Quantitation of the Efflux of Acylcarnitines from Rat Heart, Brain, and Liver Mitochondria*

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The efflux of individual short-chain and medium-chain acylcarnitines from rat liver, heart, and brain mitochondria metabolizing several substrates has been measured. The acylcarnitine efflux profiles depend on the substrate, the source of mitochondria, and the incubation conditions. The largest amount of any acylcarnitine effluxing per mg of protein was acetyl carnitine produced by heart mitochondria from pyruvate. This efflux of acetyl carnitine from heart mitochondria is almost 5 times greater with 1 mM than 0.2 mM carnitine. Apparently the acetyl-CoA generated from pyruvate by pyruvate dehydrogenase is very accessible to carnitine acetyltransferase. Very little acetyl carnitine effluxes from heart mitochondria when octanoate is the substrate except in the presence of malonate. Acetyl carnitine production from some substrates peaks and then declines, indicating uptake and utilization. The unequivocal demonstration that considerable amounts of propionylcarnitine or isobutyrylcarnitine efflux from heart mitochondria metabolizing α-ketoisovalerate and α-keto-β-methylvalerate provides evidence for a role (via removal of non-metabolizable propionyl-CoA or slowly metabolizable acyl-CoAs) for carnitine in tissues which have limited capacity to metabolize propionyl-CoA. These results also show propionyl-CoA must be formed during the metabolism of α-ketoisovalerate and that extra-mitochondrial free carnitine rapidly interacts with matrix short-chain aliphatic acyl-CoA generated from α-keto acids of branched-chain amino acids and pyruvate in the presence and absence of malate.

Several studies suggest that carnitine may have roles in intermediary metabolism besides its well established role in the β-oxidation of long-chain fatty acids (1-4). Early reports particularly by Bremer and colleagues indicate that in addition to acetylcarnitine, mitochondria have the capacity to form propionylcarnitine (5) as well as branched-chain acylcarnitines (6). These data as well as results from our laboratory (2, 7) showing that heart and liver contain a spectrum of short-chain acylcarnitines, including branched-chain ones, indicate carnitine is involved in the metabolism of the branched-chain α-keto acids derived from branched-chain amino acids. However, unequivocal evidence demonstrating a specific role for carnitine in the metabolism of these amino acids is still not available (8-10).

Three forms of carnitine deficiency have been described in humans: myopathic, systemic, and mixed; they can involve liver, skeletal muscle, heart, and kidney (11-14). Most investigators assume that the abnormal metabolism associated with carnitine deficiency is due to impaired β-oxidation of long-chain fatty acids. However, recent evidence with systemic carnitine deficiency suggests a problem with a short-chain acyl-CoA metabolism (15-18) even though in some cases ketogenesis can be normal (18). The impaired metabolism may be related to sequestration of a significant portion of the total intramitochondrial coenzyme A pool causing a partial inhibition of CoASH-dependent processes. This dual role for carnitine in intermediary metabolism, one primary and the other secondary, is similar to the obligatory role and the facilitative role proposed previously (2).

As part of our investigation of the involvement of carnitine in short-chain fatty acid metabolism, we measured the production and efflux of individual acylcarnitines from rat brain, liver, and heart mitochondria using a variety of substrates.

MATERIALS AND METHODS

Chemicals—L-[methyl-3H]Carnitine, (8 Ci/mmol) and 2-[14C]pyruvate (13.9 mCi/mmol) were obtained from Amersham (Amersham, United Kingdom). Acyl-CoA derivatives were obtained from Pharmacia P-L Biochemicals (Piscataway, NJ). l-Butanesulfonic acid, sodium salt, was obtained from Aldrich. Carnitine acetyltransferase (E.C. 2.3.1.7) was from Boehringer Mannheim (Mannheim, Federal Republic of Germany). Methanol and scintillation mixture, ScintiVerse TM-LC, were from Fisher. All other chemicals were from Sigma. L-Carnitine was a gift from Sigma Tau (Rome, Italy). The sample of tritiated L-carnitine used for these experiments contained an impurity, approximately 8.5% of the total triutium, which was metabolically inert for all of the systems tested. The impurity was not removed for these experiments. For calculations the amount of impurity in the sample was subtracted from the total radioactivity to give the disintegrations/min in L-carnitine.

Animals and Preparation of Mitochondria—White male Sprague-Dawley rats weighing 160-200 g were used. Animals were stunned before decapitation. Liver mitochondria were prepared according to Johnson and Lardy (19) from rats fasted for 24 h. The average respiratory control ratio of mitochondria with succinate was 4.4 and with 2-ketoglutarate about 7. Rat brain mitochondria were obtained from subcellular fractionation of cerebrum using the method of Both and Clark (20). Their respiratory control ratio was 3-4 for pyruvate plus malate. Heart mitochondria from fed rats were isolated with collagenase (Sigma Type VII, lot 33F-619) in 125 mM KCl, 25 mM sucrose, and 10 mM Hepes, pH 7.4, as previously described (21), with the following modifications. Minced myocardium was homogenized with a Potter-Elvejem pestle in the presence of 500 IU collagenase. The crude homogenate was allowed to incubate on ice for 5 min. Two strokes of the pestle were applied every 1.5 min to facilitate disruption of tissue. Mitochondria lost in the crude pelleted material during the first low speed spin were not recovered. Isolated mitochondria were

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1The abbreviations used are: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HFLC, high performance liquid chromatography.
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RESULTS

HPLC Profiles for Different Substrates and Different Mitochondria—Fig. 1 shows representative HPLC profiles for 5- and 15-min incubations of heart, liver, and brain mitochondria metabolizing a variety of substrates during state 3 respiration. These profiles were selected to demonstrate specific points rather than represent all of the experiments performed.

Considerable amounts of acetyl carnitine effluxed from liver, heart, and brain mitochondria when pyruvate was the substrate (see Fig. 1, A and E for heart, D and H for brain, and I and M for liver mitochondria). The \( \alpha \)-keto acid derived from isoleucine (\( \alpha \)-keto-\( \beta \)-methylvalerate) gave a different acylcarnitine efflux profile for heart mitochondria compared to liver. With heart, propionylcarnitine and small amounts of isobutyrylcarnitine were found while liver mitochondria produced primarily propionylcarnitine and acetylcarnitine; much smaller amounts of the 5-carbon acylcarnitine were produced by either mitochondria (compare Fig. 1, B, F, J, and N). After 15 min most of the initial 0.2 mM free carnitine was converted to short-chain acylcarnitines by liver mitochondria (see Fig. 1, J and N).

Octanoate was used as a substrate for \( \beta \)-oxidation because of its solubility and because it is readily oxidized by both heart and liver mitochondria. Fig. 1, C and G, show that very little acetyl carnitine effluxed from heart mitochondria metabolizing octanoate, although the respiration rate was 101 ng atoms of O/min/mg protein (0.1 µmol of O/min/mg protein) in the presence of 0.2 mM carnitine and 1.0 mM malate (data not shown). In contrast, liver mitochondria (Fig. 1, K and O) produce considerable acetyl carnitine and after 15 min more than half of the free carnitine is converted to acetylcarnitine.

The state 3 respiration rate of liver mitochondria for octanoate in the presence of 0.2 mM L-carnitine and 1.0 mM malate was 84 ng atoms of O/min/mg protein and 71 ng atoms of O/min/mg protein in the absence of malate (data not shown).

When malate, which can provide an oxidation product that acts as a sink for acyl groups, is added to liver mitochondria, a major shift in the acylcarnitine profile is observed with efflux of not only acetylcarnitine, but of butyryl-, hexanoyl-, and octanoylcarnitine as well (Fig. 1, L and P and Fig. 2B). Data similar to those shown in Fig. 1 were used for the time courses shown in Figs. 2 and 3.

Time Course for Acylcarnitine Efflux—The time courses for the efflux of individual acylcarnitines from heart and liver mitochondria metabolizing different substrates in the presence and absence of malate are given in Figs. 2 and 3. With \( \alpha \)-keto-\( \beta \)-methylvalerate, malate completely prevented the efflux of acetyl carnitine from heart mitochondria, but stimulated efflux of propionylcarnitine from both heart and liver mitochondria (Fig. 2A). When \( \alpha \)-ketoisovalerate (the \( \alpha \)-keto analog of valine) was substrate, malate had little effect on production of either propionylcarnitine or isobutyrylcarnitine in heart or liver mitochondria (Fig. 2C). Malate also had little effect on production of isovaleryl carnitine when \( \alpha \)-ketoisocaproate was a substrate (Fig. 2D). With both brain and heart mitochondria, malate prevented the accumulation of acetyl carnitine with acetoadetate as substrate (Fig. 2E).

Fig. 3 shows the production of acetyl carnitine by heart and liver mitochondria metabolizing pyruvate in the presence and absence of malate. The largest efflux of any acylcarnitine was acetyl carnitine from heart mitochondria oxidizing pyruvate. Increasing the carnitine concentration from 0.2 to 1 mM (Fig. 34) increased the efflux of acetyl carnitine almost 5-fold. Malate caused a substantial reduction in efflux of acetyl carnitine. The large efflux of acetyl carnitine from heart mitochondria indicated that much of the acetyl-CoA produced in the matrix of mitochondria via pyruvate dehydrogenase was initially converted to acetyl carnitine rather than citrate. To confirm this, experiments were performed using \( \text{\textsuperscript{14}C} \)pyruvic acid in the presence of unlabeled carnitine and malate.

The results of this experiment are shown in Fig. 4. It demonstrates that initially, much more of the radioactivity from \( \text{\textsuperscript{14}C} \)pyruvate is converted to acetyl carnitine than to CO\(_2\). The amount of \( \text{\textsuperscript{14}CO}_2 \) should give a measure of acetyl-CoA fluxing through the Kreb's cycle. Fig. 4 shows that \( \text{\textsuperscript{14}CO}_2 \) increases with time, while the acetyl carnitine increases and then decreases.

DISCUSSION

The data presented in Figs. 1–4 show that in the presence of carnitine large amounts of specific acylcarnitines exit mitochondria. This efflux is substrate-dependent, tissue-dependent, and dependent on the availability of alternative acyl-CoA utilizing reactions. For example, the lowering of acetyl carnitine production by malate is consistent with a competition for acetyl-CoA by citrate synthase in the matrix of mitochondria. Other studies have shown that the combination of mitochondrial carnitine acyltransferase with the medium-chain activity of carnitine palmitoyltransferase converts the acyl-CoAs to question to acylcarnitines (25, 26). If one assumes the carnitines are produced by carnitine acyltransferases using the respective acyl-CoA derivatives in the matrix of mitochondria, then the efflux of acetyl-, propionyl-, isobutyryl-, and isovaleryl carnitines are in accord with known
Fig. 1. HPLC profiles of effluxed acylcarnitines produced from different substrates by heart and liver mitochondria. Mitochondria were incubated as described under "Materials and Methods" for the times indicated and the acylcarnitines present in the incubation media resolved by HPLC. Abbreviations are: PYR, pyruvate; OCT, octanoate; KMV, α-keto-β-methylvalerate; MAL, l-malate; C-2, acetylcarnitine; C-3, propionylcarnitine; C-4, butyrylcarnitine; C-5, hexanoylcarnitine; C-6, octanoylcarnitine; I-C-5, isovaleryl-β-methylbutyryl, or unsaturated C-5 acylcarnitine; "Free" represents free carnitine. Substrate concentrations for α-keto-β-methylvalerate, α-ketoisovalerate, and α-ketoisocaproate were 1 mM. OCT and PYR were 2.5 mM for heart and liver mitochondria. For brain mitochondria pyruvate and acetoacetic acid concentrations were 5.0 mM. With octanoate, malate was 0.2 mM (panels L and P).
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Fig. 2. Time course for production of specific acylcarnitines by mitochondria. The abbreviations and incubation conditions are identical to those for Fig. 1. The closed symbols represent experiments performed in the absence of malate, and the open symbols represent experiments performed in the presence of 0.2 mM malate. Malate, 5 mM when brain mitochondria were used (panel E). All experiments were performed in the presence of 0.2 mM L-[methyl-3H]carnitine. KIC, α-ketoisocaproate; ACAC, acetoacetic acid; IC-4, isobutyryl and isobutenyl-carnitine.

metabolic pathways for the substrates used. In each instance the acyl-CoAs would have been produced by the known metabolic pathways for branched-chain amino acid metabolism, fatty acid oxidation, pyruvate metabolism, and acetoacetate metabolism. The one possible exception is α-ketoisovalerate, the keto acid of valine. The metabolism of this keto acid has been the subject of some debate. Our data provide strong support for the pathway previously demonstrated by Baretz and Tanaka (27) involving propionyl-CoA as an obligate intermediate.

The rate of efflux of acetylcarnitine shown herein is greater than what would be predicted from carnitine/acylcarnitine exchange rates reported by others (28, 29). However, our experimental conditions were not the same as those in which mitochondria were loaded with free carnitine or acetylcarnitine and exchange rates measured. Rather for our experiments free carnitine most likely is exchanged for newly synthesized acetyl- or other acylcarnitines during state 3 respiration. Our data show that during vigorous oxidation the carnitine/acylcarnitine translocase activity is much greater than that which occurs when exchange rates are measured. This is in accord with some recent data of Murthy and Pande who reported propionyl-CoA as an obligate intermediate.

The much larger efflux of isobutyrylcarnitine from heart mitochondria during α-ketoisovalerate oxidation (3.2 versus 1.0 nmol/mg protein/min) (39) could in part be due to a higher steady state level of isobutyryl-CoA in heart mitochondria. Propionyl-CoA is a product of both α-keto-β-methylvalerate and α-ketoisovalerate metabolism in heart. It can be carboxylated by propionyl-CoA carboxylase (31). However, it is not known whether the rate of propionyl-CoA conversion to succinyl-CoA is sufficient to dispose of all of the propionyl-CoA produced during rapid branched-chain α-keto acid metabolism, especially when the ATP/ADP ratio in the matrix may be relatively low. ATP is required for propionyl-CoA carboxylase activity. Consequently, production of propionylcarnitine from these substrates in both the presence and absence of malate is not surprising and as discussed later raises the possibility that carnitine may be required for maximum metabolism of the α-keto acids derived from the branched-chain amino acids valine and isoleucine by heart mitochondria.

In most experiments malate, most likely via conversion to oxaloacetate which can serve as a sink for the acetyl group of acetyl-CoA, lowered acetylcarnitine production (22.9 nmol/mg protein/min at 20 °C, see Table IV of Ref. 30).

We do not know the $K_m$ or relative $K_m$ values for acycarnitines on the matrix side of the inner membrane. Therefore, speculation about the contribution of the translocase relative to other routes for disposal of propionyl-CoA produced from α-ketoisovalerate by liver and heart mitochondria is not warranted. The much larger efflux of isobutyrylcarnitine from heart mitochondria during α-ketoisovalerate oxidation (3.2 versus 1.0 nmol/mg protein/min) (39) could in part be due to a higher steady state level of isobutyryl-CoA in heart mitochondria.

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In most experiments malate, most likely via conversion to oxaloacetate which can serve as a sink for the acetyl group of acetyl-CoA, lowered acetylcarnitine production (39). However, the data obtained with liver mitochondria oxidizing octanoate were unanticipated since malate promoted the production of C-4, C-6, and C-8 acylcarnitines as shown in Fig.
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Fig. 3. Time course for acetylcarnitine production from pyruvate. The symbols and incubation conditions were the same as for Fig. 1. CAR, L-carnitine.

Fig. 4. Production of $^{14}$CO$_2$ and [acetyl-$^{14}$C]carnitine from 2-$^{14}$Cpyruvic acid. The incubation conditions were the same as for Fig. 1. $^{14}$CO$_2$ was collected by incubating the samples in 25-ml Erlenmeyer flasks with alcoholic KOH to trap CO$_2$ as described elsewhere (38). Specific radioactivity of 2-$^{14}$Cpyruvate was 6.6 Bq/nmol.

1, L and P, and Fig. 2B. This result, coupled with the minor production of acetylcarnitine from octanoate by heart mitochondria, even though heart contains large quantities of carnitine acetyltransferase, indicates that acetyl-CoA generated by $\beta$-oxidation in heart is less accessible to carnitine acetyltransferase than that of pyruvate and may be more tightly coupled to the tricarboxylic acid cycle than in liver. Some acetylcarnitine production occurs with octanoate in heart mitochondria when malonate is added (4 nmol/5 min/mg protein, see Ref. 39). With palmitate as substrate, carnitine can cause an accumulation of long-chain $\beta$-hydroxyacylcarnitines in liver mitochondria in the presence of malate (32). This was attributed to an increase in the NADH/NAD$^+$ ratio in the matrix. Although octanoate can be activated both in the cytosol and the matrix of mitochondria (33), for our experiments only matrix activation must be occurring because CoASH was not added.

The most surprising data were those obtained with heart mitochondria oxidizing pyruvate. It is generally assumed that carnitine promotes $\beta$-oxidation in heart mitochondria and that acetylcarnitine is produced when excess fatty acids are oxidized (3), while most of the pyruvate enters the tricarboxylic acid cycle although some may be converted to acetate (34, 35). The data presented in Figs. 1, 3, and 4 show that during state 3 respiration, even in the presence of suboptimum concentrations of carnitine, most of the acetyl units derived from pyruvate in heart mitochondria initially are converted to acetylcarnitine. Fig. 4 shows that for the 5- and 10-min periods, more than 5 times as many of the acetyl groups from acetyl-CoA were converted to acetylcarnitine than were oxidized via the tricarboxylic acid cycle (compare the ratio of $^{14}$CO$_2$ to [acetyl-$^{14}$C]acetylcarnitine at the early time points). Recently it has been shown that glucose is a major precursor of short-chain acylcarnitines in hearts of fasted rats (36). Our data also show that the efflux of acetylcarnitine with pyruvate as substrate is dependent on the concentration of carnitine. This would be expected if the carnitine/acetylcarnitine transporter could impact on the overall process, because its $K_m$ for carnitine in heart mitochondria is above 0.2 mM (29, 28) and the $K_m$ of carnitine for carnitine acetyltransferase is near 0.2 mM. The efflux of acetylcarnitine is almost 5 times greater at 1.0 mM carnitine than 0.2 mM carnitine.

Our results show that acetylcarnitine is utilized by both heart and brain mitochondria, after an initial burst of production from pyruvate (heart mitochondria) and from acetocetate (brain mitochondria). It appears as if the increase in acetylcarnitine/free carnitine may have stimulated the influx of acetylcarnitine with subsequent metabolism. The data are consistent with the data of others (34, 37) where it was shown that both external carnitine and acetylcarnitine can alter the acetyl-CoA level in the matrix of heart mitochondria.

The data allow for the following conclusions. 1) The efflux of individual acylcarnitines from mitochondria is substrate dependent. 2) The efflux of individual acylcarnitines varies with the tissue source of mitochondria. 3) The efflux of specific acylcarnitines varies with incubation conditions, and malate can reduce acetylcarnitine production. 4) The efflux of acetylcarnitine from heart mitochondria oxidizing pyruvate is carnitine concentration dependent. 5) The efflux of propionylcarnitine and the branched-chain acylcarnitines from heart mitochondria oxidizing the branched-chain keto acids of isoleucine and valine indicate that carnitine can affect the metabolism of amino acids from which propionyl-CoA is rapidly produced. These data also indicate that most of the acetyl-CoA generated from pyruvate by pyruvate dehydrogenase is readily accessible to carnitine acetyltransferase while that generated from $\beta$-oxidation is more available to the tricarboxylic acid cycle.

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REFERENCES
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