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Effect of Carnitine on Mitochondrial Oxidation of Palmitoylcarnitine

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The effects of carnitine on the metabolism of palmitoylcarnitine were studied by using isolated rat liver mitochondria. Particular attention was given to carnitine acyltransferase-mediated interactions between carnitine and the mitochondrial CoA pool. Carnitine concentrations less than 1.25 mM resulted in an increased production of acetylcarnitine during palmitoylcarnitine oxidation. Despite this shunting of C_2 units to acetylcarnitine formation, no change was observed in the rate of oxygen consumption or major product formation (citrate or acetoacetate). Further, no changes were observed in the mitochondrial content of acetyl-CoA, total acid-soluble CoA or acid-insoluble acyl-CoA. These observations support the concept, based on studies *in vivo*, that the carnitine/acylcarnitine pool is metabolically sluggish and the acyl-group flux low as compared with the CoA/acyl-CoA pool. Acid-insoluble acyl-CoA content was decreased and CoA content increased at carnitine concentrations greater than 1.25 mM. When [^{14}C]carnitine was used in the incubations, it was demonstrated that this resulted from acid-insoluble acylcarnitine formation from intramitochondrial acid-insoluble acyl-CoA mediated by carnitine palmitoyltransferase B. Again, the higher carnitine concentrations resulted in no changes in the rates of oxygen consumption or major product formation. The above effects of carnitine were observed whether citrate or acetoacetate was the major product of oxidation. In contrast, an increase in acetyl-CoA concentration was observed at high carnitine concentrations only when acetoacetate was the product. Since the rate of acetoacetate production was not changed, these higher acetyl-CoA concentrations suggest that a new steady state had been established to maintain acetoacetate-production rates. Since there was no change in acetyl-CoA concentration when citrate was the major product, a change in the activity of the pathway utilizing acetyl-CoA for ketone-body synthesis and the potential regulation of this pathway must be considered.

Carnitine's function in the oxidation of fatty acids by mitochondria is well understood (Bremer, 1962; Fritz & Yue, 1963). However, many recent reports have suggested roles for carnitine beyond that of long-chain fatty acid transport. These functions, such as buffering and protecting the hepatic CoA pool (Pearson & Tubbs, 1967), and formation of acylcarnitines involved in branched-chain amino acid metabolism (Choi *et al.*, 1977; Paul & Adibi, 1978) require carnitine acyltransferases other than carnitine palmitoyltransferase. Further, the carnitine-mediated transfer of acyl groups out of the mitochondria has been demonstrated (Bremer & Wojtczak, 1972; Lopes-Cordozo *et al.*, 1978), but is of unknown physiological significance. Such a shunting of acyl groups (intermediates of oxidative metabolism) from the intramitochondrial CoA pool to the extramitochondrial pool might affect the rates of oxygen consumption and oxidative product formation. The mitochondrial CoA itself has been suggested to have regulatory functions (Lee & Fritz, 1972), and any carnitine-induced changes in the CoA pool could have important metabolic consequences. For these reasons an understanding of the carnitine/CoA interaction is essential in understanding carnitine's full role in metabolism.

Recent studies *in vivo* (Hoppel *et al.*, 1979) and preliminary studies *in vitro* (Brass & Hoppel, 1979) have suggested that the hepatic CoA pool is resistant to carnitine-induced perturbations. The present study examined these questions in greater detail by using isolated rat liver mitochondria. It was found that, despite enhancing acetylcarnitine formation, carnitine did not change the rates of oxygen

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consumption or major product formation (acetoacetate or citrate) during the oxidation of palmitoylcarnitine. Concentrations of carnitine above 1.25 mM did induce changes in the mitochondrial pool, but these did not cause changes in product formation. The results are consistent with the concept that the hepatic carnitine pool cannot cause major changes in the mitochondrial CoA pool or in the mitochondrial metabolism of palmitoylcarnitine.

Experimental

Mitochondrial preparation and incubations

Rats were killed by decapitation and the livers rinsed twice in cold MSM buffer (220 mM-mannitol/70 mM-sucrose/5 mM-4-morpholinepropanesulphonic acid, pH 7.4). The liver was blotted and weighed, after which it was diced and washed twice with MSM buffer. A 10% (w/v) homogenate was then prepared in MSM buffer plus 2 mM-EDTA by using a Potter–Elvehjem homogenizer. Nuclei and cell debris were removed by centrifugation at 400 *g* for 10 min. Mitochondria were isolated by centrifugation at 7000 *g* for 10 min and were washed twice in MSM buffer. Protein concentration in the final preparation was determined by the biuret method (Gornall *et al.*, 1949).

Mitochondrial incubations were carried out in a 1 ml chamber maintained at 30°C. All incubations contained 0.8 ml of a standard incubation mixture (80 mM-KCl / 50 mM-morpholinopropanesulphonic acid/1 mM-EGTA/5 mM-P_i (KH_2PO_4), at pH 7.0) and 0.2 ml of an iso-osmotic neutralized NaCl/(−)-carnitine chloride mixture to give the desired carnitine concentration in the incubation. Mitochondrial protein was added to a final concentration of 2.0 mg/ml after the incubation medium had been allowed to reach temperature. Other compounds when used were added to the following final concentrations: palmitoylcarnitine, 40 μM; malonate, 5 mM; L-malate, 2.5 mM; ADP, 2 mM. Oxygen consumption in the incubations was monitored by using a Clark-type oxygen electrode (Yellow Springs Instruments Co., no. 5331) contained in the incubation chamber.

The above system is designed to permit study of β-oxidation under controlled conditions. Garland *et al.* (1969) suggested the potential of the mitochondrial system *in vitro* when they showed that under appropriate conditions the utilization of acyl groups, the consumption of oxygen and the formation of product were very simply related by stoichiometry. This was developed by DiMarco & Hoppel (1975) into a model system using rat liver mitochondria that permitted accurate study of β-oxidation under capacity conditions. In the presence of ADP and substrate (palmitoyl-CoA + carnitine or palmitoylcarnitine) the oxidative product could be uniquely determined as citrate or acetoacetate by the presence or absence of malate. Rates of substrate utilization, oxygen consumption and product formation were demonstrated to be linear and related by the stoichiometry suggested by Garland *et al.* (1969). Thus the system as used in the present study provides a means to examine directly and quantitatively the process of β-oxidation while perturbing the system in a controlled fashion.

State 3 refers to ADP-stimulated respiration and state 4 to ADP-limited respiration as described by Chance & Williams (1955). Respiratory control ratios and ADP/O ratios were calculated by the method of Estabrook (1967). Significant departure from theory for respiratory control ratio, ADP/O ratio or state-3 rate with glutamate as a substrate indicated mitochondrial damage, and the preparation was not used for further studies.

Assays

Preparation of samples. At the times indicated a sample was removed from the incubation chamber and added to cold 60% (w/v) $HClO_4$ to a final $HClO_4$ concentration of 3%. After centrifugation (12000 *g* for 30 s) the supernatant (representing the acid-soluble fraction) was removed, diluted and neutralized (with 3 M-K_2CO_3 in 0.5 M-triethanolamine) as appropriate for particular assays. The pellet (representing the acid-insoluble fraction) was alkali-hydrolysed and further prepared as indicated in the individual assays. The total amount of CoA present per mg of mitochondrial protein varied from preparation to preparation (from 2.5 to 3.5 nmol/mg). For this reason statistical analysis was limited to experiments performed on the same day with the same preparation (and all means ± S.D. reflect this), and all Tables (except Table 6) were obtained from experiments with a single preparation. Where appropriate, the reproducibility of patterns of change are shown in the text on a percentage basis.

Recycling assay for CoA. The fluorimetric CoA assay described by Allred & Guy (1969) provides great sensitivity because the rate of NADH production over several min is used, rather than its stoichiometric production. It is important to note that the recycling CoA assay does not distinguish between CoA, oxidized CoA and acetyl-CoA, and therefore lacks specificity. For this reason the use of the recycling assay was limited to situations where total CoA measurements were desired, i.e. sum of CoA and acetyl-CoA, and for acyl-CoAs after hydrolysis. The assay was conducted by using neutralized $HClO_4$ extracts (to provide a measure of the sum of CoA and acetyl-CoA), on neutralized $HClO_4$ extracts after alkali hydrolysis (a measure of total acid-soluble CoA) and on the $HClO_4$-insoluble

pellet after alkali hydrolysis (as a measure of acid-insoluble acyl-CoA).

Endpoint assays of CoA, acetylcarnitine and acetyl-CoA. Acetylcarnitine and acetyl-CoA were directly determined by a method similar to those of Pearson & Tubbs (1967) and Decker (1974) modified as previously described (Hoppel *et al.*, 1979). This assay cannot measure acetylcarnitine in the presence of large amounts of carnitine. By using acetylcarnitine standards to which carnitine was added, it was demonstrated that the assay was not accurate in the presence of greater than 2.5mM-carnitine (results not shown). For this reason no acetylcarnitine values are reported at concentrations of carnitine above this value. CoA was determined by using the α-oxoglutarate dehydrogenase method (Tubbs & Garland, 1969). CoA concentrations in standard solution were determined by the endpoint-u.v.-absorbance method (Michal & Bergmeyer, 1974).

Other assays. β-Hydroxybutyrate (Olsen, 1971), acetoacetate (Olsen, 1971), citrate (Williamson & Corkey, 1969) and acid-soluble acylcarnitine (Brass & Hoppel, 1978) were measured as previously described.

Reagents

Chemicals and solvents used were of reagent grade. Malate dehydrogenase, citrate synthase, phosphotransacetylase and carnitine acetyltransferase were purchased from Boehringer Mannheim (Indianapolis, IN, U.S.A.). β-Hydroxybutyrate dehydrogenase was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). α-Oxoglutarate dehydrogenase was prepared from pig heart by the method of Sanadi (1969). (−)-Carnitine chloride was a gift from Otsuka Pharmaceuticals, Osaka, Japan. (−)-[*Me*-^{14}C]Carnitine chloride was prepared in two steps. *N*-Demethylation of (−)-carnitine chloride was performed by a modification of the procedure of Stokke & Bremer (1970) (S. Ingalls & C. L. Hoppel, unpublished work). The subsequent methylation of the product, 4-(*NN*-dimethylamino)-3-hydroxybutanoate, was performed as described previously (Mazzetti & Lemmon, 1957) by using [^{14}C]methyl iodide (New England Nuclear Corp., Boston, MA, U.S.A.).

Results

The state-3 incubation conditions used in this study represent optimal conditions and have been extensively examined previously (DiMarco, 1973; DiMarco & Hoppel, 1975). Thus it has been demonstrated that during the linear phase of oxygen consumption the production of the major oxidation product was also linear with time. The disappearance of palmitoylcarnitine (or other lipid substrate) could be quantitatively accounted for by the appearance of acetoacetate or citrate. Whether citrate or acetoacetate was produced under these conditions depends only on whether malate was included in the incubation to provide a source of oxaloacetate, and under no circumstances was significant CO_2 produced (less than 1% of acyl-group carbon). The demonstration of these important rate relationships was repeated for the present study with and without carnitine in the incubation (results not shown), and results were obtained qualitatively identical with those seen previously (DiMarco, 1973). Thus the data on rates of oxygen consumption and product formation are a direct representation of β-oxidation.

Table 1 shows the effect of carnitine on the state-3 oxidation of palmitoylcarnitine in the presence of malonate (therefore acetoacetate will be the major product of oxidation). No changes were observed in the rate of oxygen consumption or rate of acetoacetate formation in the presence of carnitine. The mitochondrial CoA pool from a representative experiment under these conditions is shown in Table 2. Addition of carnitine up to 1.25mM caused no change in acetyl-CoA content. Exceeding this carnitine concentration resulted in a statistically significant increase in acetyl-CoA content (acetyl-CoA content of 1.15 ± 0.20, 1.31 ± 0.30, 1.80 ± 0.20 and 1.78 ± 0.04 nmol/mg of protein at 0, 1.25, 10

Table 1. *Effect of carnitine on oxygen consumption and acetoacetate production during the state-3 oxidation of palmitoylcarnitine in the presence of malonate*

Incubation conditions and methods are those described in the Experimental section. At 60s after palmitoylcarnitine addition, samples of the incubation mixture were assayed for acetoacetate. Oxygen consumption was continuously monitored during the incubation. No significant changes (Student's unpaired *t* test) were observed in acetoacetate production or oxygen consumption ($P > 0.05$). Values are means ± S.D.

Carnitine (mM)	*n*	Acetoacetate (nmol/min per mg of protein)	Oxygen consumption (ng-atoms/min per mg of protein)
0.00	4	16.7 ± 3.8	86 ± 10
1.25	3	17.4 ± 2.0	91 ± 15
10.0	3	18.2 ± 0.9	89 ± 1
30.0	3	15.6 ± 1.6	76 ± 9

Table 2. *Effect of carnitine on the mitochondrial CoA pool during state-3 oxidation of palmitoylcarnitine in the presence of malonate*

Incubation conditions and methods are those described in the Experimental section. At 60s after the addition of palmitoylcarnitine a sample of the incubation mixture was removed and assayed for CoA, total acid-soluble CoA, acetyl-CoA, acid-insoluble CoA and total CoA.

	Content (nmol/mg of mitochondrial protein)				
Carnitine (mM)	CoA	Total acid-soluble CoA	Acetyl-CoA	Acid-insoluble CoA	Total CoA
0.00	0.00	1.36	0.90	1.31	2.67
0.05	0.02	1.48	0.91	1.29	2.77
0.50	0.07	1.45	1.02	1.23	2.68
1.25	0.13	1.54	1.09	1.13	2.67
5.00	0.18	1.90	1.28	0.89	2.79
10.0	0.18	1.87	1.40	0.87	2.74
30.0	0.31	1.96	1.21	0.84	2.80

Table 3. *Effect of carnitine on oxygen consumption and citrate production during the state-3 oxidation of palmitoylcarnitine in the presence of malate*

Incubation conditions and methods are those described in the Experimental section. At 60s after palmitoylcarnitine addition, samples of the incubation mixture were taken and assayed for citrate. Oxygen consumption was continuously monitored during the incubation. No significant changes (Student's unpaired t test) were observed in citrate production or oxygen consumption ($P > 0.05$), except for an increase in citrate production in the presence of 30mM-carnitine ($P < 0.05$). Values are means ± S.D.

Carnitine (mM)	n	Citrate (nmol/min per mg of protein)	Oxygen consumption (ng-atoms/min per mg of protein)
0.00	4	18.3 ± 1.8	97 ± 16
1.25	3	16.3 ± 2.3	100 ± 6
10.0	3	17.2 ± 1.9	103 ± 10
30.0	3	23.0 ± 0.4	103 ± 4

and 30mM-carnitine respectively, with $n = 3$; $P < 0.05$ at 10 and 30mM. These data were obtained on a separate day from those in Table 2). At these same high carnitine concentrations the acid-insoluble acyl-CoA content was decreased (to 56 ± 11% of original values, based on five different mitochondrial preparations). This decrease in acid-insoluble acyl-CoA was associated with an increase in total acid-soluble CoA (to 162 ± 45% of original values, based on four different mitochondrial preparations). The increase in total acid-soluble CoA was made up of increases in acetyl-CoA and CoA. These relationships demonstrate that major changes occurred in the variables shown as the carnitine concentration increased beyond 1.25mM. These changes in the CoA pool were not associated with changes in oxygen consumption or acetoacetate formation (Table 1).

Carnitine's effect on the oxidation of palmitoylcarnitine in the presence of malate (so that the major product will be citrate) provides a metabolic state that contrasts with the production of ketone bodies when malonate is present. As Table 3 shows, oxygen consumption was not changed by the addition of carnitine during oxidation of palmitoylcarnitine/malate. Citrate production remained constant at low concentrations of carnitine, but was significantly increased at 30mM-carnitine. This increase in acetyl-CoA seen when malonate was present was not seen in the presence of malate (Table 4). Even though the changes in acetyl-CoA were not seen, acid-insoluble acyl-CoA content decreased (to 66 ± 17% of original values, based on six different mitochondrial preparations).

The action of carnitine during state-4 oxidation was also examined, since the CoA pool is then in a new steady state because of the low activity of the respiratory chain. During state-4 oxidation, β-hydroxybutyrate was the exclusive oxidative product, and its appearance was linear with time (results not shown). Table 5 shows that the pattern of changes caused by carnitine during state-4 oxidation of palmitoylcarnitine in the presence of malonate was similar to that observed during state-3 oxidation.

Table 4. *Effect of carnitine on the mitochondrial CoA pool during state-3 oxidation of palmitoylcarnitine in the presence of malate*

Incubation conditions and methods are those described in the Experimental section. At 60s after the addition of palmitoylcarnitine, samples were taken for assay of acetyl-CoA, total acid-soluble CoA, acid-insoluble acyl-CoA and total CoA.

	Content (nmol/mg of mitochondrial protein)			
Carnitine (mM)	Total acid-soluble CoA	Acetyl-CoA	Acid-insoluble acyl-CoA	Total CoA
0.00	1.15	0.49	1.55	2.70
0.005	—	0.47	—	—
0.05	1.14	0.44	1.62	2.76
0.50	1.41	0.44	1.48	2.89
1.25	1.45	0.47	1.66	3.11
5.00	1.69	0.31	1.41	3.10
10.0	1.76	0.47	1.31	3.07
30.0	1.86	0.49	1.12	2.98

Table 5. *Effect of carnitine on mitochondrial state-4 oxidation of palmitoylcarnitine in the presence of malonate*

Incubation conditions and analysis are identical with those used in Tables 1 and 2, except that no ADP was added to the incubation. Samples were taken from the incubation 5 min after the addition of palmitoylcarnitine.

	Content (nmol/mg of mitochondrial protein)				
Carnitine (mM)	Total acid-soluble CoA	Acetyl-CoA	Acid-insoluble acyl-CoA	Total CoA	β-Hydroxybutyrate formed
0.00	0.92	0.31	1.72	2.64	41.0
0.005	0.94	0.36	1.74	2.68	51.0
0.05	0.92	0.31	1.71	2.63	39.7
0.50	1.41	0.44	1.60	3.01	42.8
1.25	1.62	0.49	1.46	3.08	41.6
5.0	2.03	0.74	1.35	3.38	43.9
10.0	2.10	0.78	1.14	3.24	33.8

Table 6. *Effect of carnitine on acetylcarnitine production*

Incubation conditions and methods are those described in the Experimental section. At the times indicated after the addition of palmitoylcarnitine, a sample of the incubation mixture was removed and assayed for acetylcarnitine.

	Acetylcarnitine (nmol/mg of mitochondrial protein)		
	State 3 (sample at 60s)		State 4 (sample at 5 min)
Carnitine (mM)	With malate	With malonate	With malonate
0.00	0.53	0.35	1.75
0.005	0.38	—	1.94
0.05	0.68	0.53	2.42
0.5	1.32	0.64	4.72
1.25	1.44	0.55	4.44

Acetyl-CoA concentrations were dramatically increased by high carnitine concentrations and acid-insoluble acyl-CoA content was decreased. Again, product formation (β-hydroxybutyrate) was not altered by carnitine addition, except at 10mM-carnitine, when a small decrease was observed.

Acetylcarnitine production from the experiments presented in Tables 2, 4 and 5 is shown in Table 6. It is clear that acetylcarnitine could be produced as a product of palmitoylcarnitine oxidation, demonstrating an interaction between the added carnitine and the intramitochondrial CoA pool. Addition of

carnitine up to 1.25mM increased the amount of acetylcarnitine formed, without, as shown in the earlier Tables, changing acetyl-CoA content, major product formation or oxygen consumption.

Formation of acid-soluble acylcarnitines from mitochondrial oxidation products was also demonstrated by assay of carnitine releasable by alkaline hydrolysis. The production of acid-soluble acylcarnitine during state-4 oxidation of palmitoylcarnitine was linear with time in the presence of malonate with a rate of 1.02nmol of acid-soluble acylcarnitine/min per mg of mitochondrial protein. During the 10min incubation more than 10nmol of acid-soluble acylcarnitines was produced/mg of mitochondrial protein. The initial carnitine concentration was only 5μM, but the carnitine pool was replenished as palmitoylcarnitine was utilized. At the end of the 10min incubation the total acid-soluble carnitine concentration was 43μM and had increased progressively during the incubation. Thus the carnitine released during the oxidation of palmitoylcarnitine was available for acid-soluble acylcarnitine formation.

The carnitine-induced decreases in acid-insoluble acyl-CoA content observed may have resulted from an increased flux of acyl-CoA out of the mitochondria through carnitine palmitoyltransferase B. Results suggesting that such efflux of acyl groups can occur have been reported (Bremer & Wojtczak, 1972; Lopes-Cordozo *et al.*, 1978). The decrease in acid-insoluble acyl-CoA content might also result from an inhibition of carnitine palmitoyltransferase B by carnitine. These possibilities were examined by including [^{14}C]carnitine in the incubation. Sodium tetrathionate (4mM) was included in the incubation to remove any extramitochondrial CoA. This concentration was shown to be effective by Skrede & Bremer (1970), and was used to prevent any palmitoylcarnitine formation through carnitine palmitoyltransferase A. The presence of sodium tetrathionate did not alter respiratory-control ratios, P/O ratios or state-3 oxidation rates (results not shown). Table 7 shows that, during oxidation of palmitoylcarnitine in the presence of malonate, [^{14}C]carnitine was incorporated into the acid-insoluble fraction, presumably as acid-insoluble acylcarnitines. As Table 7 demonstrates, acid-insoluble acyl[^{14}C]carnitines were formed even in the presence of 10mM-carnitine. From the initial specific radioactivity of the [^{14}C]carnitine added to the incubation, an estimate of the amount of acylcarnitine produced could be made. These calculations showed that much larger amounts of acid-insoluble acyl[^{14}C]carnitine were produced from intramitochondrial acyl-CoA in the presence of 10mM-carnitine than when 0.1mM-carnitine was present. These results demonstrate that in intact mitochondria carnitine palmitoyltransferase B can operate in what is normally considered the reverse direction. This acylcarnitine formation occurs at much higher rates with high extramitochondrial carnitine concentrations (up to 10mM). These results are consistent with formation of acylcarnitine from mitochondrial acyl-CoA being responsible for the decreased acid-insoluble acyl-CoA concentrations observed, rather than inhibition of carnitine palmitoyltransferase B by carnitine.

Discussion

The results reported here demonstrate that carnitine can interact with the mitochondrial CoA pool during oxidative metabolism. These interactions include the transfer of acyl group from intramitochondrial acyl-CoA (acid-soluble and acid-insoluble) to extramitochondrial carnitine. When the carnitine concentration exceeded 1.25mM this transfer of acyl groups was capable of causing concentration changes in the intramitochondrial CoA pool. Additional changes in the CoA pool, such as the increase in acetyl-CoA concentration, were observed that could not be explained by the simple transfer of acyl groups. However, these carnitine-induced changes did not result in corresponding changes in the major products or rate of oxidation.

The demonstration of carnitine-induced transfer of acetyl groups from the intramitochondrial acetyl-

Table 7. *Incorporation of [^{14}C]carnitine into acid-insoluble acylcarnitines during the state-3 oxidation of palmitoylcarnitine in the presence of malonate*

Incubation conditions are those described in the Experimental section. [^{14}C]Carnitine was present in the incubation, and at 60s after the addition of palmitoylcarnitine a sample was removed and radioactivity in the acid-insoluble fraction determined. The nmol of acid-insoluble acyl[^{14}C]carnitine produced was calculated on the basis of net acid-insoluble radioactivity and initial carnitine specific radioactivity.

Carnitine (mM)	Carnitine (c.p.m./nmol)	Acid-insoluble radioactivity (c.p.m. sample − blank)	Acid-insoluble acyl[^{14}C]carnitine (nmol)
0.007	116000	1331	0.0115
0.10	7840	2017	0.257
10.0	81.6	928	11.4

CoA pool to form acetylcarnitine was not surprising, on the basis of previous work (Pearson & Tubbs, 1967). However, several aspects of acetylcarnitine formation relate to the function of the acid-soluble acylcarnitine pool. Even with the rates of acetylcarnitine production observed with 1.25mM-carnitine (a value probably approximating to the maximal cytosolic concentrations *in vivo*), less than 5% of acetyl groups generated appear as acetylcarnitine (Tables 1 and 6). Thus it is not surprising that production of acetylcarnitine, even in large amounts on the scale of the carnitine pool, results in no change in the rate of major product formation. It is unclear what limited acetylcarnitine production, but carnitine acetyltransferase is present in relatively low activity in liver mitochondria and requires high acetyl-CoA concentrations for maximal activity.

This observed inability of acetylcarnitine production significantly to alter the major oxidative pathways is in agreement with observations made *in vivo* after carnitine administration (Hoppel *et al.*, 1979). In those studies *in vivo* dramatic changes in hepatic acylcarnitine content were seen after injection of carnitine into rats without corresponding changes in the hepatic CoA pool or apparent rates of ketone-body formation. As shown in the present experiments, liver mitochondria *in vitro* were able to produce approx. 10nmol of acid-soluble acylcarnitines/mg of mitochondrial protein in 10min. Since 1g of liver contains approx. 60mg of mitochondrial protein, this corresponds to a production rate of 600nmol of acid-soluble acylcarnitines/10min per g of liver. This production rate is sufficient to account for the increased hepatic acid-soluble acylcarnitine content (300–600nmol/10min per g of liver) observed after carnitine administration *in vivo* (Hoppel *et al.*, 1979).

Addition of carnitine to mitochondrial incubations resulted in the production of extramitochondrial acid-insoluble acylcarnitine from intramitochondrial acid-insoluble acyl-CoA with the reaction mediated by carnitine palmitoyltransferase B. At high carnitine concentrations (above 1.25mM), the rate of this reaction was sufficiently fast to lower the steady-state concentrations of intramitochondrial acid-insoluble acyl-CoAs. Despite this, oxidation rates were not decreased. This provides further evidence that the availability of acyl-CoA to the enzymes of β-oxidation cannot be rate-limiting for fatty acid oxidation in isolated mitochondria. It should be noted that the high concentrations of carnitine used to induce changes in the mitochondrial CoA pool are well above those that could be expected in liver *in vivo*. However, this does not negate the use of carnitine as a probe to study mitochondrial oxidation *in vitro*.

Several possible mechanisms exist for the increased acetyl-CoA concentrations seen after carnitine addition. Since the acetyl-CoA concentration increased without a change in oxidation rates, either product formation is not dependent on acetyl-CoA concentrations (as suggested by Lee & Fritz, 1972), or the higher acetyl-CoA concentrations represent the establishment of a new steady state to maintain the rate of product formation. This possibility is particularly intriguing, since the increased acetyl-CoA concentrations were not seen when citrate was the product, and this suggests regulation of the pathway synthesizing acetoacetate from acetyl-CoA. For example, if acid-insoluble acyl-CoAs are positive effectors for the enzymes of ketone-body synthesis, the lowering of the acid-insoluble acyl-CoA concentration would result in a decreased activity of the ketone-body-synthesis pathway. Acetyl-CoA would then build up until a new steady state was reached, and product formation could again match the rate of acetyl-CoA production from β-oxidation. Although acetyl-CoA concentrations might increase simply by mass action after the liberation of CoA from the acid-insoluble acyl-CoAs, this would also be expected to occur when malate was present and citrate was the product. An additional possibility for the differential response when acetoacetate and citrate were the products is that at least two distinct pools of intramitochondrial acetyl-CoA exist which might interact differently during metabolism.

The results presented here support the concept that the hepatic carnitine/acylcarnitine pool has a limited capacity to perturb mitochondrial oxidation. However, consistent with observations *in vivo*, the interaction between carnitine and the CoA pool is of fundamental importance in determining the status of the acylcarnitine pool. Additional information about the role of the carnitine/CoA relationship will be obtained when the specific acyl moieties of the acylcarnitine can be readily measured. In this way the role of unusual acylcarnitines can be appreciated as well as increasing the utility of carnitine as a probe of the mitochondrial CoA pool. The potential usefulness of such a probe is demonstrated by the carnitine-induced changes during mitochondrial oxidation and the question raised by the consequences of these changes.

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