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

OXIDATIVE PHOSPHORYLATION AND MITOCHONDRIAL OXIDATION OF PYRUVATE, 3-HYDROXYBUTYRATE AND TRICARBOXYLIC ACID-CYCLE INTERMEDIATES

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1. The effects of the hypoglycaemic compound pent-4-enoic acid, and of four structurally related non-hypoglycaemic compounds (pent-2-enoic acid, pentanoic acid, cyclopropanecarboxylic acid and cyclobutanecarboxylic acid), on several reactions in rat liver mitochondria were determined. 2. The use of manometric techniques for measurements of oxidations and of phosphorylation is critically discussed. 3. Pent-4-enoic acid and pentanoic acid uncoupled oxidative phosphorylation at low concentrations, but usually by not more than about 50%. 4. All the compounds, except cyclobutanecarboxylic acid, strongly inhibited the oxidation of pyruvate and 2-oxoglutarate, but the oxidations of succinate, citrate and 3-hydroxybutyrate were not strongly inhibited. 5. All the compounds, except cyclobutanecarboxylic acid, inhibited decarboxylation of [1-¹⁴C]pyruvate with ferri-cyanide as electron acceptor. 6. All the compounds, except pent-2-enoic acid, caused mitochondrial swelling after a time-lag.

Acute hypoglycaemia is a salient feature of 'vomiting sickness', a disease found in Jamaica and caused by ingestion of unripe fruits of the ackee, *Blighia sapida*. Hassall, Reyle & Feng (1954) isolated a compound, hypoglycin, from ackee fruits that caused hypoglycaemia when administered to rats. Hypoglycin, first thought to be a peptide (Hassall & Reyle, 1955*a,b*), was later shown to be L-2-amino-3-methylenecyclopropylpropionic acid (Holt & Leppla, 1958*a,b*; Wilkinson, 1958; Ellington, Hassall & Plimmer, 1958; De Ropp *et al.* 1958; Renner, Jöhl & Stoll, 1958; Anderson *et al.* 1958; Ellington, Hassall, Plimmer & Seaforth, 1959).

A series of unsaturated short-chain fatty acids related structurally to hypoglycin also cause hypoglycaemia. These include 3-methylenecyclobutanecarboxylic acid and pent-4-enoic acid (Anderson *et al.* 1958), 2-oxomethylenecyclopropylpropionic acid and methylenecyclopropylacetic acid (De Renzo *et al.* 1958). The last-named compound is formed from hypoglycin *in vivo* (Holt, 1966) and is probably the true hypoglycaemic agent.

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Anderson *et al.* (1958) found that 2-methylenecyclobutanecarboxylic acid and methylcyclopropylalanine (Table 1) were not hypoglycaemic and they proposed that the structural requirement for hypoglycaemic activity in short-chain fatty acids was a vinyl group separated by two carbon atoms from a carboxyl group or a potential carboxyl group. A cyclopropane ring was not necessary. Pent-4-enoic acid is therefore the simplest active compound (Table 1). In agreement with this we have shown that pent-2-enoic acid, pentanoic acid, cyclopropanecarboxylic acid and cyclobutanecarboxylic acid are not hypoglycaemic in rats (Senior, 1967; Senior & Sherratt, 1966), and Gray & Fowden (1962) reported that methylenecyclopropylglycine was hypoglycaemic in mice.

In victims of vomiting sickness there is severe or total depletion of liver glycogen and fatty infiltration of the liver (Hill, 1953; Jelliffe & Stuart, 1954). These effects of hypoglycin have been obtained in laboratory animals (Patrick, 1954; Leppla & Holt, 1956; Holt & Holt, 1958, 1959; Chen, Anderson, McCowen & Harris, 1957; De Renzo *et al.* 1958; McKerns, Bird, Kaleita, Coulomb & De Renzo, 1960). Hypoglycin inhibited conversion of glucose into liver glycogen in rats (Patrick, 1954), but did not affect glycogen phosphorylase (EC 2.4.1.1) or glucose 6-phosphohydrolase (EC 3.1.3.9) activity in

in skin slices. We have shown (Senior, 1967; Senior, Reay & Sherratt, 1968a) that pent-4-enoic acid raises plasma free fatty acid concentrations and causes ketosis in rats, demonstrating that it resembles hypoglycin in these ways.

The mechanisms by which these short-chain fatty acids cause hypoglycaemia were not fully understood. We decided to investigate this problem, using the simplest hypoglycaemic fatty acid, pent-4-enoic acid, assuming initially that its effects were analogous to those of hypoglycin. This compound has the advantage over hypoglycin that it has not to be metabolized (except perhaps to a CoA derivative) to give an active agent. Some non-hypoglycaemic fatty acids were also used as controls to establish which effects of pent-4-enoic acid *in vitro* could be correlated with its hypoglycaemic activity. These control acids resembled pent-4-enoic acid or hypoglycin structurally and were: pent-2-enoic acid, pentanoic acid, cyclopropanecarboxylic acid and cyclobutanecarboxylic acid (Table 1). This paper reports effects of pent-4-enoic acid and related compounds on oxidative phosphorylation, mitochondrial oxidation of pyruvate, 3-hydroxybutyrate and tricarboxylic acid-cycle intermediates, and swelling of mitochondria. Following papers describe effects on fatty acid oxidation in mitochondria (Senior, Robson & Sherratt, 1968b) and on glycolysis and gluconeogenesis and a mechanism for the hypoglycaemic action is proposed (Senior & Sherratt, 1968). The whole-animal effects of these fatty acids have been described (Senior, 1967; Senior *et al.* 1968a). Preliminary reports of some of this work have appeared (Senior & Sherratt, 1966, 1967).

METHODS

Chemicals and enzymes. Sodium succinate, sodium pyruvate, 2-oxoglutaric acid, sodium fumarate, NADP⁺, NADH, ATP and AMP were obtained from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany. DL-Lipoamide and bovine serum albumin (fraction V) were obtained from Sigma (London) Chemical Co., London, S.W. 6. Bovine serum albumin was defatted by the method of Goodman (1958). DL-3-Hydroxybutyric acid was obtained from Calbiochem, Los Angeles, Calif., U.S.A. Pent-2-enoic acid (*trans*-isomer) was obtained from Koch-Light Laboratories Ltd., Colnbrook, Bucks. Cyclopropanecarboxylic acid and cyclobutanecarboxylic acid were obtained from Aldrich Chemical Co., Milwaukee, Wis., U.S.A. Pent-4-enoic acid, butyric acid, pentanoic acid, hexanoic acid, octanoic acid and thiamine pyrophosphate were obtained from Fluka A.-G., Buchs, Switzerland. All other chemicals were obtained from British Drug Houses Ltd., Poole, Dorset. A.R. or biochemical grade chemicals were used whenever obtainable, and KH₂PO₄, glycylglycine, 2,4-dinitrophenol and EDTA (dipotassium salt) were recrystallized before use. [U-¹⁴C]Toluene standard, 2,5-diphenyloxazole and 1,4-bis-(5-phenyloxazol-2-yl)benzene were obtained from Packard Instruments Co., Downers Grove, Ill., U.S.A. Sodium

[1-¹⁴C]pyruvate was obtained from The Radiochemical Centre, Amersham, Bucks.

Hexokinase (EC 2.7.1.1) was obtained either from C. F. Boehringer und Soehne G.m.b.H. (crystalline suspension, 140 units/mg.) or from Sigma Chemical Co. (type III). Before use crystalline hexokinase suspensions were centrifuged at 0° in the 8 × 5 ml. swing-out rotor of the MSE 18 refrigerated centrifuge at 3000 rev./min. (1000g) for 5 min. to sediment the enzyme (5000g-min.). The enzyme was resuspended in 0.5 M-glucose solution, pH 7.0, and dialysed against 2 l. of 0.5 M-glucose solution. Dialysis for 6 hr., with three changes of external solution, was sufficient to remove NH₄⁺ ions (detected with Nessler's reagent). The non-diffusible material was then assayed for hexokinase activity (Slein, 1963). Sigma type III hexokinase was dissolved directly in 0.5 M-glucose solution. Both hexokinase preparations gave similar results in mitochondrial experiments. Glucose 6-phosphate dehydrogenase (EC 1.1.1.49) was obtained from C. F. Boehringer und Soehne G.m.b.H. (crystalline suspension, 140 units/mg.) and pig heart lipopamide dehydrogenase (EC 1.6.4.3) was obtained from Sigma Chemical Co.

Distilled water. All solutions were made up in water that had been boiled with KMnO₄, then distilled in a glass still, and finally deionized (British Drug Houses Ltd., Amberlite deionizer). This procedure was necessary because of the presence in the local water supply of compounds that inhibited some mitochondrial reactions. All solutions were adjusted to pH 7.0 with KOH before use.

Apparatus. Manometric measurements were made with a Braun rotary Warburg apparatus. Spectrophotometric measurements were made with a Unicam SP.500 spectrophotometer, or, where continuous recordings were required, with a Unicam SP.800 spectrophotometer with a controlled-temperature cell housing. Radioactivity measurements were made with a Packard Tri-Carb liquid-scintillation counter.

Chemical determinations. Protein was determined as described by Miller (1959), P_i as described by King (1926) and acetoacetate by the method of Walker (1954).

Animals. Male Wistar rats (180–300g.) from a local inbred strain maintained on a standard diet were used throughout.

Preparation of mitochondria. Mitochondria were prepared in 0.3 M-sucrose–1 mM-EDTA, pH 7.0, at 0–4°. A rat liver was disrupted in a loosely-fitting Potter-Elvehjem homogenizer driven by a power drill (Black and Decker U21) at 625 rev./min. with eight strokes up and down. The homogenate was centrifuged at 0–4° in the 8 × 50 ml. rotor of an MSE 18 refrigerated centrifuge at 3000 rev./min. (1000g) for 3 min. to remove the nuclear fraction, and the supernatant was centrifuged at 9000 rev./min. (9400g) for 5 min. to bring down the mitochondria. The mitochondria were washed once. The mitochondria from one liver were finally suspended in 15 ml. of homogenizing medium to give a suspension containing 10–15 mg. of protein/ml., which was used within 30 min. of preparation.

Measurement of oxidative phosphorylation. Oxygen uptake was measured by the Warburg technique. Each flask contained substrate (8 mm), hexokinase (15 units), glucose (40 mm), sucrose (48 mm), KCl (33.6 mm), MgCl₂ (12.8 mm), glycylglycine (16 mm), ATP (1 mm), AMP (1 mm), mitochondria (4–8 mg. of protein) and potassium phosphate (18 mm) in a final volume of 2.50 ml., pH 7.0, with 0.30 ml. of

2.5N-NaOH in the centre well. Flasks were incubated at 30°, the shaking rate was 100 strokes/min. and the gas phase was air.

A two-flask method (Aldridge & Parker, 1960) was used to determine P/O ratios. Two flasks had the same additions. One flask was removed at the end of thermal equilibration (zero time). A 2 ml. sample of the contents was quickly added to 2.0 ml. of 0.6N-HClO₄. The other flask was incubated for a measured time (usually 10 min.), then removed from the bath and 2.0 ml. of the contents was added to 2.0 ml. of 0.6N-HClO₄. The P_i content of each acidified sample was measured to give the P_i esterified during the incubation period.

Recently reports of very high P/O ratios have appeared (Smith & Hansen, 1964; Lynn & Brown, 1965; Gurban & Cristea, 1965). Three errors in manometric technique have been cited in explanation: (a) incomplete equilibration of O₂ between liquid and gas phase (Haslam, 1965); (b) incomplete CO₂ absorption by alkali (Koivusalo & Slater, 1966; Lenaz & Beyer, 1965); (c) incorrect estimation of O₂ uptake during thermal equilibration (Koivusalo, Currie & Slater, 1966). These errors may be very significant when the rate of oxidation is high and the incubation period is short, and lead to gross underestimation of the O₂ uptake. It is likely that some P/O ratios cited in the literature are erroneously high, even when they do not exceed the accepted maxima of 4, 3 and 2 for 2-oxoglutarate, NADH and succinate respectively. The most commonly used manometric method for determination of P/O ratios involves tipping hexokinase from the side arm into the main compartment after equilibration. This may increase the rate of O₂ uptake by the mitochondria to an extent where disequilibrium errors occur. The most accurate manometric method for finding P/O ratios is to find the rates of P_i and O₂ uptake with several flasks, the ratio of the rates being taken as the P/O ratio (Aldridge, 1957). However, the two-flask method used here is more convenient, and eliminates errors due to gas disequilibrium if the rate of O₂ uptake during the incubation period is linear and below the maximal rate possible (see Haslam, 1965).

The effect of each fatty acid was determined at various concentrations, each experiment including two control determinations of the P/O ratios.

Correction of P/O ratios for glucose 6-phosphohydrolase activity of mitochondrial preparations. The presence of glucose 6-phosphohydrolase in rat liver mitochondria, due to contamination with microsomes, would lower the apparent P/O ratios since the amount of glucose 6-phosphate remaining at the end of the experiment would be less than the amount of P_i actually incorporated into ATP. Glucose 6-phosphohydrolase was assayed in two ways under conditions similar to those used for the measurement of oxidative phosphorylation.

(1) Mitochondria (4–8 mg. of protein/ml.) were incubated at pH 7.0 and 30° with 18 mM-glucose 6-phosphate. The medium was as described above except that AMP, ATP, glucose and hexokinase were omitted. Potassium phosphate was either omitted or decreased to 4 mM. It was replaced when necessary by equimolar amounts of glycylglycine. The reaction was stopped by the addition of 2.0 ml. of 0.6N-HClO₄ and the P_i liberated was measured.

(2) Eleven Warburg flasks were prepared as for measurement of P/O ratios. Six contained 0.05 ml. of 2 mM-2,4-

dinitrophenol in the side arms, two contained 2,4-dinitrophenol (0.04 mM) in the main compartment and three had no 2,4-dinitrophenol. Mitochondria were added last, and, after thermal equilibration (4 min.) and 10 min. incubation, the six flasks that contained 2,4-dinitrophenol in the side arm were tipped (final concn. of 2,4-dinitrophenol in the main compartment 0.04 mM). The other five flasks were removed at this stage and 2.0 ml. of the contents of each flask was quickly added to 2.0 ml. of 0.6N-HClO₄. The six remaining flasks were dealt with as follows. Two were removed after a further 12 min. (total 22 min.) and two were removed after a further 19 min. (total 29 min.). The glucose 6-phosphate content of each flask was determined enzymically by the method of Hohorst (1963). This experiment yielded the following information: (i) the amount of glucose 6-phosphate formed after 4 min. thermal equilibration and after 10 min. incubation (three flasks without 2,4-dinitrophenol); (ii) confirmation that no glucose 6-phosphate was formed when 2,4-dinitrophenol was present (two flasks); (iii) the rate of hydrolysis of the glucose 6-phosphate formed after 10 min. incubation, under conditions closely resembling those used in measurement of P/O ratios.

Procedure (1) gave values of 0.02–0.03 unit/mg. of protein at 30° for the glucose 6-phosphohydrolase activity of the mitochondrial preparation. The concentration of glucose 6-phosphate was 18 mM. This is fully saturating, since liver microsomal glucose 6-phosphohydrolase has *K_m* approx. 2.5×10^{-3} M (Langdon & Weakley, 1957). The glucose 6-phosphohydrolase activity was not changed by the P_i concentration of the medium. The calculated P/O ratios would be too low by 10% (i.e. for 10 μmoles of P_i esterified by 5 mg. of mitochondrial protein) if the concentration of glucose 6-phosphate were fully saturating throughout the incubation period. Procedure (2) showed that the glucose 6-phosphate concentration was not fully saturating

Fig. 1. Rate of hydrolysis of glucose 6-phosphate formed during experiments on oxidative phosphorylation by rat liver mitochondria, measured by procedure (2) described in the Methods section.

during the whole of the incubation period: it reached 4mm at the end of the incubation period and the rate of hydrolysis of the glucose 6-phosphate formed decreased sharply with time (see Fig. 1). These results indicated that the correction to P/O ratios required was less than 4%. This was considered negligible and was not applied. Hird & Weidemann (1966) applied a 10% correction for glucose 6-phosphohydrolase activity under similar experimental conditions.

Measurement of mitochondrial oxidations. The O₂ uptake was measured under the conditions described for the determination of P/O ratios. The O₂ uptakes quoted were initial rates.

Measurement of acetoacetate formation. Acetoacetate was occasionally measured during mitochondrial oxidations. After flasks had been quickly removed from the manometers, 2.0ml. of their contents was added to 2.0ml. of 10% (w/v) HClO₄ and centrifuged to remove denatured protein. The supernatant was mixed with 2.0ml. of 1.1 M-K₂HPO₄, pH 8.2, and cooled to 0° to precipitate KClO₄, which was removed by centrifugation, and the supernatant was used for acetoacetate determinations. It was not determined whether acetoacetate formation was linear during incubation.

Assay of pyruvate decarboxylase activity of mitochondria. Pyruvate decarboxylase (2-oxo acid carboxy-lyase, EC 4.1.1.1) activity of intact rat liver mitochondria was assayed by following ¹⁴CO₂ production from [1-¹⁴C]-pyruvate with ferricyanide as electron acceptor (modified from Sanadi, 1963):

Radioactive pyruvate was stored as a stock solution in 1 N-HCl since it is unstable at higher pH values (Groth & LePage, 1955; Korff, 1964; Silverstein & Boyer, 1964). Rat liver mitochondria (4-6mg. of protein) were incubated in Warburg flasks in a medium containing [1-¹⁴C]pyruvate (8mm; specific radioactivity 0.0025 μC/μmole), sucrose (48mm), KCl (33.6mm), MgCl₂ (12.8mm), glycylglycine

(16mm), ATP (1mm), AMP (1mm) and potassium phosphate (18mm), pH 7.0. The flasks were shaken (100 strokes/min.) at 30° with air as gas phase. The centre wells contained 0.25ml. of 2 N-KOH, and the side arms 0.20ml. of 3.7 N-trichloroacetic acid (coloured with acid fuchsin). ¹⁴CO₂ was collected in the centre well and counted as described in the following paper (Senior *et al.* 1968b).

There was a slow formation of ¹⁴CO₂ from [1-¹⁴C]-pyruvate during incubation when mitochondria were omitted, though this was small compared with the rate of pyruvate decarboxylase reaction. Owing to spontaneous radiochemical breakdown of pyruvate during preparation of the medium variable amounts of ¹⁴CO₂ were evolved from the medium as soon as incubation commenced. Therefore three identical flasks were used to measure the rate of decarboxylation; one was tipped at zero time (end of 4min. thermal equilibration), one after a further 15min. and one after 30min. After being tipped the flasks were incubated for another 10min. to release retained CO₂. Production of ¹⁴CO₂ in the decarboxylation reaction was linear for 30min. and no O₂ was absorbed.

Assay of lipoamide dehydrogenase activity. This was performed spectrophotometrically at 37° essentially as described by Massey (1966).

Measurement of swelling of mitochondria. Mitochondrial swelling was measured at 25 ± 1° as the rate of change in extinction at 520mμ. Mitochondria were suspended in 3.0ml. of 0.125 M-KCl, 30mm-glycylglycine and 1mm-potassium succinate, at pH 7.4. Succinate was included to prevent spontaneous swelling, which occurred with some preparations of mitochondria.

RESULTS

Uncoupling of oxidative phosphorylation by pent-4-enoic acid and related compounds. The liver mitochondria used regularly gave P/O ratios 2.6-3.0 with citrate as substrate. Citrate was used for most experiments on oxidative phosphorylation

Table 2. *Effects of pent-4-enoic acid and related compounds on oxidative phosphorylation by rat liver mitochondria*

The results are given as the mean P/O ratios (± s.e.m. where applicable, with the numbers of observations in parentheses). At least two to four experiments were conducted at each concentration of each fatty acid. The experimental conditions are given in the Methods section.

Concn. of compound ...	P/O ratio with citrate as substrate					
	0	1.0 μM	10 μM	0.1 mm	1.0 mm	10 mm
Compound						
Pent-4-enoic acid	2.76 ± 0.02 (6)	2.39 ± 0.04 (4)	1.91 ± 0.03 (4)	1.78 ± 0.02 (4)	1.37 ± 0.03 (4)	1.99 ± 0.025 (4)
Pentanoic acid	2.77 ± 0.02 (6)	—	2.44 ± 0.03 (4)	1.92 ± 0.35 (4)	1.86 ± 0.03 (4)	1.88 ± 0.34 (4)
Pent-2-enoic acid	2.88	—	2.76	2.68	2.47	2.65
Cyclopropanecarboxylic acid	2.90	—	2.90	2.90	2.92	2.90
Cyclobutanecarboxylic acid	2.70	—	2.71	2.51	2.68	2.60
Butyric acid	2.73	—	2.50	2.05	2.14	2.05
	P/O ratio with succinate as substrate					
Pent-4-enoic acid	1.79	1.80	1.71	1.59	1.25	1.49

Table 3. *Effects of pent-4-enoic acid and related compounds on oxygen uptake by rat liver mitochondria with tricarboxylic acid-cycle intermediates as substrates*

The results are given as means of the percentages of the initial rate of oxygen uptake in the absence of added compound ($\pm$ s.e.m. where applicable, with the numbers of observations in parentheses). Usually two to four experiments were conducted at each concentration of each fatty acid and the ranges are given in parentheses where the s.e.m. values are not quoted. The experimental conditions are given in the Methods section. The 100% rates of O_2 uptake were (μ g.atoms/min./mg. of protein): succinate, 80-140; 2-oxoglutarate, pyruvate and citrate, 50-80; 3-hydroxybutyrate, 30.

Compound	Substrate (8mM)	Concn. of compound	Initial rate of O ₂ uptake (% of that in the absence of added compound)				
			1.0 μM	10 μM	0.1 mM	1.0 mM	10 mM
Pent-4-enoic acid	Citrate		103 ± 1.3 (7)	102 ± 2.5 (10)	82 ± 3.1 (9)	63 ± 2.5 (10)	62 ± 2.4 (10)
	Pyruvate + 0.8 mM-fumarate		97 ± 4.9 (6)	89 ± 7.2 (8)	31 ± 4.4 (8)	20 ± 3.3 (6)	15 ± 1.8 (7)
	Succinate		96 (97-100)	100 (92-107)	74 (70-77)	65 (60-70)	63 (60-66)
	2-Oxoglutarate		102 (98-106)	107 (106-109)	20 (0-40)	11 (10-12)	4 (0-8)
	2-Oxoglutarate + 8 mM-malonate		98 (97-99)	74 (73-75)	24 (19-29)	12 (0-24)	9 (0-17)
Pentanoic acid	3-Hydroxybutyrate		96 (86-103)	94 (98-100)	86 (75-96)	85 (75-95)	75 (73-77)
	Citrate		100	97 (92-103)	100	82 (81-82)	82 (82-83)
	Pyruvate + 0.8 mM-fumarate		95 (89-100)	90	30 (25-35)	24 (18-30)	28 (20-35)
	Succinate		104 (103-105)	100 (91-110)	88 (86-90)	90 (84-94.5)	94 (86-103)
	2-Oxoglutarate		92 (84-100)	90 (87-92)	37 (33-41)	33 (29-37)	54 (50-57)
Pent-2-enoic acid	2-Oxoglutarate + 8 mM-malonate		95 (89-99)	98 (94-102)	51 (38-63)	48 (37-60)	35 (34-37)
	3-Hydroxybutyrate		—	95	—	84 (74-94)	64 (63-65)
	Citrate		95	98 (95-100)	98 (95-100)	100	93 (92-94)
	Pyruvate + 0.8 mM-fumarate		88	43 (36-50)	35 (33-38)	24 (14-34)	34
	Succinate		100	98 (95-100)	100	92	92
Cyclopropanecarboxylic acid	2-Oxoglutarate		100	96	74	58	38
	Citrate		105 (98-112)	93 (88-100)	82 (81-84)	79 (79-82)	80 (73-87)
	Pyruvate + 0.8 mM-fumarate		101 (98-106)	67 (54-81)	44 (37-52)	33 (27-37)	21 (18-26)
	Succinate		99 (98-100)	92 (86-92)	92 (86-92)	88 (86-91)	85 (83-87)
	2-Oxoglutarate		77 (62-96)	62 (42-83)	54 (32-77)	46 (26-66)	38 (23-53)
Cyclobutanecarboxylic acid	2-Oxoglutarate + 8 mM-malonate		76 (60-92)	54 (44-63)	53 (43-63)	39 (26-53)	38 (11-66)
	3-Hydroxybutyrate		100	106	97	98	96
	Citrate		100	96 (95-96)	97 (95-100)	101 (100-101)	95 (93-98)
	Pyruvate + 0.8 mM-fumarate		100	108 (100-119)	106 (100-115)	93 (86-99)	91 (84-99)
	Succinate		99 (98-100)	99 (97-100)	93 (86-100)	98	105 (95-115)
	2-Oxoglutarate		104 (100-106)	100	93 (90-96)	97 (94-99)	91 (89-93)
	3-Hydroxybutyrate		100	104 (100-106)	99 (96-102)	88 (85-90)	83 (75-91)

because none of the compounds tested inhibited its oxidation strongly.

Pent-2-enoic acid, cyclobutanecarboxylic acid and cyclopropanecarboxylic acid did not uncouple significantly and there was good agreement between different experiments (Table 2). Pent-4-enoic acid and pentanoic acid uncoupled, but the extent varied in different experiments.

The uncoupling caused by these fatty acids is different from that caused by 2,4-dinitrophenol since it was never greater than about 50%. This suggests that it was caused indirectly, e.g. by the liberation of a fixed amount of a surface-active compound from the mitochondria. Similarly, Holt *et al.* (1966) found that methylenecyclopropylacetic acid partly uncoupled phosphorylation in mitochondria, but had no effect in submitochondrial particles where the control P/O ratio was only 0.8.

Butyrate (0.01 mM) did not affect oxygen uptake, but the P/O ratio was only 2.50. At higher concentrations (0.1, 2.0 and 10 mM) the oxygen uptake increased when the P/O ratio fell below 2.5, and it was not possible to deduce whether this fall was due to uncoupling or oxidation of the butyrate (see Hird & Weidemann, 1966).

Effects of pent-4-enoic acid and related compounds on mitochondrial oxidation of pyruvate, 3-hydroxybutyrate and tricarboxylic acid-cycle intermediates. With the shaking rate used the maximum rate of oxygen uptake possible was 0.6–0.7 $\mu\text{g}.\text{atom}/\text{min}.$ per flask. This agrees with Haslam (1965), who used similar conditions. In our experiments the rate of oxygen uptake was not greater than 0.2–0.4 $\mu\text{g}.\text{atom}/\text{min}.$ Thus any slight inhibition of oxidation was fully reflected in the rate of oxygen uptake. Rates of oxidation commonly found were 80–140 $\mu\text{g}.\text{atoms}/\text{min}./\text{mg}.$ of protein (succinate), 50–80 $\mu\text{g}.\text{atoms}/\text{min}./\text{mg}.$ of protein (2-oxoglutarate, pyruvate, citrate) or 30 $\mu\text{g}.\text{atoms}/\text{min}./\text{mg}.$ of protein (3-hydroxybutyrate).

The mitochondria showed good respiratory control with rates of oxidation in the absence of hexokinase and glucose 25–35% of the rates in their presence. There was no significant oxygen uptake in the absence of added substrate. None of the inhibitory compounds was apparently oxidized in these experiments, in either the presence or absence of 0.8 mM-fumarate or of hexokinase + glucose or both, whereas when 0.8 mM-fumarate, hexokinase and glucose were present butyrate and hexanoate were oxidized rapidly.

Table 3 shows the rates of oxidation obtained in the presence of various concentrations of the fatty acids. Pyruvate oxidation was sparked in all experiments with 0.8 mM-fumarate. 2-Oxoglutarate oxidation was also measured in the presence and absence of 8 mM-malonate. The percentage inhibition of oxidation was not significantly dependent

Fig. 2. Effect of the concentration of pent-4-enoic acid on the P/O ratio and rate of O_2 uptake by rat liver mitochondria with citrate as substrate. ○, O_2 uptake; Δ, P/O ratio. Experimental details are given in the Methods section.

on the mitochondrial protein concentration within the range used. Pent-4-enoic acid inhibited the oxidation of pyruvate and 2-oxoglutarate very strongly at higher concentrations (0.1 mM and above). The oxidation of succinate and DL-2-hydroxybutyrate was not so strongly inhibited. Pentanoic acid, cyclopropanecarboxylic acid and pent-2-enoic acid inhibited the oxidation of pyruvate and 2-oxoglutarate to approximately the same extent as pent-4-enoic acid, and, like pent-4-enoic acid, they were not so inhibitory towards citrate, succinate and 3-hydroxybutyrate oxidation. These compounds had a threshold effect on the inhibition of pyruvate and 2-oxoglutarate oxidation, the greatest increase usually occurring between 0.1 and 0.01 mM. Cyclobutanecarboxylic acid did not significantly inhibit any of the oxidations. Fig. 2 shows an experiment in which oxygen uptake and P/O ratios were compared in the presence of various concentrations of pent-4-enoic acid. The small increase in oxygen uptake caused by low concentrations of pent-4-enoic acid was probably due to uncoupling. At concentrations greater than 0.01 mM-pent-4-enoic acid, oxygen uptake was inhibited even though P/O ratios were further decreased. Inhibitory effects of pent-4-enoic acid were confirmed in preliminary experiments in which pyruvate oxidation was measured polarographically.

The effect of pent-4-enoic acid on acetoacetate production during pyruvate oxidation was measured in a few experiments. Acetoacetate formation was proportional to the oxygen uptake.

Table 4. *Effect of pent-4-enoic acid and related compounds on pyruvate decarboxylase activity of rat liver mitochondria*

The results are given as means of the percentage of the rate in the absence of added fatty acid. Three or four experiments were conducted at each concentration and the ranges are given in parentheses. The experimental conditions are given in the Methods section. The 100% rate was 9–12 μmoles of CO_2 released/min./mg. of protein.

Compound	Concn. of compound ...	Pyruvate decarboxylase activity (% of that in the absence of added compound)				
		1.0 μM	10 μM	0.1 mM	1.0 mM	10 mM
Pent-4-enoic acid	—	94 (92–95)	38 (38–40)	23 (22–24)	18 (15–20)	18 (14–21)
Pentanoic acid	—	—	96 (94–100)	—	27 (25–28)	—
Pent-2-enoic acid	—	—	76 (61–83)	—	45 (44–46)	—
Cyclopropanecarboxylic acid	—	—	48 (38–75)	—	43 (27–54)	—
Cyclobutanecarboxylic acid	—	—	100	—	81 (59–100)	—
Butyric acid	—	—	94 (89–100)	—	60 (50–70)	—
Hexanoic acid	—	—	91 (90–92)	—	58 (57–62)	—

Effects of pent-4-enoic acid and related compounds on pyruvate decarboxylase activity of mitochondria. Normal rates of carbon dioxide production were 9–12 μmoles /min./mg. of protein and there was no oxygen uptake. Pent-4-enoic acid strongly inhibited the decarboxylation at concentrations of 0.01 mM and above. Of the other compounds tested, all (including butyrate and hexanoate but except cyclobutanecarboxylic acid) inhibited the decarboxylation at 1 mM, but only cyclopropanecarboxylic acid and pent-2-enoic acid were inhibitory at 0.01 mM (Table 4). Thiamine pyrophosphate (0.02 mM) had no effect. These results are in accord with those for the inhibition of pyruvate oxidation (Table 3).

Inhibition of pyruvate decarboxylation by pent-4-enoic acid was measured at different pyruvate concentrations. The results were very variable, and since there was often a threshold effect it was not possible to define the type of inhibition. None of the compounds tested affected lipoamide dehydrogenase activity.

Mitochondrial swelling caused by pent-4-enoic acid and related compounds. Pent-4-enoic acid induced swelling at 10 mM, but not at lower concentrations (Fig. 3). There was a lag, which varied in different experiments, before swelling commenced, but was always between 20 and 35 min. EDTA (1 mM) prevented this induced swelling; bovine serum albumin (2 mg./ml.) shortened the lag before onset of swelling, and increased the rate of swelling, but it did not affect the total change in extinction (Fig. 3). This suggests that albumin does not strongly bind these short-chain fatty acid compounds.

Addition of ATP (5 mM), magnesium chloride (3 mM) and EDTA (1.0 mM) to mitochondria swollen by pent-4-enoic acid caused contraction (Fig. 3). However, whether bovine serum albumin was

present or not, this contraction was always less than 50% of the swelling. When P_i (10 mM) or calcium chloride (1.0 mM) was added to mitochondria preincubated for 15 min. in 10 mM-pent-4-enoic acid (which were therefore not yet swollen), swelling occurred immediately. The maximum contraction that could then be produced with ATP was only 20–40% of the swelling, and not 60–80% as in the absence of pent-4-enoic acid (Fig. 3).

Pent-2-enoic acid did not cause swelling at 0.01, 1.0 or 10 mM. All the other compounds tested caused swelling. Differences were noted in the duration of the lag before onset of swelling, and in the subsequent rate of swelling, but in all cases the degree of swelling was similar to that caused by pent-4-enoic acid. Butyrate (10 mM) and hexanoate (10 mM) caused swelling with a lag of 10 min., and, in contrast with pent-4-enoic acid, they caused swelling at 1 mM, the lag being 10–15 min. Cyclobutanecarboxylic acid, cyclopropanecarboxylic acid and pentanoic acid showed effects similar to pent-4-enoic acid at 10 mM, and, further, all caused slight swelling at 1 mM, with a lag of 40–60 min. These compounds inhibited the reversal of swelling similarly to pent-4-enoic acid.

DISCUSSION

Interpretation of results. If a dose of pent-4-enoic acid of 100 mg./kg. (which causes marked hypoglycaemia when given intraperitoneally to rats; Senior, 1967) were absorbed from the intraperitoneal space, and uniformly distributed throughout the tissues before any was metabolized, excreted or conjugated, the final concentration would be about 1.7 mM. In view of the assumptions made this value is certainly too high. Since hypoglycaemia after a dose of this size is corrected in 6–8 hr. (Senior, 1967)

Fig. 3. Effect of pent-4-enoic acid on the swelling of rat liver mitochondria. (a) Curve I, swelling induced by 10mM- P_i and its reversal by Mg^{2+} and ATP; curve II, swelling induced by 0.33mM- Ca^{2+} and its reversal by EDTA, Mg^{2+} and ATP (Lehninger, 1962). (b) Effect of bovine serum albumin and EDTA on swelling induced by 10mM-pent-4-enoic acid: curve I, pent-4-enoic acid and EDTA; curve II, pent-4-enoic acid and bovine serum albumin; curve III, pent-4-enoic acid alone. (c) Swelling caused by pent-4-enoic acid: curve I, no pent-4-enoic acid; curve II, 10 μ M-pent-4-enoic acid; curve III, 1mM-pent-4-enoic acid; curve IV, 10mM-pent-4-enoic acid and its reversal by EDTA, Mg^{2+} , ATP and bovine serum albumin. Experimental details are given in the Methods section.

it seems that pent-4-enoic acid is metabolized or excreted in this time. The intracellular concentration of pent-4-enoic acid might be lower than in the extracellular fluid since the acid may be bound by protein or lipid, or enter cells only slowly. When these factors are taken into account a reasonable assessment is that an effect must be readily demonstrable *in vitro* at 0.1mM or lower concentrations of pent-4-enoic acid if it is to be considered as a possible cause of hypoglycaemia. Further, the effect must be specific for a hypoglycaemic compound. When lethal doses (approx. 300mg./kg.) of pent-4-enoic acid are given to rats the effects of high concentrations (i.e. above 0.1mM) of pent-4-enoic acid shown here *in vitro* may contribute to death.

Effects of pent-4-enoic and related compounds. Only uncoupling of oxidative phosphorylation and inhibition of mitochondrial pyruvate and 2-oxoglutarate oxidation occurred at concentrations possible *in vivo*. Pentanoic acid, cyclopropanecarboxylic acid and pent-2-enoic acid all inhibited pyruvate and 2-oxoglutarate oxidation to approximately the same extent as pent-4-enoic acid, suggesting that this effect is not directly involved in the hypoglycaemic action. Indeed, inhibition of pyruvate oxidation may tend to lessen hypoglycaemia. The uncoupling of oxidative phos-

phorylation may, however, be a major causative factor (see Senior & Sherratt, 1968).

Holt *et al.* (1966) found that in rat liver homogenates methylenecyclopropylacetic acid (60mM) did not inhibit pyruvate oxidation and that 2-oxomethylenecyclopropylpropionic acid (a possible competitive inhibitor of pyruvate metabolism) only inhibited pyruvate decarboxylation at 3mM. Hypoglycin is a more powerful hypoglycaemic agent than pent-4-enoic acid in rats (Chen *et al.* 1957), and this might be partly due to the fact that hypoglycin metabolites do not inhibit pyruvate oxidation at concentrations likely *in vivo*.

Mechanism of the inhibition of pyruvate oxidation. The initial decarboxylation of pyruvate with ferricyanide as electron acceptor in mitochondrial preparations was inhibited by all the compounds tested (except cyclobutanecarboxylic acid). There was a rough correlation between this inhibition and the inhibition of the fumarate-sparked oxidation of pyruvate, though pent-4-enoic acid inhibited decarboxylation more strongly than oxygen uptake, and pent-2-enoic acid inhibited oxygen uptake more strongly.

This effect on decarboxylation could explain much of the inhibition of the mitochondrial oxidation of pyruvate by these compounds but

other mechanisms may contribute. Pyruvate oxidation is strongly inhibited during the contemporaneous oxidation of 2-oxoglutarate (Haslam, 1966; Bremer, 1966), long-chain fatty acyl-CoA, long-chain fatty acylcarnitine (Bremer, 1966; Nicholls, Shepherd & Garland, 1967), octanoate (2.0mM; Walter, Paetkau & Lardy, 1966; or 0.2mM; Nicholls *et al.* 1967) and butyrate (Nicholls *et al.* 1967). Garland & Randle (1964) showed that acetyl-CoA inhibited pyruvate dehydrogenase, whereas palmitoyl-CoA did not. There may be competition for free CoA between pyruvate and long-chain fatty acids (Bremer, 1966) or inhibition may be caused by increased reduction of NAD⁺ (Haslam, 1966; Bremer, 1966). As Nicholls *et al.* (1967) pointed out, where the total intramitochondrial pool of CoA (free or combined) or of NAD is fairly constant, it is not easy to decide whether an inhibition is of the product-inhibition type or due to competition between substrates for the same co-factor. They preferred the proposal that inhibition of pyruvate oxidation is caused by increases in the NADH/NAD⁺ and acetyl-CoA/CoA ratios.

No oxidation of pent-4-enoic acid or of any other fatty acid compound that inhibited pyruvate oxidation was detected, so they would not increase either the NADH/NAD⁺ ratio or the acetyl-CoA/CoA ratio as a consequence of their β -oxidation. Alternatively, if the inhibitory fatty acid formed a CoA derivative that was not further metabolized, this might decrease the CoA available for pyruvate oxidation. However, this explanation seems unlikely, since these compounds inhibited the oxidation of pyruvate much more strongly than the oxidation of short-chain fatty acids (Senior *et al.* 1968b), which also requires CoA. The particle-free supernatant fraction (4.5×10^6 g-min.) from rat liver homogenates forms the CoA derivatives of pent-4-enoic acid, pentanoic acid, pent-2-enoic acid and cyclobutanecarboxylic acid. The mitochondrial fraction also activates these four fatty acid compounds and as well as cyclopropanecarboxylic acid (Senior *et al.* 1968a). It is therefore possible that some of the inhibitory effects found could be caused by CoA derivatives, or even carnitine derivatives, of these fatty acid compounds.

Preliminary experiments with electron microscopy indicated that a high concentration of pent-4-enoic acid (10mM) caused complete comminution of mitochondria and that 1.0mM concentration destroyed about 30–50%, whereas at 0.01mM virtually all mitochondria remained intact (A. D. Hally, A. E. Senior & H. S. A. Sherratt, unpublished work). This suggests that at high concentrations short-chain fatty acid compounds may cause inhibition of some mitochondrial reactions by disrupting mitochondria. Experiments on swelling of mitochondria support this inter-

pretation. All the compounds tested (except pent-2-enoic acid) caused swelling at high concentrations (1.0mM or 10mM). There was always a lag before onset of swelling and invariably the ability of the mitochondria to contract on addition of ATP, magnesium chloride and EDTA was impaired. This swelling appeared to resemble that caused by u-factor (Lehninger, 1962).

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