Scheme 1.

of the phospholipids. In view of our results it is probable that the unknown isomer is $\alpha\beta'$ -GPG arising by acid-catalysed migration during drying of the acidic solution after passage through Amberlite IRC-50 (H⁺ form) resin. These authors also report that similar treatment of an authentic sample of a GPG derivative yielded, in addition to the unknown isomer, a small amount (3%) of GPGPG, thus confirming our observation of the transesterification reaction.

The formation of GPGPG from GPG under acidic conditions suggests an alternative biosynthetic route to bisphosphatidylglycerol (cf. LeCocq & Ballou, 1964). An analogous reaction between

two molecules of phosphatidylglycerol, under enzymic catalysis, would yield bisphosphatidylglycerol and glycerol.

In view of the ease with which these isomerization and transesterification reactions occur, it is necessary to emphasize the dangers of misinterpretation that could arise by the use of mild acidic conditions during structural investigations on phospholipids containing phosphodiester linkages.

Brown, D. M., Magrath, D. I., Neilson, A. H. & Todd, A. R. (1956). *Nature, Lond.*, **177**, 1124.

LeCocq, J. & Ballou, C. E. (1964). *Biochemistry*, **3**, 976.

Schwarz, H. P., Dreisbach, L., Polis, E., Polis, B. D. & Soffer, E. (1965). *Arch. Biochem. Biophys.* **111**, 422.

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The Relationship between the Rates of Conversion of Palmitate into Citrate or Acetoacetate and the Acetyl-Coenzyme A Content of Rat-Liver Mitochondria

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Changes in the acetyl-CoA content of rat livers have been implicated in the control of acetoacetate synthesis (Wieland & Weiss, 1963a; Tubbs & Garland, 1964), and increases of the acetyl-CoA content of rat-liver mitochondria during palmitate oxidation have been described (Garland, Shepherd & Yates, 1965). We now describe kinetic relationships between the acetyl-CoA content of rat-liver mitochondria and the rates of conversion of acetyl-CoA into citrate or acetoacetate, and factors controlling this process.

Methods. Reagents, preparation of mitochondria, incubation apparatus for continuous recording of oxygen pressure with rapid sampling facilities, and

fluorimetric assays for acetyl-CoA were as described by Garland *et al.* (1965). Oxaloacetate was assayed fluorimetrically. L-Carnitine and acyl-L-carnitine were assayed either by the methods described by Pearson & Tubbs (1964a,b) or by a fluorimetric modification. All mitochondrial incubations were made at 25° at a protein concentration of 2–4 mg./ml. in a medium containing initially KCl (80 mM), tris-chloride buffer, pH 7.2 (20 mM), MgCl₂ (2 mM), EDTA (1 mM), defatted bovine plasma albumin (5 mg./ml.), AMP (1.25 mM), P_i (1.25 mM) and malonate (10 mM), with further additions as indicated.

Experimental design. Rat-liver mitochondria

after being washed twice contained less than $0.1 \mu\text{mole}$ of L-carnitine (free, acetyl- or acyl-)/mg. of protein, and under the conditions used were dependent on the addition of L-carnitine for oxidation of palmitoyl-CoA. Selected and linear rates of palmitoyl-CoA oxidation were obtainable by varying the amount of carnitine added. Higher rates of oxidation were obtained by the use of $50\text{--}100 \mu\text{M}$ -palmitoyl-DL-carnitine. In all cases of linear oxidation rates serial assays for acetyl-CoA demonstrated that the concentration observed was that of a steady state and not transient state.

At low concentrations ($4\text{--}20 \mu\text{M}$) palmitoyl-DL-carnitine is rapidly oxidized with a well-marked turnover point in the oxygen-electrode trace corresponding to complete utilization of 50% of the added palmitoyl-DL-carnitine. The D-isomer is inactive (Norum, 1964). Thus it is possible to calculate the ratio $\mu\text{g. atoms of O consumed}/\mu\text{mole of palmitoyl-L-carnitine oxidized}$, and to determine which of the following reactions is operative:

The presence of fluorocitrate, kindly given by Sir R. A. Peters, prevents the further oxidation of citrate.

Results. When palmitoyl-DL-carnitine is oxidized in the presence of malonate, $\Delta\text{O}/\Delta\text{palmitoyl}$ equals 14 and reaction (1) is operative. The addition of L-malate ($0.5\text{--}5.0 \text{ mM}$) to coupled mitochondria increases the value for $\Delta\text{O}/\Delta\text{palmitoyl}$ to 16–17, and reaction (1) is still largely operative. The further addition of 2,4-dinitrophenol (0.1 mM) increases the value for $\Delta\text{O}/\Delta\text{palmitoyl}$ to 21–22 and reaction (2) is largely operative. Pentachlorophenol and 2,4-dibromophenol act similarly to dinitrophenol. Reaction (3) is demonstrable when ATP (12 mM) is added to raise the phosphorylation potential (Klingenberg, 1964) of coupled mitochondria. Thus palmitoylcarnitine oxidation in tightly coupled mitochondria proceeds mainly to acetoacetate despite the presence of malate, and proceeds mainly to citrate only if oxidative phosphorylation is uncoupled. Octanoylcarnitine oxidation shows similar features, and in each case the conclusions are verifiable by analyses for citrate and acetoacetate production.

The relationship between mitochondrial acetyl-CoA content and its rate of conversion into citrate (A in Fig. 1) exhibits a conventional shape for an enzyme-substrate interaction. As predicted by Tubbs & Garland (1964) the relationship between acetyl-CoA content and the rate of its conversion

Fig. 1. Relationship between the rate of oxidation of palmitoyl-CoA or palmitoylcarnitine and the mitochondrial acetyl-CoA content. Rat-liver mitochondria were incubated as described in the text, and samples assayed for acetyl-CoA during linear rates of oxygen uptake. In all cases either $40 \mu\text{M}$ -palmitoyl-CoA with 0.2 mM -DL-carnitine or $50\text{--}100 \mu\text{M}$ -palmitoyl-DL-carnitine was present. The lowest point on each curve corresponds to the incubation in the absence of either DL-carnitine or palmitoyl-DL-carnitine, and the upper two points correspond to incubations with 50 and $100 \mu\text{M}$ -palmitoyl-DL-carnitine. No further additions were made in incubations of curve C, and palmitoyl-L-carnitine was oxidized to acetoacetate. L-Malate (0.5 mM) and pentachlorophenol (0.01 mM) were added to incubations of curve A, and palmitoyl-L-carnitine was oxidized with malate to citrate. L-Malate (0.5 mM) was added to incubations of curve B.

into acetoacetate (C in Fig. 1) is such that there is a region where a small change of acetyl-CoA concentration can bring about a large change in the acetoacetate synthesis rate. This relationship is similar to that between acetyl-CoA concentration and the activity of rat-liver pyruvate carboxylase (EC 6.4.1.1) (Henning & Seubert, 1964), and could provide a mechanism whereby gluconeogenesis and acetoacetate synthesis are controlled in common by small changes in the mitochondrial acetyl-CoA content. Palmitoyl oxidation by coupled mitochondria in the presence of malate is associated with a complex relationship between the mitochondrial acetyl-CoA content and rate of oxygen uptake, and both the shape of the curve (B in Fig. 1) and assays for citrate and acetoacetate in the incubation mixture demonstrate that acetyl-CoA is converted into citrate at low rates of palmitoyl oxidation and to acetoacetate at high rates of palmitoyl oxidation. The ability of pentachlorophenol to promote conversion of acetyl-CoA into citrate is partially reversed by the presence of ATP and oligomycin.

Discussion. The parallel between these studies and those on oxaloacetate inhibition of succinate

dehydrogenase (reviewed by Greville, 1965) indicate that the availability of oxaloacetate to citrate synthase as well as succinate dehydrogenase is governed by the integrity of the energy-conservation mechanisms of oxidative phosphorylation and also the rate of flow through the electron-transport chain. Measurements of the concentration of oxaloacetate in the mitochondrial incubation mixture in the presence of L-malate with or without uncoupling agents showed that the relative rates of conversion of acetyl-CoA into citrate or acetoacetate are independent of the measured oxaloacetate concentration, and 'compartmentation' of oxaloacetate must be proposed.

Other mechanisms for the control of citrate synthase may be considered: (i) inhibition of citrate synthase by palmitoyl-CoA (Tubbs, 1963; Wieland & Weiss, 1963b) is rendered non-obligatory by the findings with octanoylcarnitine; (ii) reduction of oxaloacetate to malate by NADH produced during fatty acid oxidation, as suggested by Wieland, Weiss & Eger-Neufeldt (1964), is not excluded by our data but requires modification to take account of the failure of palmitoyl oxidation to alter the oxaloacetate content of the mitochondrial incubation mixture; their suggestion was based on the explicit assumption that 'compartmentation' of oxaloacetate could be disregarded.

Whatever the individual contribution towards the control of malate oxidation and intramitochondrial oxaloacetate concentration made by NAD reduction by 3-hydroxyacyl-CoA dehydrogenase, NAD reduction by acyl-CoA dehydrogenase in an energy-dependent process (Bode & Klingenberg, 1964; D. Shepherd, D. W. Yates & P. B. Garland, unpublished work), energy-dependent

oxaloacetate transfer (Tager, 1965) and effects on the hydrogen transfer from NAD available to malate dehydrogenase to NAD available to the electron-transport chain (Gamble & Lehninger, 1956), it is evident that the regulation of acetyl-CoA conversion into citrate or acetoacetate is an energy-controlled process.

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- Bode, C. & Klingenberg, M. (1964). *Biochim. biophys. Acta*, **84**, 93.
 Gamble, J. L., jun. & Lehninger, A. L. (1956). *J. biol. Chem.* **223**, 921.
 Garland, P. B., Shepherd, D. & Yates, D. W. (1965). *Biochem. J.* **97**, 587.
 Greville, G. D. (1965). *Symp. Regulation of Mitochondrial Metabolism, Bari* (in the Press).
 Henning, H. V. & Seubert, W. (1964). *Biochem. Z.* **340**, 160.
 Klingenberg, M. (1964). *Angew. Chem. (int. Ed.)*, **3**, 54.
 Norum, K. (1964). *Biochim. biophys. Acta*, **89**, 95.
 Pearson, D. J. & Tubbs, P. K. (1964a). *Biochem. J.* **91**, 2c.
 Pearson, D. J. & Tubbs, P. K. (1964b). *Biochim. biophys. Acta*, **84**, 772.
 Tager, J. M. (1965). *Symp. Regulation of Mitochondrial Metabolism, Bari* (in the Press).
 Tubbs, P. K. (1963). *Biochim. biophys. Acta*, **70**, 608.
 Tubbs, P. K. & Garland, P. B. (1964). *Biochem. J.* **93**, 550.
 Wieland, O. & Weiss, L. (1963a). *Biochem. biophys. Res. Commun.* **10**, 333.
 Wieland, O. & Weiss, L. (1963b). *Biochem. biophys. Res. Commun.* **13**, 26.
 Wieland, O., Weiss, L. & Eger-Neufeldt, I. (1964). *Advanc. Enzyme Regulation*, **2**, 35.

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The Release of Protein from the Stimulated Adrenal Medulla

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Catecholamines are secreted and can be recovered in the perfusate when isolated bovine adrenal glands are perfused with oxygenated Tyrode solution at 37° and stimulated by the injection of carbamoylcholine (Banks, 1965). For the experiments

described below the perfusion rate was 20–25 ml./min. and during the stimulation periods 0.5 ml. volumes of 10 mM-carbamoylcholine were injected at 30 sec. intervals for 3 min.

It was noticed that the perfusates collected during