spectra in solution. One of these may be the 3,4-dihydro- β -carboline (IX), suggested by Corrodi & Hillarp (1963), which has a yellow fluorescence; the other, and more readily eluted, component has a lower fluorescence maximum and has not been identified.

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The Rate-Limiting Step in the Oxidation of Palmitate or Palmitoyl-Coenzyme A by Rat-Liver Mitochondria

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It is generally agreed that the rate of β -oxidation of fatty acids in intact mammals is largely controlled by the availability of fatty acids to the tissues concerned (Fritz, 1961), but there is little information or agreement on the rate-limiting steps of fatty acid oxidation in systems supplied with non-limiting concentrations of fatty acids. Reactions catalysed by acyl-CoA synthetase (EC 6.2.1.3; Krebs, 1964; Bode & Klingenberg, 1964) and acyl-CoA dehydrogenase (EC 1.3.2.2; Bunyan & Greenbaum, 1965) have both been described as rate-limiting for fatty acid oxidation.

The low concentration of intermediates of β -oxidation in rat-liver mitochondria oxidizing palmitoylcarnitine (Garland, Shepherd & Yates, 1965) is consistent with organization of the enzymes concerned into a complex, and this possibility is further supported by studies that demonstrate a close relationship between the enzymes of β -oxidation and a barrier or membrane that requires carnitine for acyl transfer (D. W. Yates & P. B. Garland, unpublished work). The activities of enzymes extracted from mitochondria may not represent their normal intramitochondrial activities, and the studies reported below refer to intact mitochondria.

Methods. Preparation of mitochondria and substrates, measurement of mitochondrial oxygen uptake and fluorimetric assay of CoASH with oxoglutarate dehydrogenase (EC 1.2.4.2) (reaction 2) have been described by Garland *et al.* (1965). All mitochondrial incubations were made at 25° in a final volume of 2.1 ml. containing KCl (80 mM), tris-chloride buffer, pH 7.2 (20 mM), MgCl₂ (2 mM), EDTA (1 mM) and mitochondrial protein (1–3 mg./

ml.). Other additions are described below. The carnitine-palmitoyltransferase activity (EC 2.3.1.-; Norum, 1964) of intact mitochondria was assayed by coupling carnitine-dependent CoASH release from palmitoyl-CoA (reaction 1) with the fluorimetric CoASH assay:

Further additions to the incubation mixture for this assay were rotenone (0.4 μ g./ml.), KCN (0.1 mM), 2-oxoglutarate (1.0 mM), NAD (1.0 mM), oxoglutarate dehydrogenase [20 μ g. (0.06 unit)/ml.], palmitoyl-CoA (40 μ M) and DL-carnitine (2.5 mM). Carnitine was added last, and the concentration used was that which gave maximal stimulation of palmitate or palmitoyl-CoA oxidation in respiring mitochondria.

Experimental design. Under suitable conditions (Shepherd, Yates & Garland, 1965) the oxidation of palmitate, palmitoyl-CoA or palmitoylcarnitine by rat-liver mitochondria results in a stoicheiometric conversion into acetoacetate or citrate, and the rate of palmitate or palmitoyl-CoA oxidation (as μ moles of C_{16:0} oxidized/min./mg. of mitochondrial protein) can be calculated from the observed oxygen uptake rate after correction for the oxidation of endogenous substrates. Under the conditions used, palmitate or palmitoyl-CoA oxidation is absolutely carnitine-dependent, and the correction for oxidation of endogenous substrates is unlikely to include any fatty acid oxidation. The reactions resulting in palmitate oxidation may be

entered at the level of palmitate, palmitoyl-CoA or palmitoylcarnitine, and the relative rates obtained compared with each other and also with the directly measured activities of the steps palmitate $\rightarrow$ palmitoyl-CoA and palmitoyl-CoA $\rightarrow$ palmitoylcarnitine.

Results and discussion. Direct assay of the conversion of palmitoyl-CoA into palmitoylcarnitine, at concentrations of palmitoyl-CoA and carnitine that yield maximal rates of oxidation, demonstrates that this conversion proceeds at about 2 μ moles/min./mg. of mitochondrial protein. This activity

is similar to that calculated from the oxidation of palmitoyl-CoA (see Table 1). Direct assay of mitochondrial palmitoyl-CoA synthetase has shown an activity of 5–7 μ moles/min./mg. of mitochondrial protein (Yates, Shepherd & Garland, 1966), whereas the oxidation of palmitate proceeds at the same rate as that of palmitoyl-CoA. It is concluded that the palmitoyl-CoA-synthetase activity of rat-liver mitochondria is not rate-limiting for palmitate oxidation.

Palmitoylcarnitine is oxidized by rat-liver mitochondria at 3–4 μ moles/min./mg. of mitochondrial protein, and this rate is independent of whether the end product is citrate or acetoacetate. It is concluded that the conversion of palmitoyl-CoA into palmitoylcarnitine is rate-limiting for both palmitate and palmitoyl-CoA oxidation. Neither the rate of utilization of acetyl-CoA nor electron transport is rate-limiting for palmitoylcarnitine oxidation.

These conclusions may be extrapolated to the intact liver, where at least 90% of carnitine palmitoyltransferase is mitochondrial (Norum, 1965). The increased activity of this rate-limiting enzyme observed in mitochondria from the livers of fat-fed, starved or alloxan-diabetic rats (Norum, 1965) is therefore of particular significance in the regulation of fatty acid oxidation.

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Table 1. *Rate of palmitate, palmitoyl-CoA or palmitoylcarnitine utilization by rat-liver mitochondria*

Results are those of a typical experiment. For the oxidation rate studies, additions are to the incubation medium described in the Methods section, which also contained malonate (10 mM), 2,4-dinitrophenol (50 μ M) and fluorocitrate (2.5 μ M).

Direct enzymic assay

Enzyme	Product	Activity (μ moles of C _{16:0} converted/ min./mg.)
Acyl-CoA synthetase*	Palmitoyl-CoA	5–7
Carnitine palmitoyltransferase	Palmitoyl- carnitine	2.4

Oxidation rates

Substrate (with additions)	Final product	Activity (μ moles of C _{16:0} converted/ min./mg.)
Palmitate (40 μ M) [+ CoASH (10 μ M), oligomycin (5 μ g./ml.), ATP (1 mM), DL-car- nitine (2.5 mM)]	Acetoacetate	1.7
Palmitoyl-CoA (40 μ M) [+ DL-carnitine (2.5 mM)]	Acetoacetate	1.7
Palmitoylcarnitine (40 μ M)	Acetoacetate	4.0

* Yates, Shepherd & Garland (1966).