Enzymic Hydrolysis of Acetylcarnitine in Liver from Rats, Sheep and Cows

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1. The enzymic utilization of *O*-acetyl-L-carnitine other than via carnitine acetyltransferase (EC 2.3.1.7) was investigated in liver homogenates from rats, sheep and dry cows. 2. An enzymic utilization of *O*-acetyl-L-carnitine via hydrolysis of the ester bond to yield stoichiometric quantities of acetate and L-carnitine was demonstrated; 0.55, 0.53 and 0.30 μmol of acetyl-L-carnitine were utilized/min per g fresh wt. of liver homogenates from rats, sheep and dry cows respectively. 3. The acetylcarnitine hydrolysis activity was not due to a non-specific esterase or non-specific cholinesterase. *O*-Acetyl-D-carnitine was not utilized. 4. The activity was associated with the enriched outer mitochondrial membrane fraction from rat liver. Isolation of this fraction resulted in an eightfold purification of acetylcarnitine hydrolase activity. 4. The K_m for this acetylcarnitine utilization was 2 mM and 1.5 mM for rat and sheep liver homogenates respectively. 6. There was a significant increase in acetylcarnitine hydrolase in rats on starvation and cows on lactation and a significant decrease in sheep that were severely alloxan-diabetic. 7. The physiological role of an acetylcarnitine hydrolase is discussed in relation to coupling with carnitine acetyltransferase for the relief of 'acetyl pressure'.

The only known pathway to date for the production and utilization of *O*-acetyl-L-carnitine is via the enzyme, carnitine acetyltransferase (Friedman & Fraenkel, 1955; Bremer, 1962; Fritz *et al.*, 1963). Lowenstein (1964) observed a low incorporation of the acetyl group of acetylcarnitine into fatty acids. He postulated a possible hydrolysis to yield acetate as the simplest interpretation of this observation. In his investigations into the reversibility of the carnitine acetyltransferase reaction in rat liver, Bremer (1962) observed some alternative utilization of acetylcarnitine. Mahadevan & Sauer (1969), in experiments with rat liver microsomal preparations, established a hydrolytic utilization of C_6 – C_{18} acylcarnitines but did not detect any hydrolysis of C_2 – C_4 acylcarnitines. Thus a more specific investigation into the enzymic utilization of acetylcarnitine seemed warranted. In none of the above reports was the utilization of acetylcarnitine by carnitine acetyltransferase prevented. In our current investigations, carnitine acetyltransferase was completely inhibited by the addition of the specific inhibitor bromoacetyl-L-carnitine (Chase & Tubbs, 1969).

We report an alternative utilization of acetylcarnitine via hydrolysis of the ester bond to yield acetate and L-carnitine. Some of these results have been presented in a preliminary form (Costa & Snoswell, 1974).

Experimental

Methods

Animals. The rats used were hooded Wistar males weighing 250–300 g and were fed on a pelleted rat diet (Charlicks, Adelaide, S. Austral., Australia). Food was withheld from the starved rats for 48 h.

The sheep used were 4-year-old Merino wethers, weighing between 38 and 48 kg. These animals were fed *ad libitum* on lucerne-hay chaff. Tissue from Merino sheep was also obtained from the abattoirs directly after slaughter.

Non-lactating cows were predominantly Jersey and were all approx. 3 years of age. The lactating cows were all Jersey $\times$ Friesian animals in the fifth month of their second lactation. The cattle were grazed on irrigated perennial pasture consisting mainly of white clover, cocksfoot, paspallum and rye grass. No supplementary feed was given.

Alloxan-diabetic animals. Alloxan-diabetes was produced in adult Merino wethers by injecting a sterile solution of alloxan (60 mg/kg body weight) into the jugular vein. The animals were killed 5–6 days later. These animals spontaneously reduced their food intakes. Blood total acid-soluble carnitine had doubled in the 2 days before slaughter.

Tissue preparations and homogenates. Rats were killed by cervical dislocation and exsanguination. The

sheep were killed by severing the necks. Liver tissue from the non-lactating and lactating cows was obtained by biopsy as described by Baird & Heitzman (1970). Fresh samples of liver were collected directly into 0.25 M-sucrose containing 23 mM-potassium phosphate, pH 7.4. The liver tissue was homogenized with a Thomas homogenizer in either 0.25 M-sucrose containing 23 mM-potassium phosphate, pH 7.4, as the homogenizing buffer (40%, w/v) or 0.025 M-sucrose containing 2.3 mM-potassium phosphate, pH 7.4, and 0.1% Triton X-100 as the homogenizing buffer (20%, w/v).

Tissue fractionation. Rat liver was collected directly into 0.25 M-sucrose containing 23 mM-potassium phosphate, pH 7.2. Homogenates (10%, w/v) were then prepared in the same sucrose-phosphate solution with a Potter-Elvehjem homogenizer with a tight-fitting Teflon pestle. These homogenates were then centrifuged at 700 g to remove all debris and nuclei. The supernatant fractions were centrifuged at 10000 g for 10 min to sediment mitochondria. The supernatant fractions were recentrifuged at 100000 g for 1 h. The supernatants from this centrifugation were designated cytosol fractions and the pellets were resuspended in sucrose-phosphate medium to give microsomal fractions. The mitochondrial pellets were washed twice in the sucrose-phosphate medium and recentrifuged at 13000 g for 10 min.

Mitochondrial fractionation. Rat liver mitochondria were isolated in 0.44 M-sucrose-10 mM-triethanolamine (pH 7.6)-2 mM-EDTA by the method of Brdiczka *et al.* (1969). A slight modification of the method of Hoppel & Tomec (1972) was used to separate the submitochondrial fractions in that all pellet fractions were resuspended in 0.25 M-sucrose-23 mM-potassium phosphate, pH 7.4.

Enzyme activity assays. All assays were performed on a Zeiss PMQ II spectrophotometer fitted with an automatic sample changer and TE converter (Carl Zeiss, Oberkochen, Germany) connected to a Rikadenki model B140 recorder (Rikadenki Kogyo Co., Tokyo, Japan). The instrument was fitted with a temperature-controlled cell holder and the temperature was maintained at 37°C for all assays.

Carnitine acetyltransferase. Liver homogenates (20%, w/v) homogenized in 0.025 M-sucrose-2.3 mM-potassium phosphate-0.1% Triton X-100 were frozen in liquid N₂ and thawed. The homogenates were then centrifuged at 8000 g for 3 min in an Eppendorf centrifuge model 3200 (Eppendorf Geratebau Netheter and Hinz, G.m.b.H., Hamburg, Germany) and the supernatants used for assay. The assay system was similar to that described by Solberg *et al.* (1972) and contained 400 mM-Tris-HCl, pH 8.0, 100 μ M-5,5'-dithiobis-(2-nitrobenzoic acid), 100 μ M-acetyl-CoA and enzyme fraction in a total volume of 1 ml. The reaction was started by the addition of 3.3 mM-L-carnitine. The inhibition of carnitine acetyltransferase

by bromoacetylcarnitine was investigated in the same assay system. The carnitine acetyltransferase was pre-incubated with bromoacetylcarnitine (1–20 μ M).

Enzymic utilization of O-acetyl-L-carnitine. Liver homogenates (40%, w/v) homogenized in 0.25 M-sucrose-23 mM-potassium phosphate, pH 7.4, were used. Activities were measured by determining the rate of acetylcarnitine utilization and L-carnitine and acetate formation. The incubation series for each tissue consisted of a boiled-homogenate reaction, zero-time reaction, and two tubes to determine acetyl-L-carnitine utilization in the presence and absence of bromoacetyl-L-carnitine. Eppendorf tubes contained, in a total volume of 1.0 ml, 0.3 M-Tris-HCl, pH 8.0, 70 μ M-bromoacetylcarnitine, 5 mM-acetyl-L-carnitine and tissue homogenate. The tubes were incubated for 0, 5, 10, 15, 20 or 30 min at 37°C and the reaction was stopped with 200 μ l of 15% (w/v) HClO₄; after centrifugation at 8000 g for 2–5 min, the supernatant was neutralized with 3 M-KOH. The enzymic activity was linear with respect to time for 20 min and therefore a 20 min incubation period was used as a routine.

Specificity of acetylcarnitine hydrolase. Utilization of O-acetyl-D-carnitine, O-propionyl-L-carnitine and O-butyryl-L-carnitine was determined in the same manner as utilization of O-acetyl-L-carnitine.

Rotenone-insensitive NADH-cytochrome c reductase. This was assayed in the digitonin-treated submitochondrial fractions of rat liver in a system similar to that described by Sottocasa *et al.* (1967). The spectrophotometric assay was performed at 550 nm and the assay system contained 240 mM-potassium phosphate, pH 7.6, 5 mM-EDTA, 20 μ M-rotenone, 0.08 mM-cytochrome c and submitochondrial fraction in a final volume of 1.0 ml. The reaction was started with 0.1 mM-NADH.

Adenylate kinase. This was determined in the submitochondrial fractions from rat liver by spectrophotometric assay at 340 nm. The system contained, in a final volume of 1.0 ml, 50 mM-triethanolamine buffer, pH 7.6, 5 mM-EDTA, 8 mM-MgSO₄, 75 mM-KCl, 0.22 mM-NADH, 3 mM-ATP, 0.8 mM-phosphoenolpyruvate, 10 μ g of pyruvate kinase, 50 μ g of lactate dehydrogenase and enzyme fraction. The reaction was started with 3 mM-AMP.

Succinate dehydrogenase. This was assayed in the submitochondrial fractions from rat liver by spectrophotometric assay at 550 nm. The assay system contained, in a total volume of 1.0 ml, 100 mM-potassium phosphate, pH 7.6, 5 mM-EDTA, 20 μ M-rotenone, 0.1 mM-phenazine methosulphate, 0.08 mM-cytochrome c and enzyme fraction. The reaction was started with 5 mM-succinate.

Metabolite assays. Acetylcarnitine was measured by the method of Pearson & Tubbs (1964) and free carnitine by the method of Marquis & Fritz (1964).

Free acetate was determined by the kinetic method of Knowles *et al.* (1974).

Preparation of bromoacetyl-L-carnitine. The bromoacetyl derivative of L-carnitine was prepared by modification of several methods (Chase & Tubbs, 1969; Fraenkel & Friedman, 1957; Zeigler *et al.*, 1967). First 2 g (15.3 mmol) of bromoacetic acid (recrystallized) was allowed to react with 24 ml (26.4 mmol) of bromoacetyl bromide for 3 h at 75°C. Then 1 g (5.9 mmol) of L-carnitine was added and the mixture allowed to react for a further hour. The mixture was then cooled and extracted eight times with ice-cold diethyl ether. The solid mass remaining was dried with a stream of N₂ gas. The solid mass was dissolved in 4 ml of ethanol, and 3 ml of 70% (w/v) HClO₄ was added. Bromoacetyl-L-carnitine perchlorate was recrystallized from isopropyl alcohol. The resultant crystals were stored frozen *in vacuo*. A sample of bromoacetyl-L-carnitine was also kindly supplied by Dr. P. K. Tubbs, Department of Biochemistry, University of Cambridge, Cambridge, U.K.

Chemicals

L-Carnitine hydrochloride and O-acetyl-L-carnitine chloride were generously supplied by Otsuka Pharmaceuticals, Osaka, Japan, and were recrystallized from ethanol. Propionyl-L-carnitine and butyryl-L-carnitine chloride were prepared by the method of Bøhmer & Bremer (1968). CoA was obtained from Calbiochem, Los Angeles, Calif., U.S.A., enzymes were from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany, and alloxan was from Koch-Light Laboratories, Colnbrook, Bucks., U.K. Acetyl-CoA was prepared by the method of Stadtman (1957).

Results

Enzymic utilization of O-acetyl-L-carnitine by normal sheep liver homogenates

The results in Table 1 show a small amount of chemical hydrolysis of acetylcarnitine at the pH of the incubation. In addition to the chemical hydrolysis of acetylcarnitine there is a significantly greater ($P < 0.001$) enzymic utilization of acetylcarnitine. This

acetylcarnitine utilization and carnitine production may proceed *via* the carnitine acetyltransferase reaction. In the presence of bromoacetylcarnitine, a potent specific inhibitor of carnitine acetyltransferase (Chase & Tubbs, 1969), there is a small decrease in acetylcarnitine utilization ($P < 0.05$) proceeding via carnitine acetylcarnitine, but there is still a significant ($P < 0.001$) and substantial utilization of acetylcarnitine of 0.55 $\mu\text{mol/min per g}$ of fresh liver tissue. The carnitine acetyltransferase activity present in the sheep liver homogenates is completely inhibited by the concentration of bromoacetylcarnitine used in the incubations (i.e. 70 μM). This was also established by independent spectrophotometric assay of carnitine acetyltransferase inhibition as described under 'Methods'. In subsequent assays of acetylcarnitine utilization, this concentration of bromoacetylcarnitine was included in all incubations. Thus the enzymic utilization of acetylcarnitine and production of L-carnitine proceeds via an alternative enzymic mechanism to the carnitine acetyltransferase reaction.

Enzymic hydrolysis of acetylcarnitine by homogenates of normal rat, sheep and cow liver

Results in Table 2 show an overall balance between acetylcarnitine utilized and acetate and L-carnitine produced in incubations of liver homogenates from

Table 1. *Enzymic utilization of O-acetyl-L-carnitine by normal sheep liver homogenates*

Liver samples were obtained from sheep killed at the abattoirs. Incubations of acetyl-L-carnitine with sheep liver homogenates were performed in the presence and absence of bromoacetylcarnitine as described in the text. Results are means $\pm$ S.E.M. for six animals.

	Activity ($\mu\text{mol/min per g}$ of liver tissue)	
	Acetylcarnitine utilized	Carnitine produced
Normal - inhibitor	0.715 $\pm$ 0.05	0.680 $\pm$ 0.10
Normal + inhibitor	0.550 $\pm$ 0.05	0.515 $\pm$ 0.05
Boiled homogenate	0.155 $\pm$ 0.025	0.100 $\pm$ 0.020

Table 2. *Enzymic hydrolysis of acetylcarnitine by homogenates of normal rat, sheep and cow liver*

Homogenates were prepared and assayed as described in the text. N.A., Not assayed. The values shown are means $\pm$ S.E.M. with the number of determinations in parentheses.

	Activity ($\mu\text{mol/min per g}$ of fresh liver tissue) as per method of assay		
	Acetylcarnitine utilized	Carnitine produced	Acetate produced
Rat	0.50 (4) $\pm$ 0.10	0.50 (4) $\pm$ 0.05	0.45 (4) $\pm$ 0.10
Sheep	0.55 (6) $\pm$ 0.05	0.52 (6) $\pm$ 0.05	N.A.
Dry cow	0.30 (3) $\pm$ 0.05	0.30 (3) $\pm$ 0.10	0.30 (3) $\pm$ 0.05

these species. Although the amount of acetylcarnitine utilized varies between the different species, this balanced relationship holds throughout. Within each species there is no significant difference with respect to the use of any of the three compounds for determining acetylcarnitine hydrolysis activity. These results then establish a stoichiometric relationship between acetylcarnitine utilized and acetate and carnitine produced in the absence of carnitine acetyltransferase activity. Thus the enzymic utilization of acetylcarnitine in the presence of bromoacetylcarnitine in liver homogenates of these species would then appear to proceed via the hydrolytic cleavage of the ester bond of acetylcarnitine.

Enzymic specificity of acetylcarnitine hydrolysis

The hydrolysis of the ester bond of acetylcarnitine could be catalysed by a non-specific esterase. The main non-specific esterase present in liver in various species is carboxylic ester hydrolase (Shibko & Tappet, 1964; Chow & Ecobichon, 1973; Ecobichon, 1973). This enzyme is inhibited by iodoacetamide (Shibko & Tappet, 1964), but the addition of iodoacetamide had no effect on the rate of utilization of acetylcarnitine by liver homogenates. Purified preparations of acylcholine acylhydrolase (EC 3.1.1.8) did not hydrolyse acetylcarnitine to any significant extent. These results imply that acetylcarnitine hydrolysis is catalysed by a specific enzymic mechanism. Liver homogenates from rats, sheep and cows did not hydrolyse the unnatural acetyl-D-carnitine as measured by production of acetate. Thus acetylcarnitine hydrolysis appears to proceed *via* a stereospecific enzyme subsequently referred to as acetylcarnitine hydrolase.

Substrate specificity of acetylcarnitine hydrolase

Acetylcarnitine hydrolase from rat liver also hydrolysed propionyl and butyryl esters of L-carnitine. The rate of utilization of propionyl-L-carnitine by acetylcarnitine hydrolase was 9.0 nmol of carnitine liberated/min per mg of tissue protein, which was greater than the rate of utilization of acetyl-L-carni-

tine of 5.8 nmol/min per mg of tissue protein. In contrast, butyryl-L-carnitine was hydrolysed at a lower rate than acetyl-L-carnitine (2.8 and 5.8 nmol/min per mg of tissue protein respectively).

Michaelis-Menten constants for acetylcarnitine hydrolase from rat and sheep liver

The kinetic constants for acetylcarnitine hydrolase from the livers of the two species were calculated by using crude enzymic preparations from these two sources. The K_m for acetylcarnitine of rat liver acetylcarnitine hydrolase was 2 mM, and that for sheep liver acetylcarnitine hydrolase was 1.5 mM. These values were determined from Lineweaver-Burk plots by a statistical weighting method for $1/v$ described by Wilkinson (1961).

pH optimum of acetylcarnitine hydrolase

The pH optimum for acetylcarnitine hydrolysis was 8.0. There was a sharp increase in activity in the pH range from 7.4 to 8.0 with a fall in activity after pH 8.0.

Intracellular localization of acetylcarnitine hydrolase in rat liver

Preliminary fractionation of rat liver homogenates into mitochondrial, microsomal and cytosol fractions and subsequent analysis of these fractions for acetylcarnitine hydrolase activity showed the activity to be confined to the mitochondrial fraction. Submitochondrial fractions were then prepared by using the modifications described in the text. Rotenone-insensitive NADH-cytochrome *c* reductase was chosen as the marker enzyme for the outer-membrane fraction. Ernster & Kuylenskierna (1968) showed that contamination by microsomal rotenone-insensitive NADH-cytochrome *c* reductase could be significantly decreased after a few washings of the mitochondrial preparation. The advantages of using this marker enzyme instead of the more commonly used monoamine oxidase were greater ease and sensitivity of measurement. There was difficulty in preparing a rigidly pure outer-membrane fraction, as the results in Table 3 indicate. Thus the preparation was rather

Table 3. *Submitochondrial localization of acetylcarnitine hydrolase activity within rat liver mitochondria*

Submitochondrial fractions were prepared and assayed as described in the text. The values are means $\pm$ S.E.M. of three experiments. N.D., Not detectable.

	% of total activity in submitochondrial fractions		
	Outer membrane	Intermembrane space	Inner-membrane matrix
Rotenone-insensitive cytochrome <i>c</i> -succinate dehydrogenase	13 $\pm$ 6	4 $\pm$ 1	85 $\pm$ 7
Adenylate kinase	12 $\pm$ 6	88 $\pm$ 6	N.D.
Rotenone-insensitive cytochrome <i>c</i> -NADH reductase	64 $\pm$ 8	35 $\pm$ 8	N.D.
Acetylcarnitine hydrolase activity	72 $\pm$ 10	23 $\pm$ 10	N.D.

Table 4. *Enzymic hydrolysis of acetyl-L-carnitine by homogenates of rat, sheep and cow liver under various conditions*

Liver homogenates were prepared and assayed as described in the text. The results are means $\pm$ s.e.m. with the number of assays in parentheses. Significance of the difference from normal or between dry and lactating cow was determined by the Student's *t* test.

Animal	Physiological state	Activity of acetylcarnitine hydrolase (μ mol/min per g of fresh liver tissue)	
Rat	Normal	0.55 (4) $\pm$ 0.10	
	Starved	0.95 (2) $\pm$ 0.25	$P < 0.05$
Sheep	Normal	0.55 (6) $\pm$ 0.05	
	Alloxan-diabetic	0.35 (2) $\pm$ 0.10	$P < 0.01$
Cow	Dry	0.30 (3) $\pm$ 0.05	
	Lactating	0.95 (4) $\pm$ 0.05	$P < 0.01$

an enrichment of the outer-membrane fraction of the mitochondria. The results in Table 3 show that the acetylcarnitine hydrolase is associated with this fraction and its activity represented an eightfold purification over the activity in the crude homogenates from rat liver.

Effect of various physiological states on enzymic hydrolysis of acetyl-L-carnitine by homogenates of rat, sheep and cow liver

The results in Table 4 show a significant ($P < 0.05$) almost twofold increase in acetylcarnitine hydrolase in liver from rats starved for 48 h over liver from normal rats. Also the activity of acetylcarnitine hydrolase increased threefold ($P < 0.01$) in liver of cows on lactation. In direct contrast with these two results, in liver from alloxan-diabetic sheep there was a significant ($P < 0.01$) decrease in acetylcarnitine hydrolase activity when compared with the activity in normal sheep liver. All values for acetylcarnitine hydrolase activity shown in Table 4 are an overall average of measurement of acetylcarnitine utilized and L-carnitine and acetate produced.

Discussion

The results presented for sheep liver homogenates show that utilization of acetylcarnitine within the assay incubation is mainly enzymic rather than chemical. This enzymic utilization was demonstrated in the liver homogenates from at least three different species and the stoichiometry of acetylcarnitine utilization is consistent with a concept of hydrolysis of the ester bond to yield equimolar proportions of acetate and L-carnitine. These findings are not entirely inconsistent with the observations of Bremer (1962) who, using rat liver mitochondria, found some alternative utilization of acetylcarnitine other than

via the carnitine acetyltransferase reaction. Also Bremer & Davis (1974) reported acetylcarnitine concentrations lower than expected in their experiments with rat liver mitochondria, which is again consistent with an alternative utilization of acetylcarnitine.

The utilization of acetylcarnitine in the presence of iodoacetamide and non-utilization of acetyl-L-carnitine by non-specific cholinesterase indicates that hydrolysis of acetyl-L-carnitine is due to a specific enzyme entity rather than a non-specific esterase. The stereospecificity of acetylcarnitine utilization is also indicative of an enzymic rather than chemical utilization and further indicates a specific enzyme is involved. This specific hydrolase activity in rat liver cells is associated with the outer mitochondrial membrane. Thus attempts by Mahadevan & Sauer (1969) to establish a short-chain carnitine ester hydrolase in enriched microsomal fractions from rat liver were unlikely to have been successful. The isolation of an enriched outer mitochondrial membrane fraction also resulted in an eightfold purification of acetylcarnitine hydrolase activity over that present in the crude rat liver homogenate. Further attempts to purify this enzyme met with difficulty owing to its highly unstable nature.

The precise physiological role of an acetylcarnitine hydrolase within rat liver in the normal and starved state is difficult to delineate, compounded in part by the lack of a precise role for carnitine acetyltransferase. However, the increase in acetylcarnitine hydrolase activity in the starved rat is consistent with the hypothesis of the short-chain carnitine ester system buffering 'acetyl pressure' (Pearson & Tubbs, 1967) generated in starvation. The increase in acetylcarnitine hydrolase activity would allow the carnitine released to be recycled through the system or complexed with long-chain fatty acids via carnitine palmitoyltransferase. Thus the acetylcarnitine hydrolase in rat liver may maintain a high concentration of free carnitine during the starved state. The energy lost in this carnitine-maintenance system during 'acetyl pressure' would be equivalent to that lost by diverting acetyl groups into ketone-body production.

The physiological role of an acetylcarnitine hydrolase in sheep liver is also not easy to rationalize. The high activity of an acetylcarnitine hydrolase in the presence of a high carnitine acetyltransferase activity (in the normal sheep liver) could help to ease a high ratio of acetyl-CoA/CoA (Snoswell & Henderson, 1970) with the resultant bonus of producing acetate, a major metabolic fuel used in peripheral tissues in ruminants. In the severely alloxan-diabetic animal there is a significant decrease in acetylcarnitine hydrolase activity. This would firstly decrease the amount of L-carnitine recycled through the coupled system, which may be reflected in the need to synthesize more carnitine as a compensating measure, and secondly result in an accumulation of acetyl-L-carnitine (see

Snoswell & Koundakjian, 1972). In the severely diabetic state there is a 20-fold increase in total acid-soluble carnitine and a 30-fold increase in acetyl-carnitine (Snoswell & McIntosh, 1974).

The increase in acetylcarnitine hydrolase activity in livers from lactating cows relative to dry cows also suggests that the carnitine acetyltransferase-acetylcarnitine hydrolase-coupled system could operate to relieve 'acetyl pressure' in the lactating state. The carnitine acetyltransferase activity in liver from lactating cows was 2.2 μmol of CoA produced/min per g of fresh tissue (N. D. Costa, unpublished work). Thus coupling this activity with the acetylcarnitine hydrolase activity would result in a facile carnitine-recycling system in the lactating cow. The observations of Baird *et al.* (1972) where they failed to observe a significant rise in total acid-soluble carnitine could be rationalized on the basis of active carnitine recycling. This coupled-enzyme system releases a non-toxic end product, acetate, which would then be available to the mammary gland for the synthesis *de novo* of milk fatty acids, and oxidation (Annison & Linzell, 1964).

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