TRANSFER OF ACETYL-UNITS THROUGH THE MITOCHONDRIAL MEMBRANE: EVIDENCE FOR A PATHWAY DIFFERENT FROM THE CITRATE PATHWAY

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1. Introduction

The transfer of acetyl-groups of acetyl-CoA from the intramitochondrial to the extramitochondrial space is an obligatory step in the de novo synthesis of fatty acids from glycolytic precursors [1]. Citrate transport is the major form for the transfer of acetyl-units [2–4] involving the mitochondrial citrate synthase and the cytosolic citrate cleavage enzyme. But inhibition of the citrate cleavage enzyme by its competitive inhibitor (–)hydroxycitrate [2,3] or by an antibody [4] could inhibit fatty acid synthesis or the formation of extramitochondrial acetyl-CoA only up to 80–85% suggesting an additional pathway for the transfer of acetyl groups. Transport in the form of acetate, acetyl-carnitine, or acetyl-CoA has been discussed [5]. All 3 possibilities would not use the citrate synthase and the citrate cleavage reaction. In the citrate synthase reaction a loss of one of the three acetyl-CoA methyl hydrogens occurs. Therefore, a $^3H/^{14}C$ ratio of 1.0 in mitochondrial [2-^{3}H, ^{14}C]acetyl-CoA should be lowered to 0.67 in the cytosolic acetyl-CoA if the acetyl-units were transferred via the citrate pathway. Since an isotope effect $k_H/k_D = 1.4$ had been reported for the citrate synthase reaction with [2-^{2}H]acetyl-CoA [6] the drop in the $^3H/^{14}C$ ratio should be somewhat less than the theoretical value of 33%. A similar, but rather complicated approach has been used to establish the citrate transport as a transfer form of acetyl-groups through the mitochondrial membrane [7]. Intramitochondrial [2-^{3}H, ^{14}C]acetyl-CoA can be generated from [3-^{3}H, ^{14}C]L-alanine or [3-^{3}H, ^{14}C] L-lactate in mitochondrial incubations. For the determination of the radioactivity in the extramitochondrial acetyl-CoA, the following model can be used: 4-aminoantipyrine (AAP) is *N*-acetylated to 4-acetamidoantipyrine (AAAP) by arylamine transacetylase (EC 2.3.1.5) [8] which in liver is located exclusively in the cytosol [9]. Using a combined system consisting of mitochondria and high speed supernatant from rat liver with [3-^{3}H, ^{14}C]L-alanine or [3-^{3}H/^{14}C]L-lactate as substrate the radioactivity in isolated AAAP represents that in extramitochondrial acetyl-CoA. The following results are expected:

(1) Compared to the substrate the $^3H/^{14}C$ ratio in the AAAP produced should be diminished by the extent to which tritium is lost in the citrate synthase reaction.

(2) In the presence of (–)-hydroxycitrate no difference in the $^3H/^{14}C$ ratio of AAAP should be observed as compared to the substrate if now the acetyl-unit transfer involves acetate, acetyl-carnitine or acetyl-CoA.

Using this technique it is shown that C_2-units are transported out of the mitochondrial matrix not only in the form of citrate but also in a form not involving the citrate synthase reaction. Free acetate is the most likely candidate for this additional pathway.

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2. Materials and methods

2.1. *Materials*

All radioactive chemicals [3-^{3}H]L-alanine, [3-^{14}C]pyruvate, [1-^{14}C]acetyl-CoA, and [2-^{3}H]acetyl-CoA were obtained from the New England Nuclear Corp., Dreieichenhain/Germany. [3-^{14}C]L-alanine and [3-^{14}C]L-lactate were prepared enzymatically from [3-^{14}C]pyruvate by the use of glutamic pyruvic transaminase or lactate dehydrogenase resp. [3-^{3}H]L-lactate was prepared from [3-^{3}H]L-alanine by the use of glutamic pyruvic transaminase plus lactate dehyrogenase: [1-^{14}C, 2-^{3}H]citrate from [1-^{14}C, 2-^{3}H]acetyl-CoA by the use of citrate synthase. [3-^{3}H]L-lactate, [3-^{14}C]L-lactate and [1-^{14}C, 2-^{3}H]citrate were purified on a Dowex-1 × 8 column by eluting it with a linear HCl-gradient (0–0.2 N HCl); [3-^{14}C]L-alanine was purified on a Dowex-50W × 8 column by elution with a linear HCl-gradient (0–3.0 N HCl). 4-Aminoantipyrine and the silica gel thin-layer chromatogram sheets (No. 6060) came from the Eastman-Kodak Company, 4-acetamidoantipyrine from Aldrich, Milwaukee/Wisc., USA, (amino-oxy) acetate and fluorocitrate from Sigma, St. Louis/Mo., USA. (−)-Hydroxycitrate was prepared to Lewis [10], phosphoenolbutyrate according to Clark and Kirby [11]. Enzymes and cofactors were obtained from the Boehringer-Mannheim-Co, Mannheim/Germany.

2.2. *Experiments with isolated rat liver mitochondria*

Rat liver mitochondria and high speed supernatant were prepared according to Watson and Lowenstein [2]. The combined incubations contained 2.5–3.0 mg/ml mitochondrial protein and 8.0–10.0 mg/ml protein from the high speed supernatant.

AAAP was isolated from the incubation as described in detail elsewhere [12]. The isolation procedure consisted of extracting AAAP into chloroform/methanol (2/1; v/v) followed by two preparative steps on silica gel chromatogram sheets. The developing solvent in the first step was dioxane/benzene (9/1; v/v). In the second step water-saturated butanol was used in the first dimension and chloroform/methanol (2/1; v/v) in the second one. AAAP was recovered from the TLC-sheets by scraping off the parts corresponding to AAAP and extraction with methanol. The isolated AAAP was checked by its u.v. spectrum (240–320 nm) in 3.0 ml of methylene chloride, the amount calculated using the extinction coefficient at 280 nm (ϵ_{280} = 12.2 $mM^{-1} \cdot cm^{-1}$) [12]. After evaporating the methylene chloride the sample was counted in 10 ml of Bray's solution [13]. The absolute radioactivity (dpm) was evaluated by internal standardization [14].

2.3. *Experiments with citrate synthase*

Enzyme activity was assayed under the following conditions (final concentrations): Tris-HCl (pH 7.4) 0.2 M, malate 1.0 mM NAD^+ 1.0 mM, acetyl-CoA 0.1 mM, and MDH 70 units/ml in a total volume of 3.0 ml. The exchange experiments were performed as described in the legend to table 1. Water, acetate and citrate from the incubation medium were separated from each other by the following procedure: From the incubation mixture 0.1 ml was mixed with 2.0 ml of 6% $HClO_4$ and brought to pH 10.0 by 2.0 N KOH in order to hydrolyze all remaining acetyl-CoA. After neutralisation the sample was applied to a Dowex-1 × 4 column (1 × 40 cm) which was eluted by a linear HCl-gradient (100 ml water, 100 ml 0.2 N HCl). Fractions of 2.0 ml were collected. ^3H_2O, acetate and citrate from the incubation mixture appeared in different fractions. Those containing ^3H_2O, acetate or citrate were combined separately. Out of these pools the total amount of tritium and the $^3H/^{14}C$ ratio in acetate and citrate were determined.

2.4. *Experiments with citrate cleavage enzyme and arylamino transacetylase from rat liver high speed supernatant.*

Rat liver tissue was homogenized 1/5 (w/v) in 0.1 M Tris-Cl (pH 7.8) containing (final concentrations) 0.01 M EDTA and 0.03 M cysteine, and a high speed supernatant was prepared (100 000 *g*, 30 min). The exchange experiment was performed as described in the legend to table 2. AAAP was isolated as described for the mitochondrial incubations. Aliquots of the citrate used and of the isolated AAAP were counted to determine the $^3H/^{14}C$ ratio.

3. Results

3.1. *Exchange experiments with citrate synthase*

Table 1 shows the loss of tritium out of the methyl group of [1-^{14}C, 2-^{3}H]acetyl-CoA during

Table 1
Hydrogen exchange in the citrate synthase reaction

Sample	Fraction in which ^{3}H-radioactivity was determined	^{3}H-radioactivity	^{3}H-radioactivity as percent of ^{3}H-radioactivity in water + citrate
		(dpm · 10^{-6})	%
3 min	Water	2.76	23.1
	Citrate	9.16	76.9
	Water + citrate	11.92	100.0
	acetate	1.46	–
120 min	Water	2.59	22.3
	Citrate	9.00	77.7
	Water + citrate	11.59	100.0
	Acetate	1.60	–

Part A: Absolute tritium exchange.
The reaction contained (final concentrations): Tris-HCl (pH 7.4) 60.0 mM; [1-^{14}C, 2-^{3}H]acetyl-CoA (6.2 μCi ^{3}H, 0.7 μCi ^{14}C μmole) 1.0 mM; oxaloacetate 1.0 mM; and citrate synthase 7.4 units/ml in a final volume of 1.0 ml. The enzymatic activity checked at 3 min and 120 min after starting the reaction remained unchanged. Samples of 0.1 ml were analyzed for their tritium content in water, citrate and acetate at the times indicated. The tritium content is expressed per 1 ml of the reaction mixture.

Sample	Radioactivity in ^{3}H	^{14}C	^{3}H/^{14}C	Relative ^{3}H/^{14}C ratio
	(dpm)		(ratio)	(ratio)
Acetyl-CoA	11 862	1347	8.80	1.0
Citrate (3 min)	2818	409	6.89	0.78
Citrate (120 min)	3637	532	6.84	0.78

Part B: Relative tritium exchange as revealed from the ^{3}H/^{14}C ratios in acetyl-CoA and citrate. Experiment identical with that of part A. The last column shows the ^{3}H/^{14}C ratios in the substrate acetyl-CoA which was taken as 1.0.

citrate synthesis catalyzed by citrate synthase. Both the total tritium analysis as well as the ^{3}H/^{14}C ratios in the synthesized citrate compared to that in the starting acetyl-CoA show that there is a 22% loss of tritium from [1-^{14}C, 2-^{3}H]acetyl-CoA into water during citrate synthesis. The loss after 3 min and after 120 min of incubation with citrate synthase is about the same.

3.2. *Acetylation of AAAP by a rat liver high speed supernatant with [1-^{14}C, 2-^{3}H] citrate as substrate*

Table 2 shows the ^{3}H/^{14}C ratios in citrate and AAAP. The acetyl-group of AAAP came from the radioactive citrate. Both citrate cleavage enzyme and the arylamine transacetylase are required for the acetylation of AAP with citrate as substrate. There is essentially no loss of tritium during the transfer of

Table 2
Acetylation of AAP by a rat liver high speed supernatant with [1-^{14}C, 2-^{3}H]citrate as substrate

Sample	Radioactivity in ^{3}H	^{14}C	^{3}H/^{14}C	Relative ^{3}H/^{14}C ratio
	(dpm)		(ratio)	(ratio)
Citrate	1884	256	7.36	1.0
AAAP	5282	732	7.21	0.98

The reaction mixture contained (final concentrations) Tris-HCl (pH 7.8) 0.1 M, EDTA 0.01 M, and cysteine 0.0.3 M, [1-^{14}C, 2-^{3}H]citrate (0.5 μCi ^{3}H, 0.067 μCi/μmole) 1.0 mM: ATP 10.0 mM, $MgCl_2$ 5.0 mM, CoA 2.3 mM, AAP 5.0 mM and high speed supernatant protein 18.4 mg/ml in a total volume of 3.0 ml. The reaction was started by the addition of citrate and the incubation was carried out for 1 h at 37°C. The last column shows the ^{3}H/^{14}C ratio in AAAP relatively to that of the substrate citrate taken as 1.0.

radioactivity from [1-^{14}C, 2-^{3}H]citrate into the acetyl-group of AAAP.

3.3. *Acetylation of AAP during incubation of mitochondria with a high speed supernatant and with [3-^{3}H, ^{14}C]L-alanine or [3-^{3}H, ^{14}C] L-acetate as substrate*

Table 3 shows the loss of tritium out of the methyl group of radioactive L-alanine or L-lactate during conversion into the acetyl-group of AAAP. As indicated by the ^{3}H/^{14}C ratio the loss of tritium during the conversion of radioactive L-alanine and L-lactate was 95% and 40--50% resp. (Amino-oxy) acetate did not affect the tritium loss from L-lactate. In the presence of (−)-hydroxycitrate the tritium loss was diminished as can be seen from the rise of the ^{3}H/^{14}C ratios and from the rise of the specific ^{3}H-radioactivities in AAAP compared to the control experiments without (−)-hydroxycitrate. In Expt.2 the ^{3}H/^{14}C ratio in AAAP of the control sample was 0.48/0.55 = 87% of that in the presence of (−)-hydroxycitrate, in Expt.3 this ratio was 0.49/0.57 = 86%, in Expt.4 it was 0.44/0.50 = 85% or 0.43/0.50 = 86%. Similar values can be obtained if one compares the specific ^{3}H-radioactivities in AAAP. Table 4 shows the loss of tritium during conversion of [3-^{3}H, ^{14}C] L-lactate into the acetyl-group of AAAP in the absence of potassium and presence of phosphoenolbutyrate. In the absence of (−)-hydroxycitrate the ^{3}H/^{14}C ratio in AAAP dropped to 0.76–0.79 compared to that in L-lactate whereas in the presence of (−)-hydroxycitrate it was essentially the same as compared to the substrate. Similarly, the specific ^{3}H-radioactivity in AAAP rose in the presence of (−)-hydroxycitrate.

4. Discussion

During citrate synthesis from [1-^{14}C, 2-^{3}H]acetyl-CoA catalyzed by citrate synthase, 22% of the tritium is lost into water (table 1). Similar results were found by Rognstad and Katz [7]. Under the conditions used the citrate synthase reaction is practically irreversible as is also indicated by the absence of a difference between 3 and 120 min of incubation. No tritium is lost during transfer of radioactivity from [1-^{14}C, 2-^{3}H] citrate into the acetyl-group of AAAP catalyzed by citrate cleavage enzyme + arylamine transacetylase (table 2). This was a prerequistie for the anticipated investigation.

The high loss of tritium during conversion of [3-^{3}H, ^{14}C] L-alanine into the acetyl-group of AAAP (table 3) catalyzed by rat liver mitochondria + rat liver high speed supernatant can be explained by recent findings (15,16) of β-hydrogen exchange during transamination of L-alanine catalyzed by GPT. The loss of tritium from [3-^{3}H, ^{14}C] L-lactate during conversion into the acetyl-group of AAAP was also bigger than can be attributed to the citrate synthase reaction (table 3). The transaminase inhibitor (amino-oxy) acetate did not lower the tritium loss (table 3) which rules out the possibility that the endogenous GPT still causes this additional tritium loss during conversion of [3-^{3}H, ^{14}C] L-lactate into the acetyl-group of AAAP. (−)-Hydroxycitrate caused under all conditions tested a rise of about 15--20% in the ^{3}H/^{14}C ratio or in the specific ^{3}H-radioactivity in AAAP compared to the values on the absence of (−)-hydroxycitrate. This rise comes close to the value expected to occur in the citrate synthase reaction. Moreover, although the isolation of AAAP is not quantitative, clear inhibition of (−)-hydroxycitrate on the formation of AAAP was observed.

The experiments with (amino-oxy) acetate suggest

Table 3
Formation of and incorporation of radioactivity into 4-acetamidoantipyrine under various experimental conditions

Expt.	Additions	Amount of AAAP isolated	Radioactivity in ^3H	^{14}C	Specific ^3H-radioactivity	$^3H/^{14}C$	Relative $^3H/^{14}C$-ratio
		(μmole)	(dpm)		(dpm · μmole^{-1})	(ratio)	
1	GPT, 2 units	not examin.	1092	4061	not examin.	0.27	0.05
2	–	not examin.	18 383	9917	not examin.	1.85	0.48
	(–)-hydroxycitrate 1.0 mM	not examin.	12 428	5896	not examin.	2.11	0.55
3	–	0.142	425	202	2923	2.05	0.49
	(–)-hydroxycitrate 1.0 mM	0.099	361	155	3649	2.33	0.57
4	(amino-oxy) acetate 1.0	0.222	2298	2732	10 325	0.84	0.44
	(amino-oxy) acetate 1.0 mM (–)-hydroxycitrate 1.0 mM	0.040	485	483	12 221	1.0	0.52
	(amino-oxy) acetate 10.0 mM	0.184	1862	2246	10 091	0.83	0.43
	(amino-oxy) acetate 10.0 mM (–)-hydroxycitrate 1.0 mM	0.041	498	523	12 003	0.95	0.50

The reaction mixture contained (final concentrations): potassium phosphate (pH 7.2) 20 mM, sucrose 0.25 mM, ATP 10.0 mM, $MgCl_2$ 10.0 mM, malate 5.0 mM, CoA 0.6 mM, fluorocitrate 10.0 μM, AAP 5.0 mM, rat liver mitochondrial protein 2.5–3.0 mg/ml and rat liver high speed supernatant protein 8.0–10.0 mg/ml in a total volume of 10.0 ml. In addition, the incubation with L-lactate as substrate contained L-lactate 5.0 mM and NAD^+ 10.0 mM, that with L-alanine as substrate L-alanine 5.0 mM and α-ketoglutarate 5.0 mM. Further additions were as indicated. The incubations were carried out for 1 h at 37°C. The radioactive substrate, its total radioactivity, and its actual $^3H/^{14}C$ ratio as determined by a diluted aliquot were in the following experiments:
Expt.1: 5.5 μCi ^3H and 1.0 μCi ^{14}C, [3-^3H, ^{14}C]L-alanine, $^3H/^{14}C$ = 5.57
Expt.2: 19.0 μCi ^3H and 5.0 μCi ^{14}C, [3-^3H, ^{14}C]L-lactate: $^3H/^{14}C$ = 3.84
Expt.3: 0.43 μCi ^3H and 0.1 μCi ^{14}C, [3-^3H, ^{14}C]L-lactate: $^3H/^{14}C$ = 4.13
Expt.4: 1.6 μCi ^3H and 0.83 μCi ^{14}C, [3-^3H, ^{14}C]L-lactate: $^3H/^{14}C$ = 1.90
The last column shows the $^3H/^{14}C$ ratios in AAAP relatively to that of substrate L-alanine or L-lactate taken as 1.0. Incubations with mitochondria without high speed supernatant did not produce any AAAP.

Table 4
Amount of and radioactivity in 4-acetamidoantipyrine in the presence of (amino-oxy) acetate and phosphoenolbutyrate and absence of monovalent cations

Addition	Amount of AAAP isolated	Radioactivity in ^3H	^{14}C	Specific ^3H-radio-activity	$^3H/^{14}C$	Relative $^3H/^{14}C$ ratio
	(μmole)	(dpm) ($\times 10^{-3}$)		(dpm · μmole^{-1}) ($\times 10^{-3}$)	(ratio)	
–	0.130	14.4	6.9	110.9	2.09	0.79
–	0.46	15.7	7.8	108.9	2.01	0.76
(–)-Hydroxy-citrate 1.0 mM	0.062	8.9	3.4	144.1	2.62	0.99
(–)-Hydroxy-citrate 1.0 mM	0.060	8.6	3.3	142.9	2.63	0.99

The reaction mixture contained (final concentrations): Tris-HCl (pH 7.4) 20.0 mM, sucrose 0.25 mM, ATP 10.0 mM, $MgCl_2$ 10 mM, malate 5.0 mM, CoA 0.6 mM, fluorocitrate 10.0 μM, AAP 1.0 mM, [3-^{3}H, ^{14}C]L-lactate (1.62×10^5 dpm $^3H/\mu$mole, 0.61×10^5 dpm $^{14}C/\mu$mole) 1.0 mM, actual $^3H/^{14}C$ ratio = 2.65, NAD^+ 10.0 mM, (amino-oxy) acetate 1.0 mM, phospho-enolbutyrate 1.0 mM, rat liver mitochondrial protein 2.7 mg/ml and 9.7 mg/ml protein from rat liver high speed supernatant. Before use the high speed supernatant fraction was desalted on a Sephadex G-25 column. Further additions were as indicated. The incubation was carried for 1 h at 37°C. The last column shows the $^3H/^{14}C$ ratios in AAAP relatively to that in the substrate L-lactate taken as 1.0.

that there is still an additional cause for tritium loss during conversion of [3-^{3}H, ^{14}C]L-lactate into the acetyl-group of AAAP besides the loss occurring in the citrate synthase and GPT reaction. Rose [17] demonstrated that pyruvate kinase catalyzes the detritiation of [3-^{3}H]pyruvate, the exchange being dependent on potassium ion concentration. Therefore, mitochondria were incubated with a high speed supernatant in the absence of potassium. In addition, an inhibitor of pyruvate kinase, phosphoenolbutyrate [18], was used. The results of the incubation (table 4) show that under these conditions the loss of tritium during conversion of [3-^{3}H, ^{14}C]L-lactate into the acetyl-group of AAAP is reduced to that expected to occur in the citrate synthase reaction. In the presence of (–)-hydroxycitrate the specific ^{3}H-radioactivity and the $^3H/^{14}C$ ratio in AAAP rose by about 20%, the $^3H/^{14}C$ ratio being now essentially the same as that in the substrate [3-^{3}H, ^{14}C]L-lactate. These results represent additional evidence that a minor pathway for the transfer of acetyl-groups across the mitochondrial membrane exists which differs from the citrate pathway. Whether this occurs in the form of acetate, acetyl-carnitine or acetyl-CoA as discussed [5] cannot be distinguished by this approach. There is little evidence for the transfer form of acetyl-CoA [5,19]. It could be shown, however, that isolated rat liver mitochondria can release free acetate during oxidation of hexanoate (Seufert and Söling, unpulished results). Moreover the formation of free acetate in liver [20,21] and the existence of acetyl-CoA-hydrolase activity in mitochondrial membranes [22] have been reported.

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