Biochimica et Biophysica Acta, 503 (1978) 437—449

BBA 47551

THE OXIDATION OF LONG-CHAIN UNSATURATED FATTY ACIDS BY ISOLATED RAT LIVER MITOCHONDRIA AS A FUNCTION OF SUBSTRATE CONCENTRATION

WILLEM J. VAARTJES and SIMON G. VAN DEN BERGH

Laboratory of Veterinary Biochemistry, State University of Utrecht, Biltstraat 172, 3572 BP Utrecht (The Netherlands)

(Received March 1st, 1978)

Summary

1. The oxidation of linoleate by rat-liver mitochondria has been studied as a function of substrate concentration. The oxidation of other long-chain unsaturated fatty acids shows similar characteristics.

2. At low concentrations, linoleate is readily oxidized in the absence of carnitine. Its rate of activation by the intramitochondrial acyl-CoA synthetase (EC 6.2.1.2) and subsequent oxidation is limited by the availability of intramitochondrial ATP.

3. A gradual increase of the linoleate concentration leads to (i) a strong depression of the rate of linoleate oxidation, and (ii) uncoupling of respiratory-chain phosphorylation together with induction of a mitochondrial ATPase activity. At still higher linoleate concentrations this ATPase activity is lowered rather than further stimulated and, concomitantly, the rate of linoleate oxidation increases again.

4. Evidence is presented that the inhibition by linoleate of the ATPase activity occurs at the level of the ATPase complex itself. This oligomycin-like effect of linoleate allows intramitochondrial linoleate activation to take place at the expense of ATP derived from substrate-level phosphorylation.

5. At very high concentrations of linoleate, its detergent action predominates and causes a complete inhibition of respiration as well as an extensive stimulation of an oligomycin-insensitive, Mg^{2+}-dependent ATPase activity.

6. Measurement of the binding of radioactively labelled linoleate by isolated mitochondria shows that, at a given ratio of linoleate to mitochondrial protein, the ratio of bound to added linoleate is dependent on the concentration of the mitochondria.

Abbreviations: ΣAcCoA, total flux through the acetyl-CoA pool; CCCP, carbonylcyanide *m*-chlorophenylhydrazone; S-13, 5-chloro-3-*tert*-butyl-2′-chloro-4′-nitrosalicylanilide.

Introduction

The uncoupling effect of free (i.e., not bound to albumin) fatty acids has been known for many years [1—4]. This uncoupling action of fatty acids becomes stronger with increasing chain length and is especially powerful in the case of unsaturated fatty acids [5]. Fatty acids also differ from other mitochondrial respiratory substrates in that they need activation before being oxidized since the β-oxidation system accepts only acyl-CoA esters. Therefore, generation or supply of ATP is necessary in order to let fatty acid oxidation proceed.

As a consequence of these two properties, fatty acids may inhibit their own oxidation. When isolated rat-liver mitochondria oxidize fatty acids in state-3 conditions in the absence of carnitine, the P : O ratio is lowered with increasing fatty acid concentrations. When the P : O ratio drops to zero, fatty acid oxidation is strongly depressed. It has been demonstrated that at this concentration of the fatty acid, its uncoupling action prevents generation of ATP in the respiratory chain and thereby inhibits fatty acid oxidation at the level of the activation reaction [5,6]. Fatty acid oxidation is also inhibited by other uncoupling agents, provided that phosphate is present [5—8].

With unsaturated fatty acids like oleate [5,9,10] and linoleate [11], a further increase in substrate concentration will restore fatty acid oxidation in uncoupled mitochondria. When the rate of oleate oxidation is plotted against the oleate concentration (cf. Fig. 1B), two oleate concentrations can be observed at which the rate of oxidation passes through an optimum. The low rate of oxidation ("oxidation minimum") after the first oxidation optimum is explained by complete uncoupling of the respiratory chain [5]. However, the existence of a second oxidation optimum at still higher oleate concentrations has never been fully understood. Oleate activation at this second oxidation optimum is dependent on 2-oxoglutarate-linked substrate-level phosphorylation [5] and has to be ATP specific in view of the high phosphate concentrations used [8,10,12]. Still, two questions have remained unanswered: (1) Why is the ATP which is used for fatty acid activation at the second oxidation optimum not available for oleate activation at lower oleate concentrations near the oxidation minimum? (2) Why is the second oxidation optimum not observed with saturated fatty acids?

This paper deals mainly with the first question. Most of the experiments were carried out with linoleate which was arbitrarily chosen as a representative of the unsaturated long-chain fatty acids.

Parts of this work have been reported previously [11,13].

Methods and Materials

Liver mitochondria were prepared from female Wistar rats according to Myers and Slater [14] and washed once more to remove all endogenous carnitine. Mitochondrial protein was determined with the biuret method as described by Cleland and Slater [15].

In all experiments incubations were carried out at 25°C in a medium containing as standard components 15 mM KCl, 2 mM EDTA, 50 mM Tris ·

HCl (pH 7.5) and 50 mM sucrose. Unless indicated otherwise, the reactions were started by addition of the mitochondria and the final reaction volume was 2 ml.

In ATPase experiments the reactions were quenched with trichloroacetic acid to a final concentration of 5% (w/v). P_i liberated during the incubations was assayed according to Sumner [16].

In one experiment (Fig. 6) ATPase activity was estimated by measuring ADP in the neutralized supernatants [17].

In the binding experiment of Fig. 5 mitochondria were incubated for 2 min in the presence of [1-^{14}C]linoleate in 1.5 ml Eppendorf plastic cups (microtubes 3810) and subsequently spun down in 2 min in an Eppendorf 3200 centrifuge. Aliquots (0.5 ml) of the supernatants were transferred to counting vials with 10 ml Tritosol [18]. Radioactivity was measured in a liquid scintillation spectrometer (Packard Tri-Carb 2425), and quenching was monitored with the external standard.

L-Carnitine chloride was a generous gift of Dr. Masanobu Umehara, Otsuka Pharmaceutical Co., Osaka, Japan. Fatty acids, rotenone and oligomycin were obtained from Sigma; carbonylcyanide-*m*-chlorophenylhydrazone (CCCP) from Calbiochem; enzymes, ADP, ATP, phosphoenolpyruvate and CoASH from Boehringer. [1-^{14}C]Linoleate from Amersham Radiochemical Centre was diluted with unlabelled linoleate and used in a specific activity of 0.1 Ci/mol. Other chemicals were of the purest grade available.

Micellar solutions of fatty acids were prepared by neutralizing aqueous solutions of the potassium salts. Solutions of palmitate were heated to 70°C before use. Rotenone and oligomycin were added as ethanolic solutions and the ethanol concentration was kept constant at 1.5% (v/v).

Determination of the rate of β-oxidation. In liver mitochondria acetyl-CoA can be oxidized to CO_2 in the Krebs cycle as well as converted into ketone bodies and acetyl-L-carnitine. Hence, to obtain unambiguous values for the rate of β-oxidation, ΣAcCoA, as defined by Lopes-Cardozo and Van den Bergh [19], was calculated. Total acetyl flux (ΣAcCoA) through the acetyl-CoA pool during oxidation of an even numbered fatty acid can be derived from:

$$\Sigma AcCoA = 2[\Delta Acac + \Delta HB] + \Delta AcCn + \\ + \{\Delta O - [4 - (4d + 8)/n]\ \Delta Acac - [3 - (4d + 8)/n]\ \Delta HB - \\ - [2 - (2d + 4)/n]\ \Delta AcCn\}\ \{n/(6n - 2d - 4)\} \quad (1)$$

where ΔAcac, ΔHB and ΔAcCn are μmol acetoacetate, 3-hydroxybutyrate and acetyl-L-carnitine produced, ΔO is μatoms of oxygen consumed, n is the number of carbon atoms in the fatty acid and d the number of double bonds. Eqn. 1 is valid only if the medium does not contain Krebs-cycle intermediates. Accumulation of acetyl-L-carnitine only occurs when the medium is supplemented with carnitine. In case of linoleate ($C_{18:2}$) the general Eqn. 1 is reduced to:

$$\Sigma AcCoA = 9/50\ \Delta O + 36/25\ \Delta Acac + 81/50\ \Delta HB + 18/25\ \Delta AcCn \quad (2)$$

When fatty acids with different chain lengths are compared (Fig. 1), the rate

of fatty acid oxidation (ΔFA) has been given:

$$\Delta FA = \frac{2\,\Sigma AcCoA}{n} \qquad (3)$$

To determine the rate of β-oxidation, incubations were performed in a Gilson GME oxygraph with vibrating platinum electrode. At near oxygen depletion the complete reaction mixture was rapidly transferred by suction into tubes containing 1 ml ice-cold 1.2 M $HClO_4$. Acetoacetate, 3-hydroxybutyrate and acetyl-L-carnitine were measured spectrophotometrically in the neutralized supernatants using standard enzymatic methods [17].

Results and Discussion

The rate of fatty acid oxidation as a function of substrate concentration. As shown in Fig. 1A, isolated liver mitochondria readily oxidize palmitate in a carnitine-free medium supplemented with ADP and P_i. In this experiment the optimal rate of palmitate oxidation (2 nmol/min per mg protein) was measured at a concentration of 210 nmol palmitate/mg protein. Both optimal rate and optimal concentration depend, however, on the method of preparation of the micellar suspension of palmitate [20] and on the amount of mitochondrial protein. Carnitine-stimulated palmitate oxidation is much faster. Using a similar medium supplemented with low concentrations of L-malate, L-carnitine and CoASH, Lopes-Cardozo and Van den Bergh [21] observed a maximal rate of palmitate oxidation at 25°C of 5 nmol/min per mg protein.

At palmitate concentrations higher than 210 nmol/mg protein partial uncoupling of respiratory-chain phosphorylation occurs and the rate of palmitate oxidation gradually declines (see Fig. 1A). In this concentration range

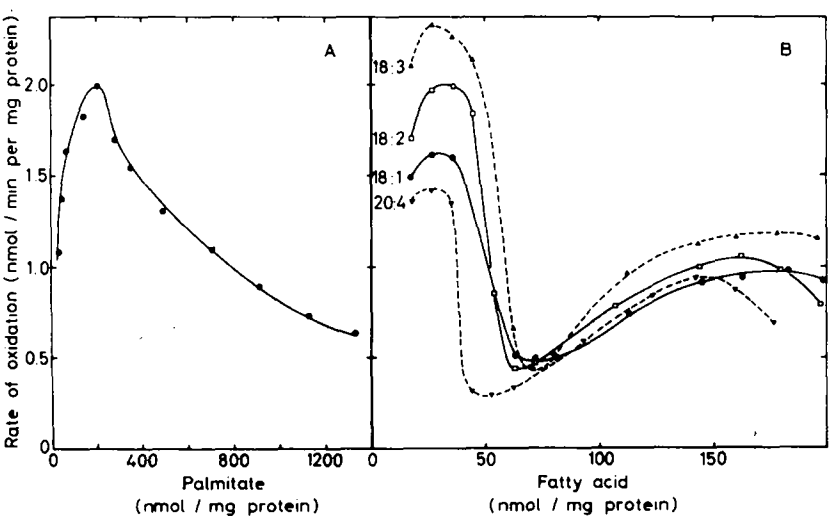

Fig. 1. Rate of oxidation of palmitate (A) and various unsaturated long-chain fatty acids (B) by rat-liver mitochondria as a function of substrate concentration (oxidation curves). The standard reaction medium was supplemented with 5 mM $MgCl_2$, 25 mM potassium phosphate (pH 7.5), 1.5 mM ADP, 2.6 mg (A) or 5.7 mg (B) mitochondrial protein and fatty acids as indicated.

the effective amount of palmitate no longer equals the amount of palmitate added, due to the poor solubility of palmitate in aqueous media at pH 7.5 and 25°C. Thus neither the existence of a second oxidation optimum at very high palmitate concentrations nor even the fully uncoupling concentration of palmitate (cf. ref. 5) can be assessed.

On the other hand, oxidation curves * obtained with a variety of unsaturated fatty acids, from $C_{14:1}$ up to $C_{20:4}$, closely agree with the one [5,9,10] originally observed with oleate ($C_{18:1}$). In Fig. 1B this is shown for linoleate ($C_{18:2}$), α-linolenate ($C_{18:3}$) and arachidonate ($C_{20:4}$). These unsaturated long-chain fatty acids are much more soluble than their saturated homologues. Similar oxidation curves have been found with *trans*-stereoisomers like elaidate and linelaidate (not shown). Hence, from a qualitative point of view neither chain length, stereoconfiguration or degree of unsaturation of the fatty acids are critical. However, their actual rate of β-oxidation depends, amongst other things, on these factors (see Fig. 1B and ref. 21).

Linoleate was arbitrarily selected for further experiments. In our usual reaction conditions (see Fig. 1) and with 3 mg mitochondrial protein present per ml, the first oxidation optimum, oxidation minimum and second oxidation optimum are observed at roughly 35, 75 and 170 nmol linoleate per mg protein, respectively.

Intramitochondrial fatty acid activation as the rate-limiting step of linoleate oxidation. The mitochondrial long-chain acyl-CoA synthetase (EC 6.2.1.3) is associated with the outer mitochondrial membrane [10,22–24]. In the absence of carnitine this enzyme cannot contribute to mitochondrial fatty-acid breakdown [25]. Groot et al. [24] demonstrated that in rat-liver mitochondria under these conditions the activation of long-chain fatty acids is catalysed by the medium-chain acyl-CoA synthetase (EC 6.2.1.2) of the matrix.

The experiment in Fig. 2 indicates that this intramitochondrial activation reaction is the rate-limiting step of linoleate oxidation in the absence of added carnitine. At any concentration of linoleate up to 200 nmol/mg protein, its rate of oxidation is greatly stimulated if ADP is replaced by ATP, carnitine and CoASH, which permit an optimal participation of the acyl-CoA synthetase in the outer membrane. From this it may be concluded that the peculiar oxidation curve of linoleate, observed in the absence of carnitine, directly reflects changes in the rate of the internal linoleate activation reaction.

In the absence of added CoASH and carnitine, the external activation reaction could still contribute to the activation of linoleate if catalytic amounts of endogenous carnitine and CoASH are present outside of the matrix. To exclude this possibility, experiments were performed (not shown) in which either any CoASH present outside the matrix was trapped with tetrathionate [26], or in which the external activating enzyme was destroyed by mild pretreatment of the mitochondria with Nagarse (EC 3.4.21.14) [27,28]. In both cases oxidation curves of linoleate were obtained which coincided with the normal one (Fig. 1B), showing that only internal activation is operative in

* The expressions "ATPase curve" and "oxidation curve" are used throughout to denote graphical representations of ATPase activity and rate of β-oxidation, respectively, as a function of fatty acid concentration.

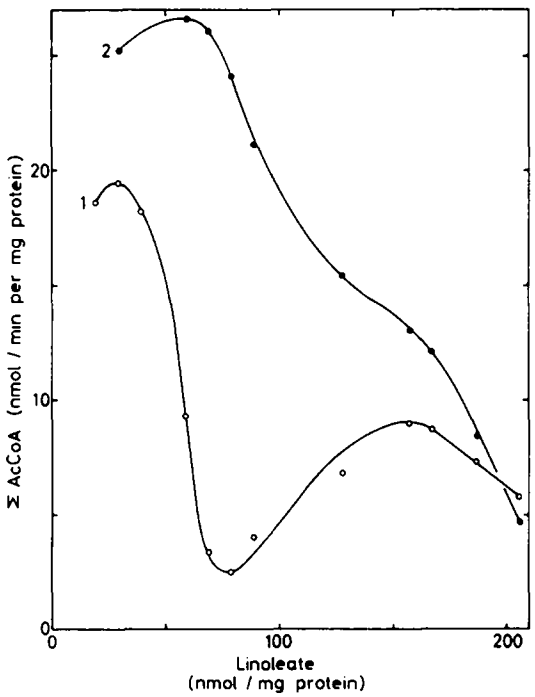

Fig. 2. Stimulation of linoleate oxidation by carnitine. Curve 1, reaction conditions as in Fig. 1; curve 2, ADP replaced by 1.5 mM ATP, 20 μM CoASH and 0.2 mM L-carnitine. Mitochondrial protein, 5.2 mg.

our normal reaction conditions. Our earlier suggestion [11] that linoleate oxidation at the second oxidation optimum is mediated by external activation is thus no longer tenable.

Stimulation of linoleate oxidation by oligomycin. At the oxidation minimum of linoleate the addition of oligomycin greatly enhances the rate of linoleate oxidation (Table I). The same stimulation occurs when CCCP is included in the medium to ensure complete uncoupling.

TABLE I

LINOLEATE OXIDATION IN THE PRESENCE AND IN THE ABSENCE OF OLIGOMYCIN AND ADP

Reaction conditions as in Fig. 1 with oligomycin (2.9 μg/mg protein) and CCCP (0.77 nmol/mg protein), if indicated. Mitochondrial protein, 5.2 mg.

Linoleate concn. (nmol/mg protein)	Position in oxidation curve	Additions	ΣAcCoA (nmol/min per mg protein)	
			with ADP	without ADP
36	1st optimum		17.2	13.9
72	Minimum		3.1	2.9
72	Minimum	Oligomycin	16.3	16.8
72	Minimum	Oligomycin + CCCP	15.9	16.0
164	2nd optimum		8.4	8.5
164	2nd optimum	Oligomycin	9.8	8.2

This strong stimulation by oligomycin indicates (1) that at the oxidation minimum a considerable amount of ATP is still being produced; (2) that in the absence of oligomycin this ATP is rapidly split by an oligomycin-sensitive ATPase activity induced by linoleate; and (3) that after the first oxidation optimum the rate of internal linoleate activation is controlled by the availability of ATP.

Table I also demonstrates that, at uncoupling concentrations of linoleate, its oxidation is not affected by removal of ADP from the medium. This indicates that ATP, possibly produced from ADP via adenylate kinase (EC 2.7.4.3) does not participate in fatty acid activation. Therefore, in uncoupled mitochondria linoleate activation is completely dependent on endogenous ATP generated in substrate-level phosphorylation.

Linoleate-induced ATPase activity. As discussed above, at the oxidation minimum linoleate oxidation can be stimulated either by addition of oligomycin or by an increase in the linoleate concentration. With increasing linoleate concentrations the stimulation by oligomycin becomes less effective (not shown) and at the second oxidation maximum oligomycin is almost completely without effect (Table I). This suggested to us that the second oxidation maximum may be explained by the fact that at higher concentrations linoleate itself inhibits the ATPase.

The effects of linoleate on the mitochondrial ATPase activity are shown in Fig. 3. Both in the absence and in the presence of Mg^{2+} (curves 1 and 2, respectively) an inhibition of the linoleate-induced ATPase activity by increasing

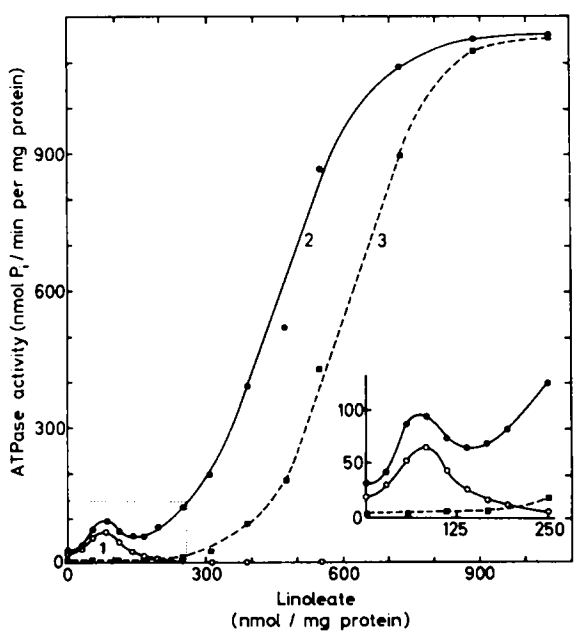

Fig. 3. Effects of linoleate on mitochondrial ATPase activity. The standard reaction medium was supplemented with 4 mM ATP and linoleate as indicated. Further additions: curve 1 (○———○), none; curve 2 (●———●), 5 mM $MgCl_2$; curve 3 (■———■), 5 mM $MgCl_2$ and oligomycin (6.8 μg/mg protein). Reaction time, 5 min; mitochondrial protein, 0.44 mg.

concentrations of linoleate is indeed observed. In both ATPase curves an optimum is found at about 80 nmol linoleate/mg protein. The two optima always appear at the same linoleate concentration, but this concentration may vary in different experiments (see below).

In the absence of added Mg^{2+} (it should be remembered that our standard medium contains 2 mM EDTA) an almost complete inhibition of the ATPase is observed at linoleate concentrations above 200 nmol per mg protein (curve 1). This ATPase curve strongly resembles dose vs. effect curves for the ATPase activities induced by uncouplers like 2,4-dinitrophenol [29], CCCP [30] and 5-chloro-3-*tert*-butyl-2′-chloro-4′-nitrosalicylanilide (S-13) [31], the main difference being that with linoleate, but not with the other uncouplers, the ATPase activity is fully inhibited at the higher concentrations.

The ATPase curve in the presence of added Mg^{2+} (curve 2 of Fig. 3) differs from that in its absence (curve 1) by a dramatic increase in ATPase activity at linoleate concentrations above 200 nmol per mg protein. At these high fatty acid concentrations a loss of endogenous Mg^{2+} and of matrix enzymes from the mitochondria [12,32–34] and an atractyloside-insensitive hydrolysis of added ATP [32] have been reported to occur. We observed that the effects of increasing concentrations of linoleate become less and less reversible by subsequent addition of serum albumin. Since it is known that unsaturated long-chain fatty acids are surface-active agents, it is likely that linoleate at these high concentrations breaks up the mitochondrial membranes. Therefore, ATPase curve 2 of Fig. 3 can be best interpreted as the summation of the uncoupler-induced ATPase activity of intact mitochondria and the detergent-stimulated, Mg^{2+}-dependent ATPase typical of mitochondrial fragments [14,35]. The structural disorganization of the mitochondria probably also accounts for the declining rate of linoleate oxidation after the second oxidation optimum.

As may be seen from curve 3 of Fig. 3, at very high linoleate concentrations the Mg^{2+}-dependent ATPase becomes oligomycin-insensitive. This oligomycin-insensitive activity remains in the supernatant after sonication (3 min) of linoleate-treated mitochondria followed by centrifugation (30 min, 40 000 × *g*). Since it turned out to be cold-stable, it may reflect the activity of membrane-associated ATPase complexes (cf. ref. 36). Its oligomycin insensitivity may be explained by denaturation of the oligomycin-sensitivity-conferring protein [37]. Alternatively, linoleate micelles may compete with oligomycin for the ATPase complex (cf. ref. 38) or, more interestingly, may cosolubilize those phospholipids [39,40] essential for oligomycin sensitivity of the ATPase complex.

ATPase curves very similar to curve 2 of Fig. 3 have previously been observed for oleate [4,10,32,41]. The inhibition of the ATPase activity by intermediate concentrations of oleate has been ascribed by Wojtczak et al. [32] to the inhibition of the adenine-nucleotide translocator by long-chain fatty acids [33,42]. As indicated previously [10], we do not agree with this interpretation. If long-chain unsaturated fatty acids inhibit the ATPase activity in the same concentration range in which they will stimulate fatty acid oxidation at the second oxidation optimum, it is clear that they inhibit the hydrolysis of both externally added ATP and of the ATP formed endogenously in

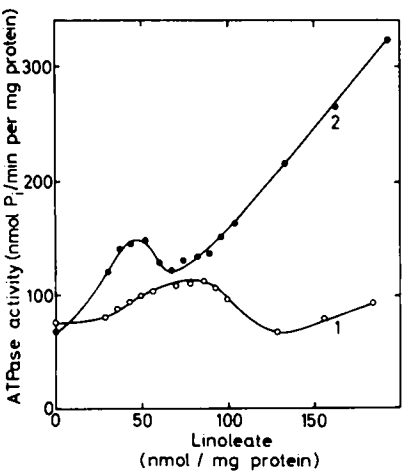

Fig. 4. Linoleate-induced ATPase activity at two different protein concentrations. Mitochondrial protein, 0.21 mg (curve 1) or 2.72 mg (curve 2). The standard reaction medium was supplemented with 5 mM $MgCl_2$, an ATP-regenerating system (4 mM phosphoenolpyruvate and 5 units pyruvate kinase), rotenone (0.85 μg/mg protein) and linoleate as indicated. Mitochondria were preincubated in the reaction mixture for 2 min, whereafter the reaction was started by adding 4 mM ATP. Final volume, 2 ml. Reaction time, 40 min (curve 1) or 4 min (curve 2).

substrate-level phosphorylation. It is also clear that this inhibition cannot be exerted at the level of the adenine-nucleotide translocator, but that the site of inhibition must be between endogenous ATP and the respiratory chain, i.e., in the region where oligomycin inhibits energy transfer.

Binding of linoleate by rat-liver mitochondria. To support our conclusion that intermediate concentrations of linoleate directly inhibit the ATPase, it is crucial to show that the minimum in the oxidation curve occurs at the same concentration as the optimum in the ATPase curve. So far, fatty acid concentrations have been expressed as relative concentrations, i.e., as nmol fatty acid/mg protein, since it is known that substantial amounts of fatty acids are adsorbed to the mitochondrial membranes [43]. However, ATPase activities are normally determined at protein concentrations over ten times lower than those used in oxidation experiments. It is uncertain whether, with such a difference in protein concentration, the relative linoleate concentration is still a reliable basis of comparison.

Fig. 4 shows that the influence of large variations in protein concentration may indeed not be overlooked. The optimum in the ATPase curve shifts to lower relative linoleate concentrations when the amount of protein is increased. Obviously linoleate becomes more effective with increasing concentrations of the mitochondria.

Direct evidence for this conclusion is given in Fig. 5, which shows that, at a given ratio of linoleate to protein, the ratio of bound to added linoleate is dependent on the protein concentration. In this experiment no distinction is made between uptake and binding of linoleate, but it has been demonstrated by others [43,44] that when unesterified fatty acids are taken up by mitochondria, they are almost completely bound to mitochondrial membranes. More-

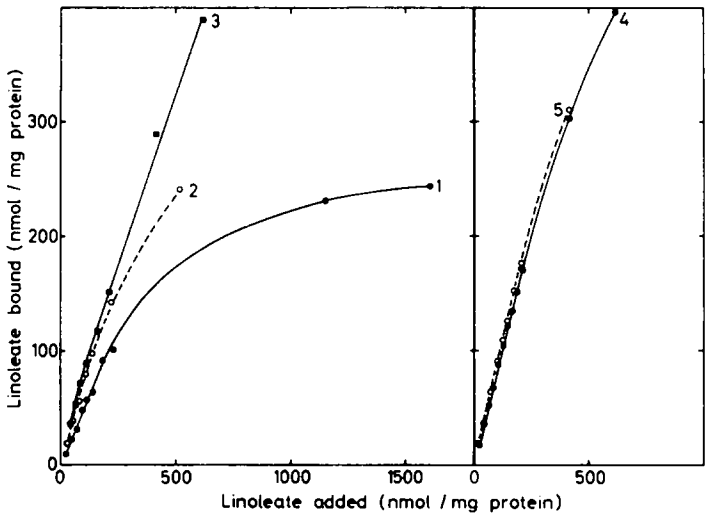

Fig. 5. Binding of linoleate by rat-liver mitochondria. Mitochondrial protein: curve 1, 0.078 mg; curve 2, 0.440 mg; curve 3, 0.840 mg; curve 4, 2.18 mg; curve 5, 4.08 mg. The standard reaction medium was supplemented with 5 mM $MgCl_2$, 25 mM potassium phosphate (pH 7.5) and rotenone (0.70 μg/mg protein). After preincubation of the mitochondria in this reaction mixture for 2 min, [1-^{14}C]linoleate was added at the concentrations indicated. Final reaction volume, 1.3 ml. After 2 min the incubations were terminated by centrifugation. Linoleate bound was calculated as the difference of linoleate added and linoleate retained in the supernatant.

over, in these experiments rotenone was added to inhibit linoleate oxidation.

From Fig. 5 it may be concluded that (i) with high protein concentrations (curves 4 and 5) almost all linoleate is bound; (ii) at linoleate concentrations up to about 160 nmol per mg protein the curves are linear, indicating that in this concentration range, binding of linoleate can be described with a partition coefficient; (iii) with low protein concentrations (curve 1) saturation of binding occurs, and (iv) all effects of linoleate on mitochondria (with the possible exception of the induction of the oligomycin-insensitive ATPase) are due to the amount of linoleate actually bound by mitochondrial membranes.

Correlation of ATPase activity and rate of linoleate oxidation. Apart from the protein concentration (Fig. 4), other factors in the incubation medium (e.g. the ATP concentration) were also found to influence the position of the optimum in the ATPase curve. In order to demonstrate that the optimum in the ATPase curve occurs at the same relative linoleate concentration as the minimum in the oxidation curve, both the rate of oxidation (ΣAcCoA) and the rate of ATP hydrolysis were, therefore, measured in the same incubations.

Fig. 6 shows the results of such an experiment. In the range of 50—140 nmol linoleate/mg protein a good inverse correlation is observed between ATPase activity and oxidation rate.

It can be seen that the optimum of the ATPase curves coincides with the minimum of the corresponding oxidation curves. At higher linoleate concentrations the ATPase activity decreases and concomitantly linoleate oxidation is stimulated. Keeping in mind that under these conditions (i) linoleate oxidation is limited by the intramitochondrial activation reaction and (ii) activation

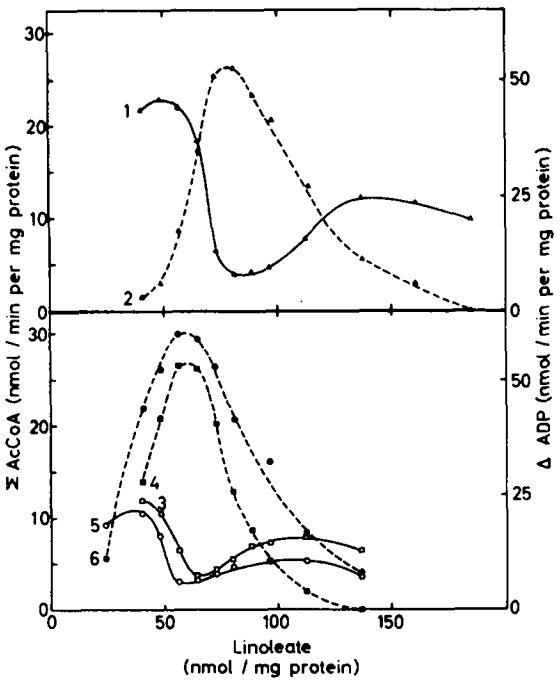

Fig. 6. Comparison of linoleate-induced ATPase activity and rate of linoleate oxidation. The standard reaction medium was supplemented with 25 mM potassium phosphate (pH 7.5), 5 mM ATP and linoleate as indicated. Solid lines and dotted lines represent oxidation curves and ATPase curves, respectively. Further additions: curves 1 and 2, none; curves 3 and 4, 5 mM malonate; curves 5 and 6, 0.5 mM arsenite. Mitochondrial protein, 2.8 mg.

is limited by the supply of ATP, the data of Fig. 6 prove unequivocally that linoleate inhibits the ATPase complex. Our original proposal that unsaturated long-chain fatty acids have an oligomycin-like effect on mitochondrial energy transfer [10] is strongly supported by these data.

The results obtained in the presence of malonate (curves 3 and 4 of Fig. 6) or arsenite (curves 5 and 6) demonstrate that the overall composition of the reaction medium also influences the position of the ATPase optimum. It should be noted that both arsenite and malonate prevent substrate-level phosphorylation. In curves 3—6 of Fig. 6 both ATPase acitivity and linoleate oxidation are, therefore, fully dependent on added ATP. Nevertheless, the same inverse correlation between the rates of oxidation and of ATP hydrolysis is observed as in curves 1 and 2. This proves again that inhibition of the ATPase at post-optimal linoleate concentrations is not due to an inhibition of the adenine-nucleotide translocator.

The mechanism by which unsaturated long-chain fatty acids inhibit the mitochondrial ATPase is not yet clear. Since the inhibition is observed both in the absence and in the presence of added Mg^{2+} (Fig. 3, curves 1 and 2), it cannot be explained by depletion of endogenous magnesium (cf. refs. 4, 10, 32).

Physiological considerations. There is little evidence for a role of fatty acids as uncouplers in rat liver in vivo. Their importance for heat production in

brown adipose tissue is well-established (for a review, see ref. 45). Moreover, in intact perfused dog hearts fatty acids diminish the working efficiency with a concomitant increase in oxygen consumption [46]. For liver the situation is much less clear. Decreases in respiratory control and P : O ratio, observed in mitochondria isolated from ischemic livers of rat [47] and rabbit [48], have been attributed to an increased mitochondrial level of long-chain fatty acids. On the other hand, perfusion with oleate did not induce ATPase activity in the isolated rat liver [49]. Species differences present a further complication. Liver mitochondria from guinea pig are much more sensitive to the uncoupling action of fatty acids than are rat-liver mitochondria [45].

In the case of rat liver the following calculation can be tentatively made, if we ignore the different species of long-chain fatty acids actually present. It has been reported [50] that the level of non-esterified fatty acids in rat liver is elevated to about 5 and 7 μequiv./g wet wt. in the case of starvation and alloxan-diabetes, respectively. Only a very small fraction of these fatty acids will be free in solution since cytosolic proteins [51—53] and mitochondrial membranes (this paper and refs. 43, 44) avidly bind long-chain fatty acids. Assuming the protein content of rat liver to be 165 mg/g wet wt. (cf. ref. 54) and assuming at random distribution of the fatty acids in the cell, one arrives at fatty acid concentrations of 30 and 42 nmol/mg protein, respectively. If the affinity of mitochondria is high enough to bind 50% of fatty acids present and if rat liver contains 60 mg mitochondrial protein/g wet wt. [55], one arrives at the figures of 42 and 58 nmol fatty acid/mg mitochondrial protein, respectively; enough to illustrate that, at least in ketotic conditions, the possibility of fatty-acid-induced uncoupling of rat-liver mitochondria in vivo cannot be excluded.

The physiological significance of the oligomycin-like effect of unsaturated long-chain fatty acids is much more doubtful, as it occurs at even higher fatty acid concentrations.

Acknowledgements

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.). The expert technical assistance of Miss T.A. Wisserhof is gratefully acknowledged.

References

1 Pressman, B.C. and Lardy, H.A. (1956) Biochim. Biophys. Acta 21, 458—466
2 Lehninger, A.L. and Remmert, L.F. (1959) J. Biol. Chem. 234, 2459—2464
3 Hülsmann, W.C., Elliott, W.B. and Slater, E.C. (1960) Biochim. Biophys. Acta 39, 267—276
4 Borst, P., Loos, J.A., Christ, E.J. and Slater, E.C. (1962) Biochim. Biophys. Acta 62, 509—518
5 Van den Bergh, S.G. (1966) in Regulation of Metabolic Processes in Mitochondria (Tager, J.M., Papa, S., Quagliariello, E. and Slater, E.C., eds.), BBA Library, Vol. 7, pp. 125—133, Elsevier, Amsterdam
6 Van den Bergh, S.G. (1967) in Mitochondrial Structure and Compartmentation (Quagliariello, E., Papa, S., Slater, E.C. and Tager, J.M., eds.), pp. 400—410, Adriatica Editrice, Bari
7 Van den Bergh, S.G. (1965) Biochim. Biophys. Acta 98, 442—444
8 Rossi, C.R., Galzigna, L., Alexandre, A. and Gibson, D.M. (1967) J. Biol. Chem. 242, 2102—2110
9 Wojtczak, L., Drahota, Z., Zaluska, H. and Zborowski, J. (1966) in Regulation of Metabolic Processes in Mitochondria (Tager, J.M., Papa, S., Quagliariello, E. and Slater, E.C., eds.), BBA Library, Vol. 7, pp. 134—142, Elsevier, Amsterdam

10 Van den Bergh, S.G., Modder, C.P., Souverijn, J.H.M. and Pierrot, H.C.J.M. (1969) in Mitochondria, Structure and Function (Ernster, L. and Drahota, Z., eds.), FEBS Symp., Vol. 17, pp. 137—144, Academic Press, New York
11 Vaartjes, W.J. and van den Bergh, S.G. (1971) in Energy Transduction in Respiration and Photosynthesis (Quagliariello, E., Papa, S. and Rossi, C.S., eds.), pp. 413—419, Adriatica Editrice, Bari
12 Drahota, Z. and Honová, E. (1968) Acta Biochim. Pol. 15, 227—233
13 Vaartjes, W.J. (1973) Langketen Vetzuren en het Mitochondriale Energiemetabolisme, Ph.D. thesis, State University of Utrecht, Elinkwijk, Utrecht
14 Myers, D.K. and Slater, E.C. (1957) Biochem. J. 67, 558—572
15 Cleland, K.W. and Slater, E.C. (1953) Biochem. J. 53, 547—556
16 Sumner, J.B. (1944) Science 100, 413—414
17 Bergmeyer, H.U. (1970) Methoden der Enzymatischen Analyse, 2nd edn., Verlag Chemie, Weinheim
18 Fricke, U. (1975) Anal. Biochem. 63, 555—558
19 Lopes-Cardozo, M. and van den Bergh, S.G. (1972) Biochim. Biophys. Acta 283, 1—15
20 Kennedy, E.P. and Lehninger, A.L. (1950) J. Biol. Chem. 185, 275—285
21 Lopes-Cardozo, M. and van den Bergh, S.G. (1974) Biochim. Biophys. Acta 357, 43—52
22 Norum, K.R., Farstad, M. and Bremer, J. (1966) Biochem. Biophys. Res. Commun. 24, 797—804
23 Aas, M. (1971) Biochim. Biophys. Acta 231, 32—47
24 Groot, P.H.E., van Loon, C.M.I. and Hülsmann, W.C. (1974) Biochim. Biophys. Acta 337, 1—12
25 Fritz, I.B. (1963) in Advances in Lipid Research (Paoletti, R. and Kritchevski, D., eds.), Vol. 1, pp. 285—334, Academic Press, New York
26 Skrede, S. and Bremer, J. (1970) Eur. J. Biochem. 14, 465—472
27 Pande, S.V. and Blanchaer, M.C. (1970) Biochim. Biophys. Acta 202, 43—48
28 De Jong, J.W. and Hülsmann, W.C. (1970) Biochim. Biophys. Acta 210, 499—501
29 Hemker, H.C. (1962) Biochim. Biophys. Acta 63, 46—54
30 Bertina, R.M. and Slater, E.C. (1975) Biochim. Biophys. Acta 376, 492—504
31 Hallman, M. and Hassinen, I. (1971) FEBS Lett. 13, 258—260
32 Wojtczak, L., Bogucka, K., Sarzata, M.G. and Zaíuska, H. (1969) in Mitochondria, Structure and Function (Ernster, L. and Drahota, Z., eds.), FEBS Symp., Vol. 17, pp. 79—92, Academic Press, New York
33 Wojtczak, L. and Zaíuska, H. (1967) Biochem. Biophys. Res. Commun. 28, 76—81
34 Chefurka, W. (1966) Biochemistry 5, 3887—3903
35 Siekevitz, P., Löw, H., Ernster, L. and Lindberg, O. (1958) Biochim. Biophys. Acta 29, 378—391
36 Pullman, M.E., Penefsky, H.S., Datta, A. and Racker, E. (1960) J. Biol. Chem. 235, 3322—3329
37 MacLennan, D.H. and Tzagoloff, A. (1968) Biochemistry 7, 1603—1610
38 Swanljung, P., Frigeri, L., Ohlson, K. and Ernster, L. (1973) Biochim. Biophys. Acta 305, 519—533
39 Palatini, P. and Bruni, A. (1970) Biochem. Biophys. Res. Commun. 40, 186—191
40 Bruni, A., van Dijck, P.W.M. and de Gier, J. (1975) Biochim. Biophys. Acta 406, 315—328
41 Bos, C.J. and Emmelot, P. (1962) Biochim. Biophys. Acta 64, 21—29
42 Vaartjes, W.J., Kemp, Jr., A., Souverijn, J.H.M. and van den Bergh, S.G. (1972) FEBS Lett. 23, 303—308
43 Spector, A.A. and Brenneman, D.E. (1972) Biochim. Biophys. Acta 260, 433—438
44 Reshef, L. and Shapiro, B. (1965) Biochim. Biophys. Acta 98, 73—80
45 Flatmark, T. and Pedersen, J.I. (1975) Biochim. Biophys. Acta 416, 53—103
46 Mjøs, O.D., Bugge-Asperheim, B. and Kiil, F. (1972) Cardiovasc. Res. 6, 23—27
47 Boime, I., Smith, E.E. and Hunter, Jr., F.E. (1968) Arch. Biochem. Biophys. 128, 704—715
48 Ozawa, K., Takasan, H., Kitamura, O., Mizukami, T., Kamano, T., Takeda, H., Ohsawa, T., Murata, T. and Honjo, I. (1971) J. Biochem. Tokyo 70, 755—764
49 Williamson, J.R., Scholz, R. and Browning, E.T. (1969) J. Biol. Chem. 244, 4617—4627
50 McLean, P., Gumaa, K.A. and Greenbaum, A.L. (1971) FEBS Lett. 17, 345—350
51 Ockner, R.K., Manning, J.A., Poppenhausen, R.B. and Ho, W.K.L. (1972) Science 177, 56—58
52 Mishkin, S. and Turcotte, R. (1974) Biochem. Biophys. Res. Commun. 57, 918—926
53 Suzue, G. and Marcel, Y.L. (1975) Can. J. Biochem. 53, 804—809
54 Spencer, A., Corman, L. and Lowenstein, J.M. (1964) Biochem. J. 93, 378—388
55 Scholz, R. and Bücher, Th. (1965) in Control of Energy Metabolism (Chance, B., Estabrook, R.W. and Williamson, J.R., eds.), pp. 393—414, Academic Press, New York