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THE EFFECTS OF COENZYME A AND CARNITINE ON STEADY-STATE ATP/ADP RATIOS AND THE RATE OF LONG-CHAIN FREE FATTY ACID OXIDATION IN LIVER MITOCHONDRIA

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Summary

1. Conditions for the optimal coupled oxidation by rat liver mitochondria of long-chain free fatty acids were defined. The fatty acids studied were in the $\omega 9$ series: oleic (18 : 1), gondoic (20 : 1) and erucic (22 : 1) acids. Carnitine (about 0.1 mM) maximally stimulated State 3 respiration due to oleic and gondoic acids about three-fold (coenzyme A present), and coenzyme A (10—20 μM) stimulated about two-fold (carnitine present). When neither coenzyme A nor carnitine was added, respiration was very slow.

2. When respiration was limited by ADP, concentrations of added CoA only slightly in excess of that required for fatty acid oxidation very significantly decreased the ATP/ADP ratio maintained at a given rate of respiration imposed by externally added ATPase, and increased the level of membrane-associated acyl-CoA. This effect was most pronounced with oleic acid, and least with erucic acid. When excess ADP was present, higher concentrations of added coenzyme A (50—200 μM) inhibited the oxidation of oleic acid in a concentration-dependent manner, whereas the oxidation of substrates other than fatty acids was essentially unaffected.

3. It is concluded that, in addition to its requirement for fatty acid oxidation, coenzyme A exerts two independent effects on mitochondrial metabolism as here determined in vitro: (a) under conditions mimicking those in the intact cell with respect to phosphorylation-dependent respiration (ADP limiting), acyl-CoA formed from added coenzyme A and fatty acid inhibits the adenine nucleotide translocase, resulting in a lowering in the extramitochondrial ATP/ADP ratio obtained at any given rate of phosphorylation-limited respiration,

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and (b) under State 3 conditions (ADP in excess) coenzyme A (<50—200 μM) specifically suppresses oxidation of long-chain fatty acids by limiting the rate of formation of intramitochondrial acyl-CoA.

Introduction

Conditions for optimum respiration in the presence of the homologous *cis* ω-9 monoenoic fatty acids oleic acid ($C_{18:1}$), gondoic acid ($C_{20:1}$), and erucic acid ($C_{22:1}$) have been studied in rat liver mitochondria. In particular we found it valuable to study carefully important factors such as carnitine and CoA. Since only scattered data are available in the literature to what extent various factors effect optimum metabolism of free fatty acids, we set out in the first instance to establish these optimal conditions in a systematic study.

Erucic acid and related long-chain monoenoic fatty acids have recently attracted considerable interest, since in high concentrations they have significant physiopathological effects [1]. In this study the metabolism of free fatty acids is investigated under State 3 conditions (excess ADP), as well as conditions close to those occurring in vivo, i.e., at phosphorylation potentials allowing about $\frac{2}{3}$ of the State 3 rate of respiration. Steady-state ATP/ADP ratios were obtained by incubating mitochondria in the presence of ATPase and ATP [2].

The future perspective of this investigation is to study the competitive oxidative metabolism of mixtures of homologous free fatty acids under optimum conditions and controlled energetic states. This has been done in State 3 with mixtures of carnitine esters of fatty acids [3,4], and mutual competition for oxidation was reported. The competitive effects of free fatty acids on heart mitochondria have been studied [5,6], but only in the resting state (State 4). Under these conditions, only a minor effect of the presence of erucic acid was found on palmitic or oleic acid oxidation.

A preliminary report of the present work has been presented [7]. A study of the competition between oxidation of free fatty acids at optimum conditions is in progress.

Materials and Methods

Materials

Enzymes, coenzymes and substrates were obtained from Sigma Chemical Company, St. Louis, Mo. Fatty acid-free bovine serum albumin (Fraction V) was purchased from Miles Laboratories, Inc., Kankakee, Ill., and DL-fluorocitrate from Calbiochem, La Jolla, Calif. Palmitoyl-carnitine was prepared according to the method of Bremer [8]. Other chemicals were of reagent grade. Mitochondrial ATPase (F_1), specific activity of about 15 enzyme units/mg protein was prepared from beef heart mitochondria [9,10]. Before use, the enzyme was twice dialyzed at room temperature for 1 h against 0.5 l of 0.25 M sucrose containing 10 mM Tris · HCl (pH 7.4) and 0.8 mM ATP. Hexokinase to be added to incubations with mitochondria was also dialyzed to remove ammonium sulfate.

Isolation of mitochondria

Liver mitochondria were prepared from 240—260-g fed male Wistar rats by the method of Johnson and Lardy [11]. The isolation medium was composed of 0.25 M sucrose-containing 1 mM EGTA/2 mM Tris · HCl (pH 7.2). The respiratory control ratio of each preparation exceeded 4.5 measured in a Gilson oxygraph with oleic acid as substrate (at optimum conditions) and 6.0 with glutamate (5 mM) and malate (1 mM) as substrates.

Incubations

The standard medium contained 60 mM Tris · HCl (pH 7.2), 40 mM KCl, 6 mM $MgCl_2$, 10 mM potassium phosphate (pH 7.2), 1 mM EGTA, 2 mM ATP and 1 mM malate unless otherwise stated. When oleic acid (0.1 mM) was tested under optimum conditions (State 3 for reference), 0.061 mM (0.4%, w/v) bovine serum albumin (molar ratio oleate/serum albumin, 1.6), 0.1 mM L-carnitine, 20 μM CoA, and 0.5 mM ADP were added. Concentration of mitochondria used was 3 mg protein/ml. Respiration experiments were performed at 30° C in 2 ml total volume. When fluorocitrate was used, mitochondria (approx. 50 mg protein/ml) were incubated with 2.5 mM fluorocitrate for 15 min before incubation [12]. Final concentration of fluorocitrate in the incubation was 0.3 mM.

In experiments using ATP and ATPase to stimulate respiration, the rate was titrated by adding controlled amounts of purified ATPase. This system [2] simulates in vivo conditions with a high energy charge, i.e., where the mitochondria are limited by the supply ADP. ATP/ADP ratios were calculated from measured nucleotide concentrations in neutralized extracts.

Results in figures and tables represent observations in a single experiment. In all cases similar results have been obtained with two or more independent mitochondrial preparations.

Metabolite assays

Glutamate, aspartate, malate, β-hydroxybutyrate, acetoacetate, citrate, α-ketoglutarate and adenine nucleotides were measured by standard enzymic procedures spectrophotometrically. The procedures used are described or referenced in previous publications [12,13].

Total acyl-CoA in the incubations was determined after acid precipitation. For determination of membrane-bound plus endogenous acyl-CoA, the mitochondria were rapidly removed by centrifugation. The mitochondrial or acid precipitations were washed in 1.5%, then 0.3% $HClO_4$ and finally stored in 10 mM DL-dithiothreitol. The acyl-CoA was hydrolyzed by treatment with 0.1 M KOH at 37° C for 30 min. The amount of CoA was then determined by a catalytic assay using phosphotransacetylase [14]. The rate was recorded in a Cary Model 118C spectrophotometer.

Protein was determined by the biuret method [15], and oxygen uptake by mitochondria was assayed in a Gilson oxygraph with vibrating electrode at 30° C.

The rate of β-oxidation in the presence of fluorocitrate and malate was calculated from the rates of formation of acetoacetate (AcAc), β-hydroxybutyrate

(βHB) and citrate (Cit), and expressed as the rate of acetyl (Ac) production, so that β-oxidation is described by the formula

$\Delta Ac = 2(\Delta AcAc + \Delta \beta HB) + \Delta Cit.$

Respiration resulting from fatty acid oxidation was then calculated to be

$-\Delta O = 4\ \Delta AcAc + 3\ \Delta \beta HB + 3\ \Delta Cit.$

Since insignificant amounts of ammonia were formed under the conditions of these experiments, oxidation of glutamate was equivalent to the amount of aspartate formed minus the decrease in malate not accounted for by formation of citrate or by side-reactions, the latter being due to aspartate formation from malate-derived oxaloacetate. Accordingly, aspartate formed independent of a change in malate concentration represents glutamate oxidation, with the reduction of three atoms of oxygen per molecule of glutamate oxidized. In addition, for every molecule of aspartate formed which does not represent glutamate oxidation, one atom of oxygen is reduced (via malic dehydrogenase). This last statement assumes that appreciable concentrations of intermediates between α-ketoglutarate and malate did not accumulate. This has been experimentally verified by comparing malate consumption and accumulation of α-ketoglutarate and citrate.

The terminology for respiratory states of Chance and Williams [16] is used.

Results

Respiration resulting from the oxidation of free fatty acids was tested in a Gilson oxygraph as a function of amounts of added bovine serum albumin, free fatty acid, malate, carnitine and CoA. The standard medium for these titrations was as described in Materials and Methods, except for the factor to be tested. After a brief preincubation period, respiration was stimulated by added ADP in order to obtain a State 3 rate.

The amount of bovine serum albumin required to protect mitochondria from the ionophoric and detergent effects of fatty acids is critical. With oleate we found the optimal molar ratio oleate/bovine serum albumin to be about 1.6 (i.e., 100 μM oleic acid/0.4%, w/v, albumin). The use of more albumin only slightly decreased respiratory rate, whereas less albumin (0.2% with 100 μM oleic acid) resulted in a decrease in respiratory control. For gondoic and erucic acids the optimal ratio was about 3.2 (100 μM fatty acid/0.2% albumin). Omission of albumin resulted in complete loss of respiratory control in presence of oleic acid while some respiratory control was still maintained in presence of gondoic or erucic acids. The use of 0.4% albumin with 100 μM gondoic or erucic acids reduced only slightly the respiratory rate and control.

Even at these high albumin/fatty acid ratios, the amount of free fatty acids had to be kept relatively low. Increasing the concentration of oleic acid to 400 μM (maintaining the same ratio) resulted in a slight decrease in respiratory control, while at higher concentrations the mitochondria were progressively uncoupled. Thus the concentration of fatty acids in the experiments reported did not exceed 300 μM, and the molar ratio of fatty acid to albumin was held at 1.6.

Malate was always present at a concentration of 1 mM. Under optimal conditions of added co-factors, malate increased the respiratory rate about 2—3-fold for the three fatty acids studied.

When neither CoA nor carnitine was added to the incubation, State 3 respiration in presence of malate was stimulated only slightly on addition of either of the three free fatty acids under study (rate increases of 8 to 16 natoms/min per mg protein). Then on addition of CoA and carnitine, respiration was further stimulated. It seems likely that this low rate of fatty acid-stimulated respiration in the absence of added CoA and carnitine is also CoA and carnitine-dependent, since isolated mitochondria contain significant endogenous amounts of these co-factors [17,18]. State 3 respiration in presence of fatty acids and malate was somewhat more rapid in the presence either of added carnitine or CoA than when both were absent.

An evaluation of dependence of respiration due to fatty acids on added carnitine, under otherwise optimal conditions (0.5 mM ADP, 20 μM CoASH present) was carried out. In the absence of added carnitine the State 3 rates of oxidation of oleic, gondoic and erucic acids were low and almost identical (data not shown). The apparent K_m for carnitine stimulation of the oxidation of oleic or gondoic acid in the presence of 20 μM CoA was about 30 μM. Carnitine stimulated the oxidation of the tested fatty acids maximally at a concentration of about 100 μM. Oxidation of oleic or gondoic acid was maximally stimulated to rates greater than three-fold those obtained in the absence of carnitine, whereas oxidation of erucic acid was only slightly elevated.

The effects of coenzyme A on fatty acid oxidation in an otherwise optimized system are shown in Fig. 1. The results are plotted as percent of the maximum respiration attained with oleate as substrate. Respiration due to the oxidation of oleic or gondoic acid was stimulated by added CoA to rates approx. 2.4-fold those obtained in the absence of added CoA, whereas the stimulation

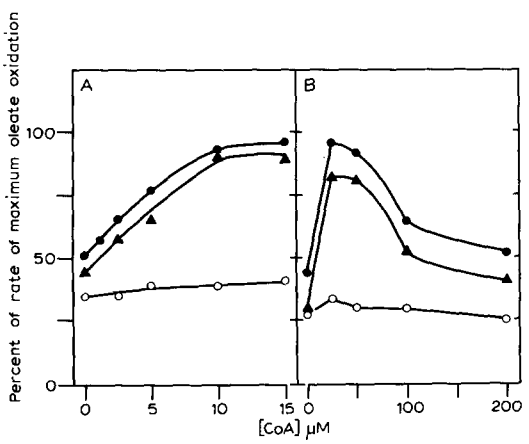

Fig. 1. The effect of [CoA] on respiration due to different monoenoic fatty acids. Mitochondria were incubated in the standard medium under optimum conditions for fatty acid oxidation as described in Materials and Methods, except that the CoA was varied. Respiration was stimulated by addition of 0.5 mM ADP after 1 min preincubation. In A, fatty acids (0.1 mM) and bovine serum albumin were added in a molar ratio 1.6 for oleic acid, and 3.2 for gondoic and erucic acids; in B, the molar ratio was 1.6 throughout. ●———●, oleic; ▲———▲, gondoic and ○———○, erucic acids.

CoA of erucic acid oxidation was very slight (Fig. 1A). Based on values from this figure the apparent K_m of CoA for stimulation of respiration due to oleic or gondoic acid was approx. 4—6 μM. Maximal stimulation of respiration was attained throughout the range of approx. 10 to 25 μM CoA. However, on further elevation of the CoA concentration, respiration was again strikingly diminished (Fig. 1B). There was already an observable inhibition with 50 μM CoA, and at a concentration of 200 μM respiration was reduced to approximately the rate obtained without any added CoA. This inhibitory pattern of CoA was further tested at two levels (0.1 and 1.0 mM) of carnitine. CoA was substantially less inhibitory when the higher carnitine concentration was used (not shown). This result would be expected if accumulation of acyl-CoA is responsible for the observed inhibtion, since a high concentration of carnitine would diminish the concentration of acyl-CoA through the formation of acyl-carnitine.

Effect of CoA on the extramitochondrial ATP/ADP ratio during oxidation of fatty acids

In order to understand the mechanism of inhibition by CoA observed above, we measured steady-state ATP/ADP ratios at different CoA concentrations, while keeping the respiratory rate constant by the presence of limiting amounts of ADP regulated by ATPase addition. In Table I, experiment 1, are presented the ATP/ADP ratios obtained during oleic acid oxidation in the presence of increasing CoA concentrations. In order to keep the same respiratory rate, more ATPase had to be added when the CoA concentration was increased. In these experiments, ADP was limiting for respiration through the use of controlled amounts of ATPase, except for erucic acid. Since the capacity for oxidation of the latter is much less than for oleic acid, the availability of ADP for stimulation of respiration with erucic acid was not rate-limiting. This conclusion is substantiated by the relatively low ATP/ADP ratio obtained under opti-

TABLE I

EFFECT OF VARIED [CoA] ON THE EXTRAMITOCHONDRIAL ATP/ADP RATIO DURING OXIDATION OF DIFFERENT MONOENOIC FATTY ACIDS

Mitochondria were incubated in the standard medium under optimum conditions for fatty acid oxidation as described in Materials and Methods, except that the CoA concentration was varied. Respiration was stimulated in each case close to 75% of State 3 with ATPase. Fatty acids (0.1 mM) and bovine serum albumin were added to a molar ratio of 1.6. The amount of ATPase used to stimulate oleic acid oxidation at 20 and 50 μM CoA, respectively was used to stimulate respiration with gondoic and erucic acids as well. In experiment 1, respiratory rate with oleic acid was 82 ngatoms/mg per min; in experiment 2 the rates were 80, 77 and 40, respectively, for oleic, gondoic and erucic acids.

	Fatty acid	CoA (μM)	ATP/ADP		
Experiment 1	Oleic acid	10	15.5	15.9	15.2
		30	8.2	7.8	8.4
		50	6.6	6.1	5.5
Experiment 2	Oleic acid	20	18.6		
		50	11.8	12.3	
	Gondoic acid	20	18.4	19.2	
		50	11.4	13.4	
	Erucic acid	20	9.4	8.7	
		50	6.4	5.6	

by mal conditions (20 μM CoA) (see also ref. 12). It can be seen that even at 30 μM CoA (respiration due to added ADP is not inhibited at this concentration of CoA), the ATP/ADP ratio was almost 50% reduced. This indicated that the adenine nucleotide translocation was inhibited as the CoA concentration was increased.

Since acyl-CoA is often considered to be a physiological inhibitor of the adenine nucleotide translocase (e.g. ref. 19), we estimated directly the concentration of acyl-CoA with increasing CoA concentrations during oxidation of fatty acids (Table II). As seen in this Table, the amount of membrane-bound acyl-CoA was elevated in each case, corresponding to the amount of added CoA. These elevated acyl-CoA levels thus correlate well with diminution in the steady-state ATP : ADP ratios when ATPase is limiting for ADP-stimulated respiration. It is interesting that the partition of acyl-CoA between the aqueous phase and the mitochondrial membranes was altered, depending on the chain-length of the fatty acid present: with oleic acid, 6% of the total acyl-CoA was membrane-associated, whereas with gondoic and erucic acids, about 9 and 55% of the total acyl-CoA, respectively, was membrane bound. This partitioning of acyl-CoA species is probably related to the increasing hydrophobic characteristics of the acyl-CoA species with increasing chain lengths. However, even though the absolute amount of membrane-associated acyl-CoA increased with increasing chain length, the extent of inhibition of adenine nucleotide translocation (based on the data from Table I) actually decreased with increasing chain length of fatty acid. These data are therefore consistent with the report [20] that longer-chain fatty acyl-CoA species are less potent inhibitors of adenine nucleotide translocase than are esters with 16 and 18 carbons in the acyl moiety.

TABLE II

EFFECT OF VARIED [CoA] ON ACYL-CoA PRODUCTION FROM DIFFERENT MONOENOIC FATTY ACIDS

Mitochondria were incubated in the standard medium under optimum conditions for fatty acid oxidation as described in Materials and Methods, except that the CoA concentration was varied. Respiration in both experiments was stimulated close to 75% of State 3 for oleate with ATPase. Fatty acids (0.1 mM) and bovine serum albumin were added in a molar ratio 1.6. The ratios of membrane-bound to total acyl-CoA were calculated from the mean of experimental values after subtraction of endogenous acyl-CoA, assuming that the latter was constant in all of the experimental conditions.

	Fatty acid	CoA (μM)	Acyl-CoA (nmol/mg)				Ratio, membrane-bound/total (%)
			Membrane-bound		Total		
Experiment 1	Oleic acid	0	0.41	0.40			
		10	0.52	0.48			
		30	0.72	0.60			
		50	1.17	1.07			
Experiment 2	Oleic acid	20	0.68		4.03	4.28	6.7
		50	0.92		7.91	8.32	6.4
	Gondoic acid	20	0.81	0.89	4.49	4.44	10.9
		50	1.05	1.36	8.63	7.37	8.7
	Erucic acid	20	1.80	1.93	2.63	2.85	53.3
		50	2.23	2.02	3.00	3.57	58.5

Studies on the site of inhibition of oleic acid oxidation by coenzyme A when ADP is present in excess

The experiment we have reported on the effects of CoA on ADP-limited oxidation of oleic or gondoic acid clearly localize the site of inhibition at the adenine nucleotide translocase. This is in agreement with conlusions made in an earlier report, in which added acyl-CoA was used to alter the steady-state phosphorylation potential [21]. However, when ADP is present in large excess (low ATP/ADP ratio), it would not be expected that the translocase would become rate limiting for respiration, even if partially inhibited.

In the first instance, the question was asked if the CoA effect under these conditions is a general phenomenon, or if it is specific for fatty acid oxidation. In preliminary experiments, it was found that ADP-stimulated oxidation of substrates other than fatty acids (succinate plus rotenone, glutamate or palmitoylcarnitine plus malate) was only slightly inhibited or unaffected by CoA concentrations up to 200 μM (not shown). Hence, the inhibitory effect of CoA appeared to be specific for fatty acid oxidation. In order to demonstrate the correctness of this conclusion, a series of experiments was carried out measur-

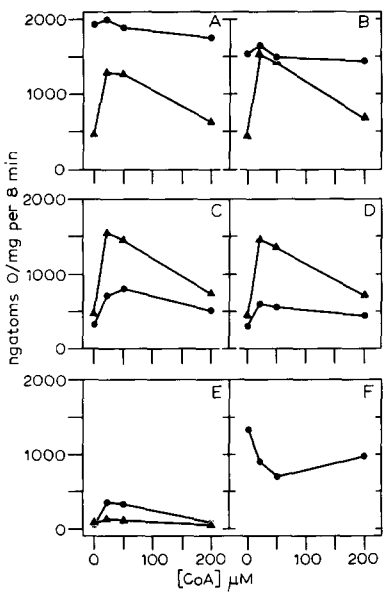

Fig. 2. The effect of [CoA] on metabolites derived from oxidation of oleate, in presence and absence of glutamate. Mitochondria were incubated in a modified standard medium as follows: 60 mM Tris-HCl (pH 7.2), 30 mM KCl, 6 mM $MgCl_2$, 12.5 mM potassium phosphate (pH 7.2), 1 mM EGTA, 2 mM ATP, 1.5 mM malate, 0.2 mM oleic acid, 0.8% bovine serum albumin (molar ratio 1.6), 0.1 mM ADP, 0.1 mM carnitine, and where indicated, 5 mM glutamate. The CoA concentration was varied as stated in figure, and the respiration was stimulated by the addition of 1 unit hexokinase/ml medium + 12.5 mM glucose. Mitochondria (2.3 mg/ml) were treated with fluorocitrate as explained in Materials and Methods. The incubation was started with a mixture of fluorocitrate-treated mitochondria and hexokinase. Incubation volume was 8 ml. Samples were taken after 4 and 8 min of incubation and reactions were terminated by acid precipitation. A. Oxygen consumption measured in oxygraph. B. Calculated total oxygen consumption from metabolic products formed. C. Calculated oxygen consumption due to acetyl production. D. Calculated oxygen consumption due to citrate production. E. Calculated oxygen consumption due to production of ketone bodies. F. Calculated oxygen consumption due to oxidation of glutamate to aspartate. Calculations are described in Materials and Methods. ●———●, oleate + glutamate; ▲———▲, oleate.

ing the respiration rate and balance of metabolic products formed (a) when oleate and malate are the sole substrate and (b) when glutamate was also present as a competing substrate. The results of such an experiment are reported in Fig. 2. As shown (Fig. 2A), the usual curve, first of stimulation of respiration, followed by an inhibition was seen with increasing concentrations of CoA when oleate was substrate, whereas CoA had only marginal effects on ADP-stimulated respiration if glutamate was also present. Calculation of the oxygen consumed from formed products gave a strikingly similar pattern (Fig. 2B). As previously observed [12], glutamate caused acetyl-units to be deflected from citrate production to the formation of ketone bodies (Figs. 2D and E), probably due to a limitation of oxaloacetate resulting from an elevated $NADH/NAD^+$ ratio (e.g. refs. 12, 22). The overall effect of glutamate was a diminution in the rate of total flux of acetyl-units (Fig. 2C). Since respiration was essentially unaltered by CoA when glutamate was present, the site of inhibition of oleate oxidation could not have been at the adenine nucleotide translocase. Furthermore, since oleic acid oxidation was suppressed when no CoA was added or was present in high concentration, it was expected that the oxidation of glutamate would be stimulated under the conditions when oleate oxidation was suppressed. That this was indeed the case is shown in Fig. 2F. That is, the curve of oxygen consumption calculated from products formed from glutamate is roughly the reciprocal to that calculated for oleate oxidation. These results lead inevitably to the conclusion that under these conditions (excess ADP), CoA inhibits oleate oxidation by limiting the availability of intramitochondrial acyl-CoA. Since the fatty acid activation step was not limiting (acyl-CoA accumulated) the results are most readily explained by the conclusion that accumulated acyl-CoA inhibits the carnitine-acyl transferase (E.C. 2.3.1.21) to such an extent that the availability of acyl-carnitine (and ultimately the availability of intramitochondrial acyl-CoA) becomes limiting for oxidation.

Discussion

The possible involvement of free fatty acids and of coenzyme A esters of fatty acids as effectors in the regulation of intracellular metabolism has been the subject of numerous studies (see, e.g., refs. 23—25 for reviews). Depending on the conditions used, relatively high concentrations of fatty acids have been shown to have marked effects on mitochondrial metabolism (e.g., refs. 26—28), but it is now generally held that these are non-specific detergent effects from which the mitochondria must be protected. On the other hand, many studies lend strong evidence that coenzyme A esters of fatty acids may play physiological regulatory roles [29].

The present study was directed in the first instance to evaluate in a systematic manner the optimal conditions required for the coupled oxidation of representative monoenoic long-chain fatty acids, and further to evaluate the effects of alteration of the concentration of components involved in their oxidation (singly or in combination) on the rates of their metabolism and on the steady-state phosphorylation potentials attained in any given respiratory state.

It was first found that a relatively low free fatty acid/serum albumin ratio

was required to prevent subtile deleterious effects of free fatty acids on the mitochondria, as judged by respiratory rates and respiratory control. This minimum required ratio was lower for oleate oxidation than for the gondoic and erucic acids. This result is probably related to the fact that, of the mono-unsaturated fatty acids, the ionophoric properties of free fatty acids increase with increasing chain-length, after which a decrease is observed on further elongation [30]. For present and future studies of distribution of metabolites derived from free fatty acids, it was also desirable to determine the conditions for maximim coupling of mitochondria in the presence of somewhat elevated concentrations of fatty acids. It was found that satisfactory results could be achieved at concentrations up to at least 300 μM of either of the three fatty acids studied, so long as a low ratio of free fatty acid/bovine serum albumin of about 1.6 was maintained.

It was found that State 3 respiration due to oleic acid by tightly-coupled mitochondria was mainly dependent (>80%) on the presence of added CoA and carnitine. Although not shown directly it seems likely that this residual oxidation of long-chain fatty acids is also carnitine- and CoA-dependent, since sub-optimal concentrations of these components are present in the sucrose-space of washed liver mitochondria [17,18]. This conclusion is in agreement with that of Bremer and Norum [31] and van Tol [32] in earlier studies.

The requirements of CoA and carnitine for maximum stimulation of respiration were then carefully evaluated for these mitochondria which retained a satisfactory degree of respiratory control in the presence of fatty acids. It was found that, in otherwise optimized conditions, (—)-carnitine maximally stimulated State 3 respiration due to oleic or gondoic acids at concentrations of about 100 μM, with apparent K_m of about 30 μM. Similarly, added CoA stimulated respiration maximally throughout the range of approx. 10—25 μM CoA, giving an apparent K_m for CoA-stimulated respiration of about 4—6 μM. It should be emphasized, however, that these operational K_m values would probably be altered by changing concentrations of mitochondria relative to those of CoA and carnitine, due to the extensive binding properties of both free fatty acids and their CoA esters to mitochondria.

The concentration of added CoA was found to be very critical. This was especially emphasized when mitochondrial respiration was limited by the rate of recycling of ADP (as it usually is in vivo). As was shown in Tables I and II, 30 or 50 μM CoA substantially diminished the steady-state extramitochondrial ATP/ADP ratio when mitochondria were respiring at 75% of the State 3 rate in the presence of oleate. When, for example, 30 μM CoA was present, this effect was seen when the concentration of membrane-bound acyl-CoA was approx. 0.25 nmol/mg of mitochondrial protein. If one assumes the value of 60 mg of mitochondrial protein per g of liver, this strongly inhibitory value of acyl-CoA represents a concentration of about 15 nmol per g tissue. Although unambigous measurements of the intracellular distribution of acyl-CoA cannot with present methods be done, the concentrations of oleyl-CoA which alter the extramitochondrial ATP/ADP ratio in vitro (present results and ref. 21) are very substantially less than the reported values of the total cellular acyl-CoA [33]. Furthermore, concentrations of CoA required for maximum stimulation of

respiration, and those which begin to have an effect on the steady-state ATP/ADP ratio are indeed very close, and may even overlap. Thus it remains a likely possibility that the level of acyl-CoA plays a regulatory role on the adenine-nucleotide translocase, and therefore on the cytosol ATP/ADP ratio, when respiration is limited by the availability of ADP. It is also noteworthy that the observed decrease in the ATP/ADP ratio by CoA in the presence of gondoic and erucic acids was less extensive as the chain-length was increased, even though the partition to membrane-bound acyl-CoA also increased with increasing chain-length.

We should like to emphasize that these relatively low concentrations of added CoA (up to at least 30 μM) had no inhibitory effect whatsoever on State 3 respiration (excess ADP). However, on increasing the [CoA] in the range of 50—200 μM also increasingly inhibited State 3 respiration due to oleate plus malate. This inhibition was shown to coincide with accumulation of acyl-CoA, and to be specific for fatty acid-dependent respiration. Since long-chain acyl-CoA accumulated under these conditions, it is concluded that the conversion of extramitochondrial acyl-CoA to intramitochondrial acyl-CoA became limiting for fatty acid oxidation. Bremer and Norum [34] studied the reaction kinetics of isolated carnitine palmitoyl-transferase and suggested that accumulated acyl-CoA may limit this enzyme for the formation of intramitochondrial acyl-CoA. More recently, van Tol [32] reported a pattern of respiratory stimulation and then of inhibition as a function of concentration of palmitoyl-CoA in presence of carnitine. However, van Tol interpreted this effect to be due to inhibition of adenine nucleotide translocase. In a recent report, Wood et al. [35] reported a lag period in the State 3 oxidation of palmitoyl-CoA in presence of carnitine, and have arrived at the conclusion that formation of acyl-carnitine was limiting during the lag period for oxidation of added palmitoyl-CoA. In contrast to the present results, however, Wood et al. reported that palmitoyl-CoA also produced significant inhibition of respiration by mitochondria oxidizing glutamate plus malate. Thus, the effect of CoA concentrations in excess of that needed for maximum oleate oxidation in State 3, which we report, is specific for the oxidation of fatty acids.

That the effects of high concentrations of CoA were not due to non-specific detergent effects of the acyl-CoA formed is attested to by the facts that (a) increasing the CoA concentration as high as 200 μM had no effect on State 4 respiration, and (b) respiration of the mitochondria responded in the normal way to ADP when substrates other than fatty acids were also present. It should also be noted that uncoupling effects of free fatty acids have been carefully prevented in these experiments. On the other hand, the inhibitory effects of lower concentrations of CoA (<50 μM) were seen only when the supply of ADP was limiting for respiration (or the ATP/ADP ratio was high), whereas the usual phosphorylation-coupled respiration due to added ADP was observed.

In conclusion, two sites of interaction of added CoA in excess of that required for maximum oxidation of fatty acids are described: (a) when the availability of ADP is limiting for coupled respiration, increasing concentrations of CoA cause a diminution in the steady-state ATP/ADP ratio at any given respiratory rate. This coincides with increasing concentrations of membrane-bound acyl-CoA. The site of this action of CoA is interpreted to be

limitation of the activity of the adenine nucleotide translocase by accumulated acyl-CoA; inhibition of respiration is compensated by an elevated level of ADP; and (b) in the presence of excess ADP, elevated concentrations of CoA cause a limitation to the availability of intramitochondrial acyl-CoA. The accumulated data strongly suggest that the first of these effects is of physiological importance in cellular regulation, whereas the significance of the latter effect will require further study.

Acknowledgements

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