A carnitine/acylcarnitine translocase assay applicable to biopsied muscle specimens without requiring mitochondrial isolation

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A simple method for assaying the mitochondrial carnitine/acylcarnitine translocase of muscles that needs only few milligrams of fresh tissue is described. The procedure involves monitoring of the sulphobetaine (an inhibitor of the translocase)-sensitive acetylation of sub-saturating concentrations of carnitine in the medium, linked to the oxidation of [2-¹⁴C]pyruvate in the presence of malonate. Conditions affecting the reliability of the outlined procedure and the ancillary information to be collected, namely the activities of pyruvate oxidase system and carnitine acetyltransferase, for detecting possible deficiency of the translocase are described, together with data on the translocase activity in human skeletal muscle, in rat red and white skeletal muscles and in rat heart. The concepts outlined should allow development of assays of other mitochondrial transporters that also would require neither isolation of mitochondria nor availability of a large quantity of tissue, both of which are otherwise needed at present.

INTRODUCTION

Various congenital disorders of energy metabolism have been identified in myopathic individuals. Defects of carbohydrate metabolism were recognized first, but several other kinds of defects have been found subsequently. Growing understanding of fatty acid metabolism and availability of methods that could be applied clinically have led to the identification of defects of fatty acid oxidation, such as the deficiencies of carnitine, carnitine palmitoyltransferase and of various acyl-CoA dehydrogenases (DiMauro, 1979; Deufel *et al.*, 1984; Stanley *et al.*, 1983). It is likely that other defects will be identified in cases of unknown etiology as simple methods to allow clinical screening of those steps become available (Saudubray *et al.*, 1982; Gohil *et al.*, 1983).

The possibility that a myopathy with evidence of defective mitochondrial fatty acid oxidation may result at times from the deficiency of carnitine/acylcarnitine translocase, an essential step in the mitochondrial oxidation of long-chain fatty acids, has been considered (Clark *et al.*, 1983; Sengers *et al.*, 1984); in one study this was based on the observation of abnormally high concentrations of long-chain acylcarnitines in muscles (Glasgow *et al.*, 1983). Elevated concentrations of long-chain acylcarnitines in serum and muscles of patients showing carnitine deficiency have been found (DiDonato, 1984), but why this paradoxical increase occurs is obscure. Assays of mitochondrial carnitine/acylcarnitine translocase in such cases and where an unknown defect in oxidizing palmitoylcarnitine seems to be involved (Gohil *et al.*, 1983) could be informative, but these have not been performed.

Carnitine/acylcarnitine translocase is currently assayed by monitoring either the uptake of carnitine into mitochondria or the efflux of mitochondrial carnitine (Halperin & Pande, 1979; Pande & Parvin, 1980). For clinical use these procedures are technically difficult and theoretically unsound, because, as described elsewhere

(Murthy & Pande, 1985), (a) the translocase-catalysed exchange-diffusions do not constitute the rate-limiting step in the above assays, (b) the observed influx/efflux rates are markedly influenced by changes in matrix carnitine concentration, (c) isolation of milligram quantities of mitochondria needed for these assays, as in other procedures for evaluating functions of mitochondria (Black *et al.*, 1975; Land *et al.*, 1981), requires grams of muscle and use of techniques not within the reach of many laboratories (Byrne, 1983). A simple translocase assay that does not suffer from the above limitations and needs only a few milligrams of fresh muscle is described here, together with data on the translocase activity of skeletal muscles of human and rat and of rat heart muscle. This assay is based on the [¹⁴C]acetylation of carnitine in the medium, coupled to the oxidation of [2-¹⁴C]pyruvate in the mitochondrial matrix. Therefore, this assay also requires the simultaneous measurements of pyruvate oxidation and carnitine acetyltransferase to ensure that their deficiencies do not invalidate the translocase assay.

MATERIALS AND METHODS

Materials

All the chemicals of high purity were obtained as before (Murthy & Pande, 1984). Commercial [¹⁴C]pyruvate was stored at 4 °C at pH near 2 (with HCl) to enhance stability (Silverstein & Boyer, 1964). Occasionally batches of [¹⁴C]pyruvate obtained were grossly impure; in one such lot only about 25% of the total ¹⁴C present was as [¹⁴C]pyruvate. Pyruvic acid solutions were standardized out by using lactate dehydrogenase (Silverstein & Boyer, 1964) and/or glutamate-pyruvate transaminase (for [¹⁴C]pyruvate only); the two procedures gave identical results. The transaminase assay contained 0.1 M-potassium phosphate, pH 7.4, 25 mM-glutamate, 2 units of glutamate-pyruvate transaminase (none in controls) and 0.25 mM test [¹⁴C]pyruvate in a final volume

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of 200 μ l at 30 °C. Then 1 h later 100 μ l of 7.5% (w/v) HClO_4 was added. After neutralization with KOH, the [^{14}C]alanine was separated by using an AG 2-X8 (Cl^- form) exchanger column.

Preparation of muscle mitochondria

Mitochondria from rat skeletal muscle, prepared as described by Rigault & Blanchaer (1970), and from rat heart, prepared as described by Pande & Blanchaer (1971a), were suspended in 210 mM-mannitol/70 mM-sucrose/0.1 mM-EDTA/10 mM-Tris/HCl, pH 7.4.

Preparation of muscle homogenates

Homogenates were prepared by Polytron treatment (Ultra-Turrax; Janke und Kunkel, Staufen i. Br., West Germany) with a microprobe (Tekmar, Cincinnati, OH, U.S.A.). Portions (about 100 mg) of skeletal muscle from the legs of male adult fed rats or from muscularis tibialis of patients (40–71 years, $n = 6$) undergoing coronary bypass surgery were finely minced on a chilled plate, and then transferred to a tube containing 1 ml of 210 mM-mannitol/70 mM-sucrose/0.1 mM-EDTA/10 mM-Tris/HCl, pH 7.4, and homogenized at 0 °C for 10 s four times, with 30 s cooling between the Polytron-treatment steps. Polytron treatment was carried out at 50% of the total power output setting, and the resulting whole homogenate was used. The use of a 500 g, 30 s supernatant of the homogenates was tried, but was abandoned when the results showed that most (> 70%) of the Triton-releasable citrate synthase and carnitine-acetylation activity of the homogenates was sedimented by such a centrifugation.

Procedure for carnitine/acetylcarnitine translocase assay

The translocase activity was measured in the homogenate or in mitochondrial fraction of muscles by monitoring (Murthy & Pande, 1984) added-carnitine-dependent formation of [^{14}C]acetylcarnitine from [2- ^{14}C]pyruvate. The assay system at 30 °C, in a final volume of 200 μ l, contained 225 mM-mannitol, 25 mM-Hepes, pH 7.4, 0.05 mM-EDTA, 3 mM-ADP, 5 mM-potassium phosphate, pH 7.4, 2 mM-dichloroacetate and carnitine at indicated concentrations (0.5 mM for human muscles). Control tubes, in addition, contained 25 mM-sulphobetaine (SB_8), an inhibitor of the translocase. After preincubation of mitochondria (5–10 μ g of protein) or homogenate (0.2–0.6 mg wet wt. of muscle) in the above medium for 15 min, the reaction was initiated by adding 0.2 mM-[2- ^{14}C]pyruvate and 2 mM-malonnate. At the end of the incubation period (10–60 min), the reaction was terminated by the addition of 800 μ l of methanol. A sample of the clear supernatant was applied on a column (0.3 cm $\times$ 6 cm, in water) of exchanger resin (AG 2-X8; 200–400 mesh; Cl^- form), and 60% (v/v) methanol (5 $\times$ 0.2 ml) was passed through the column. This eluted the [^{14}C]acetylcarnitine quantitatively, the radioactivity of which was measured.

Measurement of activity of the pyruvate oxidase system

For this, the production of $^{14}\text{CO}_2$ from [1- ^{14}C]pyruvate or of ^{14}C -labelled citric acid-cycle intermediates from [2- ^{14}C]pyruvate was monitored. The incubation system was as for acetylcarnitine formation, except that carnitine and malonnate were replaced by malate (3 mM or as shown). Reactions were terminated by 50 μ l of 2 M-HCl. The tubes with [1- ^{14}C]pyruvate were capped and the

$^{14}\text{CO}_2$ formed was trapped in Hyamine hydroxide (0.2 ml).

To the tubes containing [2- ^{14}C]pyruvate, 24 μ l of 5 M-KOH was added to neutralize to pH 7.0. Then 25 μ l of about 240 mM- H_2O_2 was added, and the tubes were kept at 30 °C for 20 min to convert the unchanged [2- ^{14}C]pyruvate into [^{14}C]acetate (Kent, 1972). Excess H_2O_2 was decomposed by adding 1 μ l of 2 M-KOH and then leaving the tubes at room temperature for 10 min. Then 250 μ l was transferred to 25 ml glass counting vials and mixed with 25 μ l of a mixture of 6 M-HCl and 2% acetic acid. The vials were left on a hot-plate at a low heat setting (without charring), and air was blown to dry the contents. When dried to completion, the vials were brought to room temperature and water and scintillant were added for liquid-scintillation counting.

The oxidation procedure for [2- ^{14}C]pyruvate outlined above gave results identical with those for [1- ^{14}C]pyruvate conversion into $^{14}\text{CO}_2$. We have preferred the former, however, as this has obviated the need to buy and standardize another solution of radioactive pyruvate.

Assay of overt and latent citrate synthase activity

Citrate synthase activity of freshly prepared homogenates and mitochondria was measured without or with their prior incubation with Triton X-100. For prior incubations, the medium in 1 ml, at 0 °C, contained 210 mM-mannitol, 70 mM-sucrose, 0.1 mM-EDTA, 10 mM Tris/HCl, pH 7.4, whole homogenate corresponding to 1–3 mg of muscle or mitochondria (0.1–0.15 mg of protein) and 0 or 0.5% Triton X-100. After 5 min, portions from this were used for citrate synthase assay (Shepherd & Garland, 1969). Triton-releasable citrate synthase activity (the difference between that with and without exposure to Triton X-100) was taken to represent the activity that was residing in intact mitochondria, and the activity found without exposure to Triton X-100 was considered to be due to mitochondrial breakage in the process of homogenization etc. The above Triton exposure conditions were those found best for obtaining the maximal citrate synthase activity.

Carnitine acetyltransferase activity

The conversion of 100 μ M-[^{14}C]acetyl-CoA into [^{14}C]acetylcarnitine before and after treating the homogenates with 0.5% Triton X-100 was measured as described previously (Parvin & Pande, 1977).

RESULTS

We have shown with rat heart mitochondria that greater import rates of carnitine are detected by monitoring the [2- ^{14}C]pyruvate + malonnate-dependent (Pande, 1975) acetylation of carnitine that enters the matrix than those observed as the uptake of radioactive carnitine, because the latter is unable to reflect import of those carnitine molecules that after reaching a micro-environment on the inner side of the inner membrane exit in exchange for the other carnitine molecules moving in (Murthy & Pande, 1984, 1985). The same also applied for the skeletal-muscle mitochondrial carnitine transport in the present experiments (results not shown). This indicated that a simpler assay of the translocase in muscle homogenates might become possible by employing the acetylation approach, provided that the acetylation of carnitine was exclusively intramitochondrial and that the

translocase constituted the rate-limiting step. The use of pyruvate oxidation to provide acetyl-CoA meets the former requirement, because pyruvate dehydrogenase is an exclusively intramitochondrial enzyme. Moreover, under the conditions selected, the carnitine acetylation activity in homogenates and in isolated mitochondria from skeletal muscles and heart of rat was similar when related to the Triton-releasable citrate synthase activity (Table 1), a marker of intact mitochondria. This established that the carnitine acetylation activity being measured in the homogenates was exclusively linked to the operation of the carnitine/acylcarnitine translocase of intact mitochondria.

Meeting the second objective required use of a sub-saturating carnitine concentration in the medium (≤ 3 mM and ≤ 0.5 mM for rat and human muscles respectively), so that the rates of acetylcarnitine formation were not limited by the activities of the pyruvate oxidase system or of mitochondrial inner carnitine acetyltransferase (Fig. 1). Under these conditions, linear rates of acetylcarnitine formation were observed with the amount of homogenate protein and time of incubation (Fig. 2). The rates of carnitine acetylation in the homogenates were inhibited by the inclusion of known inhibitors of the carnitine/acylcarnitine translocase [$\geq 99\%$ inhibition by 25 mM-SB₈ or 50 μ M-palmitoyl(+)-carnitine], and the overall process showed a high temperature-dependence (mean of 5.4-fold increase in activity for every 10 °C rise in temperature for the 0–30 °C range), in agreement with the previously reported values (Ramsay & Tubbs, 1976; Pande & Parvin, 1980; Murthy & Pande, 1984). These findings showed that, under the conditions selected, the translocase activity limited carnitine acetylation rates and that the contribution of pyruvate oxidase and carnitine acetyltransferase, derived from the broken mitochondria in the homogenates, to acetylcarnitine formation was negligible.

Conditions affecting reliability of the present procedure

Because the present assay is intended for muscle-biopsy specimens, the effect of preparing homogenates at different times after tissue excision was examined. With both red and white skeletal muscles of rat, higher activities were obtained with tissue homogenized immediately after excision than with that homogenized after storage for 2 h at 0 °C. However, once the tissue was homogenized, the carnitine acetylation activity proved

Fig. 1. Effect of carnitine concentration in the medium on the acetylation of carnitine in rat and human skeletal-muscle homogenates

Rat skeletal-muscle homogenate (25–30 μ g of protein; ●) or human skeletal-muscle homogenate (50–60 μ g of protein; ■) was preincubated with various concentrations of carnitine at 30 °C for 15 min. Reactions were then started by adding 0.2 mM-[2-¹⁴C]pyruvate plus 2 mM-malonate and were terminated after 20 min by adding methanol. [¹⁴C]Acetylcarnitine formed was measured as described in the Materials and methods section. Values are means $\pm$ S.E.M. for four to six observations.

relatively stable to storage at 0 °C (Table 2). Therefore it is necessary to homogenize the muscles immediately after excision and to match closely the storage conditions of the tissues and homogenates being compared. Unlike with skeletal muscles, no loss of the activity was observed for the heart muscle stored for up to 2 h at 0 °C (Table 2). This relative stability of a heart mitochondrial function to storage of tissue at 0 °C is intriguing.

Optimal carnitine acetylation activity for both skeletal and heart muscles of rat was obtained for tissue homogenized between 20 and 60 s. In 5 s, only $60 \pm 3\%$ of the acetylation activity was released, and the released activity declined to $80 \pm 2\%$ on increasing the polytron treatment time to 120 s, showing that the breakage of

Table 1. Carnitine/acylcarnitine translocase activity measurements in whole homogenates and isolated mitochondria yielded similar values when expressed on the basis of Triton-releasable citrate synthase activity

Mitochondria (4–6 μ g of protein) isolated from red skeletal muscle or heart of the rat and 20–30 μ g of the whole homogenate protein of these tissues were used. Carnitine concentration was 3 mM, and the incubations were at 30 °C for 10–30 min. Formation of [¹⁴C]acetylcarnitine and Triton X-100-releasable citrate synthase were measured as described in the Materials and methods section. Values shown are means $\pm$ S.E.M. for seven observations.

	Acetylcarnitine formed (nmol/min per mg of protein) (A)	Triton X-100-releasable citrate synthase (nmol/min per mg of protein) (B)	Acetylcarnitine formed Triton-releasable citrate synthase ($10^3 \times A/B$)
Red muscle homogenate	4.9 ± 0.2	270 ± 16	18.4 ± 1.1
Red muscle mitochondria	46.8 ± 3.7	2710 ± 130	17.3 ± 0.9
Heart homogenate	12.5 ± 0.7	720 ± 35	17.4 ± 0.8
Heart mitochondria	52.2 ± 4.6	2900 ± 230	18.0 ± 1.0

Fig. 2. (a) Effect of incubation period on the formation of acetylcarnitine by human and rat skeletal-muscle homogenates, and (b) effect of human (●) and rat (■) skeletal-muscle homogenate protein content on acetyl-carnitine formation

(a) Incubations of human skeletal-muscle homogenate (50–60 μg of protein) with 1 mM-carnitine (●) or with 0.5 mM-carnitine (▲) and of rat skeletal-muscle homogenate (25–30 μg of protein) with 0.5 mM-carnitine (■) were carried out. Reactions were started by adding 0.2 mM-[2-¹⁴C]pyruvate plus 2 mM-malonate and were terminated at the times shown. [¹⁴C]Acetylcarnitine formed was measured as described in the Materials and methods section. Values are means ± S.E.M. of four to six observations. (b) Incubations were with 0.5 mM-carnitine for 20 min with the protein amounts shown. Other details were as given in (a).

prereleased mitochondria then dominated over the release of more mitochondria. Total and Triton X-100-releasable citrate synthase activities (nmol/min per mg of protein) in rat skeletal muscles were 419 ± 33 and 231 ± 18 respectively, and the corresponding values for human skeletal muscles were 109 ± 9 and 75 ± 6 . This shows that the proportion of intact mitochondria in muscles homogenized for 40 s averaged 55% for the rat and 71% for humans.

As previously observed for rat heart mitochondria (Murthy & Pande, 1985), for skeletal-muscle mitochondria also the carnitine-transport rates derived from carnitine acetylation remained relatively independent of

matrix carnitine concentration over a wide range (Table 3); in rat heart mitochondria the acetylation rates declined only when the matrix carnitine decreased to the unusually low values (to 7% of normal; Murthy & Pande, 1985). To exclude the possibility that the decreased carnitine acetylation rates at times may result solely from such a deficiency of matrix carnitine, a 15 min incubation of homogenate with carnitine at 30 °C was allowed in the present procedure before addition of [¹⁴C]pyruvate to initiate the acetylation process. This prior incubation would allow a sufficient build-up of mitochondrial-matrix carnitine, owing to the ability of the translocase to catalyse concentration-gradient-dependent unidirectional transport (Pande & Parvin, 1980), so that the subsequently monitored carnitine acetylation rates would reflect the activity of the translocase itself.

DISCUSSION

This paper provides a simple procedure of carnitine/acylcarnitine translocase assay that is suitable for clinical use and needs only a few milligrams of muscle. This should now make possible an examination of whether a deficiency or abnormality of this translocase may be the cause of defective mitochondrial fatty acid oxidation in certain patients, as speculated (Clark *et al.*, 1983; Sengers *et al.*, 1984).

The present assay is essentially like a coupled enzyme assay, and is based on the observations (Murthy & Pande, 1984, 1985) that, in intact mitochondria, the translocase, the mitochondrial inner carnitine acetyltransferase, the pyruvate dehydrogenase system and the associated respiratory-chain activities seem to be efficiently linked, owing to their sharing of a micro-compartment. Thus any observed decreased carnitine acetylation rates with suspected muscle homogenates should be ascribed to the deficiency/abnormality of the carnitine/acylcarnitine translocase only when the possible abnormality of other associated steps has been excluded. Thus a concurrent determination of total carnitine acetyltransferase and [¹⁴C]pyruvate+malate oxidation as described in the Materials and methods section is essential to ascertain that the carnitine acetylation rates are not limited by deficiencies of carnitine acetyltransferase, the pyruvate transporter/pyruvate oxidase system, or those of the respiratory chain, several of which have been described (DiMauro, 1979; Carafoli & Roman, 1980).

To minimize the possibility that decreased carnitine acetylation may result from an increased proportion of pyruvate dehydrogenase in the inactive state, we allow a 15 min substrate-free preincubation of homogenates at 30 °C to activate this enzyme fully (Fuller & Randle, 1984). Additionally, we have included 2 mM-dichloroacetate in situations such as diabetes and starvation, where full pyruvate dehydrogenase activation otherwise might not proceed (Fuller & Randle, 1984), although its omission has not made any difference to the pyruvate oxidation rates of the muscles analysed so far. The possibility of interference from a defect in the pyruvate transport/pyruvate oxidase system is, however, eliminated when higher pyruvate oxidation rates are seen in the presence of 10 mM-carnitine or with 3 mM-malate (Table 4), compared with those with limiting (0.5 mM) carnitine employed during the translocase assay. The possibility of the decreased carnitine acetylation activity resulting from a decreased tissue mitochondrial content would be cor-

Table 2. Effect of storage of rat muscles or their whole homogenates on the subsequent assay of carnitine/acylcarnitine translocase activity

Freshly excised muscle tissues from the rat were homogenized either immediately or after storing portions of the muscle mince in homogenizing medium at 0 °C for 2 h. Homogenates made from the freshly isolated tissues were either assayed immediately or after being kept at 0 °C for 2 h. Incubations were at 30 °C for 20 min with 3 mM-carnitine and 20–40 µg of homogenate protein. Values shown are means $\pm$ S.E.M. for six observations.

Tissue	Tissue homogenized	Homogenate assayed	Acetylcarnitine formed (nmol/min per mg of protein)
White muscle	Immediately	Immediately	1.15 $\pm$ 0.03
	After storage	Immediately	0.96 $\pm$ 0.03*
	Immediately	After storage	1.10 $\pm$ 0.08
Red muscle	Immediately	Immediately	5.09 $\pm$ 0.21
	After storage	Immediately	3.58 $\pm$ 0.40*
	Immediately	After storage	5.67 $\pm$ 0.26
Heart	Immediately	Immediately	12.28 $\pm$ 0.52
	After storage	Immediately	13.01 $\pm$ 0.41
	Immediately	After storage	12.72 $\pm$ 0.21

* $P < 0.05$ as compared to the tissue homogenized and assayed immediately.

Table 3. Effect of carnitine content of skeletal-muscle mitochondria on acetylcarnitine formation

Freshly isolated muscle mitochondria were incubated at 37 °C in the presence of carnitine concentrations in the medium as shown, and then assayed for acetylcarnitine formation as described in the Materials and methods section with 5–6 µg of mitochondrial protein and 6 mM-carnitine in the medium. The total carnitine content of mitochondria after the preincubation step was determined by sedimenting mitochondria through a dextran-containing wash layer and silicone oil etc., as described previously (Pande & Parvin, 1980). Values shown are means $\pm$ S.E.M. for five observations.

Preincubation conditions		Resultant mitochondrial carnitine content (nmol/mg of protein)	Acetylcarnitine formed (nmol/min per mg)
[Carnitine] in medium (mM)	Time at 37 °C (min)		
0	0	1.1	124 $\pm$ 6
0	15	0.2	116 $\pm$ 5
0.25	15	0.6	115 $\pm$ 8
0.5	15	1.1	123 $\pm$ 10
1.0	15	1.7	125 $\pm$ 5
3.0	15	4.3	134 $\pm$ 12

rected for by monitoring the citrate synthase activity and by relating the carnitine acetylation activity to the Triton-releasable citrate synthase (Table 1).

The distribution of the translocase in muscles paralleled that of the various carnitine acyltransferases, the concentration of carnitine (Baldwin & Tipton, 1972; Kerner & Bieber, 1983) and the ability to respire with different acylcarnitines (Pande & Blanchaer, 1971b) and to oxidize palmitate and pyruvate (Baldwin *et al.*, 1972) inasmuch as the activity was higher in rat heart than in red skeletal muscle, and white muscles had the lowest activity (Table 2). In human skeletal muscles, however, the carnitine/acylcarnitine translocase assayed with 0.5 mM-carnitine is as active (on a tissue-protein basis) as in rat skeletal muscles (Fig. 1). The carnitine acetylation value reached 'saturation' at much lower carnitine concentration in the medium in human muscle homogenate than in rat muscle homogenate (Fig. 1). This stems from the relatively lower ability of the former to oxidize

pyruvate (Table 4). The carnitine acetyltransferase activity in human skeletal-muscle homogenates was, however, higher than that in the rat muscle when expressed on the basis of total citrate synthase activity (0.32 ± 0.05 and 0.09 ± 0.01 unit/unit of citrate synthase, respectively).

Finally, it should be stressed that the concepts and the approaches outlined here are likely to allow simpler assay of other mitochondrial transporters in homogenates that would require availability of only a few milligrams of tissue. Import of several metabolites into mitochondria seems to follow their rapid metabolic conversion by enzyme(s) of the inner-membrane/matrix region (Murthy & Pande, 1985), and specific inhibitors of many transporters are now available, so that more reliable assays than the conventional influx/efflux assays should become possible for other transporters also. Such assays, based on the functional determination of translocase activities, are likely to reveal abnormalities caused not

Table 4. Comparison of pyruvate oxidation with malate or with malonate + carnitine in the homogenates of skeletal muscles of human and rat and of rat heart

Homogenates of human skeletal muscle (50–60 μ g of protein), rat skeletal muscle (30–50 μ g of protein) and rat heart (10–20 μ g of protein) were incubated at 30 °C with 0.2 mM-[2- 14 C]pyruvate and other additions as indicated. Formation of [14 C]acetylcarnitine in tubes containing malonate plus carnitine and of 14 C-labelled citric acid-cycle intermediates in tubes containing malate was determined as described in the Materials and methods section. Triton X-100-releasable citrate synthase activities (in units of nmol/min per mg of protein) for skeletal muscles of human and rat and of rat heart were 75 ± 6 , 231 ± 18 and 710 ± 63 respectively. Values shown are means $\pm$ S.E.M. for six or seven observations.

Tissue	Additions to the assay system	Pyruvate oxidation rate	
		On the basis of protein (nmol/min per mg of protein)	On the basis of Triton-releasable citrate synthase ($\times 10^3$)
Human skeletal muscle	3 mM-Malate	1.8 ± 0.1	25 ± 2.0
	0.5 mM-Carnitine + 2 mM-malonate	$1.1 \pm 0.2^*$	$17 \pm 2.5^*$
	10 mM-Carnitine + 2 mM-malonate	$2.4 \pm 0.2^*$	$34 \pm 3.0^*$
Rat skeletal muscle	3 mM-Malate	6.7 ± 0.1	29 ± 0.7
	3 mM-Carnitine + 2 mM-malonate	$5.6 \pm 0.2^*$	$25 \pm 1.1^*$
	10 mM-Carnitine + 2 mM-malonate	$7.9 \pm 0.2^*$	34 ± 3.5
Rat heart muscle	3 mM-Malate	17.5 ± 1.4	25 ± 2.2
	3 mM-Carnitine + 3 mM-malonate	$12.8 \pm 0.6^*$	$18 \pm 0.9^*$
	10 mM-Carnitine + 2 mM-malonate	$15.7 \pm 1.3^*$	22 ± 1.6

* $P < 0.05$ as compared with the respective malate values.

only by the mere deficiency of the translocase protein but also by the improper interaction of the translocase with the membrane lipids and associated proteins, if any.

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