STIMULATION OF PHOSPHOENOLPYRUVATE FORMATION BY OLEIC ACID AND OTHER UNCOUPLERS OF OXIDATIVE PHOSPHORYLATION IN RABBIT LIVER MITOCHONDRIA*

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SUMMARY

Oleic acid, like DNP, stimulates the oxidation of α-ketoglutarate and pyruvate by rabbit liver mitochondria, and at the same time accelerates the formation of phosphoenolpyruvate from these substrates. The net production of malate, citrate and acetoacetate similarly depends on the concentration of uncoupler.

Mudge et al.(1954) demonstrated that DNP** stimulated the formation of PEP from α-ketoglutarate by rabbit liver mitochondria. PEP production was dependent on DNP concentration although the response was polyphasic under the conditions employed (Stanbury and Mudge, 1954). These observations have been repeated and extended in recent years with liver mitochondria isolated from guinea pig (Nordlie and Lardy, 1963), rat (Scholte and Tager, 1965), and rabbit (Gamble and Mazur, 1965). Scholte and Tager (1965) emphasized that the requirement for P_i during the oxidation of α-ketoglutarate or glutamate in the presence of DNP was due to the formation of PEP, a process which depleted mitochondria of endogenous P_i.

It is well established that unsaturated long-chain fatty acids function efficiently as uncouplers of respiratory chain-linked oxidative phosphorylation.

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** Abbreviations: DNP, 2,4-dinitrophenol; PEP, phosphoenolpyruvate.

Several laboratories have shown that oleic acid can uncouple respiration and stimulate ATPase activity (Pressman and Lardy, 1956; Lehninger and Remmert, 1959; and Borst et al., 1962). Although the oxidation of oleic acid itself by rat liver mitochondria is partially uncoupled, the process is self-sustaining if concentrations of oleic acid are employed which are less than .05 µmoles per mg of mitochondrial protein (Rossi et al., 1967; van den Bergh, 1966).

The present study was undertaken to determine whether fatty acids could stimulate the formation of PEP from various substrates by isolated liver mitochondria. It was found that oleic acid, like DNP, greatly enhanced the generation of PEP from α-ketoglutarate and pyruvate in rabbit liver mitochondria. Since PEP is a key intermediate in gluconeogenesis (Utter et al., 1964; Krebs, 1964), and since net glucose formation may be signalled by perfusing fatty acids through liver tissue (Struck et al., 1966; Teuffel et al., 1967), it is postulated that this effect of unsaturated fatty acids in isolated liver mitochondria may play a supporting role in gluconeogenesis***.

METHODS: Rabbit liver mitochondria were prepared as described by Myers and Slater (1957). Incubations were carried out at 30° in Warburg vessels having 6-8 ml capacity. The standard incubation medium contained 50 mM KCl, 25 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$ in a final volume of 1.0 ml. Reactions were started by the addition of mitochondria and terminated by the addition of 0.1 ml 2 M $HClO_4$. After removing the precipitated protein, the samples were neutralized with KOH and assayed spectrophotometrically for pyruvate and PEP, as described by Kornberg and Pricer (1951). Pyruvate was estimated by the addition of lactate dehydrogenase (free of pyruvic kinase), and PEP by the subsequent addition of crystalline pyruvic kinase. Acetoacetate was measured spectrophoto-

*** Elevated free fatty acid levels of the order of 0.1-1.0 µmoles/gram of liver tissue are observed in rats during starvation (Y. Yugari, personal communication). Even higher values have been obtained following administration of glucagon or anti-insulin serum to rats (J. Ashmore, personal communication). Assuming a yield of 10 mg of mitochondrial protein per gm of liver, the intrahepatic fatty acid levels correspond to 0.01-0.1 µmoles of free fatty acid per mg of mitochondrial protein.

metrically using D(-)-β-hydroxybutyric dehydrogenase. Malate plus fumarate was estimated by measuring the appearance of NADH in the presence of excess acetyl-CoA, malic dehydrogenase, fumarase, and citrate synthase. Citrate was measured with citrate lyase (E.C. 4.1.3.6) and malic dehydrogenase (Gruber and Moellering, 1966). Protein was determined by the Biuret method. Nucleotides and enzymes were obtained from the Sigma Chemical Co., St. Louis, Missouri. Pyruvic acid was purified as described previously (Davis, 1967).

RESULTS: Oxygen uptake and production of PEP by mitochondria in the presence of α-ketoglutarate are dependent on the concentration of added DNP or oleate (Table I). At concentrations of oleate in excess of .05 μmoles per mg of mitochondrial

TABLE I

The Effect of DNP and Oleate on the Oxidation of α-ketoglutarate and on PEP Synthesis by Rabbit Liver Mitochondria

[DNP] (mM)	[oleate] (mM)	-Δ O (μ atoms)	+Δ PEP (μmoles)
0		1.0	0.06
0.1		12.0	3.51
1.0		12.1	3.57
	0.1	3.0	0.21
	0.2	5.5	0.76
	0.4	8.8	1.69
	0.8	8.3	1.54

The standard incubation mixture included 2 mM potassium phosphate (pH 7.4), 9.5 mg. mitochondrial protein, 10 mM α-ketoglutarate, plus DNP or oleate as indicated, in a final volume of 1.0 ml. Incubation time: 33 min.

protein both respiration and PEP formation were progressively diminished. Similar results were obtained with glutamate and citrate as substrates. Palmitate and stearate were much less effective than oleate in stimulating PEP production.

In contrast to earlier experiments (Stanbury and Mudge, 1954; Nordlie and Lardy, 1963) pyruvate alone can provide for PEP formation (Table II and Figure 1).

TABLE II

The Effect of DNP and Oleate on Pyruvate Oxidation and PEP Formation

Exp. 1	[DNP] (μM)	-Δ O (μ atoms)	+Δ PEP (μmoles)
	0	1.8	0.32
	10	8.2	1.28
	20	13.0	1.66
	40	5.6	0.12
Exp. 2	**[oleate] (μM)**	**-Δ O (μ atoms)**	**+Δ PEP (μmoles)**
	0	2.8	0.47
	200	6.9	0.68
	300	10.0	1.32
	500	12.5	1.04
	1000	3.9	0.08

The standard incubation mixture included 2 mM potassium phosphate, 5.0 μmoles sodium pyruvate, and other additions as indicated. In the experiments cited 8.2 and 7.5 mg of mitochondrial protein were added, respectively. Correspondingly, the incubation times were 44 and 48 min.

With supraoptimal levels of DNP or oleate, PEP formation fails and acetoacetate production is initiated suggesting that insufficient ATP is available for continuing oxaloacetate generation from pyruvate through pyruvate carboxylase (pyruvate:CO_2 ligase (ADP), E.C. 6.4.1.1) (Utter *et al.*, 1964). Production of oxaloacetate from pyruvate is clearly not rate-limiting at relatively low concentrations of uncouplers, since both malate and citrate accumulate under these conditions (Fig. 1).

As previously observed with systems containing DNP (Mudge *et al.*, 1954; Scholte and Tager, 1965) P_i is necessary for oleate-stimulated PEP formation from α-ketoglutarate, glutamate, citrate, or pyruvate. Malonate inhibits the production of PEP from these substrates.

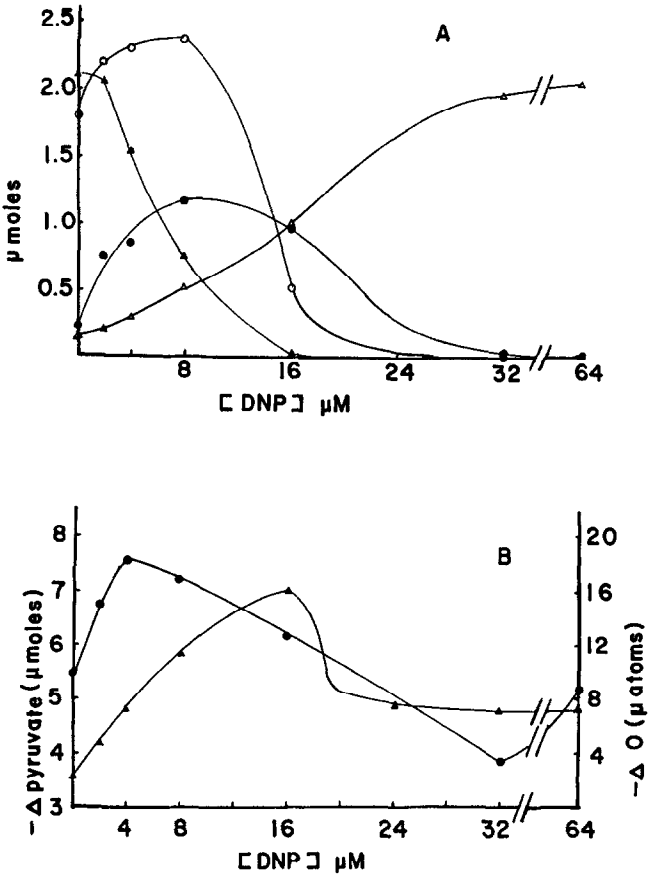

Fig. 1: Stoichiometry of metabolite accumulation from pyruvate in presence of various concentrations of DNP. Basic medium included 2 μmoles of phosphate, 8.75 μmoles of pyruvate, 10.2 mg mitochondrial protein, and DNP at the concentrations indicated in a final volume of 1.0 ml. Incubation time, 32 min.
Fig. 1A: citrate, ○; malate plus fumarate ▲; PEP, ●; and acetoacetate, Δ.
Fig. 1B: pyruvate, ●; total oxygen consumed, ▲.

DISCUSSION: Current hypotheses explaining the manner in which fatty acids stimulate gluconeogenesis are primarily based on the following considerations: (a) β-oxidation of fatty acids generates high levels of acetyl-CoA which in turn activate pyruvate carboxylase (Wieland and Weiss, 1963; Utter and Keech, 1963). Enhancement of this system leads to an increased supply of oxaloacetate (derived from pyruvate or alanine) which is then available for PEP synthesis. (b) Acetyl-

CoA inhibits pyruvic dehydrogenase (Garland and Randle, 1964). This action would presumably encourage a shunting of pyruvate carbon toward the formation of oxaloacetate. (c) Fatty acid oxidation is accompanied by an elevation in the concentration of malate under certain conditions, and an increase in the ratios, NADH/NAD^+, lactate/pyruvate and malate/oxaloacetate (Walter et al., 1966). Both Walter et al. (1966) and Krebs (1967) have emphasized the possible importance of malate in gluconeogenesis, both as a carbon source and as a source of extramitochondrial reducing equivalents. On the other hand, Williamson et al. (1967) failed to reveal a consistent change in the NADH/NAD^+ ratio due to starvation or diabetes.

The data presented here suggest that elevated levels of free fatty acids in liver*** could also enhance gluconeogenesis from certain amino acids, (as well as pyruvate, α-ketoglutarate and citrate) in a different manner. Partial uncoupling of oxidative phosphorylation by fatty acids would increase the rate of flow of intermediates to oxaloacetate, as well as stimulate production of GTP through the substrate-level phosphorylation coupled to the oxidation of α-ketoglutarate (Sanadi et al., 1956). When higher levels of uncoupler are present, PEP formation from pyruvate is not maintained since the concentration of ATP, per se, required for formation of oxaloacetate via pyruvate carboxylase becomes limiting. Consequently the concentration of oxaloacetate needed for PEP formation and citrate synthesis is inadequate, and pyruvate is diverted largely to acetoacetate. When liver mitochondria are partially uncoupled, at lower levels of uncoupler, the anaplerotic maintenance of oxaloacetate from pyruvate is adequate for rapid formation of PEP, and there is very little accumulation of acetoacetate. It appears that most of the energy required for PEP formation can be mediated through GTP.

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