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Methionine metabolism by rat muscle and other tissues

Occurrence of a new carnitine intermediate

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Perfused rat hindquarter preparations were shown to incorporate radioactivity from [U-^{14}C]methionine into citrate-cycle intermediates, lactate, alanine, glutamate, glutamine and CO_2. During perfusion, large amounts of methionine were also oxidized to methionine sulphoxide. The capacity for transamination of methionine or its oxo analogue, 4-methylthio-2-oxobutyrate, by muscle extracts was demonstrated. Rat skeletal muscle, heart, liver and kidney mitochondria, when incubated with the latter plus radiolabelled carnitine, formed a newly identified carnitine derivative, 3-methylthiopropionylcarnitine. It is concluded that the capacity for oxidation of methionine by a trans-sulphuration-independent pathway occurs in several mammalian tissues. The extent of inter-organ handling of intermediates in this pathway(s) is discussed.

INTRODUCTION

A series of studies evaluating the ability of skeletal muscle to metabolize amino acids resulted in balance studies which indicated that, of the non-dispensable amino acids, not only the branched-chain amino acids were broken down, but methionine was as well [1,2]. Methionine is generally assumed not to be metabolized by muscle since the enzymes of the accepted catabolic pathway, the trans-sulphuration–trans-methylation pathway, are very weak, if not totally absent [3,4]. In recent years Benevenga and co-workers have championed the existence (studied in liver only) of an alternative pathway involving an initial transamination (see, e.g., [5] for review and earlier references). A trans-sulphuration-independent pathway of methionine metabolism is not mentioned in modern biochemistry texts, and is therefore largely ignored. The importance, or even existence, of such a pathway is met by some reviewers with scepticism [6,7]. However, for the present authors, evidence for the physiological importance of an alternate pathway of methionine catabolism (at least in liver) involving transamination and oxidative decarboxylation is compelling. This evidence is lucidly summarized by Livesey [8].

In the present study we show that a rat skeletal-muscle preparation converts methionine carbon into citrate-cycle intermediates, lactate, glutamate, alanine and CO_2. In addition, we report that incubation of 'oxomethionine' (4-methylthio-2-oxobutyrate) together with carnitine and mitochondria from four different rat tissues results in formation of 3-methylthiopropionylcarnitine. Hence it appears that 3-methylthiopropionyl-CoA is an intermediate in the breakdown of methionine in at least several tissues.

EXPERIMENTAL

Perfusions and metabolite separation

Hindquarters from 48 h-starved male Wistar rats (200–250 g) were perfused for 2 h without added substrate as previously reported [1]. L-[U-^{14}C]Methionine ($9 \times 10^6 - 12 \times 10^6$ d.p.m.; 300 mCi/mmol) was added to the perfusate after an initial 'washout' of 50 ml. Recovery of total radioactivity in acid-soluble fractions from tissue plus perfusate after 120 min was 56 ± 9%, showing that a substantial portion of endogenous methionine was oxidized to CO_2 (see ref. [2]). Acid-soluble extracts of tissue and perfusate were prepared as in [9]. Separation of amino acids from organic acids, followed by analysis of some organic acids, was as described [10]. Analysis of the amino acid fraction was done by t.l.c. on silica-gel plates in two systems. (*a*) Methionine was separated from methionine sulphoxide with butan-1-ol/acetic acid/water (4:1:1, by vol.) as solvent. Methionine and methionine sulphoxide were well separated in this system (R_F 0.65 and 0.25 respectively). (*b*) The second solvent system contained butan-1-ol/butan-2-one/2-methylpropan-2-ol/acetic acid/water (4:3:3:1:1, by vol.). R_F values for methionine, alanine, glutamate, glutamine and methionine sulphoxide were 0.62, 0.41, 0.31, 0.27 and 0.18 respectively.

Purification of methionine

It was found that L-[U-^{14}C]methionine from all available sources was contaminated very substantially with methionine sulphoxide (20–60%). Therefore [^{14}C]-methionine was purified by t.l.c. before use [system (*a*) above]. There was no autoxidation of the methionine, stored frozen, within 72 h.

Abbreviations used: since the formal nomenclature of the various chemical intermediates is cumbersome, the following descriptive names are used and defined: oxomethionine, 4-methylthio-2-oxobutyrate; oxomethionine sulphone, 4-methylsulphonyl-2-oxobutyrate; oxomethionine sulphone, 4-methylsulphonyl-2-oxobutyrate; oxoisoleucine, 3-methyl-2-oxovalerate.

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Measurement of transaminase activities in skeletal muscle

Extracts were obtained by homogenizing leg muscle in 3 vol. of 50 mM-potassium phosphate/2 mM-EDTA/0.5% Triton X-100 adjusted to pH 7.4. The homogenates were centrifuged at 12000 *g* for 10 min, and the pellet was resuspended and homogenized in the original volume. The supernatant fractions were pooled and used for enzymic assays. Assays for 'glutamine transaminases', types L and K, were carried out as described by Cooper & Meister [11], and asparagine transaminase was assayed as described by Cooper [12].

Experiments with mitochondria

Mitochondria from heart and kidney [13], liver [14] and skeletal muscle [15] of 12 h-starved rats, were prepared by published procedures indicated. The final incubation volume was 1 ml and contained (in mM): Hepes, 20; KCl, 60; potassium phosphate, 5; ADP, 2; substrates as indicated in legends, 4; and (−)-[*methyl*-^{3}H]carnitine, 0.05 or 1 mM (6×10^6 d.p.m.), final pH being 7.4. The labelled carnitine was purified to > 99% radiochemical purity by chromatography on silica-gel t.l.c. plates developed with the same mobile phase as described below. Incubations were started with mitochondria, and after 15 or 20 min the incubations were stopped by addition of 40 μl of 70% (v/v) $HClO_4$. Neutralization was with 2 M-KOH containing 0.5 M-triethanolamine.

Synthesis of methylthiopropionylcarnitine

Methylthiopropionic acid was prepared by methylating thiopropionic acid dissolved in methanol/KOH with an equivalent amount of methyl iodide. After dilution with water and acidification with HCl, the methylthiopropionic acid was extracted with diethyl ether. Evaporation of the ether (rotavaporator) left the methylthiopropionic acid as an oil (brown from some iodine). G.l.c. (see below) showed one main peak. Methylthiopropionyl chloride was prepared by treating methylthiopropionic acid with excess oxalyl chloride at room temperature. Excess oxalyl chloride was evaporated *in vacuo*. Methylthiopropionylcarnitine was prepared by treating (−)-carnitine (300 mg) dissolved in trifluoroacetic acid (0.5 ml) with 3–4-fold molar excess of methylthiopropionyl chloride at 45 °C overnight. The methylthiopropionylcarnitine was obtained as a sticky mass after precipitation from the reaction mixture with ether and drying *in vacuo*. T.l.c. (see below) showed one spot and no remaining free carnitine after exposure to iodine vapour. Reaction with alkaline hydroxylamine, acidification and addition of $FeCl_3$ showed the presence of close to the expected amount of ester bonds [16].

Determination of acylcarnitines found in mitochondria from various tissues

The neutralized extracts were chromatographed on silica-gel t.l.c. plates. The plates were developed with methanol/chloroform/water/conc. ammonia/formic acid (22:20:4:3:1, by vol.) [4], cut into pieces (5 mm × 30 mm) and counted for radioactivity in a scintillation counter after the addition of scintillation fluid. The identification of acylcarnitines was based on their R_F values determined by including the corresponding synthetic derivatives, and spotting in a chamber with iodine vapour.

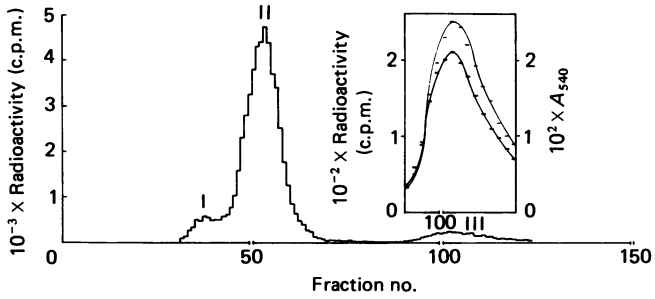

Fig. 1. Ion-exchange separation and identification of carnitine derivatives

Heart mitochondria were incubated for 15 min (30 °C) with (−)-[*methyl*-^{3}H]carnitine (1 mM) and oxomethionine (4 mM) as described in the text. After deproteinization and neutralization, 10 mg of synthetic methylthiopropionylcarnitine was added to the sample. The sample was chromatographed on a Dowex 50 column (H^+ form) eluted with 0.2 M-ammonium formate, pH 4.2; 250 μl portions of each 8 ml fraction were counted for radioactivity. A 2 ml portion of every second fraction between fractions 91 and 119 was freeze-dried, and methylthiopropionylcarnitine was assayed by the hydroxamate reaction at 540 nm. I, Acetylcarnitine; II, carnitine; III, methylthiopropionylcarnitine. In the inset, the upper and lower curves are absorbance (hydroxamate reaction) and radioactivity respectively.

Identification of methylthiopropionylcarnitine as reaction product

Extracts from incubations with carnitine and 4-methylthio-2-oxobutyrate with heart mitochondria were pooled, and approx. 10 mg of synthetic methylthiopropionylcarnitine was added. The sample was chromatographed on a Dowex 50 column (0.8 cm × 30 cm) eluted with 0.2 M-ammonium formate (pH 4.2). The samples collected were 8 ml each. Radioactivity was measured in 250 μl samples. A 2 ml portion of each fraction was freeze-dried, and methylthiopropionylcarnitine was assayed by the hydroxamate reaction [16]. Fig. 1 shows that the synthetic derivative exactly co-chromatographs with one of the radioactive peaks.

Exact co-chromatography of the added carrier and the radioactive peak with the highest R_F value was also found in the silica-gel t.l.c. system (Fig. 2).

The fractions from the Dowex column with positive hydroxamate reaction were freeze-dried. The residue was dissolved in 1 M-KOH and hydrolysed at 80 °C for 10 min. After acidification with HCl (to pH 1), the sample was extracted with 3 × 1 ml of diethyl ether, methylated with diazomethane [17] and chromatographed on a Pye Unicam gas–liquid chromatograph (GCV chromatograph) equipped with a glass column [1.83 m (6 ft)] filled with 8% butanediol succinate on Chromosorb WAW (Supelco). The oven temperature was 160 °C. The flame ionization detector was connected to an integrator (Hewlett–Packard 3385A automation system). The gas chromatogram showed one main peak with the same retention time as the original methylthiopropionic acid.

Materials

Male albino rats (approx. 200 g) starved overnight were used. Methyl iodide, 3-thiopropionic acid and

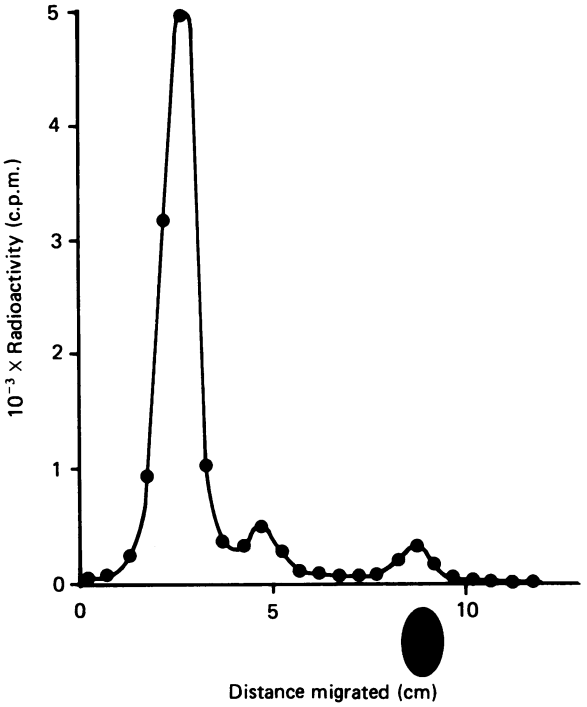

Fig. 2. T.l.c. separation and identification of carnitine derivatives

Muscle mitochondria were incubated with (−)-[*methyl*-^{3}H]carnitine and oxomethionine as described in the Experimental section. After deproteinization and neutralization, 50 μg of synthetic methylthiopropionylcarnitine was added to 10 μl of the neutralized sample. The sample was chromatographed on silica-gel t.l.c. plates developed with chloroform/methanol/water/conc. ammonia/formic acid (22:20:4:3:1, by vol.). The plates were cut into pieces (5 mm × 30 mm) and the radioactivity (●) was counted. The added carnitine derivative was spotted in a chamber with iodine vapour (iodine blot indicated below graph). Radioactive peaks, from the left, were carnitine, acetylcarnitine and methylthiopropionylcarnitine.

oxalyl chloride were supplied by Fluka A.G., Buchs, Switzerland. Silica-gel (Kieselgel 60) t.l.c. plates were from E. Merck (Darmstadt, Germany). Bio-Rad Laboratories (Richmond, CA, U.S.A.) provided Dowex 50 W-X8 (200–400 mesh). Chromosorb WAW (80–100 mesh) was supplied from Applied Science Laboratories (State College, PA, U.S.A.). (−)-Carnitine was a gift from Otsuka Pharmaceutical Factory (Otsuka, Japan). (−)-[*methyl*-^{3}H]Carnitine was prepared as reported previously [18]. All substrates, coenzymes and enzymes were from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

RESULTS AND DISCUSSION

Table 1 shows the distribution of radioactivity derived from trace [U-^{14}C]methionine into some organic and amino acids after perfusion of rat hindquarters for 2 h. About 12% of total remaining radioactivity was recovered as organic acids, and the remainder as amino acids. Of the organic acids, about 35% of radioactivity recovered was found in lactate plus the citrate-cycle intermediates which were identified. Hence methionine carbon enters the citrate cycle. Some of this carbon, in turn, leaves the cycle by decarboxylation, since radioactivity was recovered in both lactate and alanine (Table 1). A surprising result was that a large percentage of the amino acid fraction was identified as methionine sulphoxide. The possibility that this conversion was due simply to autoxidation was excluded. Circulation of the perfusates without the hindquarters for 2 h did not lead to formation of methionine sulphoxide. The mechanism of formation of methionine sulphoxide and of its disposition is a subject of continuing studies.

Transaminase activities of skeletal muscle

The perfusion studies reported here and in balance studies [1,2] established that methionine is catabolized. Owing to the virtual absence of the trans-sulphuration pathway we sought other routes of methionine catabolism. As shown in Table 2, the oxo analogue of methionine is a substrate for both the so-called glutamine transaminase types K and L, by using the definitions and assay conditions of Cooper & Meister [11]. It is of interest that the sulphoxide and sulphone of oxomethionine were also transaminated under the type-K assay conditions. It was shown many years ago [19] that liver contains enzymes capable of transaminating with asparagine. On purification of the enzyme [12], the original nomenclature of 'asparagine transaminase' was retained. Cooper [12] showed, however, that the purified enzyme has a broad range of specificity toward both oxo acids and amino acids, including methionine. Using the assay conditions of Cooper [12], we were able to demonstrate detectable, but extremely low, activity with respect to methionine in muscle extracts. Also transamination with pyruvate or 2-oxoglutarate was very weak.

Metabolism of oxomethionine and oxoisoleucine by mitochondria

Since significant transamination with methionine was demonstrable with muscle extracts, we questioned if and how oxomethionine could be further degraded. The trans-sulphuration-independent pathway of methionine catabolism in liver homogenates involves 3-methylthiopropionic acid as an intermediate [20]. Although the CoA ester of this acid has not been identified, its existence seemed to us likely. Dixon & Benevenga [21] had previously shown that oxomethionine is decarboxylated by liver mitochondria.

In previous studies we identified carnitine esters as intermediates in the catabolism of branched-chain amino acids by perfused hearts [22] and skeletal muscle [23], thereby demonstrating their metabolism and the formation of CoA esters. We chose to use a similar strategy for methionine metabolism. Oxoisoleucine was chosen as a control, as its metabolism to carnitine intermediates has been demonstrated. At the outset, liver mitochondria were chosen as a control tissue source, since, in view of published work, we could predict that methylthiopropionylcarnitine formation from oxomethionine would be detected.

The data in Table 3 summarize the results of three representative experiments in which skeletal muscle, heart, liver and/or kidney mitochondria were incubated with (−)-[^{3}H]carnitine and with no substrate, oxoisoleucine or oxomethionine. After the incubations, the neutralized extracts were chromatographed on t.l.c. plates, and carnitine and its acyl derivatives were identified (see the Experimental section). Without added substrate, only acetylcarnitine was formed. With oxo-

Table 1. Distribution of radioactivity among amino acid and organic acid fractions from hindquarter perfusions with L-[U-^{14}C]methionine

Hindquarters from 48 h-starved rats were perfused without added substrate, but with trace-labelled L-[U-^{14}C]methionine, for 2 h. Total remaining radioactivity in tissue plus perfusate was $4.7(\pm 0.6) \times 10^6$ d.p.m. ($n = 5$). Separation of amino acid and organic acid fractions, followed by identification of selected amino and organic acids, was carried out as described in the Experimental section. Results are means ± S.D. ($n = 5$).

Source	Percentage of total radioactivity in: Amino acids	Organic acids
Tissue	22.0±1.4	2.4±0.3
Perfusate	66.2±5.7	9.5±0.3

Source	Percentage of total remaining radioactivity in the amino acid fraction: Methionine	Methionine sulphoxide	Glutamate plus glutamine	Alanine
Tissue	46.1±5.7	42.5±4.2	3.0±0.8	1.4±0.4
Perfusate	46.7±7.5	44.7±7.0	3.4±0.6	3.6±0.6

Source	Percentage of total radioactivity of the organic acid fraction: Lactate	Malate	Citrate
Tissue + perfusate	25.6±4.7	2.6±0.6	5.4±0.8

Table 2. Transamination activities perhaps involved in utilization of methionine by skeletal muscle

Extracts from leg muscle were made as described in the Experimental section. Assays for 'glutamine transaminases', types L and K, were carried out as described by Cooper & Meister [11], and asparagine transaminase was assayed as described by Cooper [12]. Results (in μmol/h per g of tissue) are means ± S.D. ($n = 6$); N.D., not detectable.

Substrate	Glutamine transaminase: Type K	Type L
Oxomethionine	6.9±2.6	23.0±6.6
Oxomethionine sulphoxide	N.D.	20.3±5.9
Oxomethionine sulphone	N.D.	7.7±3.3

	Amination of pyruvate	Amination of oxoglutarate
Methionine	0.30±0.08	0.62±0.15
Methioine sulphoxide	0.24±0.09	N.D.
Methionine sulphone	0.13±0.04	N.D.

	Asparagine transaminase
Oxomethionine	0.78+0.15
Oxomethionine sulphoxide	0.97±0.35
Oxomethionine sulphone	0.92±0.37

isoleucine, propionyl- and 2-methylbutyryl-carnitines were formed, as expected from previous studies with perfused muscle and heart [22,23]. But now, with oxomethionine as substrate, a carnitine ester not previously identified was formed by mitochondria from all four tissues. As outlined in the Experimental section, this carnitine intermediate was identified as 3-methylthiopropionylcarnitine in two chromatographic systems. In a few experiments (as exemplified in Table 3, Expt. 2) incubations were with a lower carnitine concentration (50 μM instead of 1 mM, but with 20-fold higher specific radioactivity), resulting in an elevated labelling of propionyl- and 2-methylbutyryl-carnitines (from oxoisoleucine) and of methylthiopropionylcarnitine (from oxomethionine). The higher labelling of acylcarnitines with the low carnitine concentration is probably due to two opposing effects: with lower carnitine concentration, one would expect (by mass action) a lower amount of acylcarnitines: and, since the specific radioactivity of the carnitine in this case was elevated 20-fold, the radioactivity in acylcarnitines would be elevated.

Our present and recently published [1,2] work clearly demonstrates that skeletal muscle metabolizes methionine. It further shows that at least four rat tissues are capable of metabolizing methionine (at least partially) by a trans-sulphuration-independent pathway, with 3-methylthiopropionyl-CoA as an intermediate. The latter as an intermediate with liver, although not directly demonstrated, was strongly indicated by previous work: (*a*) Dixon & Benevenga [21] had previously shown that rat liver mitochondria oxidatively decarboxylate oxomethionine, and that other oxo acids inhibit this process;

Table 3. Formation of acylcarnitine by heart, skeletal muscle, kidney and liver mitochondria incubated with oxomethionine, oxoisoleucine or without added substrate

Conditions were as described in the Experimental section. The carnitine concentration was 1 mM, except for 'low carnitine', in which case carnitine was 50 μM, with a 20-fold higher specific radioactivity. The amount of mitochondria (mg of protein/ml) was as follows: Expt. 1, 0.88; Expt. 2, liver, kidney and heart, 2.1, 1.5, and 1.0 respectively; Expt. 3, liver, kidney and heart, 1.9, 0.6 and 0.6 respectively. Incubation time was 15 min (Expts. 1 and 2) or 20 min (Expt. 3). Values are radioactivity (c.p.m.) on t.l.c.. plates after separation.

			Radioactivity distribution on t.l.c. plates (c.p.m.)			
Expt. No.	Tissue	Substrate	Carnitine	Acetyl-carnitine	Propionyl- + 2-methyl-butyryl-carnitine	Methylthio-propionyl-carnitine
1	Heart	None	17682	26770		
		Oxoisoleucine	8829	1095	1826	
		Oxomethionine	7826	2798		129
	Muscle	None	8554	858		
		Oxoisoleucine	8364	2246	364	
		Oxomethionine	10458	1014		149
2	Liver	None	9314	391		
		Oxomethionine	7319	686		1027
	Kidney	None	9023	526		
		Oxomethionine	7620	988		553
	Heart	None	7474	224		
		Oxoisoleucine, + low carnitine	5344	4257	1993	
		Oxoisoleucine	6756	964	551	
		Oxomethionine, + low carnitine	9383	537		658
		Oxomethionine	6804	119		196
3	Liver	None	6649	351		
		Oxoisoleucine	4501	495	1403	
		Oxomethionine	4618	129		152
	Kidney	None	10273	139		
		Oxoisoleucine	5451	1050	899	
		Oxomethionine	2938	153		72
	Heart	None	6343			
		Oxoisoleucine	5278	186	238	
		Oxomethionine	6160	13		114

(*b*) Livesey & Lund [24] showed that, with isolated liver cells, ^{14}C-labelled oxomethionine could form methionine by transamination, and was oxidatively decarboxylated; (*c*) 3-methylthiopropionate is a product of methionine metabolism by liver homogenates [20]; and (*d*) since the present work with mitochondria was completed, Jones & Yeaman [25] reported that oxomethionine is a good substrate for highly purified bovine kidney branched-chain oxo acid dehydrogenase.

The metabolism of 3-methylthiopropionate appears to be almost exclusively an hepatic process, on the basis of studies with homogenates of rat tissues [25]. However, this result can be interpreted in two ways. Considering data in references cited, and the present work, free 3-methylpropionate appears to be on a side path of methionine metabolism resulting from deacylation of its CoA ester, as appears to be the case with intermediates in the catabolism of isoleucine and leucine (see ref. [2]). The first possibility, then, is that the enzyme responsible for re-activation of this acid is present exclusively in the liver. In this case the results of Steele & Benevenga [26] could be interpreted incorrectly, since our present results show that mitochondria from at least three other tissues form its carnitine (and therefore CoA) ester. The second possibility is that enzymes carrying out subsequent steps in the catabolism of 3-methylthiopropionyl-CoA are absent from extra-hepatic tissues, which would probably result in release of the free acid and subsequent processing taking place in the liver. A prototype for inter-organ disposition of amino acids is that of valine, and probably of isoleucine and leucine as well [2,27]. Livesey [8] made the important point that it is a paradox that methionine catabolism (via the accepted trans-sulphuration–trans-methylation pathway) should be compulsorily linked to and constrained by transmethylation (anabolic) reactions, and he also recognized an alternative degradative pathway as a conceptual necessity. The present work emphasizes the need for further studies on the inter-organ metabolism of methionine by trans-sulphuration-independent pathways.

Since this paper was submitted, an abstract has appeared [28] in which methylthiopropionyl-CoA is

identified as an intermediate in the metabolism of radiolabelled oxomethionine or methylthiopropionate by pig heart mitochondria. These and our results are confirmatory. In addition, our results show that this pathway, at least up to the CoA ester, exists also in several extra-hepatic tissues.

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