Biochem. J. (1973) **136**, 157–171
Printed in Great Britain

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

EFFECTS OF THE FREE ACIDS AND THEIR CARNITINE ESTERS ON COENZYME A-DEPENDENT OXIDATIONS IN RAT LIVER MITOCHONDRIA

By P. C. HOLLAND* and H. S. A. SHERRATT
Department of Pharmacology, Medical School, University of Newcastle upon Tyne, Newcastle upon Tyne NE1 7RU, U.K.

(*Received* 6 *February* 1973)

1. The synthesis of pent-4-enoyl-L-carnitine, cyclopropanecarbonyl-L-carnitine and cyclobutanecarbonyl-L-carnitine is described. 2. Pent-4-enoate strongly inhibits palmitoyl-L-carnitine oxidation in coupled but not in uncoupled mitochondria. Pent-4-enoyl-L-carnitine strongly inhibits palmitoyl-L-carnitine oxidation in uncoupled mitochondria. Prior intramitochondrial formation of pent-4-enoyl-CoA is therefore necessary for inhibition. 3. There was a small self-limiting pulse of oxidation of pent-4-enoyl-L-carnitine during which the ability to inhibit the oxidation of subsequently added palmitoyl-L-carnitine developed. 4. Pent-4-enoate and pent-4-enoyl-L-carnitine are equally effective inhibitors of the oxidation of all even-chain acylcarnitines of chain length C_4–C_{16}. Pent-4-enoyl-L-carnitine also inhibits the oxidation of pyruvate and of 2-oxoglutarate. 5. Pent-4-enoate strongly inhibits the oxidation of palmitate but not that of octanoate. This is presumably due to competition between octanoate and pent-4-enoate for medium-chain acyl-CoA ligase. 6. There was less inhibition of the oxidation of pyruvate by pent-4-enoyl-L-carnitine, and of palmitoyl-L-carnitine by cyclopropanecarbonyl-L-carnitine, after pre-incubation with 10mM-arsenate. This suggests that these inhibitions were caused either by depletion of free CoA or by increase of acyl-CoA concentrations, since arsenate deacylates intramitochondrial acyl-CoA. There was little effect on the inhibition of palmitoyl-L-carnitine oxidation by pent-4-enoyl-L-carnitine. 7. Penta-2,4-dienoate strongly inhibited palmitoyl-L-carnitine oxidation in coupled mitochondria; acrylate only inhibited slightly. 8. Pent-4-enoate (0.1mM) caused a rapid and almost complete decrease in free CoA and a large increase in acid-soluble acyl-CoA when incubated with coupled mitochondria. Cyclopropanecarboxylate caused a similar decrease in CoA, with an equivalent rise in acid-soluble acyl-CoA concentrations. *n*-Pentanoate caused extensive lowering of CoA and a large increase in acid-soluble acyl-CoA and acetyl-CoA concentrations. Octanoate caused a 50% lowering of CoA and an increase in acid-soluble acyl-CoA and acetyl-CoA concentrations. 9. Cyclopropanecarboxylate and *n*-pentanoate were less potent inhibitors of palmitate oxidation than was pent-4-enoate. 10. It is concluded that pent-4-enoate causes a specific inhibition of β-oxidation after the formation intramitochondrially of its metabolites.

Pent-4-enoic acid is the simplest hypoglycaemic fatty acid structurally related to methylenecyclopropylacetic acid, the active metabolite of hypoglycin from the fruit of the ackee, *Blighia sapida*. Ingestion of unripe ackee fruits may cause vomiting sickness, a disease characterized by severe hypoglycaemia (Anderson *et al.*, 1958; De Renzo *et al.*, 1958; von Holt, 1966; Senior & Sherratt, 1968*a*,*b*; Sherratt *et al.*, 1971) and probably by isovalericacidaemia (Tanaka *et al.*, 1972). Both pent-4-enoic acid and hypoglycin are thought to cause hypoglycaemia by impairing gluconeogenesis secondarily to inhibiting fatty acid oxidation (Senior, 1967; Sherratt *et al.*, 1971) and pent-4-enoic acid has been used to demonstrate the dependence of gluconeogenesis on fatty acid oxidation in perfused rat liver (Ruderman *et al.*, 1968; Toews *et al.*, 1970; Williamson *et al.*, 1970; Menahan & Williams, 1971).

The mechanism of the inhibition of fatty acid oxidation by pent-4-enoic acid, first described by

* Present address: Institute for Enzyme Research, University of Wisconsin, Madison, Wis. 53706, U.S.A.

Yardley (Yardley, 1964; Yardley & Godfrey, 1963, 1967), is therefore of considerable interest (Corredor *et al.*, 1967; Senior *et al.*, 1968; Bressler *et al.*, 1969; Williamson *et al.*, 1969; Fukami & Williamson, 1971; Sherratt *et al.*, 1971). McKerns *et al.* (1960) originally proposed that inhibition of fatty acid oxidation by compounds related to hypoglycin might be caused either by the accumulation of their metabolically inert CoA derivatives, which sequester CoA necessary for normal metabolism, or by the specific inhibition of an enzyme of β-oxidation. Most authors have concluded that inhibition of oxidation of fatty acid and pyruvate by pent-4-enoic acid is caused by sequestration of CoA as pent-4-enoyl-CoA and as its oxidation product, acryloyl-CoA (Bressler *et al.*, 1969; Fukami & Williamson, 1971). Measurements of the effects of pent-4-enoic acid on the concentrations of CoA and its acyl derivatives in mitochondria by Fukami & Williamson (1971) are consistent with this interpretation. However, some of the changes that were found cannot be assumed to be caused specifically by pent-4-enoic acid, since many non-metabolizable simple fatty acids could also decrease CoA and increase short-chain acyl-CoA concentrations because of the broad specificity of medium-chain acyl-CoA synthetase (Mahler *et al.*, 1953). Indeed we had reported several years ago that some simple non-hypoglycaemic fatty acids which do not inhibit β-oxidation as strongly as does pent-4-enoic acid were equally effective in inhibiting mitochondrial oxidation of pyruvate and 2-oxoglutarate with the experimental conditions used, indicating that inhibition of fatty acid oxidation was not simply due to depletion of CoA (Senior & Sherratt, 1968*a*; Senior *et al.*, 1968). For these reasons we had emphasized the importance of using chemically similar but non-hypoglycaemic fatty acids as controls in this type of investigation (Senior & Sherratt, 1968*a*).

Since it is now apparent that formation of pent-4-enoyl-CoA must precede inhibition of fatty acid oxidation (Sherratt *et al.*, 1971) we reinvestigated the effects of pent-4-enoic acid and of some control fatty acids. We also investigated the effects of penta-2,4-dienoic acid and acrylic acid, which are metabolites of pent-4-enoic acid. This paper describes their effects and those of most of their L-carnitine esters on oxidation of fatty acids, pyruvate and 2-oxoglutarate and on the concentrations of free CoA and its acyl derivatives. The results indicated that there is a specific block in β-oxidation caused by pent-4-enoic acid or its metabolites, and the next paper (Holland *et al.*, 1973) describes the effects of their CoA derivatives on the enzymes of fatty acid oxidation and shows that penta-2,4-dienoyl-CoA, a metabolite of pent-4-enoic acid, strongly inhibits 3-oxoacyl-CoA thiolase (EC 2.3.1.9). Preliminary accounts of some of this work have appeared (Sherratt *et al.*, 1971; Holland & Sherratt, 1969, 1970, 1972).

Experimental

Materials

Crystalline carnitine acetyltransferase (EC 2.3.1.7) and malate dehydrogenase (EC 1.1.1.37) and most other biochemicals were purchased from Boehringer Corp. (London), London W5 2TZ, U.K., except phosphate acetyltransferase (EC 2.3.1.8), citrate synthase (EC 4.1.3.7) and CoA, which were obtained from P-L Biochemicals Inc., Milwaukee, Wis., U.S.A. 5,5'-Dithiobis-(2-nitrobenzoic acid) and dithiothreitol were obtained from Sigma Chemical Co. (London) Ltd., Kingston-upon-Thames, Surrey KT2 7BH, U.K. L-Carnitine chloride, oxalyl chloride and acrylic acid were obtained from Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K. Cyclopropanecarboxylic acid and cyclobutanecarboxylic acid were obtained from the Aldrich Chemical Co., Milwaukee, Wis., U.S.A. Cyclopropanecarbonyl chloride and cyclobutanecarbonyl chloride were obtained from R. N. Emanuel Ltd., Wembley, Middx., U.K., and decatetraenoic acid from K & K Laboratories Inc., New York, N.Y., U.S.A. Pent-4-enoic acid, crotonic acid and all saturated fatty acids and their acid chlorides and anhydrides were purchased from Fluka A.-G., Buchs, Switzerland. *N*-Ethylmaleimide and all other chemicals were obtained from BDH Ltd., Poole, Dorset, U.K., and were of A.R. grade where possible.

Penta-2,4-dienoic acid and pent-4-enoyl-CoA were prepared as described by Holland *et al.* (1973). Pent-4-enoyl chloride was prepared by reaction of dry pent-4-enoic acid (1 vol.) with oxalyl chloride (2 vol.) at room temperature. After 24h the mixture was distilled and the fraction boiling between 115° and 128°C redistilled to give a 50–60% yield.

Methods

Preparation of carnitine esters. Carnitine esters of straight-chain saturated fatty acids were prepared from the acid chlorides by the method of Bohmer & Bremer (1968) by using 900mg quantities of L-carnitine chloride. Esters of fatty acids of chain length greater than C_8 were precipitated from the reaction mixture by addition of 20ml of dry diethyl ether at 0°C. After stirring of the mixture at room temperature for 10min, the precipitate was collected, dissolved in 3ml of propan-2-ol at 50–60°C and centrifuged to remove any unchanged L-carnitine. The acylcarnitine was precipitated from hot propan-2-ol with acetone. Esters of fatty acids of chain length C_8 or less were purified as described by Bohmer & Bremer (1968). Carnitine esters were finally washed with ether and dried *in vacuo* over P_2O_5. Cyclopropanecarbonyl-L-carnitine and cyclobutanecarbonyl-L-carnitine were also prepared essentially as described by Bohmer & Bremer (1968), except that it

Table 1. *Characterization of acylcarnitine esters*

Chromatograms were run by descent on Whatman no. 1 paper in (1) propan-2-ol–ethyl methyl ketone–0.1 M-HCl (5:3:2, by vol.) (Freidman *et al.*, 1955) and in (B) ethanol–aq. NH_3 (sp.gr. 0.88)–water (18:1:1, by vol.) (Strack & Lorentz, 1954). The compounds were detected with iodine vapour. Melting points are uncorrected.

Acylcarnitine ester	R_F		
Solvent ...	A	B	M.p. (°C)
Pent-4-enoyl-L-carnitine	0.56	0.61	157–160
Cyclobutanecarbonyl-L-carnitine	0.56	0.59	155–160
Cyclopropanecarbonyl-L-carnitine	0.52	0.46	153–158
Butyryl-L-carnitine	0.55	0.59	142–145
n-Pentanoyl-L-carnitine	0.59	0.61	147–150
Hexanoyl-L-carnitine	0.68	0.70	—
Octanoyl-L-carnitine	0.68	0.74	155–159
Decanoyl-L-carnitine	0.72	0.76	—
Lauroyl-L-carnitine	0.74	0.77	156–159
Myristoyl-L-carnitine	0.77	0.79	—
Palmitoyl-L-carnitine	0.74	0.77	169–173
Stearoyl-L-carnitine	0.78	0.77	—
L-Carnitine chloride	0.29	0.16	—

was necessary to allow cyclopropanecarbonyl chloride to react with L-carnitine chloride for 48h at 40°C. Yields of 50–60% of all acylcarnitines were obtained. Attempts to prepare pent-4-enoyl-L-carnitine by standard methods always gave a yellow oil. A procedure was developed which gave a 60% yield of crystalline product. L-Carnitine chloride (900mg) was dissolved in a mixture of 1.5ml of pent-4-enoyl chloride and 6.0ml of pent-4-enoic acid. Moisture was excluded with a $CaCl_2$ tube and the mixture was stirred magnetically for 48h at room temperature. It was then added to 20ml of acetone at 0°C, after 2–3h traces of unchanged L-carnitine had precipitated and the supernatant was decanted and dry ether added to it until turbidity was incipient (about 20ml). The mixture was kept at 0°C and after crystallization had begun a further 20ml of ether was added, and the flask was kept at 0°C overnight to complete crystallization. The product was recrystallized twice from ethanol–acetone (3:20, v/v) by the careful addition of ether and finally washed with ether and dried.

Characterization of carnitine esters. All preparations of acyl-L-carnitine chlorides were at least 97% pure when assayed for ester-bond content by the ferric hydroxamate method (Snyder & Stephens, 1959). Contamination with L-carnitine, determined by the method of Chase & Tubbs (1966) by using carnitine acetyltransferase, revealed 5% contamination for cyclopropanecarbonyl-L-carnitine and less than 2% for all other esters. All esters gave one spot on paper chromatography (Table 1) and no free carnitine was detected by this technique. The melting points (uncorrected) are recorded in Table 1. Carnitine esters were also characterized by their i.r.-absorption spectra in Nujol. L-Carnitine has an OH stretching band at 3260 cm^{-1}, a C–O stretching band characteristic of a secondary alcohol at 1100cm^{-1} and a C=O stretching band due to the carboxylic acid function at 1725cm^{-1}. With carnitine esters the bands at 3260 and 1100cm^{-1} disappeared, but the C=O stretching band was displaced to 1710–1720cm^{-1} (cf. Ziegler *et al.*, 1967). In addition, however, C–O stretching bands of the acyl group were found at about 1200cm^{-1} and an absorption band due to the acyl keto group appeared at around 1740cm^{-1}. The nuclear-magnetic-resonance spectrum of pent-4-enoyl-L-carnitine showed a chemical shift of $\tau = 4.7$ compatible with the presence of a terminal methylene function, and solutions of pent-4-enoyl-L-carnitine discoloured $KMnO_4$, indicating the presence of a double bond. The other acyl-L-carnitines did not discolour $KMnO_4$ solutions, showing that no contamination with crotonbetaine had occurred during their preparation.

Preparation of solutions of potassium salts of fatty acids. The potassium salts of short-chain fatty acids were prepared by adjusting solutions of the free acids to pH7.0 with KOH. Those of fatty acids with chain lengths of C_{10} or more were prepared as aqueous 1–2mM solutions at pH10–12 at 60°C, and then adjusted to pH7.0 with HCl, and continually stirred at 40°C.

Apparatus. Polarographic measurement of oxygen activity was made with an oxygen electrode (Yellow Springs Instrument Co., Yellow Springs, Ohio, U.S.A.). Fluorimetric measurements of NADH concentrations were made with a Zeiss PMQ II spectrophotometer fitted with the ZFM fluorimeter attachment containing an Hg lamp and an M365 filter, with a temperature-controlled cell-holder and connected to a Servoscribe chart recorder with a back-off circuit.

Preparation of mitochondria. Mitochondria were prepared from the livers of male albino rats (200–300g), from a local inbred strain maintained on a standard diet, in 0.3M-sucrose–2mM-EDTA, pH7.0, as described by Senior & Sherratt (1968*a*). Protein was determined as described by Layne (1957) with dried bovine serum albumin used as standard.

Measurement of oxygen uptake by mitochondria. Oxygen concentration was recorded at pH7.0 and 30°C in a final volume of 3.0 or 6.0ml, by using 1–3mg of mitochondrial protein/ml. The basal medium contained 80mM-KCl, 6mM-$MgCl_2$, 20mM-Tris, 2mM-EDTA and 2.5mM-P_i, adjusted to pH7.0 with

KOH. In many experiments, 20mM-malonate, 10mM-arsenate and 20μM-2,4-dinitrophenol were included. When malonate or arsenate was omitted additional concentrations of 30mM-KCl or 15mM-KCl respectively were added to maintain osmolarity.

Determination of the concentrations of CoA and its acylated derivatives in mitochondria. CoA was determined by the procedure of Allred & Guy (1969), which involves recycling CoA:

$$\text{Acetyl phosphate} + \text{CoA} \rightarrow \text{acetyl-CoA} + P_i$$

$$\text{Acetyl-CoA} + \text{oxaloacetate} \rightarrow \text{citrate} + \text{CoA}$$

$$\text{Malate} + NAD^+ \rightleftharpoons \text{oxaloacetate} + NADH + H^+$$

where the formation of NADH is followed fluorimetrically. This method only requires a sensitivity of the fluorimeter corresponding to a full-scale deflexion of the recorder of 10μM-NADH and can determine accurately as little as 50pmol of CoA.

Mitochondrial suspensions were quenched with $HClO_4$ as described in the legend to Fig. 9 and subsequently treated as described by Williamson & Corkey (1969) to obtain the fraction containing CoA, acetyl-CoA and acid-soluble short-chain acyl-CoA and that containing acid-insoluble long-chain acyl-CoA. Free CoA plus acetyl-CoA was determined directly and acetyl-CoA was determined alone after reaction of free CoA with *N*-ethylmaleimide in the acid-soluble fraction as described by Allred & Guy (1969). The total CoA in each fraction was determined after alkaline hydrolysis (Williamson & Corkey, 1969). Acid-soluble acyl-CoA means the total CoA present in the acid-soluble fraction minus that present as free CoA and as acetyl-CoA. Acid-insoluble acyl-CoA means the total CoA present in the acid-insoluble fraction.

Results

Requirement for the conversion of pent-4-enoate into pent-4-enoyl-CoA for the inhibition of mitochondrial CoA-dependent oxidations

Pent-4-enoate strongly inhibits the CoA-dependent oxidation of fatty acids, pyruvate and 2-oxoglutarate but has little effect on the oxidation of succinate, citrate or 3-hydroxybutyrate (Senior & Sherratt, 1968*a*; Senior *et al.*, 1968). This suggested that pent-4-enoyl-CoA rather than pent-4-enoate could be the inhibitory species. Therefore we first investigated the effects of pent-4-enoate on palmitoyl-L-carnitine oxidation in uncoupled mitochondria where pent-4-enoyl-CoA cannot be formed.

Mitochondria were uncoupled and depleted of ATP by incubation with 20μM-2,4-dinitrophenol and 10mM-arsenate. This concentration of 2,4-dinitrophenol prevents ATP synthesis and is optimum for stimulation of acylcarnitine oxidation (Levitsky & Skulachev, 1972). 2,4-Dinitrophenol alone does not necessarily abolish all mitochondrial ATP-dependent fatty acid activation, since stimulation of the citrate cycle would cause formation of GTP, which can then phosphorylate ADP (Van Tol *et al.*, 1969). Malonate (20mM) was therefore included to block the citrate cycle and arsenate was used to prevent the formation of GTP by causing the arsenolysis of succinyl-CoA, catalysed by the GTP-dependent succinyl-CoA synthetase. Further, 2.5mM-phosphate, also included, inhibits the GTP-dependent acyl-CoA synthesis (Galzigna *et al.*, 1967). In the presence of 2,4-dinitrophenol, arsenate and malonate, the theoretical amount of oxygen necessary for the oxidation of even-numbered straight-chain acylcarnitines to acetoacetate was consumed, so these acyl-CoA esters and intermediates of fatty acid oxidation were not effective substrates for deacylation by the GTP-dependent enzyme. However, it will be shown below that there is some arsenolysis of non-metabolizable or slowly metabolizable acyl-CoA derivatives under these conditions.

Pent-4-enoate (0.1mM, in the presence of 20mM-malonate) inhibited palmitoyl-L-carnitine oxidation by about 80% in coupled mitochondria but had only a weak effect (about 10% inhibition) in uncoupled mitochondria (Fig. 1). Additional evidence that the formation of intramitochondrial pent-4-enoyl-CoA is obligatory for strong inhibition of palmitoyl-L-carnitine oxidation was provided by the strong inhibition obtained under uncoupling conditions after preincubation with pent-4-enoyl-L-carnitine (Fig. 2). Further, preincubation with pent-4-enoyl-CoA does not cause inhibition unless L-carnitine is also added (Fig. 2). These derivatives of pent-4-enoate presumably give intramitochondrial pent-4-enoyl-CoA by reactions catalysed by the carnitine acyltransferases associated with the mitochondrial inner membrane (see Fritz & Yue, 1963; West *et al.*, 1971; Holland *et al.*, 1973). Omission of arsenate in the presence of 2,4-dinitrophenol, phosphate and malonate did not increase the slight inhibition of palmitoyl-L-carnitine oxidation by pent-4-enoate.

Characteristics of the inhibition of fatty acid oxidation by pent-4-enoate and by pent-4-enoyl-L-carnitine

Preincubation of mitochondria with 0.1mM-pent-4-enoyl-L-carnitine, under either coupling or uncoupling conditions, was essential to obtain inhibition of fatty acid oxidation (Fig. 2). Pent-4-enoyl-L-carnitine caused no inhibition when added during the rapid oxidation of a pulse of palmitoyl-L-carnitine. However, the oxidation of a second addition of palmitoyl-L-carnitine made 3min after completion of the oxidation of the first was strongly inhibited. By contrast, 0.1mM-pent-4-enoate strongly inhibited the oxidations of palmitate, palmitoylcarnitine and octanoylcarnitine, but not that of octanoate itself (Fig. 3);

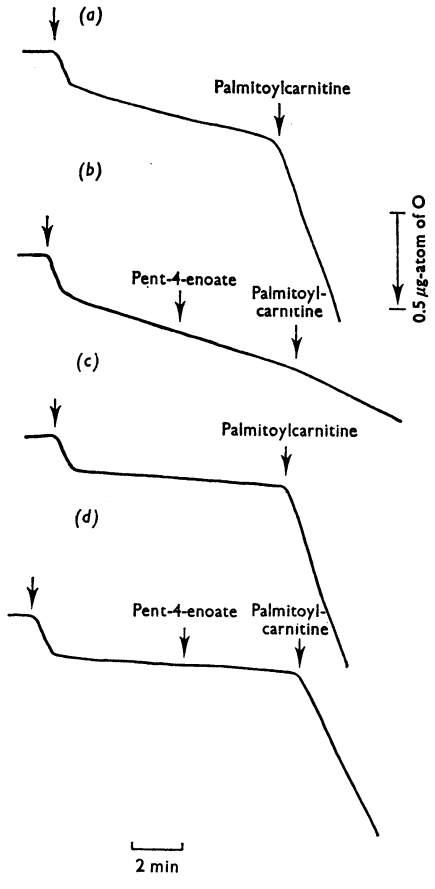

Fig. 1. *Effect of pent-4-enoate on the oxidation of palmitoyl-L-carnitine in coupled and in uncoupled mitochondria*

Mitochondria (8.5mg of protein) were added where indicated (by unlabelled arrows) to 6.0ml of medium at 30°C containing 20mM-malonate. In (*a*) and (*b*) 0.4mM-ADP was present, and in (*c*) and (*d*) 20μM-2,4-dinitrophenol and 10mM-arsenate were also present. 40μM-Palmitoyl-L-carnitine and 0.1mM-pent-4-enoate were then added as shown. Other details are given in the text.

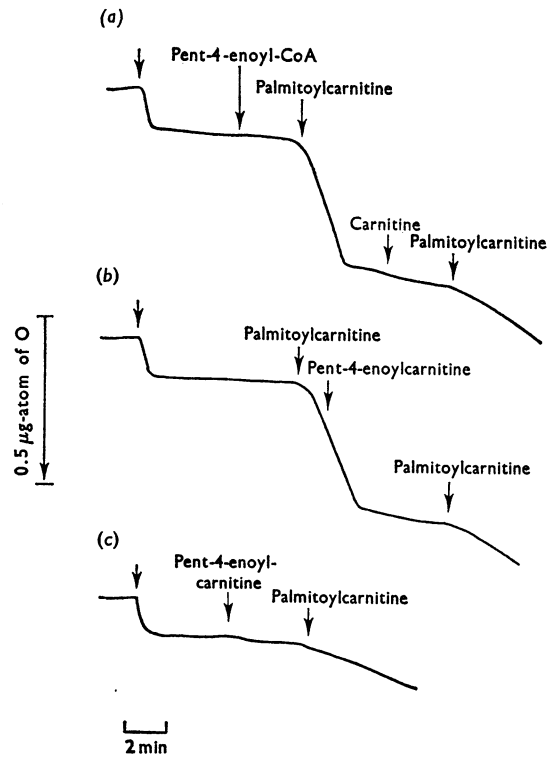

Fig. 2. *Effects of pent-4-enoyl-CoA and of pent-4-enoyl-L-carnitine on palmitoyl-L-carnitine oxidation in uncoupled mitochondria*

Mitochondria (4.7mg of protein) were added where indicated (by unlabelled arrows) to 6.0ml of medium at 30°C containing 20μM-2,4-dinitrotrophenol, 10mM-arsenate and 20mM-malonate. 20μM-Palmitoyl-L-carnitine, 0.1mM-pent-4-enoyl-CoA, 0.5mM-L-carnitine and 0.1mM-pent-4-enoyl-L-carnitine were then added as shown. Other details are given in the text.

---

the inhibition did not depend on the order of addition of inhibitor and substrate. Octanoate oxidation was only inhibited by pent-4-enoate after 2–3min of pre-incubation, as was also found by Fukami & Williamson (1971). This difference between the effects of pent-4-enoate and of pent-4-enoyl-L-carnitine may be explained on the basis of competition between substrate and inhibitor for intramitochondrial CoA. Octanoate and pent-4-enoate are probably both substrates for the same intramitochondrial ATP-dependent acyl-CoA synthetase (EC 6.2.1.2), and octanoate will impair activation of pent-4-enoate. The K_m for octanoate (0.15mM) of ox liver medium-chain acyl-CoA synthetase is the lowest for its normal substrates (Mahler *et al.*, 1953). Further, Garland *et al.* (1970) have presented evidence that octanoate is the best substrate for this enzyme in rat liver mitochondria.

Addition of pent-4-enoyl-L-carnitine to coupled or uncoupled mitochondria resulted in a slight stimulation of the rate of oxygen uptake for about 3min, which then gradually declined until eventually the endogenous rate was lightly inhibited (Fig. 4). The size of the pulse of oxidation was directly proportional to the amount of mitochondrial protein, with a

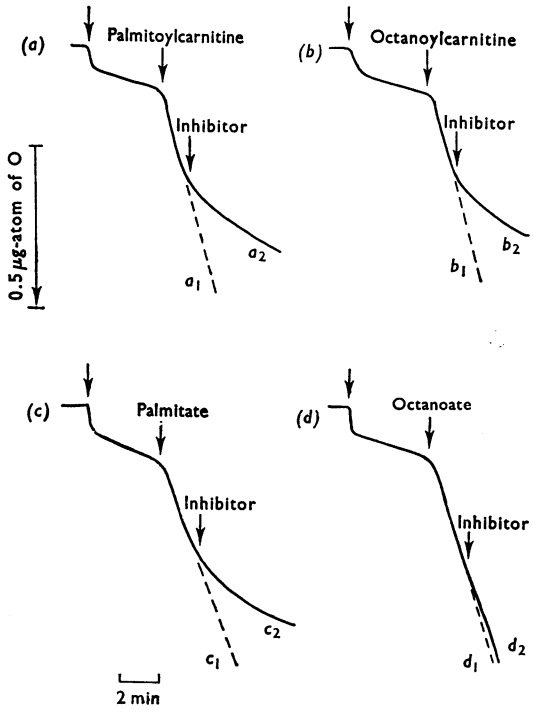

Fig. 3. *Comparison of the effects of pent-4-enoyl-L-carnitine and of pent-4-enoate on the oxidation of palmitoyl-L-carnitine, octanoyl-L-carnitine, palmitate and octanoate in coupled mitochondria*

Mitochondria (5.1 mg of protein) were added where indicated (by unlabelled arrows) to 1.0 ml of medium containing 20 mM-malonate and 0.4 mM-ADP, and (*c*) also contained 0.5 mM-L-carnitine. 40 μM-Palmitoyl-L-carnitine (*a*), 40 μM-octanoyl-L-carnitine (*b*), 40 μM-palmitate (*c*) and 40 μM-octanoate (*d*) were added, followed by the inhibitor: 0.2 mM-pent-4-enoyl-L-carnitine (a_1, b_1, c_1 and d_1: broken lines) or 0.2 mM-pent-4-enoate (a_2, b_2, c_2 and d_2: solid lines). Other details are given in the text.

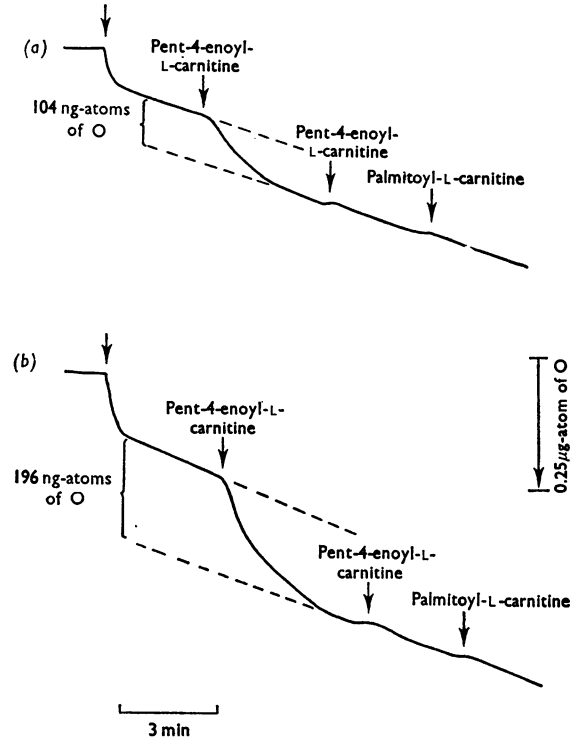

Fig. 4. *Self-limiting oxidation of pent-4-enoyl-L-carnitine in uncoupled mitochondria*

Mitochondria [5.1 mg (*a*) or 10.2 mg (*b*) of protein] were added where indicated by unlabelled arrows to 6.0 ml of medium at 30°C containing 20 μM-2,4-dinitrophenol, 10 mM-arsenate and 20 mM-malonate. 0.17 mM-Pent-4-enoyl-L-carnitine and 0.17 mM-palmitoyl-L-carnitine were added where indicated. This and other similar experiments show that the size of the pulse of oxygen uptake (between the broken lines) is proportional to the amount of mitochondrial protein added and not the amount of pent-4-enoyl-L-carnitine. Other details are given in the text.

mean value of about 20 ng-atoms of O/mg of protein. This probably represents a self-limiting oxidation of pent-4-enoyl-CoA. The total amount of intramitochondrial CoA is about 2.6 nmol/mg of protein (Fig. 9); complete conversion of the maximum amount of pent-4-enoyl-CoA that could be formed (2.6 nmol) into acryloyl-CoA and acetyl-CoA would require the uptake of 5.2 ng-atoms of O/mg of protein. If it is assumed that CoA in the form of acryloyl-CoA is irreversibly sequestered then only recycling of CoA esterified as acetyl-CoA (1.3 nmol) (to give acetoacetate and free CoA) could occur and would eventually allow a maximum formation of another 2.6 nmol of pent-4-enoyl-CoA. Under these circumstances therefore a maximum uptake of 10.4 ng-atoms of O/mg of protein should occur. The observed uptake of 20 ng-atoms of O/mg of protein indicates some recycling of CoA bound as acryloyl-CoA. This pulse of oxygen uptake is seen most clearly when 2,4-dinitrophenol and arsenate are present and endogenous respiration is minimized. It was not detected by Senior & Sherratt (1968*a*) because it was below the sensitivity of the manometric methods used. The extent of inhibition of palmitoyl-L-carnitine oxidation increased with increasing times of preincubation of mitochondria with pent-4-enoyl-L-carnitine for

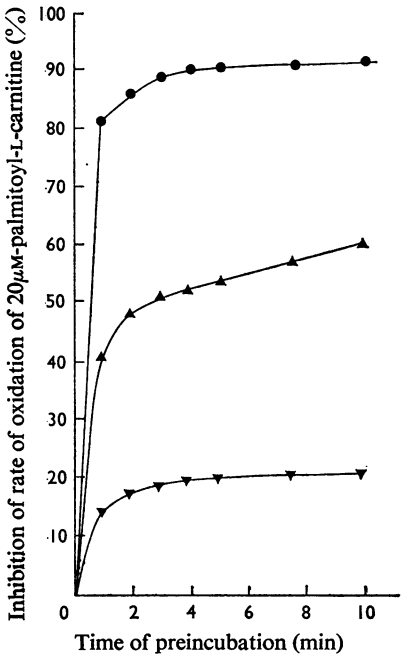

Fig. 5. *Effect of different times of preincubation of uncoupled mitochondria with pent-4-enoyl-L-carnitine, cyclopropanecarbonyl-L-carnitine or with cyclobutanecarbonyl-L-carnitine on the oxidation of palmitoyl-L-carnitine*

In a series of experiments mitochondria (4–6mg of protein) were preincubated with acyl-L-carnitines (●, 0.1mM-pent-4-enoyl-L-carnitine; ▲, 1.0mM-cyclopropanecarbonyl-L-carnitine; ▼, 1.0mM-cyclobutanecarbonyl-L-carnitine) in 6.0ml of a medium containing 20μM-2,4-dinitrophenol, 10mM-arsenate and 20mM-malonate at 30°C for various times as indicated on the abscissa. 20μM-Palmitoyl-L-carnitine was then added and the initial rate of oxygen uptake recorded; this was expressed as the percentage of the rate in the absence of an added acyl-L-carnitine. Other details are given in the text.

up to about 3min, coinciding with the end of the self-limiting pulse of pent-4-enoyl-L-carnitine oxidation (Fig. 5).

The L-carnitine esters of cyclopropanecarboxylic acid and cyclobutanecarboxylic acid inhibited palmitoyl-L-carnitine oxidation by uncoupled mitochondria, just as the corresponding free acids inhibited palmitate oxidation by coupled mitochondria (Senior *et al.*, 1968). As with pent-4-enoyl-L-carnitine, preincubation for 3–4min was required for maximum inhibition (Fig. 5), although no pulse of oxygen uptake was detected. A comparison of the effects of these three carnitine esters on the rates of oxidation of palmitoyl-L-carnitine, pyruvate and 2-oxoglutarate in uncoupled mitochondria is shown in Fig. 6. Pent-4-enoyl-L-carnitine was by far the most effective and cyclobutanecarbonyl-L-carnitine the least effective inhibitor of oxidation of palmitoyl-L-carnitine and pyruvate. The oxidation of 2-oxoglutarate was less sensitive to inhibition by pent-4-enoyl-L-carnitine than that of the other two substrates.

Chain-length specificity of inhibition of fatty acid oxidation by pent-4-enoyl-L-carnitine and by pent-4-enoate

The rates of oxidation followed polarographically of all carnitine esters (butyryl- to stearoyl-) were inhibited to similar extents by both pent-4-enoyl-L-carnitine and by pent-4-enoate in uncoupled and in coupled mitochondria respectively (Table 2). By contrast, earlier work from this laboratory (Senior *et al.*, 1968) reported an apparent chain-length specificity of inhibition of the oxidation of free fatty acids by pent-4-enoate. The oxidation of high concentrations (1mM) of 1-^{14}C-labelled myristate, palmitate or stearate, but not of fatty acids of shorter-chain lengths, was strongly inhibited when measured manometrically in the presence of a citrate-cycle intermediate, DL-carnitine and an ADP-regenerating system and in the absence of malonate (Senior *et al.*, 1968). It was therefore necessary to compare the effects of pent-4-enoate on the oxidation of free fatty acids and on their carnitine esters under identical conditions (Table 3) to ensure that these apparent discrepancies were not due to experimental differences. The results confirmed that pent-4-enoate (after a 3min preincubation) is a stronger inhibitor of palmitate oxidation than it is of octanoate oxidation, and showed conclusively that under identical conditions pent-4-enoate inhibits oxidation of palmitoyl-L-carnitine and octanoyl-L-carnitine to the same extent. However, the oxidation of high concentrations of free fatty acids was inhibited more strongly than that of low concentrations. Further, there was less difference between the extent of inhibition of palmitate oxidation compared with that of octanoate at higher substrate concentration (Table 3).

Pent-4-enoyl-L-carnitine was a less potent inhibitor than pent-4-enoate in coupled mitochondria (Table 3). If the formation of pent-4-enoyl-CoA from exogenous pent-4-enoyl-L-carnitine is more impeded by competition for mitochondrial CoA with endogenous fatty acids than is pent-4-enoyl-CoA formation from pent-4-enoate, then pent-4-enoate would be a more potent inhibitor of fatty acid oxidation. In support of this suggestion it was found that pent-4-enoyl-L-carnitine was a more potent inhibitor of acylcarnitine oxidation in uncoupled mitochondria (which cannot

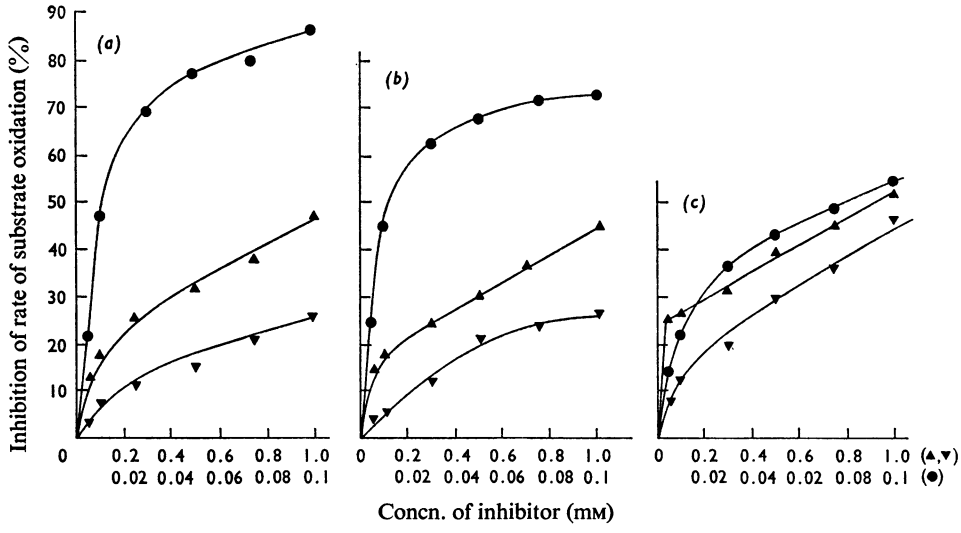

Fig. 6. *Effects of the concentration of pent-4-enoyl-L-carnitine, cyclopropanecarbonyl-L-carnitine and of cyclobutanecarbonyl-L-carnitine on the β-oxidation of palmitoyl-L-carnitine, and on the oxidation of pyruvate and of 2-oxoglutarate in uncoupled mitochondria*

Mitochondria (4–6mg of protein) were preincubated for 3min with the appropriate concentrations of acyl-L-carnitines (●, pent-4-enoyl-L-carnitine; ▲, cyclopropanecarbonyl-L-carnitine; ▼, cyclobutanecarbonyl-L-carnitine) in 6.0ml of medium at 30°C containing 20μM-2,4-dinitrophenol and 10mM-arsenate. The substrate [(*a*) 20μM-palmitoyl-L-carnitine; (*b*) 10mM-pyruvate; (*c*) 10mM-oxoglutarate] was then added and the initial rate of oxygen uptake recorded and expressed as the percentage of that in the absence of added acyl-L-carnitine. Mixture (*a*) also contained 20mM-malonate and (*b*) and (*c*) contained an extra concentration of 30mM-KCl in place of malonate. Control rates of oxidation of pyruvate and 2-oxoglutarate were in the range 120–150 and 200–250ng-atoms of O/min per mg of protein respectively. Other details are given in the text.

Table 2. *Inhibition of the oxidation of L-carnitine esters by pent-4-enoate in coupled mitochondria and by pent-4-enoyl-L-carnitine in uncoupled mitochondria*

Mitochondria (6–10mg of protein) were incubated in 6.0ml of medium containing 20mM-malonate; 0.4mM-ADP was added to obtain coupling conditions or 10mM-arsenate and 20μM-2,4-dinitrophenol to obtain uncoupling conditions. They were preincubated with the inhibitor for 3min and the subsequent rate of oxygen uptake was recorded for 3min after the addition of the substrate. Other details are given in the text. All results are given as the mean ± S.D. for four experiments. Control rates of O_2 uptake were in the range 50–80mg-atoms of O/min per mg of protein.

Substrate	Inhibition of the control rate of oxygen uptake (%)	
	10μM-Pent-4-enoate in coupled mitochondria	10μM-Pent-4-enoyl-L-carnitine in uncoupled mitochondria
100μM-Butyryl-L-carnitine	49 ± 6	46 ± 10
40μM-Hexanoyl-L-carnitine	43 ± 8	50 ± 6
40μM-Octanoyl-L-carnitine	55 ± 6	57 ± 3
20μM-Decanoyl-L-carnitine	49 ± 6	58 ± 8
20μM-Lauroyl-L-carnitine	43 ± 7	55 ± 5
20μM-Myristoyl-L-carnitine	50 ± 12	56 ± 14
20μM-Palmitoyl-L-carnitine	65 ± 4	61 ± 15
20μM-Stearoyl-L-carnitine	58 ± 6	43 ± 8

activate endogenous fatty acids) than it was in coupled mitochondria (Table 4). This interpretation implies that the intramitochondrial acyl-CoA synthetase(s) has a greater affinity for CoA than the carnitine acyltransferases, in accord with the conclusions of Bremer (1966, 1968). Finally, this competition for CoA would also explain why the oxidation of acylcarnitines is more strongly inhibited by pent-4-enoate in coupled mitochondria than that of the corresponding free fatty acids (Table 3).

Effects of arsenate, L-carnitine and CoA on the inhibition of mitochondrial oxidation of fatty acids and pyruvate by pent-4-enoyl-L-carnitine

Inhibition of palmitoyl-L-carnitine oxidation by pent-4-enoyl-L-carnitine was largely unaffected by the presence of arsenate. There was a slight relief at the shorter preincubation times with 10μM-pent-4-enoyl-L-carnitine (Fig. 7*a*). By contrast, inhibition of pyruvate oxidation was considerably less in the presence of arsenate than in its absence, and this difference was maintained even after prolonged incubation of mitochondria with inhibitor (Fig. 7*b*), whereas inhibition of oxidation of both palmitoyl-L-carnitine and pyruvate by cyclopropanecarbonyl-L-carnitine was significantly less in the presence of arsenate (Figs. 7*c* and 7*d*).

Galzigna *et al.* (1967) reported the arsenolysis of acyl-CoA catalysed by the purified GTP-dependent acyl-CoA synthetase, and Garland *et al.* (1970) demonstrated rapid arsenolysis of deca-2,4,6,8-tetraenoyl-CoA in intact mitochondria by this enzyme. Pent-4-enoate is probably a substrate for the GTP-dependent synthetase since it inhibited palmitoyl-L-carnitine oxidation by about 50% in mitochondria

Table 3. *Effect of* 0.1 mM-*pent-4-enoate and of* 0.1 mM-*pent-4-enoyl-L-carnitine on the oxidation of* 20μM *and of* 1.0 mM *concentrations of octanoate and palmitate and of their L-carnitine esters by coupled mitochondria*

Mitochondria (6–10mg of protein) were incubated in 6.0ml of medium containing 20mM-malonate and 0.5mM-L-carnitine; 0.5mM-ATP was included when octanoate or palmitate were substrates and 0.4mM-ADP when the carnitine esters were used. They were preincubated with inhibitor for 3min and the subsequent rate of oxygen uptake was recorded for 3min after addition of the substrate. Other details are given in the text. Results are given as the mean±S.E.M. with the number of experiments in parentheses. Mean values for the control rates of oxygen uptake (ng-atoms of O/min per mg of protein) are: 20μM-octanoate, 57; 1.0mM-octanoate, 59; 20μM-palmitate, 64; 1.0mM-palmitate, 39; 20μM-octanoyl-L-carnitine, 76; 20μM-palmitoyl-L-carnitine, 86.

	Inhibition of the control rate of oxygen uptake (%)	
Substrate	0.1mM-Pent-4-enoate	0.1mM-Pent-4-enoyl-L-carnitine
20μM-Octanoate	19.2±2.7 (14)	12.0±3.2 (16)
1.0mM-Octanoate	31.9±3.2 (14)	32.2±2.9 (16)
20μM-Palmitate	45.6±4.1 (14)	27.2±3.9 (16)
1.0mM-Palmitate	49.7±4.2 (14)	39.5±3.4 (16)
20μM-Octanoyl-L-carnitine	75.6±2.8 (10)	53.6±4.9 (10)
20μM-Palmitoyl-L-carnitine	70.4±3.2 (10)	47.4±3.3 (10)

Table 4. *Potency of* 10μM-*pent-4-enoyl-L-carnitine as an inhibitor of acyl-L-carnitine oxidation by coupled and uncoupled mitochondria*

Mitochondria (2.5–5.0mg of protein) were suspended in 6.0mg of medium containing 20mM-malonate; 0.4mM-ADP was added to obtain coupling conditions or 10mM-arsenate and 20μM-2,4-dinitrophenol to obtain uncoupling conditions. They were preincubated with 10μM-pent-4-enoyl-L-carnitine for 3min and the subsequent rate of oxygen uptake was recorded for 3min after the addition of the substrate. Other details are given in the text. Results are given as the mean±S.D. with the number of experiments in parentheses.

	Inhibition of the control rate of oxygen uptake (%)	
Acylcarnitine	In coupled mitochondria	In uncoupled mitochondria
100μM-Butyryl-L-carnitine	17±6 (6)	42±11 (4)
40μM-Octanoyl-L-carnitine	23±6 (5)	46±12 (5)
20μM-Lauroyl-L-carnitine	19±9 (6)	51±4 (6)
20μM-Palmitoyl-L-carnitine	27±4 (6)	57±7 (4)

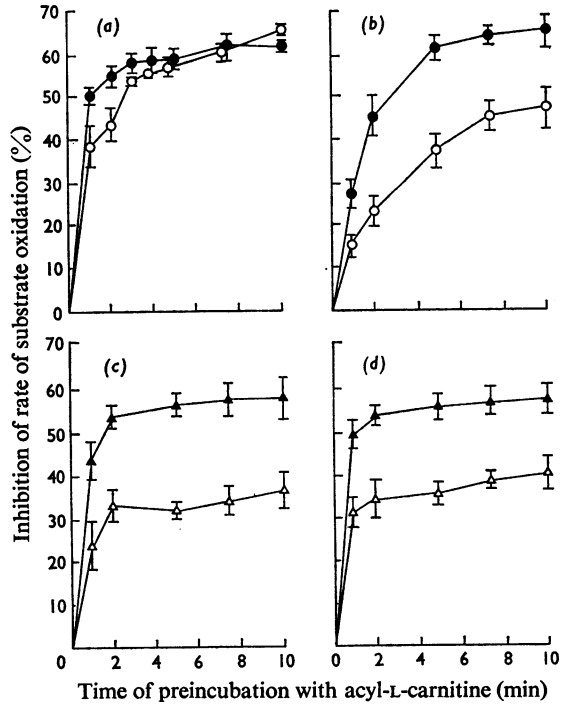

Fig. 7. *Effects of different times of preincubation of uncoupled mitochondria with* 10 μM-*pent-4-enoyl-*L-*carnitine or with* 1.0 mM-*cyclopropanecarbonyl-*L-*carnitine in the presence or in the absence of* 10 mM-*arsenate on the inhibition of the oxidation of* 20 μM-*palmitoyl-*L-*carnitine or of* 10 mM-*pyruvate*

Mitochondria (4–6 mg of protein) were preincubated with 10 μM-pent-4-enoyl-L-carnitine (*a* and *b*), or with 1.0 mM-cyclopropanecarbonyl-L-carnitine (*c* and *d*), in the presence (○, △) or absence (●, ▲) of 10 mM-arsenate for various times as indicated on the abscissae, in 6.0 ml of medium at 30°C. (*a*) and (*c*) also contained 20 mM-malonate. 20 μM-Palmitoyl-L-carnitine (*a* and *c*) or 10 mM-pyruvate (*b* and *d*) was then added and the initial rate of oxygen uptake recorded and expressed as the percentage of that in the absence of inhibitor. Each point is the mean ± S.E.M. of four experiments with different preparations of mitochondria. Other details are given in the text.

uncoupled by 2,4-dinitrophenol in the absence of phosphate, arsenate and malonate when only GTP-dependent activation of fatty acids should occur. Our results therefore suggest that arsenolysis occurs of intramitochondrial acyl-CoA formed from exogenous acylcarnitines (and possibly of acylated metabolites of pent-4-enoyl-CoA) and that inhibition of pyruvate oxidation by pent-4-enoyl-L-carnitine is mainly due to sequestration of intramitochondrial CoA or to formation of pent-4-enoyl-CoA, or both. On the other hand the lack of effect of arsenate on the inhibition of palmitoyl-L-carnitine oxidation by pent-4-enoyl-L-carnitine indicates that sequestration of CoA is not the primary cause in this case, although it may explain the slight relief of inhibition with low concentrations of inhibitor and short times of preincubation.

Brendel *et al.* (1969) claimed that addition of CoA and carnitine to pigeon liver homogenates prevented the inhibitory effects of pent-4-enoate on fatty acid oxidation. However, we have found that neither 2 mM-L-carnitine nor 0.2 mM-CoA, nor both, prevented or reversed the inhibition of the oxidation of 20 μM-palmitate by 50 μM-pent-4-enoate in rat liver mitochondria. However, addition of 2 mM-L-carnitine to mitochondria oxidizing 10 mM-pyruvate decreased the inhibition caused by the prior addition of 50 μM-pent-4-enoate. Similar partial relief of inhibition was obtained when 2 mM-L-carnitine was added after pent-4-enoate.

*Effects of penta-2,4-dienoate and of acrylate on palmitoyl-*L-*carnitine oxidation*

Penta-2,4-dienoate inhibited palmitoyl-L-carnitine oxidation as effectively as pent-4-enoate in coupled

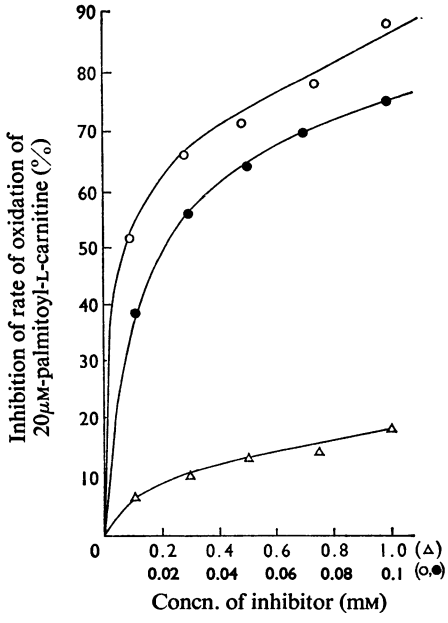

Fig. 8. *Comparison of the effects of pent-4-enoate, penta-2,4-dienoate and acrylate on the oxidation of 20μM-palmitoyl-L-carnitine in coupled mitochondria*

Mitochondria (7.0mg of protein) were preincubated for 3min in 6.0ml of medium at 30°C containing 3mM-ADP and 20mM-malonate with various concentrations of pent-4-enoate (○), pent-2,4-dienoate (●) or acrylate (△); 20μM-palmitoyl-L-carnitine was then added and the initial rate of oxygen uptake recorded and expressed as a percentage of that in the absence of inhibitor. Other details are given in the text.

mitochondria (Fig. 8), although it was only very weakly inhibitory with uncoupling conditions. Acrylate was a poor inhibitor (Fig. 8).

Effects of pent-4-enoate and related compounds on the concentrations of CoA, acetyl-CoA, acid-soluble acyl-CoA and acid-insoluble acyl-CoA in mitochondria

The intramitochondrial concentrations of free CoA and its acylated forms found (Fig. 9) agree very well with those reported by Garland *et al.* (1965), Fukami & Williamson (1971) and Chase & Tubbs (1972), considering the different techniques and strains of animals used and that the mitochondria were incubated under similar but not identical conditions.

Pent-4-enoate (0.1mM) produced a rapid and almost complete decrease in free CoA concentrations accompanied by an increase in acid-soluble acyl-CoA and by small increases in acetyl-CoA and acid-insoluble acyl-CoA (Fig. 9*a*). The accumulation of acid-soluble acyl-CoA indicates the formation of non-metabolizable or slowly metabolizable short-chain acyl-CoA derivatives of pent-4-enoate; the accumulation of acid-insoluble acyl-CoA presumably results from the inhibition of the oxidation of endogenous long-chain fatty acids, in agreement with the results of Fukami & Williamson (1971). Extensive depletion of free CoA and formation of acid-soluble acyl-CoA and some acetyl-CoA was also caused by incubating mitochondria with 0.1mM-pent-4-enoyl-L-carnitine. A similar profound depletion of free CoA was caused by 0.1mM-cyclopropanecarboxylate (Fig. 9*b*), although it had much less effect on the oxidation of palmitate than did 0.1mM-pent-4-enoate (Table 5), and this was accompanied by an almost equivalent increase in the concentration of acid-soluble acyl-CoA, whereas those of acetyl-CoA and acid-insoluble acyl-CoA were unchanged. These results agree with the findings that [1-^{14}C]cyclopropanecarboxylate (Duncombe & Rising, 1968) and cyclopropanecarbonyl-L-carnitine are not oxidized and that cyclopropanecarbonyl-CoA is not a substrate for butyryl-CoA dehydrogenase (Holland *et al.*, 1973).

Similarly, 0.1mM-*n*-pentanoate caused a rapid depletion of the CoA concentration with some increase in acetyl-CoA and acid-soluble acyl-CoA (Fig. 9*c*) consistent with the slow oxidation of *n*-pentanoate to acetyl-CoA and propionyl-CoA. By contrast, 0.1mM-octanoate (which is rapidly oxidized) caused only a 60% depletion of CoA with a nearly equivalent rise in the concentration of acetyl-CoA, and with little increase with acid-soluble acyl-CoA (Fig. 9*d*) (which was, however, already somewhat higher in this experiment). The CoA content increased again after 3min owing to conversion of octanoyl-CoA into acetoacetate and CoA.

In the presence of 20mM-malonate, 0.1mM-penta-2,4-dienoate caused a similar acylation of mitochondrial CoA to that caused by pent-4-enoate and deca-2,4,6,8-tetraenoate caused about 60% acylation, in general agreement with the results of Garland *et al.* (1970).

In other experiments (not shown) both 0.1mM-acylate and 1.0mM-acrylate had relatively small effects on the concentrations of intramitochondrial CoA derivatives. There was a decrease (up to 30%) in free CoA and an equivalent increase in acid-insoluble acyl-CoA after 6min, indicating a slight inhibition of long-chain fatty acid oxidation without apparent formation of acid-soluble acyl-CoA. This result with rat liver mitochondria was unexpected, since acrylate is a substrate for the medium-chain acyl-CoA synthetase from ox liver (Mahler *et al.*, 1953).

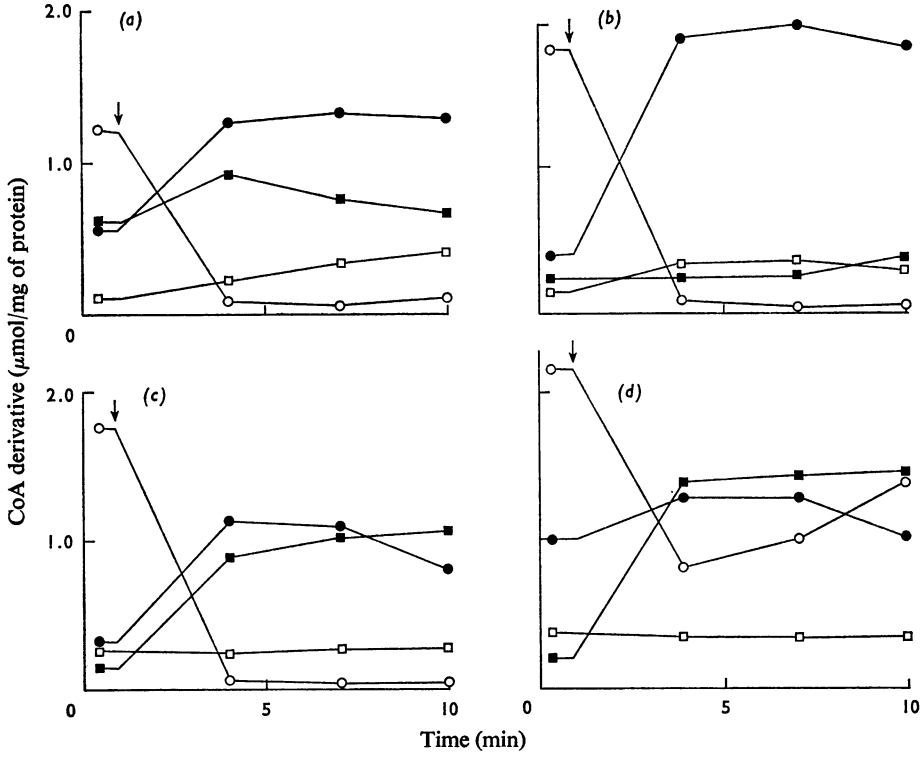

Fig. 9. *Effects of pent-4-enoate, cyclopropanecarboxylate, n-pentanoate and octanoate on the concentrations of CoA and its acyl derivatives in coupled mitochondria*

Mitochondria (30–70mg of protein) were incubated aerobically at 30°C in 12.0ml of medium containing 80mM-KCl, 50mM-sucrose, 20mM-Tris–HCl, 20mM-malonate, 5mM-$MgCl_2$, 2mM-EDTA, 2.5mM-P_i and 3.3mM-ADP, pH7.0. 0.1mM-Pent-4-enoate (*a*), 0.1mM-cyclopropanecarboxylate (*b*), 0.1mM-*n*-pentanoate (*c*) and 0.1mM-octanoate (*d*) were added at the times indicated (by unlabelled arrows). At various times 2.0ml samples of the incubation mixtures were quickly quenched with 1.0ml of ice-cold 10% (w/v) $HClO_4$ and were treated as described by Williamson & Corkey (1969) for the determination of free CoA (○), acetyl-CoA (■), acid-soluble acyl-CoA (●) and acid-insoluble acyl-CoA (□). Other details are given in the text.

Table 5. *Comparison of the effect of pent-4-enoate, n-pentanoate and cyclopropanecarboxylate on palmitate oxidation*

Mitochondria (6–10mg of protein) were incubated in 6.0ml of medium containing 20mM-malonate, 0.5mM-L-carnitine and 0.5mM-ATP; 3min after addition of the inhibitory fatty acid, 100μM-palmitate was added and the rate of oxygen uptake recorded for 3min. Other details are given in the text. Results are quoted for two experiments.

Fatty acid	Concn. of inhibitory fatty acid (mM) ...	Inhibition of the control rate of oxygen uptake (%)		
		0.1	1.0	10
Pent-4-enoate		85, 75	—	—
n-Pentanoate		14	22	56, 56
Cyclopropanecarboxylate		37	40	53, 38

Discussion

Oxidation of fatty acids by mitochondria

In this investigation fatty acid oxidation by rat liver mitochondria was measured as oxygen uptake, usually in the presence of malonate when fatty acids are oxidized quantitatively to acetoacetate. This permits direct evaluation of fatty acid oxidation; in the absence of an inhibitor of the citrate cycle the interpretation is difficult since the nature of the products (CO_2, citrate, acetoacetate etc.) depends on the exact conditions (Garland, 1968).

Bressler and co-workers (Corredor *et al.*, 1967; Brendel *et al.*, 1969) maintain that the effects of pent-4-enoate and of hypoglycin are due to the sequestration of CoA and carnitine as non-metabolizable or slowly metabolizable acyl derivatives. Corredor *et al.* (1969) found that an established inhibition of gluconeogenesis was reversed by CoA plus carnitine in pigeon liver homogenates, although Brendel *et al.* (1969) reported that inhibition of fatty acid oxidation was only prevented but not reversed by these cofactors in this system. However, Brendel *et al.* (1969) found that the inhibition of gluconeogenesis was largely reversed by addition of butyrate, hexanoate, octanoate or palmitoyl-L-carnitine, implying that the oxidation of these substrates was not inhibited. This is in direct contrast with the findings of the present study and that of Fukami & Williamson (1971), who also used rat liver mitochondria, that palmitoyl-L-carnitine is one of the most sensitive substrates to inhibition. Certainly many of the results obtained by Bressler and his associates cannot be obtained with isolated rat liver mitochondria, or with isolated pigeon liver mitochondria (H. S. A. Sherratt, unpublished work). A full discussion of the work published by Bressler and his co-workers requires more space than is justified here but some points may be made. 1. Fatty acid oxidation was assessed only by $^{14}CO_2$ formation from [1-^{14}C]palmitate so it is not apparent whether fatty acid oxidation always remained proportional to $^{14}CO_2$ release. 2. These workers have not reported any investigations of the effects of varying the concentrations of inhibitors, substrates or cofactors, and our results point to the necessity of such studies (Fig. 6, Table 3). 3. The claim that only hypoglycaemic fatty acids form CoA esters (Corredor *et al.*, 1967; Brendel *et al.*, 1969) is clearly untenable (Senior *et al.*, 1968). 4. We have failed to confirm reports (Corredor *et al.*, 1967; Entman & Bressler, 1967) that administration of L-carnitine to mice, stated to reverse inhibition of fatty acid oxidation *in vivo*, antagonizes the hypoglycaemic effects of pent-4-enoate or of hypoglycin (Marley & Sherratt, 1973). 5. Finally, no investigations appear to have been made of the effects of pent-4-enoate on blood glucose concentrations in pigeons; hypoglycin had little hypoglycaemic effect in this species although it caused fatty infiltration of the liver and sometimes death (Chen *et al.*, 1957).

Metabolism of pent-4-enoate

Pent-4-enoate is converted by β-oxidation into acetyl-CoA and acrylolyl-CoA. Williamson *et al.* (1970) suggested that some further metabolism of acryloyl-CoA occurs by at least two pathways. Rendina & Coon (1957) demonstrated its hydration to 3-hydroxypropionyl-CoA by enoyl-CoA hydratase (EC 4.2.1.17) and subsequent deacylation of this by a deacylase from pig heart. Vagelos *et al.* (1959) showed hydration of acryloyl-CoA by an enzyme from heart muscle to give lactoyl-CoA. Williamson *et al.* (1969) found that 0.5mM-pent-4-enoate was oxidized to completion by perfused rat liver and that higher concentrations were oxidized incompletely, although to an extent greater than the total CoA and carnitine content of the liver. Fukami & Williamson (1971) reported that pent-4-enoyl-CoA was oxidized slowly by sonicated rat liver mitochondria. Some very limited oxidation of [1-^{14}C]pent-4-enoate by mouse heart homogenates (Corredor *et al.*, 1967) and by pigeon liver homogenates (Brendel *et al.*, 1969) has been described. Brendel *et al.* (1969) also claimed that [2,3-^{3}H]acryloylcarnitine was formed from [4,5-^{3}H]pent-4-enoate by pigeon liver homogenates fortified with CoA and L-carnitine. Finally, all the reactions necessary for the conversion of pent-4-enoyl-CoA into acryloyl-CoA are catalysed by purified enzymes of β-oxidation (Holland *et al.*, 1973).

Effects of pent-4-enoate and related compounds on the concentrations of CoA-derivatives in mitochondria

Free CoA was barely detectable after mitochondria were incubated in the presence of 20mM-malonate with 0.1mM-pent-4-enoate, 0.1mM-cyclopropanecarboxylate, or with 0.1mM-*n*-pentanoate (Fig. 9). Free CoA concentrations were low in mitochondria rapidly oxidizing palmitoyl-L-carnitine (about 0.2nmol/mg of mitochondrial protein) both in the presence of malonate (Garland *et al.*, 1965) and in its absence (Fukami & Williamson, 1971), indicating a concentration of free CoA of 200μM assuming that there is only one pool of CoA within the matrix space (see Garland *et al.*, 1970) and that this contains 1μl of water/mg of mitochondrial protein. There were similar concentrations of free CoA in mitochondria incubated with inhibitory concentrations (20–200μM) of pent-4-enoate (Fukami & Williamson, 1971). The maximum concentration of any acyl-CoA species that could be formed intramitochondrially is about 2.6mM (cf. Fig. 9). Further, as pointed out by Garland *et al.* (1970), the reactions catalysed by the acyl-CoA synthetases are readily reversible so that

the extent of acylation of intramitochondrial CoA also depends on the concentrations of pyrophosphate and AMP (Ciman *et al.*, 1972) and of phosphate and GTP. These concentrations and their variations with different conditions are unknown (cf. Stucki *et al.*, 1972), but they may sometimes be such as to prevent complete acylation of CoA. Indeed there may be biochemical mechanisms to maintain free CoA concentrations in the presence of large amounts of short-chain fatty acids, as for example in adult ruminants (Armstrong, 1965).

Mechanism of the inhibition of fatty acid oxidation by pent-4-enoate

We contend that the strong inhibition of β-oxidation by pent-4-enoate is due to a specific enzyme block by its metabolites rather than by sequestration of free CoA, and that formation of pent-4-enoyl-CoA is a necessary prerequisite for the development of this inhibition. It is not, of course, denied that severe lowering of free CoA concentrations could also contribute to this inhibition. Other non-metabolizable or slowly metabolizable fatty acids are weaker inhibitors (Senior *et al.*, 1968) and, in contrast with the effects of pent-4-enoyl-L-carnitine, the inhibition of palmitoyl-L-carnitine oxidation by cyclopropanecarbonyl-L-carnitine can be partly reversed by arsenate. Similarly, addition of arsenate or L-carnitine, procedures that can deacylate intramitochondrial CoA (Garland *et al.*, 1970), partly reversed inhibition of pyruvate oxidation by pent-4-enoyl-L-carnitine or by pent-4-enoate respectively. It cannot, however, be assumed that reversal of inhibition necessarily means that the inhibition is simply due to CoA depletion. Thus competitive inhibition by an acyl-CoA derivative would be relieved by lowering the intramitochondrial concentration of the inhibitor. Conversely, failure to relieve an inhibition with carnitine or arsenate would not necessarily indicate irreversible inhibition of an enzyme if the inhibitory CoA species is a poor substrate for the carnitine acyltransferases or for the GTP-dependent acyl-CoA synthetase respectively. Finally, coincidence between depletion of CoA and inhibition of fatty acid oxidation may result from inhibition of an enzyme at a stage which causes an accumulation of earlier acyl-CoA derivatives in the β-oxidation sequence.

Inhibitions by acylcarnitines cannot be directly compared with inhibitions by the same concentrations of the corresponding free acids (Senior & Sherratt, 1968*a*) since different enzymes are involved in the formation of inhibitory CoA species intramitochondrially (see Holland *et al.*, 1973). In neither case is the effective concentration of the inhibitory acyl-CoA species at its site of action a simple function of the initial overall concentration of inhibitory fatty acid or of its carnitine derivative added to the system.

There are many similarities between the effects of 2-bromopalmitate [which is also hypoglycaemic in the rat (Burges *et al.*, 1968)] and of pent-4-enoate. The CoA and carnitine esters of 2-bromopalmitate are very potent inhibitors of mitochondrial CoA-dependent oxidations (Chase & Tubbs, 1972). 2-Bromopalmitoyl-CoA, generated intramitochondrially from 2-bromopalmitoylcarnitine by the action of the inner pool of carnitine palmitoyltransferase (EC 2.3.1.23), inhibits the oxidation of palmitoyl-L-carnitine, pyruvate, 2-oxoglutarate and hexanoate. The inhibition of the oxidation of the last three substrates, but not that of palmitoyl-L-carnitine, was relieved by addition of L-carnitine. It was suggested that 2-bromopalmitoyl-CoA inhibited one or more enzymes specific for long-chain intermediates of β-oxidation, and that its formation also sequestered free CoA required for the other oxidations (Chase & Tubbs, 1972).

Conclusions

The work described in the present paper leaves little doubt that pent-4-enoate causes a specific inhibition of fatty acid oxidation. It is very difficult to explain in other ways the requirement for the $CH_2{=}C{-}C{-}C{-}CO_2H$ grouping necessary for hypoglycaemic activity in compounds related to or derived from hypoglycin (Anderson *et al.*, 1958), where there are compelling reasons to believe that this activity depends on inhibition of fatty acid oxidation by this series of compounds (see Sherratt *et al.*, 1971). If inhibition is simply due to sequestration of CoA as non-metabolizable or slowly metabolizable esters it is not apparent why this should occur with hypoglycaemic fatty acids (sometimes derived *in vivo* from their amino acid precursors) but not with their structurally very similar non-hypoglycaemic analogues (Sherratt *et al.*, 1971). It is much more likely that the CoA derivatives or the unique metabolites of hypoglycaemic compounds specifically inhibit an enzyme of β-oxidation and this interpretation is strongly supported by the finding that penta-2,4-dienoyl-CoA, formed from pent-4-enoyl-CoA, is an effective and specific inhibitor of 3-oxoacyl-CoA thiolase (Holland *et al.*, 1973).

We thank Mrs. Freda Hardy for excellent technical assistance, Dr. G. A. Swan for help with the synthesis and characterization of acylcarnitines, and the British Diabetic Association for generous financial help.

References

Allred, J. B. & Guy, D. G. (1969) *Anal. Biochem.* **29**, 293–299

Anderson, H. V., Johnson, J. L., Nelson, J. W., Olson, E. C., Spector, M. E. & Vavra, J. J. (1958) *Chem. Ind. (London)* 330

Armstrong, D. G. (1965) in *Physiology of Digestion in the Rumen* (Doughtery, R. W., ed.), pp. 272–288, Butterworths, London

Bohmer, T. & Bremer, J. (1968) *Biochim. Biophys. Acta* **152**, 559–567
Bremer, J. (1966) *Biochim. Biophys. Acta* **116**, 1–11
Bremer, J. (1968) in *Cellular Compartmentation and Control of Fatty Acid Metabolism* (Gran, F. C., ed.), pp. 65–88, Academic Press, London and New York
Brendel, K., Corredor, C. F. & Bressler, R. (1969) *Biochem. Biophys. Res. Commun.* **34**, 340–347
Bressler, R., Corredor, C. & Brendel, K. (1969) *Pharmacol. Rev.* **21**, 105–130
Burges, R. A., Butt, W. D. & Baggaley, A. (1968) *Biochem. J.* **109**, 38P–39P
Chase, J. F. A. & Tubbs, P. K. (1966) *Biochem. J.* **99**, 32–40
Chase, J. F. A. & Tubbs, P. K. (1972) *Biochem. J.* **129**, 55–65
Chen, K. K., Anderson, R. C., McCowen, M. C. & Harris, P. N. (1957) *J. Pharmacol. Exp. Ther.* **121**, 272–285
Ciman, M., Rossi, C. R. & Siliprandi, N. (1972) *FEBS Lett.* **22**, 8–10
Corredor, C., Brendel, K. & Bressler, R. (1967) *Proc. Nat. Acad. Sci. U.S.* **58**, 2299–2306
Corredor, C., Brendel, K. & Bressler, R. (1969) *J. Biol. Chem.* **244**, 1212–1219
De Renzo, E. C., McKerns, K. W., Bird, H. H., Cekleniak, W. P., Coulomb, B. & Kaleita, E. (1958) *Biochem. Pharmacol.* **1**, 236–237
Duncombe, W. G. & Rising, T. J. (1968) *Biochem. J.* **109**, 449–455
Entman, M. & Bressler, R. (1967) *Mol. Pharmacol.* **3**, 333–340
Freidman, S., McFarlane, J. E., Battacharyya, P. K. & Fraenkel, G. (1965) *Arch. Biochem. Biophys.* **59**, 484–490
Fritz, I. B. & Yue, K. T. N. (1963) *J. Lipid Res.* **4**, 279–288
Fukami, M. H. & Williamson, J. R. (1971) *J. Biol. Chem.* **246**, 1206–1212
Galzigna, L., Rossi, C. R., Sartorelli, L. & Gibson, D. M. (1967) *J. Biol. Chem.* **242**, 2111–2115
Garland, P. B. (1968) *Biochem. Soc. Symp.* **27**, 41–60
Garland, P. B., Shepherd, D. & Yates, D. W. (1965) *Biochem. J.* **97**, 587–594
Garland, P. B., Yates, D. W. & Haddock, B. A. (1970) *Biochem. J.* **119**, 553–564
Holland, P. C. & Sherratt, H. S. A. (1969) *Biochem. J.* **114**, 67P
Holland, P. C. & Sherratt, H. S. A. (1970) *Biochem. J.* **118**, 4P–5P
Holland, P. C. & Sherratt, H. S. A. (1972) *Biochem. J.* **127**, 79P
Holland, P. C., Senior, A. E. & Sherratt, H. S. A. (1973) *Biochem. J.* **136**, 173–184
Layne, E. (1957) *Methods Enzymol.* **3**, 447–454
Levitsky, D. O. & Skulachev, V. P. (1972) *Biochim. Biophys. Acta* **275**, 33–50
Mahler, H. R., Wakil, S. J. & Bock, R. M. (1953) *J. Biol. Chem.* **204**, 453–468
Marley, J. & Sherratt, H. S. A. (1973) *Biochem. Pharmacol.* **22**, 281–284
McKerns, K. W., Bird, H. H., Kaleita, E., Coulomb, B. S. & De Renzo, E. C. (1960) *Biochem. Pharmacol.* **3**, 305–315
Menahan, L. A. & Williams, R. H. (1971) *Eur. J. Biochem.* 448–493
Rendina, G. & Coon, M. J. (1957) *J. Biol. Chem.* **225**, 523–534
Ruderman, N. B., Shafrir, E. & Bressler, R. (1968) *Life Sci.* **7**, 1083–1089
Senior, A. E. (1967) Ph.D. Dissertation, University of Newcastle upon Tyne
Senior, A. E. & Sherratt, H. S. A. (1968*a*) *Biochem. J.* **110**, 499–509
Senior, A. E. & Sherratt, H. S. A. (1968*b*) *Biochem. J.* **110**, 521–527
Senior, A. E., Robson, B. & Sherratt, H. S. A. (1968) *Biochem. J.* **110**, 511–519
Sherratt, H. S. A., Holland, P. C., Marley, J. & Senior, A. E. (1971) in *A Symposium on Mechanisms of Toxicity* (Aldridge, W. N., ed.), pp. 205–218, Macmillan, London
Snyder, F. & Stephens, N. (1959) *Biochim. Biophys. Acta* **34**, 244–245
Strack, E. & Lorentz, I. (1954) *Hoppe-Seyler's Z. Physiol. Chem.* **298**, 27–33
Stucki, J. W., Brawand, F. & Walter, P. (1972) *Eur. J. Biochem.* **27**, 181–191
Tanaka, K., Isselbacher, K. J. & Shih, V. (1972) *Science* **175**, 69–71
Toews, C. J., Lowy, G. & Ruderman, N. B. (1970) *J. Biol. Chem.* **245**, 818–824
Vagelos, P. R., Earl, J. M. & Stadtman, E. R. (1959) *J. Biol. Chem.* **234**, 765–769
Van Tol, A., De Jong, J. W. & Hulsman, W. C. (1969) *Biochim. Biophys. Acta* **176**, 414–416
von Holt, C. (1966) *Biochim. Biophys. Acta* **125**, 1–10
West, D. W., Chase, J. F. A. & Tubbs, P. K. (1971) *Biochem. Biophys. Res. Commun.* **42**, 912–918
Williamson, J. R. & Corkey, B. E. (1969) *Methods Enzymol.* **13**, 434–513
Williamson, J. R., Fukami, M. H., Peterson, M. J., Rostand, S. G. & Schaltz, R. (1969) *Biochem. Biophys. Res. Commun.* **36**, 407–413
Williamson, J. R., Rostand, S. G. & Peterson, M. J. (1970) *J. Biol. Chem.* **245**, 3242–3251
Yardley, H. J. (1964) *Abstr. Int. Congr. Biochem. 6th* **7**, 166
Yardley, H. J. & Godfrey, G. (1963) *Biochem. J.* **88**, 16P–17P
Yardley, H. J. & Godfrey, G. (1967) *Arch. Dermatol.* **96**, 89–93
Ziegler, H. J., Bruckner, P. & Binon, F. (1967) *J. Org. Chem.* **32**, 3989–3991