# The Activation of Fatty Acid Oxidation by Kidney and Liver Mitochondria

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Lehninger (1945) has shown that the initiation of fatty acid oxidation in cell-free preparations of animal tissues must be accompanied by oxidative phosphorylation. Grafflin & Green (1949) considered that the co-oxidation of a citric-acid cycle intermediate is also necessary to provide oxaloacetic acid to condense with the two-carbon fragments formed in the breakdown of fatty acids. The condensation thus permits the complete oxidation of the fatty acid via the citric acid cycle. Kennedy & Lehninger (1951), however, have demonstrated that the oxidation of reduced diphosphopyridine nucleotide will prime the oxidation of octanoate and palmitate by rat-liver mitochondria. This oxidation gives rise to phosphorylation generating adenosine triphosphate, but no citric-acid cycle intermediate is involved.

The present paper considers these apparently conflicting views of fatty acid oxidation in mitochondrial systems. During these investigations 2:4-dinitrophenol (DNP) and fluoroacetic acid have proved useful reagents for the analysis of the fatty acid oxidase system.

## EXPERIMENTAL

### *Methods*

*Enzyme preparations.* Preparations of mitochondria from liver and kidney were made by the method of Schneider (1948) using 0·25 M-sucrose as the medium.

*Estimations.* Measurement of oxygen uptake was by the method of Warburg. Acetoacetate was estimated by an unpublished method of Dr A. L. Greenbaum.

### *Materials*

Adenosine 5′-phosphate (AMP), obtained commercially, was crystallized twice from hot water. On spectrophotometric analysis (Kalckar, 1947) and by paper chromatography (Hanes & Isherwood, 1949) it was pure.

Adenosine diphosphate (ADP), prepared as described by LePage (1949), was used occasionally as phosphate acceptor; by chemical and spectrophotometric analysis it was found to be approximately 95% pure.

Adenosine triphosphate (ATP) was prepared by the method of Dounce, Rothstein, Beyer, Meier & Freer (1948). By chemical and spectrophotometric analysis it was found to be 98% pure.

Creatine phosphate was prepared by the method of Ennor & Stocken (1948).

Creatine phosphokinase was prepared by the method of Racker & Krimsky (1948).

Sodium citrate and sodium L-malate were commercial preparations.

Octanoic acid was obtained commercially and distilled *in vacuo*.

Sodium fluoroacetate was provided by Dr R. A. E. Galley of the Agricultural Research Council.

Cytochrome *c* was prepared by the method of Keilin & Hartree (1937) and dialysed against distilled water.

The medium had the following composition (final concentration in brackets): phosphate buffer (0·025 M), pH 7·4, adenosine 5′-phosphate (0·001 M), $MgSO_4$ (0·0067 M), KCl (0·025 M), cytochrome *c* (10 $\mu$M), mitochondrial suspension in 0·25 M-sucrose, and water to a final volume of 3 ml. It is referred to as the 'standard' medium.

Gas phase in all experiments was air, and the temperature of incubation was 38°.

## RESULTS

### *Oxidation of octanoic acid by mitochondria*

It was necessary to obtain a system in which the priming of octanoate oxidation could be achieved by a substrate which is not an intermediate in the citric acid cycle. $\beta$-Hydroxybutyrate is such a substrate (Lehninger & Smith, 1949), its oxidation giving rise to the phosphorylations shown to be necessary for the priming reaction. We have tested the ability of $\beta$-hydroxybutyrate to prime the oxidation of octanoate, in mitochondria prepared from three tissues. Table 1 shows the effect of L-malate, a citric acid cycle intermediate, and $\beta$-hydroxybutyrate with rabbit-liver and rabbit-kidney mitochondria. In both cases $\beta$-hydroxybutyrate failed to activate fatty acid oxidation.

Rat liver was next investigated. In preliminary experiments rat-liver mitochondria, washed four times with isotonic sucrose (0·25 M), required no primer to initiate octanoate oxidation as was also found by Kennedy & Lehninger (1951). There was no residual respiration in these particles and we are unable to explain the results. In further experiments it was found that washing the mitochondria with isotonic (0·15 M) potassium chloride removed the ability of the particles to oxidize octanoate without a primer. After this treatment $\beta$-hydroxybutyrate effectively primed the oxidation of

Table 1. *The priming of octanoate oxidation in rabbit-kidney and rabbit-liver mitochondria*

(The standard incubation medium was used. 5 μmoles octanoate added and primer, as indicated. Duration of experiments: 40 min. with rabbit-kidney mitochondria; 20 min. with rabbit-liver mitochondria.)

| Expt. no. | Tissue | Primer | Oxygen uptake (μl.) Complete system | No octanoate | No primer |
|---|---|---|---|---|---|
| 1 | Rabbit kidney | β-Hydroxybutyrate (3 μmoles) | 53 | 27 | 40 |
| | | L-Malate (3 μmoles) | 300 | 108 | 40 |
| 2 | Rabbit kidney | β-Hydroxybutyrate (10 μmoles) | 34 | 47 | 10 |
| 3 | Rabbit liver | β-Hydroxybutyrate (10 μmoles) | 14 | 14 | 2 |
| | | L-Malate (3 μmoles) | 51 | 18 | 2 |

Table 2. *The priming of octanoate oxidation by rat-liver mitochondria*

(The mitochondria were washed twice with isotonic KCl after two sedimentations from isotonic sucrose. The standard incubation medium was used, with octanoate (5 μmoles), β-hydroxybutyrate (10 μmoles), L-malate (3 μmoles) and ATP (5 μmoles) where indicated.)

| Expt. no. | Duration of Expt. (min.) | Primer | Oxygen uptake (μl.) Complete system | No octanoate | No primer |
|---|---|---|---|---|---|
| 1 | 30 | β-Hydroxybutyrate | 170 | 46 | 2 |
| | | β-Hydroxybutyrate | 119 | 29 | Nil |
| | | L-Malate | 214 | 85 | Nil |
| 2 | 20 | ATP | 218 | Nil | Nil |
| | | β-Hydroxybutyrate | 236 | 29 | Nil |

Table 3. *The effect of DNP on fatty acid oxidation by mitochondria from rat liver and rabbit kidney*

(The standard incubation medium was used. Further additions as indicated, at the following concentrations: octanoate (5 μmoles), acetate (30 μmoles), β-hydroxybutyrate (10 μmoles), ATP (5 μmoles), L-malate (5 μmoles).)

| Tissue | Washing of mitochondria | Primer | Substrate | $O_2$ uptake (μl./10 min.) $2 \times 10^{-5}$ M-DNP | No DNP |
|---|---|---|---|---|---|
| Rat liver | KCl | β-Hydroxybutyrate | Octanoate | 10 | 70 |
| Rat liver | KCl | ATP | Octanoate | 59 | 66 |
| Rat liver | KCl | Creatine phosphate (20 μmoles), ADP (3 μmoles) and creatine phosphokinase (0·3 ml.) | Octanoate | 91 | 98 |
| Rat liver | Sucrose | ATP | Octanoate | 12 | 77 |
| Rabbit kidney | Sucrose | L-Malate | Acetate | 67 | 130 |
| Rabbit kidney | Sucrose | ATP + L-malate | Acetate | 69 | 128 |

octanoate by rat-liver mitochondria. Table 2 shows the results of a typical experiment. We decided, therefore, to test the effect of ATP on the oxidation of octanoate by rat-liver mitochondria. Table 2 also shows that in the presence of particles washed with potassium chloride ATP was as effective as β-hydroxybutyrate in the priming reaction.

One possible explanation of the failure of β-hydroxybutyrate to prime fatty acid oxidation in rabbit-kidney mitochondria is that it is due to the small ability of these preparations to form acetoacetate from octanoate. For this reason the co-oxidation of a tricarboxylic acid cycle intermediate is essential, since the fatty acid oxidation must proceed via the cycle.

### *The effect of 2:4-dinitrophenol (DNP) on fatty acid oxidation*

Table 3 shows the effect of DNP on octanoate oxidation in rat-liver mitochondria. In the presence of a large excess of ATP, there was no inhibition of the oxygen uptake. Moreover, the generation of ATP from creatine phosphate, creatine phosphokinase and adenosine diphosphate substituted for ATP in this system.

When the action of DNP was tested on sucrose-washed mitochondria oxidizing octanoate or acetate, ATP failed to prevent the inhibition (Table 3). It is presumed that ATP failed to reach the system in the sucrose-washed mitochondria, and that potassium

chloride washing in some way rendered the system more accessible to ATP. The action of DNP on the oxidation of fatty acid by mitochondria is probably due to an inhibition of the synthesis of ATP, which latter takes part in the priming reaction.

*The effect of fluoroacetate on fatty acid oxidation*

The most reasonable explanation for the inhibition of fatty acid oxidation by fluoroacetate (Kalnitsky & Barron, 1948) is that this compound diminishes the oxidation of the primer and subsequent entry of $C_2$ units into the citric acid cycle. We wished to ascertain whether fluoroacetate or its activation products could in some other way interfere with the oxidation of fatty acids.

Kennedy & Lehninger (1951) have shown that rat-liver mitochondria are able to oxidize octanoate to acetoacetate in the absence of a citric acid cycle intermediate. If it were possible to test the action of fluoroacetate in such a system, any further effects of the inhibitor should become apparent. However, it was first necessary to determine whether the preparation was capable of activating fluoroacetate. Fluoroacetate is converted to a fluoro-tricarboxylic acid, probably fluorocitric acid, which is the actual inhibitor (Buffa, Peters & Wakelin, 1951). This process probably follows the same path as the conversion of acetate to citrate.

Preliminary experiments with rat-liver mitochondria showed that fluoroacetate was activated very poorly by this preparation as indicated by the experiments shown in Table 4. This is in remarkable agreement with *in vivo* experiments of Buffa & Peters (1949). It will be seen that the oxidation of citrate by rat-liver mitochondria was inhibited approximately 16%, whereas with rabbit-kidney mitochondria the inhibition was 57%.

These experiments set a problem in that rat-liver mitochondria, which convert octanoate to acetoacetate, were unable to activate fluoroacetate, whereas the converse was true of rabbit-kidney mitochondria. We therefore decided to make use of these special properties of the different preparations. The plan of the experiment was the biosynthesis of 'fluorocitrate' (Buffa *et al.* 1951) by rabbit-kidney mitochondria, followed by the removal of these particles by centrifugation and substitution by rat-liver mitochondria. It is clear that this experiment should demonstrate any inhibitory action of fluoroacetate on fatty acid oxidation, other than interference with the entry of $C_2$ units into the citric acid cycle.

Table 5 shows that a powerful inhibitor for citrate oxidation was formed in the presence of fluoroacetate, and that the apparent inhibition of fatty acid oxidation was approximately 20%. Acetoacetate analysis showed that this apparent inhibition was due to the complete oxidation of a small portion of octanoate in the control flask, i.e. the inhibitor had no action on the oxidation of octanoate to acetoacetate.

It may be concluded that the action of fluoroacetate on fatty acid oxidation is solely due to its interruption of the citric acid cycle. It has been found that fluoroacetate had no effect on oxidative phosphorylation in any tissue tested.

Table 4. *Fluoroacetate inhibition of citrate oxidation by rat-liver and rabbit-kidney mitochondria*

(The standard incubation medium was used. Citrate (30 $\mu$moles) in the side arm, was added after equilibration (10 min.). Fluoroacetate (3 $\mu$moles) and L-malate (3 $\mu$moles) were present in main chamber.)

| | $O_2$ uptake ($\mu$l./45 min.) | | |
|---|---|---|---|
| Tissue | No fluoroacetate | Fluoroacetate present | Inhibition (%) |
| Rat liver | 328 | 273 | 16 |
| Rabbit kidney | 201 | 87 | 57 |

Table 5. *The effect of the active inhibitor ('fluorocitrate') on citrate and octanoate oxidation by rat-liver mitochondria*

(Rabbit-kidney mitochondria, prepared from 16 g. of tissue, were suspended in 0·15 M-KCl (final vol. of suspension, 12 ml.). The particle suspension was incubated in open flasks at 38°. The medium contained (final concentrations in brackets): AMP ($2 \times 10^{-3}$ M), phosphate buffer pH 7·4 (0·028 M), $MgSO_4$ (0·0067 M), cyt. *c* ($3 \times 10^{-5}$ M), fumarate (0·006 M), KCl (0·025 M), fluoroacetate when present (0·0033 M), and mitochondria equivalent to 5 g. of fresh tissue; final vol. in flasks, 35 ml. A simultaneous manometric experiment was made to determine cessation of oxygen uptake (approx. 4 hr.). The flask contents were then centrifuged at 20 000 *g* for 30 min. 2 ml. of the clear supernatants were added to the Warburg flasks. In addition, there were present 0·03 ml. of 0·2 M-$MgSO_4$, 0·015 ml. of 1·5 M-KCl, 0·5 ml. of rat-liver mitochondrial suspension (in 0·25 M-sucrose) and water to 3 ml. $O_2$ uptakes and acetoacetic acid accumulation corrected for oxidation of $\beta$-hydroxybutyrate.)

| | Oxygen uptake ($\mu$l./40 min.) | | Acetoacetate accumulated ($\mu$moles) | |
|---|---|---|---|---|
| Substrate | Control medium | Fluoroacetate medium | Control medium | Fluoroacetate medium |
| Citrate (30 $\mu$moles) | 175 | 44 | — | — |
| Octanoate (5 $\mu$moles) + $\beta$-hydroxybutyrate (5 $\mu$moles) | 213 | 170 | 4·8 | 5·0 |

*Activation of fluoroacetate*

Since it seems reasonable to suppose that the activation of fluoroacetate is analogous to that of acetate (Liebecq & Peters, 1949), we have tested the effect of DNP on the capacity of fluoroacetate to stop citrate oxidation by rabbit-kidney mitochondria. Table 6 shows the results of a typical experiment. It can be seen that DNP protected the oxidation of citrate. When DNP, citrate and fluoroacetate were incubated together the same protective effect was observed. Table 7 shows an experiment in which DNP was placed in the side arm while citrate and fluoroacetate were in the main compartment; the DNP was tipped after the inhibition became apparent. No reversal of the inhibition was observed under these circumstances.

It is concluded that, as expected, DNP abolishes the activation of fluoroacetate. Once the inhibitor is formed it appears to be stable in that added DNP fails to reverse its action.

Table 6. *The effect of DNP on the inhibition by fluoroacetate of citrate oxidation*

(The standard incubation medium was used, with rabbit-kidney mitochondria. Citrate (30 $\mu$moles) in the side arm; fluoroacetate (3 $\mu$moles), L-malate (3 $\mu$moles) and DNP ($2 \times 10^{-5}$ M) in the main chamber.)

| DNP | Fluoroacetate | $O_2$ uptake ($\mu$l./60 min.) |
|---|---|---|
| Absent | Absent | 550 |
| Present | Absent | 534 |
| Absent | Present | 318 |
| Present | Present | 540 |

Table 7. *The effect of DNP on an established inhibition of citrate oxidation by fluoroacetate*

(The standard incubation medium was used, with rabbit-kidney mitochondria. Citrate (30 $\mu$moles), fluoroacetate (3 $\mu$moles) in main chamber. The figures refer to the last 10 min. of a 40-min. experiment, DNP ($2 \times 10^{-5}$ M) having been added from the side arm at 30 min.)

| DNP | Fluoroacetate | $O_2$ uptake ($\mu$l.) |
|---|---|---|
| Absent | Present | 92 |
| Present | Present | 88 |
| Absent | Absent | 202 |
| Present | Absent | 199 |

Table 8. *The reversal by ATP of the effect of DNP on the fluoroacetate inhibition of citrate oxidation*

(All flasks contained standard medium, 30 $\mu$moles citrate, 5 $\mu$moles ATP and $10^{-4}$ M-DNP. Further additions as shown. Rabbit-kidney mitochondria prepared as usual and then washed once in 0·15 M-KCl and resuspended in 0·25 M-sucrose.)

| | $O_2$ uptake ($\mu$l.) | | | |
|---|---|---|---|---|
| Fluoro-acetate | 1st 30 min. | Inhibition (%) | 2nd 30 min. | Inhibition (%) |
| Absent | 162 | — | 171 | — |
| Present | 119 | 27 | 103 | 40 |

*Reversal of the action of DNP on fluoroacetate*

Rabbit-kidney mitochondria were prepared and washed twice with isotonic potassium chloride. Table 8 shows that the protective effect of DNP is abolished in the presence of ATP. This result accords well with those reported earlier in this paper.

## DISCUSSION

Kennedy & Lehninger (1951) demonstrated that the oxidation of reduced diphosphopyridine nucleotide (in the absence of added citric acid cycle intermediate) by rat-liver mitochondria activated fatty acid oxidation. They considered that the activation process did not appear to consist simply of a mechanism for generating ATP continuously, ATP alone not sufficing to activate the oxidation. In the present investigations, it has been found that with rat-liver mitochondria, which had been washed with potassium chloride, fatty acid oxidation could be initiated either by the addition of ATP or by ATP produced from creatine phosphate and creatine phosphokinase.

Cross, Taggart, Covo & Green (1949) demonstrated that DNP inhibits fatty acid oxidation. We have found that the inhibition by DNP can be abolished by the addition of ATP itself or by an external generation of ATP via the creatine phosphate-phosphokinase system. Thus DNP inhibition of fatty acid oxidation is probably an inhibition of the generation of ATP rather than a direct effect of DNP on the fatty acid oxidase system.

Grafflin & Green (1949) considered that fatty acid oxidation depended on the simultaneous oxidation of an intermediate of the citric acid cycle. We have shown that this is not true of rat-liver mitochondria. Mitochondrial preparations from rabbit kidney have been found to oxidize $\beta$-hydroxybutyrate with the generation of ATP (Judah & Williams-Ashman, 1951), but such systems failed to activate fatty acid oxidation. Thus in the case of rabbit-kidney preparations the co-oxidation of a citric acid cycle intermediate is essential for fatty acid oxidation, since the oxidation of this intermediate provides oxaloacetate for condensation with $C_2$ units derived from the fatty acid.

Grafflin & Green (1949) have shown that rabbit liver can form acetoacetate from octanoate, and it is surprising that $\beta$-hydroxybutyrate, which is oxidized by rabbit-liver mitochondria with the generation of high-energy phosphate bonds, is unable to prime the oxidation of octanoate in this tissue.

Kalnitsky & Barron (1948) demonstrated an inhibition of fatty acid oxidation by fluoroacetate in rabbit-kidney suspensions. We have shown that this inhibition is due solely to the block of citrate oxidation. Fluoroacetate appears to be activated

similarly to acetate in that its activation is inhibited by DNP and that this inhibition may be reversed by ATP.

Judah (1951) demonstrated that the DNP inhibition of pyruvate activation by sucrose-washed rat-liver mitochondria was reversed by ATP. This was in contrast to the DNP inhibition of octanoate activation. It was therefore considered that the activation of octanoate and pyruvate followed different pathways. Our present investigations have shown that DNP inhibition of fatty acid activation may be reversed if the mitochondria are washed with potassium chloride. In the case of sucrose-washed mitochondria it appears that ATP is unable to reach the active centre of the fatty acid oxidase system.

Until we possess a greater knowledge of the organization of such mitochondrial systems, great care must be taken in interpretation of such results.

## SUMMARY

1. It has been demonstrated that the oxidation of octanoate by rat-liver mitochondria may be activated by adenosine triphosphate or by a system generating adenosine triphosphate.

2. Rabbit-kidney and rabbit-liver mitochondria are unable to activate octanoate oxidation in the presence of adenosine triphosphate and require the co-oxidation of an intermediate of the citric acid cycle. The different requirements of the three tissues are discussed.

3. The action of 2:4-dinitrophenol on fatty acid oxidation is shown to be due to the inhibition of adenosine triphosphate synthesis. Reversal of this inhibition by adenosine triphosphate has been demonstrated.

4. The activation of fluoroacetate has been studied. 2:4-Dinitrophenol prevents its activation and this effect can be suppressed by adenosine triphosphate.

5. The effect of fluoroacetate on fatty acid oxidation is due solely to its inhibition of the citric acid cycle.

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