laurate	7 (0–14)	1 (0–2)	48 (13–84)	43 (34–86)	88 (87–88)
	Palmitate	9 (8–19)	11 (0–26)	23 (22–24)	20 (14–29)	39 (30–49)
Pent-2-enoic acid	Butyrate	0	0	11 (10–12)	14 (10–18)	49 (36–62)
	Octanoate	1 (0–3)	2 (0–5)	6 (6–7)	18 (14–21)	64 (51–77)
	Laurate	3 (0–6)	14 (0–29)	11 (0–32)	88 (87–90)	89 (88–90)
	Palmitate	6 (0–9)	6 (0–12)	15 (9–22)	28 (26–30)	43 (43–45)
Cyclopropanecarboxylic acid	Butyrate	0	4 (4–5)	28 (22–35)	28 (28, 28)	41 (39–42)
	Octanoate	6 (5–8)	4 (0–12)	20 (17–23)	26 (23–25)	50 (47–52)
	Laurate	4 (4, 4)	21 (4–31)	22 (14–35)	84 (75–90)	88 (87–90)
	Palmitate	5 (0–13)	12 (0–37)	33 (26–39)	42 (37–49)	49 (46–53)
Cyclobutanecarboxylic acid	Butyrate	0	0	21 (20–22)	44 (43–45)	57 (50–62)
	Octanoate	0	4 (0–8)	21 (20–22)	24 (18–29)	54 (50–58)
	Laurate	4 (0–8)	5 (5, 5)	8 (5–11)	62 (14–86)	86 (84–86)
	Palmitate	6 (0–11)	20·5 (0–38)	4 (0–8)	16 (7–32)	49 (48–50)

Table 4. *Effect of pent-4-enoic acid on the oxidation of serum albumin–[1-^{14}C]palmitate complex by rat liver mitochondria*

The results are given as means of the percentage inhibition in two experiments and the ranges are given in parentheses. The experimental conditions are given in the Methods section. The 100% rates were within the ranges quoted in Tables 1 and 3.

	Inhibition (%)				
Concn. of pent-4-enoic acid ...	1·0 μM	10 μM	0·1 mM	1·0 mM	10 mM
Initial rate of O_2 uptake	0	36 (32,40)	54 (51,57)	71 (61,81)	79 (76,84)
Total $^{14}CO_2$ production	17	56 (54,58)	75 (70,80)	90 (89,91)	95 (94,96)

variation from these mean values and the differences between our two series of experiments were much less than the differences reported by Holt *et al.* (1966). Inhibition of $^{14}CO_2$ production by lower concentrations of methylenecyclopropylacetic acid was not as great as that found in this work with pent-4-enoic acid, but Holt *et al.* (1966) do not appear to have used an acid tip to release retained carbon dioxide from the medium and may not therefore have accurately determined the percentage inhibition (see Fig. 1). [1-^{14}C]Acetyl units, derived from 1-^{14}C-labelled fatty acids, yield $^{14}CO_2$ from their oxidation by the tricarboxylic acid cycle at the 2-oxoglutarate dehydrogenase (EC 1.2.4.2) reaction only after their carbon atoms have once completely traversed the cycle (Lowenstein, 1967). Variable results may therefore be expected if the operation of this cycle has to depend on endogenous dicarboxylic acids present in the mitochondrial preparations.

The percentage inhibition of oxygen uptake by 0·01 mM-methylenecyclopropylacetic acid was lower than with pent-4-enoic acid at these concentrations (Holt *et al.* 1966). However, these workers found 94% inhibition with 6 mM-methylenecyclopropylacetic acid. Holt *et al.* (1966) found that methylenecyclopropylacetic acid inhibited most strongly the oxidation of myristate, palmitate and stearate,

Table 5. *Effect of* DL-*carnitine on the oxidation of saturated* 1-^{14}C-*labelled fatty acids by rat liver mitochondria*

The rate of initial O_2 uptake is expressed as mμg.atoms/min./mg. of protein and $^{14}CO_2$ production as 10^{-3} × counts/min./flask/40min. DL-Carnitine (1·0mM) was added or omitted, and other experimental details are given in the Methods section.

		Rate of O_2 uptake		$^{14}CO_2$ production	
Saturated fatty acid	Expt. no.	No carnitine	With carnitine	No carnitine	With carnitine
Butyrate	1	42·6	42·6	12·2	11·9
Octanoate	1	82·0	82·0	19·6	22·8
	2	96·0	90·0	7·34	7·10
Laurate	1	64·0	62·6	3·90	4·71
	2	64·0	61·8	4·22	5·26
Palmitate	1	32·6	53·4	3·04	5·80
	2	33·0	49·1	1·20	2·52

Table 6. *Effect of* DL-*carnitine on the inhibition by pent-4-enoic acid of* [1-^{14}C]*palmitate oxidation in rat liver mitochondria*

The results are quoted for a representative experiment and are given as the percentage inhibitions of $^{14}CO_2$ production and of O_2 uptake. The experimental conditions are given in the Methods section. The 100% rates were similar to those indicated in Table 5.

	Inhibition of O_2 uptake (%)				
Concn. of pent-4-enoic acid ...	1·0μM	6·0μM	10μM	0·1mM	1·0mM
1·0mM-Palmitate	0	3	56	72	72
1·0mM-Palmitate + 1·0mM-DL-carnitine	0	0	50	69	68
	Inhibition of $^{14}CO_2$ production				
1·0mM-Palmitate	3	17	77	82	83
1·0mM-Palmitate + 1·0mM-DL-carnitine	11	27	81	85	85

Table 7. *Effect of pent-4-enoic acid and related compounds on oxygen uptake by rat liver mitochondria with fumarate as substrate*

The results are given as the percentage inhibitions (mean of at least two experiments) of the initial rate of oxidation of 0·4mM-fumarate and the ranges are given in parentheses. The experimental conditions are given in the Methods section. The 100% rates of O_2 uptake were 8–15mμg.atoms/min./mg. of protein.

	Inhibition of initial rate of O_2 uptake (%)				
Concn. of compound ...	1·0μM	10μM	0·1mM	1·0mM	10mM
Compound					
Pent-4-enoic acid	4 (0–16)	1·5 (0–5)	10 (6–16)	13·5 (0–21)	36 (28–39)
Pentanoic acid	0	0	0	21 (16–26)	39 (38–40)
Pent-2-enoic acid	0	0	0	2 (0–4)	43 (36–50)
Cyclopropanecarboxylic acid	0	0	10	23 (18–28)	43 (37–48)
Cyclobutanecarboxylic acid	0	0	0	40 (40–41)	—

similarly to pent-4-enoic acid (Fig. 2). Methylenecyclopropylacetic acid differed from pent-4-enoic acid in that it apparently specifically inhibited acetoacetate formation at 0·06 and 0·6mM. In the present work only cyclopropanecarboxylic acid specifically inhibited acetoacetate formation in this concentration range (Table 8).

DISCUSSION

Inhibition of long-chain fatty acid oxidation by pent-4-enoic acid. The only significant inhibitions of fatty acid oxidations found were those of myristate, palmitate and stearate by pent-4-enoic acid. Yardley & Godfrey (1967) also found that

Table 8. *Effect of pent-4-enoic acid and related fatty acids on acetoacetate formation during the oxidation of saturated fatty acids by rat liver mitochondria*

The results are given as the means of the percentage inhibitions in two to six experiments of total acetoacetate formation during 40min. incubation and the ranges are given in parentheses. The experimental conditions are given in the Methods section. The 100% rates of acetoacetate formation were (mμmoles/min./mg. of protein): butyrate and laurate, 0·05–0·10; octanoate, 0·10–0·20.

		Inhibition of acetoacetate formation (%)				
Concn. of compound ...		1·0μM	10μM	0·1mM	1·0mM	10mM
Compound	Substrate					
Pent-4-enoic acid	Butyrate	8 (0–16)	8 (4–12)	14 (14–15)	55 (50–60)	91 (85–94)
	Octanoate	0	7 (0–14)	14 (12–16)	46 (41–51)	82 (78–87)
	Laurate	7 (0–14)	10 (5–15)	21 (17–25)	74 (72–75)	97 (96–100)
Pentanoic acid	Butyrate	14 (0–28)	14 (0–27)	27 (23–31)	66 (60–72)	68 (59–77)
	Octanoate	+16	0	+11	21 (18–25)	54 (40–68)
	Laurate	11 (0–22)	4 (0–8)	3 (3, 3)	93 (93–94)	95 (93–97)
Pent-2-enoic acid	Butyrate	+16	4·5 (0–9)	25 (23–27)	36 (36, 36)	67·5 (64–71)
	Octanoate	7 (5–9)	11 (0–15)	5 (0–11)	33 (32–34)	74 (63–85)
	Laurate	6 (0–13)	7 (0–15)	12 (10–14)	96 (96–97)	97 (96–97)
Cyclopropanecarboxylic acid	Butyrate	8 (8, 8)	17 (15–19)	40·5 (36–45)	97 (95–97)	98 (96–99)
	Octanoate	4 (0–19)	26 (8–31)	66 (62–73)	69·5 (65–77)	96 (93–98)
	Laurate	4 (0–8)	33 (25–41)	43 (36–48)	92 (88–95)	91 (90–92)
Cyclobutanecarboxylic acid	Butyrate	0	0	0	39 (39, 39)	71 (71, 71)
	Octanoate	0	6 (0–21)	49 (41–58)	53·5 (49–57)	98 (97–98)
	Laurate	8 (4–13)	10 (4–15)	26 (20–32)	91 (86–97)	96 (94–98)

pent-4-enoic acid inhibited the formation of $^{14}CO_2$ from [U-^{14}C]palmitate by skin slices.

Inhibition of 2-oxoglutarate dehydrogenase could cause inhibition of $^{14}CO_2$ production from 1-^{14}C-labelled fatty acids (Lowenstein, 1967). However, $^{14}CO_2$ production from short-chain 1-^{14}C-labelled fatty acids was not strongly inhibited at 0·1mM concentrations of any of the inhibitory compounds tested, yet these compounds at 0·1mM (except cyclobutanecarboxylic acid) all inhibit oxidation of added 2-oxoglutarate (Senior & Sherratt, 1968*b*). One must therefore conclude that the remaining rate of oxidation of 2-oxoglutarate endogenously generated in mitochondria is not sufficiently impaired by these compounds to be rate-limiting.

A possible site of inhibition of long-chain fatty acid oxidation by pent-4-enoic acid is at a stage of β-oxidation, which is catalysed by enzymes with different chain-length specificity. Some possible mechanisms are now considered.

Acyl-CoA–carnitine O-acyltransferases. Acetyl-CoA–carnitine *O*-acyltransferase (EC 2.3.1.7) (Fritz, Schultz & Srere, 1963) and palmitoyl-CoA–carnitine *O*-acyltransferase (EC 2.3.1.–) (Norum, 1964) are active with short-chain and long-chain acyl-CoA derivatives respectively.

Entman & Bressler (1967) have claimed that hypoglycin or its metabolites inhibit palmitoyl-CoA–carnitine acyltransferase. Homogenates of hearts from hypoglycin-treated mice had an impaired ability to oxidize palmitate that was partly restored by 1·5mM-L-carnitine. Simultaneous administration of L-carnitine with hypoglycin decreased the hypoglycaemic effects of the latter compound. Entman & Bressler (1967) attempted to assay palmitoyl-CoA–carnitine acyltransferase by an isotope-exchange method depending on [^{3}H]-carnitine release from palmitoyl[^{3}H]carnitine. They reported that its activity was decreased in mouse heart after hypoglycin treatment and that 1·5mM-L-carnitine largely reversed the inhibition. Their assay was derived from that of Norum (1964), who measured the reaction in the direction of palmitoylcarnitine formation and who also found that the rate increased with increasing carnitine concentration when the enzyme was not saturated by 3mM-carnitine. Therefore more data are required to establish the validity of the assay used by Entman & Bressler (1967).

With an adequate supply of long-chain fatty acids the palmitoyl-CoA–carnitine acyltransferase is rate-limiting in fatty acid oxidation (Shepherd, Yates & Garland, 1965). Since hypoglycin (De Renzo *et al.* 1958) and pent-4-enoic acid (Senior, 1967) raise plasma free fatty acid concentrations in rats, inhibition of the palmitoyl-CoA–carnitine acyltransferase would limit long-chain fatty acid oxidation, if this is carnitine-dependent. The

extent of the carnitine requirement for palmitate oxidation *in vitro* depends on the experimental conditions, since relatively high concentrations of palmitate (1mM) partly remove the carnitine requirement for its own oxidation (Yates, Shepherd & Garland, 1966). In our manometric experiments about 50% of palmitate oxidation by rat liver mitochondria was carnitine-dependent and DL-carnitine did not reverse the inhibition of palmitate oxidation by pent-4-enoic acid. Further, in experiments in which oxygen uptake was measured polarographically, 10mM-L-carnitine did not reverse the strong (80%) inhibition of palmitate oxidation in the presence of 1·0mM-ATP and 10mM-malonate by 0·01mM-pent-4-enoic acid with conditions where the oxidation was 95% carnitine-dependent (H. S. A. Sherratt, unpublished work).

Acyl-CoA dehydrogenases. There are three acyl-CoA dehydrogenases in liver that catalyse the oxidation of acyl-CoA derivatives of different chain lengths (Beinert, 1963). Holt & Holt (1958, 1959) and Holt *et al.* (1966) have given circumstantial evidence that an acyl-CoA dehydrogenase was inhibited by hypoglycin metabolites *in vivo*, for administration of riboflavine phosphate to alloxan-diabetic rats or normal mice prevented the hypoglycaemic effects of hypoglycin and decreased the toxicity of hypoglycin. No measurements appear to have been made on the effects of hypoglycin, methylenecyclopropylacetic acid or pent-4-enoic acid on any acyl-CoA dehydrogenase.

Acyl-CoA ligases. Three ATP-linked acyl-CoA ligases show chain-length specificity (Jencks, 1962). A fourth GTP-linked enzyme activates fatty acids of various chain lengths (Galzigna, Rossi, Sartorelli & Gibson, 1967). Its significance is unknown and since it is inhibited by P_i (Galzigna *et al.* 1967), present in all experiments in the present work, this GTP-linked enzyme probably may be discounted as a site of action of pent-4-enoic acid. Circumstantial evidence points to a lack of a major inhibition of acyl-CoA ligases by methylenecyclopropylacetic acid. Incorporation of [1-^{14}C]palmitate into triglycerides in mouse heart is doubled by hypoglycin treatment (Entman & Bressler, 1967).

Other possible mechanisms of inhibition. The soluble high-speed supernatant fraction of rat liver homogenates forms CoA derivatives of pent-4-enoic acid, pent-2-enoic acid, pentanoic acid and cyclobutanecarboxylic acid; the mitochondrial fraction forms CoA derivatives of these compounds and also of cyclopropanecarboxylic acid (Senior, Reay & Sherratt, 1968). Therefore pent-4-enoyl-CoA, and possibly pent-4-enoylcarnitine, may cause some of the inhibitory effects of pent-4-enoic acid. Methylenecyclopropylacetyl-CoA is formed *in vivo* from hypoglycin (Holt, 1966).

Formation of pent-4-enoyl-CoA may decrease the amount of CoA available for the activation of fatty acids, as first suggested for hypoglycin metabolites by McKerns, Bird, Kaleita, Coulomb & De Renzo (1960), though it is difficult to explain the differential effects of pent-4-enoic acid (and of methylenecyclopropylacetic acid) on the oxidation of fatty acids of different chain lengths on this basis. Further, the metabolism of hypoglycin in rat liver requires CoA (Holt, 1966).

It is possible that more than one reaction of fatty acid oxidation is inhibited. Though pent-4-enoic acid and methylenecyclopropylacetic acid appear to belong to the same series of hypoglycaemic compounds each may act predominantly on a different stage of fatty acid oxidation. It is most curious that administration of carnitine (Entman & Bressler, 1967) or of riboflavine phosphate (Holt & Holt, 1958, 1959) appears to prevent hypoglycaemia in mice after hypoglycin treatment. Injection of DL-carnitine (800mg./kg.) or riboflavine phosphate (12·5mg./kg.), or both, in mice had no effect on the toxicity of pent-4-enoic acid (Senior & Sherratt, 1968*a*). The available, and to some extent contradictory, data do not yet allow the site of inhibition of fatty acid oxidation by pent-4-enoic acid or by methylenecyclopropylacetic acid to be defined.

The present results with pent-4-enoic acid and four structurally related non-hypoglycaemic compounds, together with those of Holt *et al.* (1966) with hypoglycin metabolites, disclose a correlation between hypoglycaemic activity and the inhibition of long-chain fatty acid oxidation in this series of compounds (Senior, 1967; Senior & Sherratt, 1967). The mechanism of this hypoglycaemic effect is discussed in the following paper (Senior & Sherratt, 1968*c*).

Since this work was completed a report on the hypoglycaemic effect of pent-4-enoic acid has appeared (Corredor, Brendel & Bressler, 1967). These workers observed an inhibition of [1-^{14}C]-palmitate oxidation in homogenates of hearts from mice treated with pent-4-enoic acid that was reversed by the addition of L-carnitine. They also reported that only hypoglycaemic fatty acids were activated by the medium-chain acyl-CoA ligase from ox heart and that pent-4-enoyl-CoA was a substrate for palmitoyl-CoA–carnitine acyltransferase. Administration of pent-4-enoic acid caused a decrease in tissue concentrations of L-carnitine with an increase in acid-soluble acylcarnitine. Some of these results are difficult to reconcile with our findings.

We thank Mr C. G. Chambers for excellent technical assistance. This work was supported by a grant from the Medical Research Council and A. E. S. was in receipt of a Science Research Council training grant.

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