BBA 56150

# THE MECHANISM OF INHIBITION BY FLUORIDE OF MITOCHONDRIAL FATTY ACID OXIDATION

J. J. BATENBURG AND S. G. VAN DEN BERGH

*Laboratory of Veterinary Biochemistry, State University of Utrecht, Biltstraat 172, Utrecht (The Netherlands)*

(Received June 16th, 1972)

SUMMARY

The following evidence is presented in favour of the old hypothesis that $F^-$ inhibition of fatty acid oxidation in intact, coupled rat-liver mitochondria is due to an accumulation of pyrophosphate in the mitochondrial matrix:

1. Addition of fatty acid to mitochondria oxidizing malate in the presence of $F^-$ initially causes an increased rate of $O_2$ uptake, followed by a gradual decrease, indicating the accumulation of an inhibitor as a result of fatty acid oxidation.

2. This inhibition is only found when the fatty acid substrate is activated in the mitochondrial matrix.

3. The matrix acyl-CoA synthetase (acid:CoA ligase (AMP), EC 6.2.1.3) is strongly inhibited by pyrophosphate.

4. Mitochondrial pyrophosphatase (pyrophosphate phosphohydrolase, EC 3.6.1.1) is inhibited by $F^-$ and is localized mainly in the matrix.

5. The mitochondrial inner membrane is impermeable to pyrophosphate.

6. Pyrophosphate accumulates in mitochondria oxidizing fatty acid in the presence of fluoride.

Oxidation of fatty acids by uncoupled mitochondria in the absence of inorganic phosphate also leads to pyrophosphate accumulation when $F^-$ is added, showing that under these conditions too, an ATP-dependent acyl-CoA synthetase is active.

## INTRODUCTION

It has been known for a long time that low concentrations of fluoride inhibit the carnitine-independent oxidation of fatty acids by tissue homogenates or mitochondria[1-4]. Aisenberg and Potter[4] found that acetate oxidation by a rat-liver homogenate was inhibited by $F^-$, whereas pyruvate oxidation was unimpaired. They concluded that the inhibition occurred at the level of the activation reaction. Since they

Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; EGTA, ethyleneglycol-bis-(2-aminoethyl)-tetraacetate.

were unable to demonstrate any accumulation of pyrophosphate in the $F^-$-inhibited system, they rejected the idea that $F^-$ depression of acetate activation is secondary to $F^-$ depression of inorganic pyrophosphatase (pyrophosphate phosphohydrolase, EC 3.6.1.1). The latter had been observed earlier by Lehninger and Smith[5].

No further studies on the mechanism of the inhibition by $F^-$ of fatty acid oxidation in coupled mitochondria have appeared in the literature.

Galzigna *et al.*[6] reported that fatty acid oxidation by uncoupled rat-liver mitochondria is also inhibited by $F^-$. They attributed this to the observed inhibition by $F^-$ of the purified GTP-specific acyl–CoA synthetase which is supposed to be operative in uncoupled mitochondria in the absence of added inorganic phosphate[7–10].

Van den Bergh[11] reported that carnitine-dependent fatty acid oxidation by rat-liver mitochondria is insensitive to $F^-$. In this case the activation of the fatty acid substrate is effected by the ATP-specific acyl–CoA synthetase localized in the outer mitochondrial membrane.

It seemed interesting to investigate more closely the mechanism of the inhibition by $F^-$ of fatty acid oxidation. On the one hand we found a close similarity in the sensitivity towards $F^-$ of fatty acid oxidation in coupled and uncoupled mitochondria, suggesting that the inhibition in both cases is brought about *via* the same mechanism. On the other hand we were unable to demonstrate an inhibition by $F^-$ of the ATP-dependent acyl–CoA synthetase (acid:CoA ligase (AMP), EC 6.2.1.3) of the mitochondrial matrix.

Part of these results have been briefly presented in abstract form[12].

## EXPERIMENTAL

### *Reagents*

Nucleotides and enzymes were obtained from Boehringer. [1-$^{14}$C]Palmitic acid was supplied by NEN chemicals. $(NH_4)_4P_2O_7$ was prepared from commercial $Na_4P_2O_7$ by ion-exchange chromatography over a Dowex 50W-X4 (50–100 mesh) column at 3 °C. The scintillation mixture Unisolve 1 was obtained from Koch Light. Lubrol WX was a kind gift of Dr P. L. Pedersen.

### *Methods*

Liver mitochondria were prepared from female Wistar rats according to Myers and Slater[13].

Subfractionation of mitochondria was carried out as follows. The outer membrane, the intermembrane space and the inner membrane–matrix fractions were separated by treatment of the intact mitochondria with digitonin as described by Schnaitman and Greenawalt[14], using 0.25 M sucrose as the fractionation medium. The inner membrane–matrix fraction was further fractionated with Lubrol WX according to Chan *et al.*[15]. Prior to enzyme assay the fractions were sonicated (2 min at 20 kHz, 6.2 $\mu$m from peak to peak, in 2 ml portions at 0–4 °C).

In one experiment (Fig. 2) a crude matrix fraction was used. It was obtained by freezing and thawing 5 times a suspension of mitochondria (150 mg protein/50 ml) in 10 mM Tris–HCl buffer (pH 7.5) with 0.5 mM ATP followed by centrifugation at 150000 $\times g$ for 1 h. The supernatant contained no detectable outer-membrane contamination (as judged by the monoamine oxidase (EC 1.4.3.4) activity), whereas only

6.6% of its palmitoyl-CoA synthetase activity could be attributed to microsomal contamination (as judged by the NADPH–cytochrome *c* reductase activity).

Microsomes were obtained from the same livers from which the fractionated mitochondria were isolated. The supernatant of the nuclei- and debris-free homogenate from which the mitochondria had been sedimented, was centrifuged again at 27000 × *g* for 10 min. From the resulting supernatant the microsomes were sedimented by centrifugation at 144000 × *g* for 1 h.

Palmitoyl–CoA synthetase activity was determined from the rate of formation of [1-$^{14}$C]palmitoyl–CoA from [1-$^{14}$C]palmitate. The incubation mixture contained in a final volume of 0.1 ml: 1.2 mM EDTA, 10 mM $MgCl_2$, 300 mM Tris, 11 mM ATP, 1.7 mM CoA and 0.2 μmole potassium palmitate (spec. act. 0.49 μCi/μmole) at pH 8.0. The reaction was started by addition of the enzyme preparation. After 10 min at 37 °C the reaction was stopped by addition of 0.6 ml 1.5% $HClO_4$. The excess palmitate was removed by extracting the aqueous medium 5 times with 5-ml portions of diethyl ether. The water phase containing the [1-$^{14}$C]palmitoyl–CoA was transferred to a counting vial with 15 ml of Unisolve 1. The radioactivity was measured in a Tracerlab Coru/Matic 25 liquid scintillation counter.

Inorganic pyrophosphatase activity was assayed at 25 °C in 1.5 ml of a reaction mixture containing 15 mM KCl, 2 mM EDTA, 5 mM $MgCl_2$, 50 mM Tris–HCl buffer (pH 7.5), 50 mM sucrose, 30 mM glucose and 3 mM sodium pyrophosphate. The reaction was started by addition of the enzyme preparation. After 20 min the reaction was stopped by addition of 1.5 ml ice-cold 10% trichloroacetic acid. Inorganic phosphate formation was determined in deproteinized samples as described by Sumner[16].

Monoamine oxidase activity was measured according to Tabor *et al.*[17], using benzaldehyde as the substrate.

Malate dehydrogenase (EC 1.1.1.37) activity was assayed according to Ochoa[18] in the presence of 5 μg rotenone to inhibit oxidation of NADH.

Adenylate kinase (EC 2.7.4.3) activity was measured as described by Schnaitman and Greenawalt[14], but with a Tris–HCl buffer instead of glycylglycine.

Cytochrome *c* oxidase (EC 1.9.3.1) activity was measured polarographically according to Sottocasa *et al.*[19].

NADPH-cytochrome *c* reductase activity was assayed according to Sottocasa *et al.*[19]. 27.5 Units malate dehydrogenase and 0.25 mM oxaloacetate were added to prevent interference by NAD(P) transhydrogenase (EC 1.6.1.1) plus NADH-cytochrome *c* reductase.

Oxidation of palmitate was studied manometrically at 25 °C in 2 ml of a medium containing as standard components: 15 mM KCl, 5 mM $MgCl_2$, 2 mM EDTA, 50 mM Tris–HCl buffer (pH 7.5), 30 mM glucose and 50 mM sucrose.

In one experiment (Fig. 1) the oxidation of palmitate was studied with [1-$^{14}$C]-palmitate as the substrate. The experiment was performed in closed Warburg vessels under the same conditions as in the manometric experiments. The centre well contained 0.2 ml 2 M KOH. After 20 or 40 min the reaction was stopped by addition of 0.2 ml 45% $HClO_4$ from the side-arm. Shaking was continued for 1 h to complete the uptake of $CO_2$ by the KOH. The contents of the centre well were then transferred to a counting vial. Complete transfer was achieved by rinsing with 0.8 ml water. The contents of the main compartment were centrifuged to remove protein. Excess palmitate was removed by extracting the supernatant 3 times with 5-ml portions of water-

saturated *n*-pentane. 1 ml of the water layer was transferred to a counting vial. To the counting vials 15 ml of Unisolve 1 was added.

The pyrophosphate content of mitochondria was determined after separation of the mitochondria from the incubation medium. This separation was necessary because of the high phosphate concentration in the medium, which would otherwise have made the determination of pyrophosphate impossible. After the separation the mitochondria were extracted with $HClO_4$. Separation and extraction were performed according to the method of Garber and Ballard[20], except that the syringes were rapidly changed instead of using a 3-way valve. In the $HClO_4$ extract neutralized with NaOH, pyrophosphate was determined by the method of Grindey and Nichol[21]. If the medium contained no phosphate (Fig. 6) the separation procedure could be omitted and pyrophosphate could be determined directly in the deproteinized and neutralized medium.

Protein was determined as described by Cleland and Slater[22], except for the fractionation studies in which it was determined according to the method of Gornall *et al.*[23].

## RESULTS

### *The effect of $F^-$ on palmitate oxidation by intact coupled mitochondria*

To gain an insight into the extent of the inhibition by fluoride of palmitate activation in intact coupled mitochondria, the mitochondria were incubated with labelled palmitate. After termination of the incubation the water-soluble counts and the labelled $CO_2$ were measured. The rate of formation of water-soluble counts and labelled $CO_2$ during a period of linear $O_2$ uptake provided a good measure of palmitate activation. The results of a representative experiment are given in Fig. 1. It can be seen that low concentrations of $F^-$ inhibit palmitate activation considerably. As

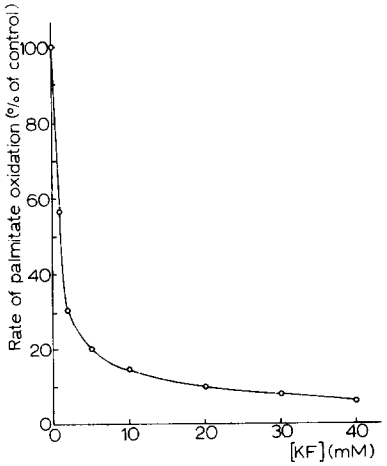


Fig. 1. The inhibition by $F^-$ of palmitate oxidation in intact coupled mitochondria. The medium contained besides the standard components 10 mM L-malate, 2 $\mu$moles of labelled palmitate (spec. act. 0.058 $\mu$Ci/$\mu$mole), 1 mM ADP, 30 mM phosphate, 6 units hexokinase (EC 2.7.1.1), mitochondria (4.74 mg protein) and a variable concentration of KF. The rate of palmitate oxidation is calculated from the differences in the amounts of water-soluble counts and labelled $CO_2$ present at the end of 40 and 20 min incubation, respectively. In the absence of KF the rate was 3.41 nmoles/min per mg protein.

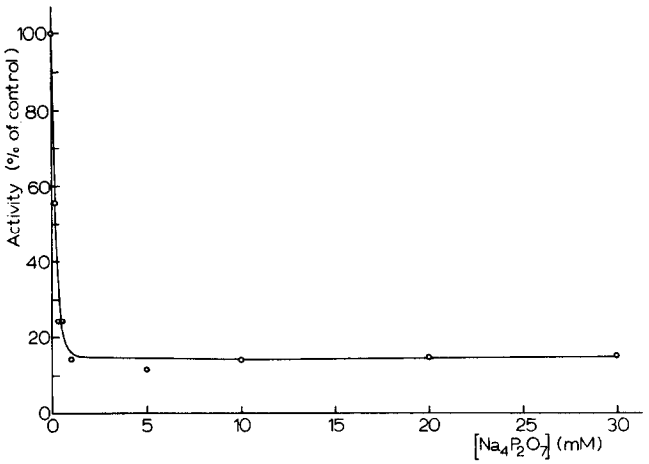


Fig. 2. The inhibition of matrix acyl–CoA synthetase by inorganic pyrophosphate. A crude matrix fraction was used, as described in *Methods*. In the absence of inorganic pyrophosphate the activity was 7.27 nmoles/min per mg protein.

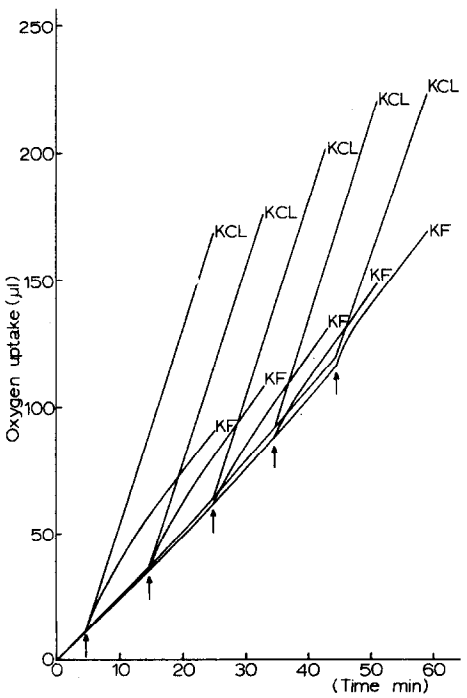


Fig. 3. The lack of an effect of preincubation of the mitochondria with $F^-$ upon the inhibition of mitochondrial palmitate oxidation by $F^-$. To the standard medium 10 mM L-malate, 1 mM ADP, 30 mM phosphate, 6 units hexokinase, mitochondria (5.06 mg protein) and 5 mM KF or KCl were added. At the times indicated by the arrows 2 µmoles of palmitate were added from the side-arm. Manometers were read every 2 min; for reasons of clarity the experimental points could not be included in the figure.

demonstrated earlier[11], the inhibition by $F^-$ of palmitate activation is not found when the fatty acid is activated by the activating enzyme of the mitochondrial outer membrane.

*The inhibition by pyrophosphate of the acyl-CoA synthetase of the mitochondrial matrix*

According to the old hypothesis (see *e.g.* refs. 6 and 24) the inhibition of fatty acid activation by $F^-$ is caused by a pyrophosphate accumulation. The depression by pyrophosphate of the activation of palmitate by a crude matrix fraction of mitochondria is shown in Fig. 2. It can be seen that low pyrophosphate concentrations cause a profound inhibition. This is not an effect of the added $Na^+$, as 100% of the control activity is found in the presence of 120 mM NaCl.

*The effect of preincubation of the mitochondria with $F^-$*

In the experiment shown in Fig. 3 mitochondria first oxidized malate in the presence of $F^-$ or $Cl^-$. At various times palmitate was added. In the absence of $F^-$, addition of palmitate resulted in a rapid rate of respiration. If $F^-$ was present, an initial increase in the rate of $O_2$ uptake was noted, followed by a gradual decrease. This phenomenon was observed independently of the time of palmitate addition, indicating that the gradual decrease in the rate of oxidation did not reflect a slow penetration of $F^-$ into the mitochondrial matrix. It is a first indication that in the presence of $F^-$ an inhibitor accumulates as a consequence of fatty acid oxidation.

*The inhibitory effect of $F^-$ on inorganic pyrophosphatase*

The inorganic pyrophosphatase activity of sonicated mitochondria (spec. act. 102 nmoles $PP_i$/min per mg protein) was strongly inhibited by $F^-$. In the presence of 0.5 and 5mM NaF the activity was inhibited for 95.3 and 98.2%, respectively.

*The intramitochondrial localization of inorganic pyrophosphatase*

An inhibition by $F^-$ of pyrophosphatase can only give rise to an accumulation of pyrophosphate in the inner compartment of the mitochondria if the pyrophosphatase itself is, at least partly, localized in that compartment. Studies of the submitochondrial distribution of inorganic pyrophosphatase, as shown in Table I, demonstrated that this is indeed the case.

TABLE I

THE DISTRIBUTION OF INORGANIC PYROPHOSPHATASE AND VARIOUS MARKER ENZYMES OVER SUBMITOCHONDRIAL FRACTIONS

| *Fraction* | *Protein (mg)* | *Spec. act. (nmoles/min per mg protein)* | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | *Monoamine oxidase* | *Adenylate kinase* | *Cyto-chrome c oxidase* | *Malate dehydro-genase* | *NADPH–cytochrome c reductase* | *Inorganic pyrophosphatase* | |
| | | | | | | | *I** | *II** |
| Mitochondria | 296 | 17.1 | 241 | 440 | 2640 | 5.86 | 66.4 | — |
| Inner membrane | 63.7 | 10.3 | 0 | 1915 | 958 | 2.41 | 12.8 | −19.6 |
| Matrix | 166 | 1.01 | 0 | 25.6 | 4460 | 1.63 | 118 | 117 |
| Outer membrane | 28.9 | 108 | 43.5 | 520 | 646 | 19.7 | 63.9 | 34.1 |
| Intermembrane space | 43.5 | 41.9 | 1220 | 0 | 2170 | 9.15 | 19.9 | −66.2 |
| Microsomes | — | — | — | — | — | 81.9 | 35.5 | — |
| Recovery | 102% | 114% | 76.3% | 109% | 117% | 80.2% | 117% | — |

* I, uncorrected; II, corrected for mutual and microsomal contamination.

*The permeability of mitochondria towards inorganic pyrophosphate*

Pyrophosphate can only exert an inhibitory action on matrix enzymes if it is accumulated in the matrix. The permeability of the mitochondrial inner membrane towards pyrophosphate was tested according to Chappell and Crofts[25]. No swelling occurs in an iso-osmolar solution (91 mM $(NH_4)_4P_2O_7$ with 20 mM Tris, 1 mM EGTA, 1.4 mM sucrose and 0.42 $\mu$M rotenone). Swelling in the pyrophosphate medium could not be induced by addition of 2 mM ammonium phosphate and 2 mM ammonium malate. These observations indicate that $P_2O_7^{4-}$ is unable to pass the inner membrane.

*Pyrophosphate accumulation during fatty acid oxidation in the presence of $F^-$ by coupled mitochondria*

That pyrophosphate accumulation actually occurs in mitochondria oxidizing

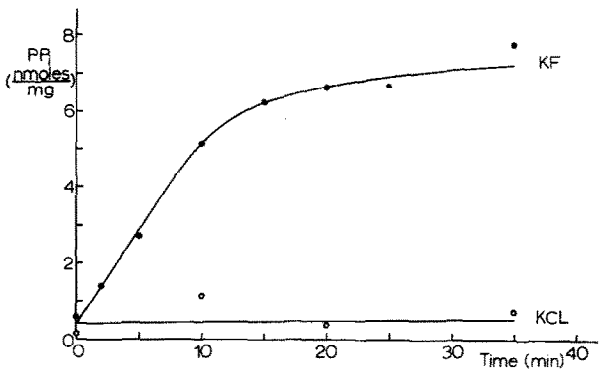


Fig. 4. The pyrophosphate content of coupled mitochondria during fatty acid oxidation. To the standard medium 1 mM ADP, 20 mM phosphate, 6 units hexokinase, 10 mM KF or KCl, mitochondria (5.44 mg protein) and, except for the zero-time control, 2 $\mu$moles of palmitate were added. At the end of the respective incubation times 1-ml samples of the incubation media were taken, from which the mitochondria were analyzed for their pyrophosphate content.

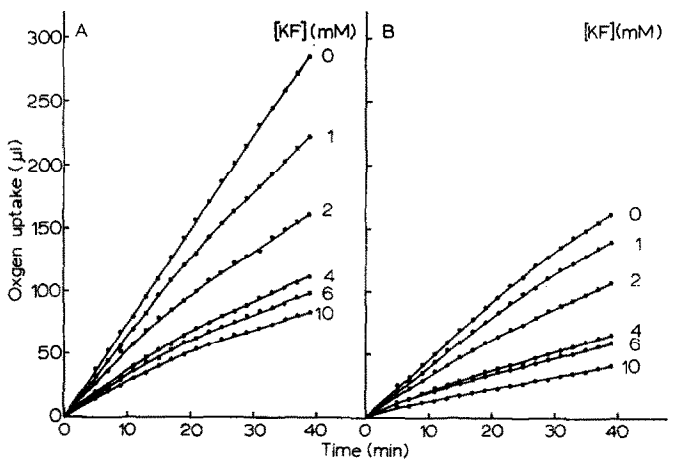


Fig. 5. The inhibition by $F^-$ of palmitate oxidation by coupled (A) and uncoupled (B) mitochondria. Besides the standard components the medium contained 10 mM L-malate, 2 $\mu$moles of palmitate and mitochondria (4.64 mg protein). Moreover, in Expt A, 30 mM phosphate, 1 mM ADP and 6 units hexokinase were added; in Expt B, 1 $\mu$M CCCP.

fatty acid in the presence of $F^-$ is shown in Fig. 4. In the absence of $F^-$ no pyrophosphate accumulation occurs, whereas in the presence of $F^-$ the intramitochondrial pyrophosphate concentration increases considerably.

*Comparison of the inhibition by $F^-$ of fatty acid oxidation by coupled and uncoupled mitochondria*

The inhibition by $F^-$ of the $O_2$ uptake during palmitate oxidation by coupled and uncoupled mitochondria is shown in Fig. 5. It is clear from this experiment that equal concentrations of $F^-$ cause about the same degree of inhibition in both systems.

*Pyrophosphate accumulation during fatty acid oxidation in the presence of $F^-$ by uncoupled mitochondria*

From Fig. 6 it can be seen that palmitate oxidation in the presence of $F^-$ by uncoupled mitochondria in the absence of phosphate also gives rise to pyrophosphate accumulation.

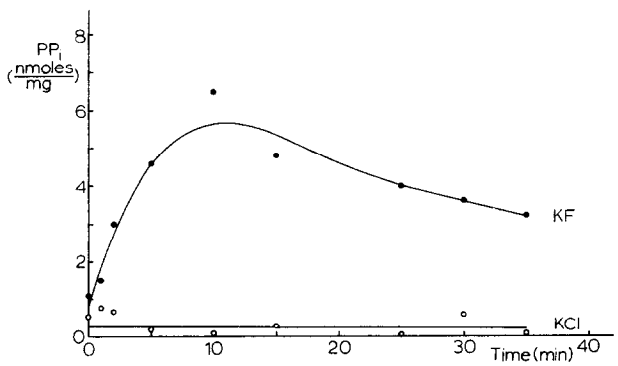


Fig. 6. Pyrophosphate accumulation during palmitate oxidation by uncoupled mitochondria. The medium contained besides the standard components 10 mM L-malate, 2 $\mu$moles of palmitate, 1 $\mu$M CCCP, mitochondria (4.56 mg protein) and 10 mM KF or KCl. The reaction was stopped by addition of 1 ml 1.2 M $HClO_4$.

## DISCUSSION

In the preceding section it was shown that palmitate oxidation by coupled mitochondria in the presence of $F^-$ gives rise to an accumulation pyrophosphate in the mitochondrial matrix. This accumulation of pyrophosphate was not found during oxidation of substrates other than fatty acids, indicating that the pyrophosphate probably results from the fatty acid-activation reaction. Its accumulation in the matrix can be explained by the observations that $F^-$ inhibits an internally localized inorganic pyrophosphatase and that the inner mitochondrial membrane is impermeable towards pyrophosphate. This demonstrated pyrophosphate accumulation, together with the observations that in the presence of $F^-$ an inhibitor is accumulated as a consequence of fatty acid oxidation and that the matrix acyl–CoA synthetase is inhibited by pyrophosphate, makes the hypothesis that $F^-$ inhibition of carnitine-independent coupled fatty acid oxidation is secondary to a $F^-$ inhibition of the inorganic pyrophosphatase, highly likely.

The observation that $F^-$ does not inhibit fatty acid activation in the outer membrane can be explained by the fact that in this case the pyrophosphate is homogeneously distributed over the whole reaction medium.

The question of whether the observed depression by pyrophosphate of palmitate activation is due to a true inhibition of the activating enzyme or merely to a shift of equilibrium cannot be answered unambiguously. Since the fatty acid substrate in the experiment shown in Fig. 2 is not homogeneously distributed, the actual substrate concentration is unknown. From experiments with octanoate which, unlike palmitate, is in true solution in aqueous media, it may be concluded that inhibition by pyrophosphate occurs long before equilibrium is reached. It should be noted, however, that octanoate and palmitate activation may be effected by different enzymes. Our results with octanoate activation are in agreement with those obtained by Graham and Park[26] working with beef-liver mitochondrial octanoyl-CoA synthetase and by Webster and co-workers[27–30] working with beef-heart mitochondrial acetyl-CoA synthetase and beef-heart and beef-liver mitochondrial butyryl-CoA synthetase.

Schick and Butler[31] studying the intramitochondrial localization of rat-liver inorganic pyrophosphatase found that the enzyme is localized at the inner face of the inner membrane. They reported that the enzyme could be detached from the membrane by mild sonication, indicating that it is relatively loosely bound to the membrane. Perhaps the binding is so loose that the enzyme is detached from the membrane by detergents. This might explain why we, using the detergent Lubrol WX to resolve the inner membrane–matrix fraction into a matrix and an inner membrane fraction, find the pyrophosphatase in the soluble fraction. Our results are, however, in agreement with those of Schick and Butler[31] with respect to a localization in the inner mitochondrial compartment. Such a localization is necessary in order to explain a pyrophosphate accumulation in the matrix in the presence of fluoride.

The inhibition by $F^-$ of rat-liver mitochondrial inorganic pyrophosphatase is in agreement with earlier observations[5,31,32].

Rossi and co-workers[6–8] have demonstrated an enzyme catalyzing a GTP-dependent acyl-CoA synthesis according to Eqn 1:

$$RCOOH + GTP + CoA \rightleftharpoons RCOS\text{–}CoA + P_i + GDP \qquad (1)$$

to be present in mitochondria from rat and beef liver. The enzyme was reported to be inhibited by phosphate. This sensitivity to phosphate, together with the finding by Van den Bergh[9,10] that fatty acid oxidation in uncoupled mitochondria is inhibited by phosphate and is dependent on substrate-level phosphorylation, has led to the conclusion that in uncoupled mitochondria in the absence of phosphate fatty acid activation is effected by the GTP-dependent enzyme. The subsequent findings that only low palmitoyl–CoA synthetase activity is observed when ATP is substituted by GTP[33–35] and that the inhibitory effect of phosphate can also be explained by a phosphate-induced loss of citric acid-cycle intermediates from the mitochondria[33,36] raised some doubts as to the importance of the GTP-dependent enzyme.

Palmitate oxidation by uncoupled mitochondria in the absence of phosphate and in the presence of $F^-$ also results in a pyrophosphate accumulation. In this case too, pyrophosphate accumulation was specific for fatty acid oxidation. This indicates that also under these conditions fatty acids are activated by an enzyme liberating

pyrophosphate from nucleoside triphosphate. Since no enzyme has been described catalyzing acyl-CoA synthesis according to Eqn 2:

$$RCOOH + GTP + CoA \rightleftharpoons RCOS\text{-}CoA + GMP + PP_i \quad (2)$$

this triphosphate is most probably ATP formed in substrate-level phosphorylation *via* GTP and nucleoside diphosphate kinase (EC 2.7.4.6).

The fact that ATP-dependent fatty acid activation takes place in uncoupled mitochondria in the absence of phosphate may explain the equal sensitivity towards $F^-$ of fatty acid oxidation by coupled and uncoupled mitochondria.

Our conclusion that in uncoupled mitochondria in the presence of $F^-$ fatty acid activation is brought about by an ATP-dependent enzyme does not mean that in the absence of $F^-$ all activation is ATP-dependent. It is possible that in the absence of $F^-$ both ATP- and GTP-dependent activation takes place. In that case addition of $F^-$ would first inhibit the GTP-dependent enzyme *via* a direct action[6], and then gradually inhibit the ATP-dependent enzyme *via* pyrophosphate accumulation. The mechanism of fatty acid activation in uncoupled mitochondria is at present under investigation in this laboratory.

ACKNOWLEDGEMENTS

The technical assistance of Mr C. H. Obispo is gratefully acknowledged. This investigation was supported in part by the Netherlands Foundation for Chemical Research (S.O.N.) with financial aid from the Netherlands Organization for the Advancement of Pure Research (Z.W.O.).

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