Specific Inhibition of Mitochondrial Fatty Acid Oxidation by 2-Bromopalmitate and its Coenzyme A and Carnitine Esters

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1. The CoA and carnitine esters of 2-bromopalmitate are extremely powerful and specific inhibitors of mitochondrial fatty acid oxidation. 2. 2-Bromopalmitoyl-CoA, added as such or formed from 2-bromopalmitate, inhibits the carnitine-dependent oxidation of palmitate or palmitoyl-CoA, but not the oxidation of palmitoylcarnitine, by intact liver mitochondria. 3. 2-Bromopalmitoylcarnitine inhibits the oxidation of palmitoylcarnitine as well as that of palmitate or palmitoyl-CoA. It has no effect on succinate oxidation, but inhibits that of pyruvate, 2-oxoglutarate or hexanoate; however, the oxidation of these substrates (but not of palmitate, palmitoyl-CoA or palmitoyl-carnitine) is restored by carnitine. 4. In damaged mitochondria, added 2-bromopalmitoyl-CoA does inhibit palmitoylcarnitine oxidation; pyruvate oxidation is unaffected by the inhibitor alone, but is impaired if palmitoylcarnitine is subsequently added. 5. The findings have been interpreted as follows. 2-Bromopalmitoyl-CoA inactivates (in a carnitine-dependent manner) a pool of carnitine palmitoyltransferase which is accessible to external acyl-CoA. This results in inhibition of palmitate or palmitoyl-CoA oxidation. A second pool of carnitine palmitoyltransferase, inaccessible to added acyl-CoA in intact mitochondria, can generate bromopalmitoyl-CoA within the matrix from external 2-bromopalmitoylcarnitine; this reaction is reversible. Such internal 2-bromopalmitoyl-CoA inactivates long-chain β -oxidation (as does added 2-bromopalmitoyl-CoA if the mitochondria are damaged) and its formation also sequesters intramitochondrial CoA. Since this CoA is shared by pyruvate and 2-oxoglutarate dehydrogenases, the oxidation of their substrates is depressed by 2-bromopalmitoylcarnitine, unless free carnitine is available to act as a 'sink' for long-chain acyl groups. 6. These effects are compared with those reported for other inhibitors of fatty acid oxidation.

The CoA derivatives of fatty acids are only oxidized when added to intact mitochondria if carnitine is present, whereas fatty acyl esters of carnitine are excellent substrates by themselves. Such observations suggest that carnitine esters are obligatory intermediates in the mitochondrial metabolism of externally formed acyl-CoA. When free fatty acids are used as substrates, carnitine is required with long-chain acids such as palmitate provided that the mitochondria are undamaged, although short-chain acids such as hexanoate are also oxidized in its absence; this difference is a consequence of the locations of the relevant acyl-CoA synthetases with respect to the mitochondrial inner membrane (Van den Bergh *et al.*, 1969; Lippel & Beattie, 1970).

The details of the role which carnitine esters play in the transport of fatty acyl groups into mitochondria are not fully understood. Fritz & Yue (1963) proposed that this involves the sequential action of two pools of carnitine palmitoyltransferase (EC 2.3.1.23), only one of which is accessible to external acyl-CoA. Evidence in support of this has accumulated (Bremer,

1963; Garland & Yates, 1967; Hoppel & Tomec, 1972). We have investigated this problem with the aid of inhibitory substrate analogues, based on the finding that the bromoacetyl esters of CoA and carnitine are very powerful inhibitors of carnitine acetyltransferase (EC 2.3.1.7) (Chase & Tubbs, 1969, 1970). A partially purified carnitine palmitoyltransferase was found to be strongly inhibited by the CoA derivatives of 2-bromo-substituted fatty acids (Tubbs & Chase, 1967) and we also briefly reported that these compounds prevent the mitochondrial oxidation of palmitoyl-CoA, but not that of palmitoylcarnitine. An acyl-carnitine analogue, 2-bromomyristoylthiocarnitine, was, however, found to inhibit the oxidation of both substrates (Tubbs & Chase, 1970). The present paper reports the effects of 2-bromopalmitate and its CoA and carnitine esters on the oxidation of various mitochondrial substrates. These compounds are potent and specific inhibitors of fatty acid oxidation. The results indicate the existence in rat-liver mitochondria of two forms of carnitine palmitoyltransferase, only one of which is inhibited by the 2-bromoacyl esters

of CoA or carnitine; two such transferases have been obtained from ox liver (West *et al.*, 1971). Evidence is presented that the intramitochondrial pool of CoA that is acylated by externally added palmitoylcarnitine is shared by the pyruvate and 2-oxoglutarate dehydrogenases, as well as by the enzymes of β -oxidation.

Materials

Carnitine acetyltransferase was obtained from Boehringer Corp. (London) Ltd., London W.5, U.K. 2-Oxoglutarate dehydrogenase (EC 1.2.4.2) was prepared from pig heart by a modification of the method of Sanadi *et al.* (1952).

L-Carnitine hydrochloride was bought from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K. and converted into the perchlorate form as described by Chase & Tubbs (1970). Palmitic acid and 2-oxoglutaric acid were obtained from BDH Chemicals Ltd., Poole, Dorset, U.K., as was sodium DL-3-hydroxybutyrate. Sodium pyruvate, CoA, NAD^+ , ATP, ADP, acetylpyridine-adenine dinucleotide and cytochrome *c* were bought from Boehringer Corp. (London) Ltd.; hexanoic acid and DL-2-bromopalmitic acid were from Fluka A.G., Buchs, Switzerland, and L-malic acid ('A' grade) was from Calbiochem, Los Angeles, Calif., U.S.A. Bovine serum albumin, from Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex, U.K., was defatted by the method of Chen (1967).

Other chemicals were of the highest quality available, usually from BDH Chemicals Ltd.

Methods

CoA derivatives

Palmitoyl-CoA was prepared by treating CoA with excess of *S*-palmitoyl thioglycollate. The latter compound was prepared as follows: palmitic acid (5g; 20.3mmol) was dissolved in 15ml of trifluoroacetic acid and 2.5ml (about 18mmol) of trifluoroacetic anhydride was added. After 1 h at room temperature, 1.4ml (20mmol) of thioglycollic acid (redistilled and stored at -20°C ; see White, 1960) was added, followed 30min later by a few millilitres of water. The mixture was evaporated to dryness *in vacuo*, and the palmitoyl thioglycollate was recrystallized from hexane. To prepare palmitoyl-CoA, 175mg (530 μmol) of palmitoyl thioglycollate was dissolved in 1.8ml of 2-methylpropan-2-ol and 1.6ml of water at 30°C , and 0.5ml of 1M-NaOH was added. CoA (50mg), dissolved in 1ml of aq. 50% (v/v) 2-methylpropan-2-ol was added, followed by 1M-NaOH to give a monophasic solution at pH8.5. After 45min at 30°C , the solution was acidified with HClO_4 to pH3 and the 2-methylpropan-2-ol was largely removed with N_2 . More HClO_4 was

added to give pH1–2 and the mixture extracted six times with ether. After the ether had been evaporated and the product cooled, the insoluble palmitoyl-CoA was washed twice with cold 2% (w/v) HClO_4 and finally dissolved in 25mM-potassium phosphate buffer, pH7; the yield based on CoA was 80%.

DL-2-Bromopalmitoyl-CoA was made by treating CoA with the acyl chloride (Seubert, 1960) in aq. 50% 2-methylpropan-2-ol adjusted to pH8 with NaOH. Acylation was monitored by the nitroprusside method (Stadtman, 1957); when this was complete, the mixture was acidified with HClO_4 and the insoluble bromopalmitoyl-CoA purified as described for palmitoyl-CoA. Bromopalmitoyl chloride was prepared by treating the acid [twice recrystallized from light petroleum (b.p. $40\text{--}60^\circ\text{C}$)] in dry benzene at room temperature for 4–6h with excess of oxalyl chloride, in the presence of a trace of dimethylformamide as catalyst (Bosshard *et al.*, 1959). Volatile material was then removed by rotary evaporation.

Concentrations of palmitoyl- and bromopalmitoyl-CoA were calculated by assuming a ϵ_{260} of $16.4 \times 10^3 \text{ cm}^{-1}$ (Stadtman, 1957).

Palmitoyl-L-carnitine perchlorate

The method of Bremer (1968) was modified as follows. The mixed anhydride of palmitic acid and trifluoroacetic acid, in trifluoroacetic acid solution, was prepared as described above for the preparation of palmitoyl thioglycollate. Instead of the thioglycollic acid, 0.75equiv. (based on the palmitic acid used) of L-carnitine perchlorate was added, dissolved in trifluoroacetic acid. After 1 h the mixture was cooled to 0°C , an equal volume of cold water was added, and after 30min the mixture was extracted four times with light petroleum (b.p. $40\text{--}60^\circ\text{C}$) and evaporated to dryness by rotary evaporation; a drop of octan-2-ol was added to minimize frothing. The residue was dissolved in dilute NaHCO_3 soln., and palmitoyl-L-carnitine was precipitated by acidification with HClO_4 and chilling. After being washed twice with cold 2% (w/v) HClO_4 and then with a small volume of cold water, the palmitoylcarnitine perchlorate was dried over P_2O_5 .

DL-2-Bromopalmitoyl-L-carnitine perchlorate

L-Carnitine perchlorate (1.2g) was dissolved in a small volume of dry, redistilled acetonitrile; bromopalmitoyl chloride (4.5ml), prepared as described above, was added, followed by dry 2-methylpropan-2-ol to give a monophasic solution. The flask was placed in a desiccator containing KOH pellets, which was then partially evacuated and kept at 37°C for 4 days. After cooling, a small volume of water was added and the mixture was left for 1 h before being

evaporated to dryness by rotary evaporation. The residue was dissolved in aq. 50% (v/v) ethanol, 2 drops of 60% (w/v) HClO_4 were added and the solution was extracted four times with light petroleum. The aqueous layer was adjusted to pH 5 with 1M-NaOH, dried by rotary evaporation and storage over P_2O_5 , and the residue was extracted with warm ethanol. After concentration by rotary evaporation, several volumes of cold aq. 2% (w/v) HClO_4 were added; the precipitate was washed once with dilute HClO_4 and twice with a little ice-cold water, and then dried *in vacuo* over P_2O_5 . The resulting material, dissolved in ethanol, gave a single spot on t.l.c. in methanol-acetone-conc. HCl (1:9:1, by vol.; Linstedt & Linstedt, 1965), with an R_f close to that of palmitoylcarnitine. Purified 'outer' carnitine palmitoyltransferase (West *et al.*, 1971) was totally inactivated when incubated for 2 min at pH 8.0 with the material at a final concentration of $5 \mu\text{g}/\text{ml}$, provided that CoA was also present (Chase & Tubbs, 1969). For these reasons it is assumed that the product was DL-2-bromopalmitoyl-L-carnitine perchlorate; concentrations given are based on mol.wt. 577.

Mitochondrial experiments

Mitochondria were prepared from livers of albino rats by conventional differential centrifugation in 0.25M-sucrose containing 10mM-tris-HCl, pH 7.4; the mitochondria were twice washed in the same medium and finally resuspended to give a protein concentration of about 70mg/ml. Mitochondrial protein was determined by the method of Gornall *et al.* (1949).

Rates of oxygen consumption at 30°C were measured polarographically with an oxygen electrode (Rank Bros., Bottisham, Cambs., U.K.). Except where otherwise indicated in the text, mitochondria (5–10mg of protein) were suspended in 3ml of a medium containing 80mM-KCl, 20mM-tris-HCl, pH 7.4, 3.3mM- MgCl_2 , 3.3mM-potassium phosphate, 3.3mM-potassium malonate, 1mM-ADP and 4mg of defatted bovine serum albumin/ml. Palmitic acid and bromopalmitic acid and their carnitine esters were added as ethanol solutions; other substrates and inhibitors were added as neutral aqueous solutions. All substrate and inhibitor solutions were stored at –20°C.

The methods used for assay of mitochondrial contents of CoA, acetyl-CoA and long-chain acyl-CoA have been described by Tubbs & Garland (1969). CoA was measured spectrophotometrically by using oxoglutarate dehydrogenase and acetylpyridine-adenine dinucleotide; acetyl-CoA and long-chain acyl-CoA were measured similarly after cleavage by carnitine acetyltransferase and alkaline hydrolysis respectively.

Results

Effects of DL-2-bromopalmitate on mitochondrial respiration

Fig. 1 shows the results obtained with mitochondria that scarcely oxidized palmitate in the absence of added CoA (the requirement for CoA, unlike that for carnitine, is variable; Garland *et al.*, 1965). When such mitochondria were preincubated with $20 \mu\text{M}$ -DL-2-bromopalmitate in the presence of ATP, CoA and carnitine, the oxidation of palmitate or palmitoyl-CoA was virtually abolished. The oxidation of palmitoylcarnitine, or of pyruvate plus malate (not shown), was unaffected by this treatment, whereas that of 0.2mM-hexanoate was about 25% inhibited.

The inhibitory effect of bromopalmitate on palmitoyl-CoA oxidation was only observed if both CoA and ATP were available during the preincubation

Fig. 1. Effects of bromopalmitate on mitochondrial fatty acid oxidation

(a) Rat-liver mitochondria (6.7mg of protein) were added to the standard incubation system (see the Methods section) containing, in addition, 2mM-ATP, 70mM-CoA and $20 \mu\text{M}$ -bromopalmitate. Then 1mM-carnitine was added as shown, followed by $40 \mu\text{M}$ -palmitate and $20 \mu\text{M}$ -palmitoylcarnitine. The broken line shows the course of oxygen uptake seen on the addition of palmitate in the absence of bromopalmitate. (b) As for (a), except that $12.5 \mu\text{M}$ -palmitoyl-CoA was added instead of palmitate. (c) As for (a), except that the CoA concentration was $23 \mu\text{M}$ and $200 \mu\text{M}$ -hexanoate was added instead of palmitate. The following abbreviations are used in the Figures and Scheme 1: Alb, bovine serum albumin; Cn, L-carnitine; AcCoA, acetyl-CoA; Hex, hexanoate; Hex-CoA, hexanoyl-CoA; Pm, palmitate; PmCoA, palmitoyl-CoA; PmCn, palmitoyl-L-carnitine; BrPm, DL-2-bromopalmitate; BrPmCoA, DL-2-bromopalmitoyl-CoA; BrPmCn, DL-2-bromopalmitoyl-L-carnitine; Dnp-OH, 2,4-dinitrophenol; OG, 2-oxoglutarate; Pyr + Mal pyruvate + L-Malate; Succ, succinate.

(Fig. 2); in the absence of added CoA, bromopalmitate caused progressive inhibition, presumably after release of CoA from palmitoyl-CoA. This suggests that bromopalmitoyl-CoA, rather than bromopalmitate itself, was the true inhibitor. For palmitate oxidation, acyl-CoA synthetase may be an additional site of inhibition by bromopalmitate (Sauer *et al.*, 1971; Mahadevan & Sauer, 1971).

Effects of DL-2-bromopalmitoyl-CoA on respiration

Figs. 3 and 4 show that preincubation of intact mitochondria with bromopalmitoyl-CoA in the presence of carnitine inhibited the subsequent carnitine-dependent oxidation of palmitate or palmitoyl-CoA; palmitoylcarnitine oxidation was, however, unaffected. Similar results were obtained with bromolauroyl-CoA and bromostearoyl-CoA.

Inhibition of palmitoyl-CoA oxidation was only observed when carnitine, as well as bromopalmitoyl-CoA, was present during preincubation of the mito-

Fig. 2. CoA and ATP requirement for the inhibition of palmitoyl-CoA oxidation by bromopalmitate

(a) Mitochondria were incubated with bromopalmitate and ATP as in Fig. 1, but without added CoA; carnitine, palmitoyl-CoA and palmitoylcarnitine were added as in Fig. 1(b). The broken line (---) shows the result when bromopalmitate was absent; the dotted line (····) was obtained when CoA ($70\mu\text{M}$) and bromopalmitate were both present initially. (b) Mitochondria were depleted of endogenous ATP by incubation with $50\mu\text{M}$ -2,4-dinitrophenol in the standard medium, which contained $70\mu\text{M}$ -CoA but no serum albumin. After 2 min albumin (4mg/ml) was added, followed by $20\mu\text{M}$ -bromopalmitate, 1mM -carnitine and $12.5\mu\text{M}$ -palmitoyl-CoA. The broken line (---) shows the result when bromopalmitate was absent; the dotted line (····) was obtained when ATP (2mM) and bromopalmitate were both present initially. Abbreviations are as used in Fig. 1.

chondria (Figs. 3a and 3b). Palmitoyl-CoA itself afforded considerable protection against the inhibitor. The concentrations of bromopalmitoyl-CoA used in these experiments (up to $14\mu\text{M}$; Figs. 4a and 4b) had no effect on the oxidation of pyruvate, 2-oxoglutarate, succinate or 3-hydroxybutyrate, whether or not carnitine was also present. Respiratory control with pyruvate as substrate was unimpaired. With 0.2mM -hexanoate as substrate very little inhibition was observed (Figs. 4b).

The amount of bromopalmitoyl-CoA needed to produce a given degree of inhibition of palmitoyl-CoA oxidation was found to depend on the quantity of mitochondria used; thus with 4.9mg and 9.8mg of mitochondrial protein 50% inhibition was caused by 0.25nmol and 0.45nmol respectively under conditions similar to those of Fig. 4(a).

Although palmitoyl-CoA oxidation by intact mitochondria is entirely carnitine-dependent, acyl-CoA oxidation may be observed without the addition of carnitine in mitochondria that have been damaged by exposure to hypo-osmotic conditions (Fig. 5).

Fig. 3. Effects of bromopalmitoyl-CoA on mitochondrial oxidation of palmitoyl-CoA and palmitate

(a) Mitochondria (4.6mg of protein) were added to the standard incubation medium, containing in addition 2.5mM -L-carnitine and $0.34\mu\text{M}$ -bromopalmitoyl-CoA. After 2 min, $12.5\mu\text{M}$ -palmitoyl-CoA was added, followed by $20\mu\text{M}$ -palmitoylcarnitine as shown. The broken line (---) shows the course of oxygen uptake when substrate was added in the absence of bromopalmitoyl-CoA. (b) As for (a), except that carnitine was omitted from the original incubation mixture. Palmitoyl-CoA ($12.5\mu\text{M}$) and L-carnitine (2.5mM) were added as shown. (c) As for (a), except that 2mM -ATP and $100\mu\text{M}$ -CoA were present originally and $40\mu\text{M}$ -palmitate was added instead of palmitoyl-CoA. Abbreviations are as used in Fig. 1.

Fig. 4. Effects of different concentrations of bromopalmitoyl-CoA on mitochondrial respiration

(a) ▲, Rates of oxygen uptake when mitochondria (4.6 mg of protein) were preincubated for 3 min in the standard medium containing bromopalmitoyl-CoA (BrPmCoA) (as shown) plus 2.5 mM-L-carnitine before the addition of 12.5 μM-palmitoyl-CoA as substrate; ●, rates of oxygen uptake after the further addition of 20 μM-palmitoyl-carnitine to the above systems (cf. Fig. 3a). (b) ■, Rates of oxygen uptake when mitochondria (7.0 mg of protein) were preincubated for 3 min in the standard medium containing bromopalmitoyl-CoA (as shown), L-carnitine (2.5 mM) and CoA (100 μM), before the addition of palmitate (40 μM) as substrate; ○, as above, except that CoA was omitted from the preincubation and hexanoate (200 μM) was added as substrate. All oxidation rates shown have been corrected for the small blank rate found in the absence of added substrate.

Preincubation of damaged mitochondria with bromopalmitoyl-CoA had inhibitory effects additional to those found when intact mitochondria were used. The oxidation of palmitoylcarnitine was almost abolished and that of pyruvate became severely inhibited if palmitoylcarnitine was present; the latter inhibition was relieved by the subsequent addition of free carnitine (Fig. 5c). These effects of bromopalmitoyl-CoA on damaged mitochondria strongly resemble the results, described in the following section, of treating intact mitochondria with bromopalmitoylcarnitine.

Effects of DL-2-bromopalmitoylcarnitine on respiration

Figs. 6 and 7 show that preincubation of mitochondria with bromopalmitoylcarnitine abolished the oxidation of palmitoylcarnitine as well as the carnitine-dependent oxidation of palmitate or palmitoyl-CoA. The oxidation of succinate (or DL-3-hydroxybutyrate, not shown) was unaffected, but bromopalmitoylcarnitine strongly inhibited the oxidation of pyruvate plus malate, 2-oxoglutarate or hexanoate. The oxidation of these substrates (but not of palmitate, palmitoyl-CoA or palmitoylcarnitine) was largely restored by the further addition of carnitine (Figs. 6 and 7). Palmitoylcarnitine, however, enhanced the inhibition of 2-oxoglutarate (or pyruvate) oxidation caused by preincubation with low con-

centrations of bromopalmitoylcarnitine (Fig. 6c). With pyruvate as substrate in the presence of carnitine, even large concentrations (30 μM) of bromopalmitoylcarnitine had no effect on mitochondrial respiratory control, suggesting that it caused very little damage to the mitochondria.

In some experiments, DL-2-bromomyristoyl-DL-thiocarnitine (Tubbs & Chase, 1970) was used as an inhibitor; the results obtained were similar to those reported here for bromopalmitoylcarnitine.

The inhibition of mitochondrial pyruvate (or oxoglutarate or hexanoate) oxidation by bromopalmitoylcarnitine, and its at least partial reversal by subsequently added carnitine, were surprising, since these effects would not result from the expected inactivation of carnitine palmitoyltransferase. Indeed, the reversal of inhibition by carnitine (Figs. 6 and 7) and its potentiation by palmitoylcarnitine (Figs. 6c) indicated that part at least of the mitochondrial carnitine palmitoyltransferase was still active in the presence of bromopalmitoylcarnitine. Further evidence that the apparently irreversible inhibition of long-chain β-oxidation by bromopalmitoylcarnitine was not due to inactivation of the transferase is provided by the results in Fig. 8. In this experiment, mitochondria were deliberately damaged by exposure to 40 μM-palmitate in the absence of serum albumin, so that they oxidized palmitate without the addition of carnitine. Even such carnitine-independent fatty

Fig. 5. Effects of bromopalmitoyl-CoA on respiration of mitochondria damaged by exposure to hypo-osmotic conditions

(a) Mitochondria (0.15 ml; 11.2 mg of protein) were added to 2.65 ml of water at 30°C. After 2 min, 3.3 mM-potassium phosphate, pH 7.0, 3.3 mM-MgCl₂, 3.3 mM-potassium malonate, 20 mM-tris-HCl, pH 7.4, 1 mM-ADP, 0.48 mg of cytochrome *c*/ml and 0.12 mM-NAD⁺ were added to give a final volume of 3.0 ml. Recording of the oxygen content of the medium was begun and palmitoyl-CoA (23 μM), L-carnitine (1 mM) and palmitoylcarnitine (20 μM) were added as shown. (b) Mitochondria were preincubated as in (a). After recording was begun, bromopalmitoyl-CoA (56 μM) was added, followed after 3 min by palmitoyl-CoA (23 μM), L-carnitine (1 mM) and pyruvate (5 mM) plus L-malate (1 mM) as shown. (c) As in (b), except that palmitoylcarnitine (20 μM) was added, followed by pyruvate plus malate and L-carnitine. The broken line (---) shows the rate of pyruvate plus malate oxidation when palmitoylcarnitine was omitted. Abbreviations are as used in Fig. 1.

acid oxidation, in which the palmitoyltransferase presumably plays no part, was inhibited by bromopalmitoylcarnitine.

Fig. 9 shows that the amounts of bromopalmitoylcarnitine that gave 50% inhibition of oxidation of either palmitoylcarnitine or pyruvate were proportional to the quantity of mitochondria used.

A reasonable interpretation of the above findings with bromopalmitoylcarnitine is illustrated in Scheme 1. According to this scheme, bromopalmitoylcarnitine fails to inhibit the 'inner' pool of carnitine palmitoyltransferase, for which indeed it is a substrate. Thus treatment of mitochondria with bromopalmitoylcarnitine gives rise to bromopalmitoyl-CoA within the inner membrane, which, in the absence of carnitine, is impermeable to fatty acyl groups (Yates & Garland, 1966). Bromopalmitoyl-CoA formed within the mitochondrial matrix space might well act as a powerful inhibitor of one or more of the enzymes of long-chain β-oxidation. Further

Fig. 6. Effects of bromopalmitoylcarnitine on the mitochondrial oxidation of succinate, 2-oxoglutarate and palmitoylcarnitine

(a) Mitochondria were preincubated for 2 min together with 5.5 μM-bromopalmitoylcarnitine in the standard medium from which malonate had been omitted. Subsequent additions of palmitoylcarnitine (20 μM), 2-oxoglutarate (2 mM) and L-carnitine (2.5 mM) were made as shown. The broken line (---) and dotted line (···) show the rates of oxygen uptake when palmitoylcarnitine or 2-oxoglutarate respectively were added in the absence of bromopalmitoylcarnitine. (b) and (c). As for (a), but the order of addition of substrates was as shown. The broken line (---) in (b) shows the rate of oxygen uptake resulting from the addition of succinate (2 mM); this was unaltered by preincubation of the mitochondria with bromopalmitoylcarnitine. Abbreviations are as used in Fig. 1.

additions of either bromopalmitoyl- or palmitoylcarnitine would then cause extensive acylation of the matrix CoA pool, thereby depriving CoA-dependent enzymes, such as pyruvate and 2-oxoglutarate dehydrogenases, of this substrate. As the carnitine palmitoyltransferase reaction is freely reversible, the subsequent addition of free carnitine provides a 'sink' for long-chain acyl groups and results in deacylation of the matrix CoA and restoration of the CoA-dependent oxidation of pyruvate and oxoglutarate.

Such a scheme is compatible with the results reported in Figs. 6-9, and that it is substantially correct is confirmed by the experiment described below.

Acylation state of CoA in mitochondria treated with bromopalmitoylcarnitine

The acylation state of mitochondrial CoA was investigated by using an apparatus similar to that described by Garland *et al.* (1965); this permits the repeated removal of samples for analysis from a mito-

Fig. 7. Effects of various concentrations of bromopalmitoylcarnitine on mitochondrial respiration

(a) ●, Rates of oxygen uptake observed after mitochondria (5.5 mg of protein) were preincubated for 2 min in the standard medium containing various concentrations of bromopalmitoylcarnitine (BrPmCn) (as shown), before the addition of palmitoylcarnitine (20 μM) as substrate; ○, rates on the further addition of pyruvate (5 mM) plus L-malate (1 mM) to the above system; △, rates on the addition of L-carnitine (3.33 mM) to a system already containing palmitoylcarnitine and pyruvate plus malate (see Fig. 6a for an analogous experiment in which oxoglutarate was added in place of pyruvate plus malate). (b) ■, Rates of oxygen uptake on the addition of hexanoate (200 μM) to mitochondria (5.0 mg of protein) preincubated with bromopalmitoylcarnitine as in (a); □, rates observed on the further addition of L-carnitine (3.3 mM) after hexanoate. All oxidation rates shown have been corrected for the small blank rate found in the absence of added substrate.

chondrial incubation system, the oxygen consumption of which is continuously monitored.

Fig. 10 shows that, in agreement with Garland *et al.* (1965), mitochondria oxidizing palmitoylcarnitine contain considerable amounts of long-chain acyl-CoA and especially of acetyl-CoA, and their content of free CoA is correspondingly decreased. Exposure of the mitochondria to bromopalmitoylcarnitine caused the virtual disappearance of free CoA; this was accompanied by the formation of large amounts of long-chain acyl-CoA (presumably bromopalmitoyl-CoA) and a decrease in the total CoA content. As would be expected, no acetyl-CoA was formed from bromopalmitoylcarnitine or from palmitoylcarnitine by mitochondria that had been treated with the inhibitor. Addition of free carnitine to mitochondria treated with bromopalmitoylcarnitine caused the reappearance of free CoA at the expense of long-chain acyl-CoA; in accord with Scheme 1 this was accompanied by the restoration of rapid pyruvate oxidation. Carnitine did not, however, reverse the decrease of the total assayable CoA that was caused by bromopalmitoylcarnitine.

Discussion

Randle (1969) found that perfusion of hearts from alloxan-diabetic rats with 2-bromostearate restored their impaired glucose utilization to normal values, apparently owing to inhibition of fatty acid oxidation. Sauer *et al.* (1971) have reported that bromopalmitate inhibits the oxidation of palmitate, but not of palmitoylcarnitine, by isolated liver cells; in later work (Mahadevan & Sauer, 1971) this effect was primarily ascribed to competitive interference with palmitate uptake by the cells.

In the present work we have shown that bromopalmitate inhibits the oxidation by rat-liver mitochondria of palmitate and of palmitoyl-CoA, although not that of palmitoylcarnitine. Mahadevan & Sauer (1971) proposed that bromopalmitate prevents mitochondrial oxidation of palmitate by inhibiting palmitoyl-CoA synthetase; we have not investigated this, but, since both CoA and ATP are necessary for the inhibition of palmitoyl-CoA oxidation, it would appear that in this case the effective inhibitory agent is bromopalmitoyl-CoA; presumably this is formed,

Fig. 8. Inhibitory effects of bromopalmitoylcarnitine on carnitine-independent palmitate oxidation

The solid line shows the oxygen uptake observed when $40\mu\text{M}$ -palmitate (Pm) was added to mitochondria (8.0mg of protein) incubated in the standard medium from which serum albumin had been omitted. The broken line shows the result of a similar experiment in which the mitochondria were preincubated with $21\mu\text{M}$ -bromopalmitoylcarnitine for 3 min before the addition of palmitate.

at least in small amounts, by an acyl-CoA synthetase situated outside the mitochondrial inner membrane, which is impermeable to CoA (Yates & Garland, 1966).

In confirmation of this, added bromopalmitoyl-CoA also inhibits oxidation of palmitate or palmitoyl-CoA without affecting the oxidation of palmitoylcarnitine or other substrates by undamaged mitochondria. With intact mitochondria, carnitine is necessary not only for the oxidation of palmitate and palmitoyl-CoA, but also for the inhibitory effects of bromopalmitoyl-CoA. The latter finding suggests that the site of inhibition is carnitine palmitoyltransferase; West *et al.* (1971) have found that ox-liver mitochondria contain two types of palmitoyltransferase, one of which is inhibited by bromopalmitoyl-CoA in a carnitine-dependent fashion. The mechanism of this inhibition is presumably analogous to that described by Chase & Tubbs (1969) for the action of bromoacetyl-CoA on carnitine acetyltransferase. The fact that bromopalmitoyl-CoA inhibits the oxidation of palmitoyl-CoA but not of palmitoylcarnitine indicates that the transferase that

Fig. 9. Dependence of the inhibitory effects of bromopalmitoylcarnitine on the quantity of mitochondria used

The effects of preincubating mitochondria with various concentrations of bromopalmitoylcarnitine (BrPmCn) on their subsequent oxidation of palmitoylcarnitine (▲), or pyruvate plus malate (●), were investigated as described for Fig. 7(a).

is inhibited corresponds to the 'outer' pool of Fritz & Yue (1963), that is the enzyme that is accessible to extramitochondrial acyl-CoA. The very small amount of bromopalmitoyl-CoA required for 50% inhibition of palmitoyl-CoA oxidation, about 0.05nmol/mg of mitochondrial protein, is comparable with the quantity of antimycin A needed to inhibit the respiratory chain (Ernster *et al.*, 1963).

The effects of bromopalmitoylcarnitine on mitochondrial respiration appear to be adequately described by Scheme 1, in which the formation of non-metabolizable bromopalmitoyl-CoA within the inner membrane leads to both inhibition of long-chain β -oxidation and depletion of free CoA in the matrix. The resulting impairment of the CoA-dependent oxidation of substrates such as pyruvate is reversed by the addition of carnitine, indicating that the 'inner' pool of carnitine palmitoyltransferase is not inhibited by the 2-bromoacyl derivatives of CoA or carnitine and, indeed, accepts them as substrates. A form of carnitine palmitoyltransferase that is tightly bound to membrane material and has the properties ascribed to the 'inner' pool in Scheme 1 has been obtained from ox-liver mitochondria (West *et al.*, 1971).

Scheme 1. *Effects of bromopalmitate and its CoA and carnitine esters on the oxidation by intact mitochondria of palmitate and other CoA-dependent substrates*

Palmitate (Pm) is first converted into palmitoyl-CoA (PmCoA), and the palmitoyl group is then passed to carnitine (Cn) by an 'outer' pool of carnitine palmitoyltransferase (●). An 'inner' pool of this enzyme (◄) reacts with palmitoylcarnitine (PmCn), giving rise to palmitoyl-CoA within the mitochondrial matrix. Oxidation of this and other CoA-dependent substrates via β -oxidation and the tricarboxylic acid cycle proceeds as indicated. The fate of added bromopalmitate (BrPm), bromopalmitoyl-CoA (BrPmCoA) and bromopalmitoylcarnitine (BrPmCn) is shown by broken arrows ($\longleftrightarrow$). Outside the mitochondrial inner membrane, bromopalmitoyl-CoA (added as such or formed from bromopalmitate) inactivates the 'outer' carnitine palmitoyltransferase (broad arrow). Bromopalmitoyl-CoA within the matrix, formed only from added bromopalmitoylcarnitine by the 'inner' transferase, is non-metabolizable and, in addition, inhibits long-chain β -oxidation (broad arrow). Matrix CoA, which is thus sequestered in the form of bromopalmitoyl-CoA, may be released on the addition of excess of free carnitine. Hex, Hexanoate; Pyr, pyruvate; OGI, 2-oxoglutarate.

Experiments with damaged mitochondria provide further support for the suggestion that the effects of bromopalmitoylcarnitine depend on the formation of bromopalmitoyl-CoA in the mitochondrial matrix, and for other features of Scheme 1. Thus when mitochondria are exposed to hypo-osmotic conditions, palmitate and palmitoyl-CoA are oxidized in the absence of carnitine, which is also no longer required for inhibition by bromopalmitoyl-CoA. Further, palmitoylcarnitine oxidation is now inhibited by bromopalmitoyl-CoA, as is that of pyruvate, provided that palmitoylcarnitine is added to sequester mitochondrial CoA (Fig. 5c); this impairment of pyruvate oxidation is reversed by carnitine. The effects of bromopalmitoylcarnitine on intact mitochondria can therefore be reproduced by using bromopalmitoyl-CoA and damaged mitochondria.

The exact site of inhibition of β -oxidation by bromopalmitoyl-CoA in the matrix has not yet been

established; results obtained with hexanoate (Fig. 7b) show that inhibition is more severe with long-chain substrates. The apparent destruction of some of the mitochondrial CoA by incubation with bromopalmitoylcarnitine (Fig. 10) has not been investigated.

The observations that treatment of mitochondria with bromopalmitoylcarnitine inhibits their utilization of pyruvate, 2-oxoglutarate and hexanoate by decreasing the availability of CoA indicates, in agreement with the findings of Garland *et al.* (1970), that the 'inner' carnitine palmitoyltransferase, pyruvate and oxoglutarate dehydrogenases, the β -oxidation enzymes and short-chain acyl-CoA synthetase all share the same CoA pool, namely that of the mitochondrial matrix. The quantity of bromopalmitoylcarnitine causing 50% inhibition of pyruvate oxidation, about 2 nmol/mg of mitochondrial protein (Fig. 9), is comparable with the total CoA content of the mitochondria [Fig. 10; Garland *et al.* (1965)], most

Fig. 10. Effects of bromopalmitoylcarnitine on the acylation state of mitochondrial CoA

Mitochondria (5.4mg of protein/ml) were suspended in the standard medium at 19°C. Additions of palmitoylcarnitine (20 μ M), bromopalmitoylcarnitine (48 μ M), L-carnitine (2mM) and pyruvate (5mM) plus L-malate (1mM) were made as shown. At the points marked (o) in the oxygen-electrode traces (upper diagrams), samples (3.0ml) of the mitochondrial incubation mixtures were taken for analysis of their CoA content as described in the text. The corresponding points on the lower diagrams show the results of these analyses for free CoA (●), acetyl-CoA (▲) and long-chain acyl-CoA (■). Abbreviations are as used in Fig. 1.

Scheme 2. Sites of inhibition of mitochondrial fatty acid oxidation by bromopalmitate, bromopalmitoyl-CoA, bromopalmitoylcarnitine, acyl-D-carnitine and pent-4-enoate

The vertical double line represents the mitochondrial inner membrane. Added substrates and inhibitors are shown in capitals and italics respectively. Broad arrows indicate irreversible inhibition, broken arrows reversible inhibition. Bromopalmitoylcarnitine and pent-4-enoate also lead to sequestration of matrix CoA.

of which is probably in the matrix. Incubation of mitochondria with 9.8nmol of bromopalmitoylcarnitine/mg of protein decreased the concentration of free CoA to negligible values (Fig. 10). These amounts of bromopalmitoylcarnitine are maximal values because, quite apart from questions of purity, DL-2-bromopalmitic acid was used in its synthesis and the stereospecificity of the observed effects is not known.

The use of two other inhibitors of fatty acid oxidation has been reported. These are fatty acyl-D-carnitine and pent-4-enoate (see Scheme 2). The metabolic effects observed on perfusion of organs with acyl-D-carnitine derivatives [abolition of long-chain fatty acid oxidation in rat heart (Fritz & Marquis, 1965); decreased gluconeogenesis in rat liver (Williamson *et al.*, 1968)] are probably entirely attributable to the known effects of such compounds as reversible competitive inhibitors of the carnitine acyltransferases (Fritz *et al.*, 1963; Fritz & Marquis, 1965). Treatment of liver preparations with pent-4-enoate also leads to impaired fatty acid oxidation and gluconeogenesis (Corredor *et al.*, 1969; Williamson *et al.*, 1970); experiments with isolated mitochondria have shown that pent-4-enoate only inhibits palmitoylcarnitine oxidation after conversion into pent-4-enoyl-CoA, presumably within the matrix. Pent-4-enoyl-CoA is incompletely oxidized, and it has been suggested (Holland *et al.*, 1972) that its derivative pent-2,4-dienoyl-CoA is the actual inhibitor of fatty acid oxidation, since this compound is rapidly inactivated a purified 3-oxoacyl-CoA thiolase. The oxidation of pyruvate or 2-oxoglutarate is also inhibited by pent-4-enoate, owing to sequestration of free CoA (Fukami & Williamson, 1971). Thus the effects of pent-4-enoate (and in particular of pent-4-enoylcarnitine; Holland & Sherratt, 1970) on mitochondria resemble in certain respects those of 2-bromopalmitoylcarnitine, although the latter compound is considerably more potent.

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