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Relationships between Carnitine and Coenzyme A Esters in Tissues of Normal and Alloxan-Diabetic Sheep

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1. The total acid-soluble carnitine concentrations of four tissues from Merino sheep showed a wide variation not reported for other species. The concentrations were 134, 538, 3510 and 12900nmol/g wet wt. for liver, kidney cortex, heart and skeletal muscle (M. biceps femoris) respectively. 2. The concentration of acetyl-CoA was approximately equal to the concentration of free CoA in all four tissues and the concentration of acid-soluble CoA (free CoA plus acetyl-CoA) decreased in the order liver > kidney cortex > heart > skeletal muscle. 3. The total amount of acid-soluble carnitine in skeletal muscle of lambs was 40% of that in the adult sheep, whereas the concentration of acid-soluble CoA was 2.5 times as much. A similar inverse relationship between carnitine and CoA concentrations was observed when different muscles in the adult sheep were compared. 4. Carnitine was confined to the cytosol in all four tissues examined, whereas CoA was equally distributed between the mitochondria and cytosol in liver, approx. 25% was present in the cytosol in kidney cortex and virtually none in this fraction in heart and skeletal muscle. 5. Carnitine acetyltransferase (EC 2.3.1.7) was confined to the mitochondria in all four tissues and at least 90% of the activity was latent. 6. Acetate thiokinase (EC 6.2.1.1) was predominantly (90%) present in the cytosol in liver, but less than 10% was present in this fraction in heart and skeletal muscle. 7. In alloxan-diabetes, the concentration of acetylcarnitine was increased in all four tissues examined, but the toatl acid-soluble carnitine concentration was increased sevenfold in the liver and twofold in kidney cortex. 8. The concentration of acetyl-CoA was approximately equal to that of free CoA in the four tissues of the alloxan diabetic sheep, but the concentration of acid-soluble CoA in liver increased approximately twofold in alloxan-diabetes. 9. The relationship between CoA and carnitine and the role of carnitine acetyltransferase in the various tissues is discussed. The quantitative importance of carnitine in ruminant metabolism is also emphasized.

It has been shown (Snoswell & Henderson, 1970) that the [free carnitine]/[acetylcarnitine] ratio in sheep liver varies widely with different dietary conditions, whereas much smaller variations in the ratio [free CoA]/[acetyl-CoA] were observed. In view of these results, plus the fact that acetate, a major fuel in ruminants, is metabolized mainly in extrahepatic tissues (Mayfield *et al.*, 1966), we have investigated the relationships between various carnitine and CoA fractions in several tissues of normal sheep. Also, in order to understand more fully the role played by carnitine acetyltransferase (EC 2.3.1.7) in the conversions between acetylcarnitine, CoA, acetyl-CoA and carnitine, we have examined the intracellular distribution of carnitine acetyltransferase, carnitine and CoA and also the intracellular distribution of acetate thiokinase in these tissues.

A marked increase in acetylcarnitine concentration in sheep liver occurs on starvation (Snoswell & Henderson, 1970). However, this increase does not occur at the expense of free carnitine (as observed in the rat by Pearson & Tubbs, 1967), for the concentrations of free and total acid-soluble carnitine also increased markedly on starvation (Snoswell & Henderson, 1970). Alloxan-diabetes causes an even more striking increase in the total acid-soluble carnitine content of sheep liver. Mehlman *et al.* (1969) reported that the carnitine content of rat skeletal muscle is approximately halved in alloxan-diabetes. Thus, we have extended our observations on the effects of alloxan-diabetes in sheep and have examined carnitine and CoA fractions in various

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sheep tissues in order to investigate intra-tissue relationships between carnitine and CoA under conditions of severe metabolic stress.

Experimental

Methods

Animals. The adult sheep used were Merino wethers, approx. 4 years old, and weighing between 35 and 45kg; they were given a diet of lucerne–hay chaff. The lambs were between 5 and 16 days old. They were bottle-fed on reconstituted skim-milk powder with a vitamin supplement. Tissues from Suffolk sheep were obtained from the abattoirs at Bristol directly after slaughter.

Alloxan-diabetic animals. Alloxan-diabetes was produced in adult Merino wethers by injecting a sterile solution of alloxan (60mg/kg body wt.) into the jugular vein. The animals were killed 3 days later. Results are presented only for those animals in which the blood glucose concentration had risen to 200mg/100ml.

Tissue preparations. The sheep were killed by severing the necks, and samples of liver, kidney cortex, heart and skeletal muscle (M. biceps femoris and M. sternothyreoidus) from adult animals, and liver and skeletal muscle (M. biceps femoris) from lambs, were immediately freeze-clamped with aluminium-faced tongs previously cooled in liquid N_2 (Wollenberger *et al.*, 1960). The $HClO_4$ extracts of the frozen tissues were prepared as described for liver (Snoswell & Henderson, 1970).

Homogenates and subcellular fractions. Fresh samples of sheep liver and kidney cortex were collected directly into 0.25M-sucrose containing 23mM-potassium phosphate (pH7.2). Homogenates (10%, w/v) were then prepared in the same sucrose–phosphate solution with a Potter–Elvehjem homogenizer. These homogenates were then centrifuged at 700*g* to remove cell debris and nuclei. The supernatant fractions were centrifuged at 10000*g* for 10min to sediment mitochondria. The mitochondrial pellets were washed twice in the sucrose–phosphate medium and re-centrifuged at 13000*g* for 10min. Microsomal and supernatant (cytosol) fractions were prepared by centrifugation (for 1h at 100000*g*) of the supernatants obtained after sedimentation of the mitochondria. The mitochondrial and microsomal fractions were finally suspended in the sucrose–phosphate medium described above.

Homogenates (10%, w/v) of sheep heart and skeletal muscle were prepared in the electrolyte buffer described by Chappell & Perry (1954) by using a ground-glass homogenizer.

The preparation of subcellular fractions from sheep heart and skeletal muscle was much more difficult than the preparation of similar fractions from sheep liver and kidney cortex. Most methods for preparing subcellular fractions from heart and skeletal muscle involve the use of a bacterial proteinase. However, there have been a number of reports of the destruction of mitochondrial enzymes, particularly carnitine acyltransferases, by these methods (see, e.g., De Jong & Hülsmann, 1970). Thus we have avoided using fractionation methods involving the use of a proteinase. Unfortunately, all other methods for preparing subcellular fractions from heart and skeletal muscle require more extensive homogenization in a ground-glass homogenizer. These methods led to extensive loss of the mitochondrial-matrix marker enzyme, citrate synthase, into the cytosol fractions. Thus a direct preparation of subcellular fraction from sheep heart and skeletal muscle was not practicable. Instead, an indirect method was employed to prepare cytosol fractions of these tissues relatively free of mitochondrial contamination. 'Press' fractions of heart and skeletal muscle (M. sternothyreoidus) were prepared by direct centrifugation of whole muscle (cut into small pieces with scissors) at 100000*g* for 4h (skeletal muscle) or 33000*g* for 1½h (heart) in a procedure similar to that described by Amberson *et al.* (1964). The small amounts of supernatants thus obtained were designated 'press' fractions.

Homogenates, mitochondrial and microsomal fractions were disrupted by sonic disintegration for 3min at 0°C (in 15s intervals with 15s cooling in between) by using a Soniprobe (Dawe Instruments Ltd., London W.3, U.K.) at 20kHz and 2A. The preparations were subsequently centrifuged at 20000*g* for 2min and the supernatants were used for the assay of enzymes.

Immediately after isolation, samples (1.5ml) of the various homogenates and subcellular fractions were treated with 0.25ml of 30% (w/v) $HClO_4$ and the supernatants were neutralized with saturated $KHCO_3$ before assay of carnitine and CoA.

Enzyme assays. All assays were done at 30°C with a Hilger–Gilford recording spectrophotometer or a Unicam spectrophotometer (model SP.500) fitted with a Gilford automatic cell-positioner and recorder.

Acetate thiokinase (*EC* 6.2.1.1). This was assayed in sonic extracts of the various subcellular fractions by coupling the reaction with that catalysed by arylamine acetyltransferase (EC 2.3.1.5) and measuring the change in extinction at 460nm caused by acetylation of (*p*-aminophenylazo)benzene-*p*-sulphonic acid in an assay system similar to that used for pyruvate dehydrogenase (EC 1.2.4.1) by Denton *et al.* (1971). The arylamine acetyltransferase was prepared from acetone-dried powder of pigeon liver by the method of Tabor *et al.* (1953) and was kindly supplied by Dr. R. M. Denton. The assay system contained 100mM-tris–HCl (pH7.8), 0.5mM-EDTA, 1mM-$MgCl_2$, 5mM-mercaptoethanol, 100mM-potassium acetate, 10mM-ATP and 0.3 unit of

arylamine acetyltransferase plus the sample in total volume of 1.0ml. The reaction was started by the addition of 0.3μmol of CoA and a linear rate of reaction was observed after 5 to 10min. A molar extinction coefficient (substrate–acetylated substrate) at 460nm of 7.11×10^6 litre·mol^{-1}·cm^{-1} (Jacobson, 1961) was used to calculate enzyme activity, which was expressed as nmol of acetyl-CoA produced/min at 30°C. All subcellular fractions were assayed for acetate thiokinase immediately after preparation, as it was found that the activity of this enzyme decreased rapidly after isolation of these fractions, particularly in the cytosol fractions.

Carnitine acetyltransferase. This was assayed in the various subcellular fractions that had been exposed to hypo-osmotic sucrose (0.025M) plus 0.1% Triton X-100 for 30min. Activity was also assayed in whole mitochondrial suspensions. The assay system was similar to that described by Barker *et al.* (1968) but included 0.18M-sucrose, and the acetyl-CoA was added 5min before the addition of 5,5′-dithiobis-(2-nitrobenzoic acid) to ensure maximum activity.

Citrate synthase (*EC* 4.1.3.7). This was assayed spectrophotometrically by using dithiobis(nitrobenzoic acid) as described by Shepherd & Garland (1969). Enzyme activity was assayed in sonic extracts of the various subcellular fractions of heart and skeletal muscle.

Glutamate dehydrogenase (*EC* 1.4.1.3). This was assayed in sonic extracts of various subcellular fractions from kidney cortex and liver by the method of Barker *et al.* (1968), but with 1.5mM-ADP to ensure maximum activity and 10μM-rotenone in place of 3mM-KCN and P_i in place of tris–HCl buffer. Recovery of the glutamate dehydrogenase present in homogenates in the various subcellular fractions could only be achieved if P_i was included in the homogenizing medium and in the assay medium (Walter & Anabitarte, 1971).

Lactate dehydrogenase (*EC* 1.1.1.27). This was assayed in sonic extracts of the various subcellular fractions by spectrophotometric assay at 340nm. The system contained 200mM-tris–HCl (pH7.4), 0.2mM-NADH, 10μM-rotenone and enzyme fraction in a final volume of 1.0ml. The reaction was started by addition of 1mM-pyruvate.

Metabolite assays. Acetylcarnitine was measured by the method of Pearson & Tubbs (1964), and free carnitine by the method of Marquis & Fritz (1964). High blank values in the latter assay, due to endogenous free thiol groups, were decreased to acceptable values by adjusting the extracts to pH8.5 with 1M-tris base and heating at 90°C for 5min before assay, as suggested by Marquis & Fritz (1964). Pearson *et al.* (1969) suggest that significant hydrolysis of short-chain carnitine esters may occur under these conditions. We could detect no breakdown of a 0.2mM solution of *O*-acetyl-L-carnitine in 20mM-tris–HCl, pH8.5, at 90°C after 15min heating. However, owing to the temperature coefficient of the tris the actual pH at 90°C would have been nearer 6.5. The total amounts of acid-soluble carnitine and acid-insoluble carnitine were measured by the method of Pearson & Tubbs (1967).

Acetyl-CoA and acetyl-CoA plus free CoA were measured by the kinetic method of Allred & Guy (1969); free CoA was determined by difference. In these kinetic determinations a standard curve was prepared on each occasion by using a CoA standard solution, the concentration of which was determined with phosphotransacetylase (EC 2.3.1.8) by the method of Michal & Bergmeyer (1963). However, as this method only assays reduced CoA, the standards were preincubated for 10min at 25°C with 2μl of 0.2M-dithiothreitol to ensure that all the CoA was in the reduced form.

Instrumentation was as described by Snoswell & Henderson (1970).

Table 1. *Free carnitine, carnitine esters, free CoA and acetyl-CoA in sheep tissues*

Merino wethers that had been given a diet of lucerne chaff were killed and tissue samples were immediately frozen with aluminium-faced tongs previously cooled in liquid N_2. The frozen tissue powders were extracted with $HClO_4$ and assayed as described in the text. Results are means ± S.E.M. for four animals.

	Concentration (nmol/g wet wt.)					
Tissue	Acetylcarnitine	Free carnitine	Total acid-soluble carnitine	Acid-insoluble carnitine	Acetyl-CoA	Free CoA
Liver	3.5±2.6	74±7	134±9	<0.1	46±3	50±6
Kidney cortex	67±18	415±45	538±64	4±1	31±1	34±3
Heart	812±83	2060±323	3510±143	12±8	12±3	16±1
Skeletal muscle (M. biceps femoris)	1820±478	9860±1380	12900±880	17±5	1.0±0.1	1.3±0.2

Chemicals

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

Results

Free carnitine, carnitine esters, free CoA and acetyl-CoA in normal sheep tissues

The results in Table 1 show that acetylcarnitine constitutes a relatively small portion of the total acid-soluble carnitine in all tissues shown except the heart, where it represents approx. 25%. Acid-insoluble carnitine (long-chain fatty-acyl carnitine esters) constitutes a very minor fraction of the total carnitine in all the sheep tissues examined. The total carnitine content of the four tissues examined had a wide range (Table 1). The concentrations of the various carnitine fractions in sheep liver recorded in Table 1 are comparable with those in the liver of sheep fed on lucerne *ad libitum*, as reported by Snoswell & Henderson (1970).

The concentrations of acetyl-CoA are approximately equal to the concentrations of free CoA in all four sheep tissues (Table 1) and the acid-soluble CoA content of these tissues decreases in the order liver > kidney > heart > skeletal muscle.

The carnitine and CoA concentrations in different muscles were found to vary and also in the same muscle from different species, e.g. M. sternothyreoidus from Merino sheep was found to contain 3.3 and 8390nmol of acid-soluble CoA and total acid-soluble carnitine/g wet wt. respectively, whereas the same muscle from Suffolk sheep contained 9.5 and 2260nmol/g wet wt. respectively. M. biceps femoris from Merino sheep contained 2.3 and 12900nmol/g wet wt. of acid-soluble CoA and total acid-soluble carnitine respectively (Table 1). These results, together with those for M. biceps femoris for Merino lambs (Table 2), suggest an inverse relationship between CoA and carnitine concentrations in sheep muscles.

Free carnitine, carnitine esters, free CoA and acetyl-CoA in lamb tissues

The concentration of total acid-soluble carnitine in the liver of lambs (Table 2) is comparable with that of adult sheep (Table 1) although the proportion of acetylcarnitine is considerably greater in the lamb. Similarly the lamb liver contains a greater proportion of acid-insoluble carnitine (compare Table 2 with Table 1), which undoubtedly reflects the milk diet of the lambs. The total acid-soluble carnitine content of skeletal muscle in the lamb (Table 2) is significantly ($P<0.01$) less than that of the adult sheep (Table 1), being only about 40% of the adult value. The concentrations of acetyl-CoA and free CoA in the lamb liver (Table 2) are very similar to those of the adult sheep (Table 1), but the total concentration of acid-soluble CoA in lamb skeletal muscle is about 2.5 times ($P<0.05$) that of the adult sheep.

Subcellular distribution of carnitine, CoA, carnitine acetyltransferase and acetate thiokinase in sheep tissues

The subcellular distribution of these metabolites and enzymes, together with the marker enzymes, glutamate dehydrogenase and lactate dehydrogenase, in sheep liver is shown in Table 3. As it has been reported that sheep liver is rather difficult to fractionate (Taylor *et al.*, 1971), the fact that 96% of the mitochondrial marker glutamate dehydrogenase, was found in the mitochondrial fraction indicates a very satisfactory fractionation.

Table 2. *Free carnitine, carnitine esters, free CoA and acetyl CoA in liver and skeletal muscle of lambs*

Merino lambs 5 to 16 days old were bottle-fed on reconstituted skim-milk powder with vitamin supplement. Tissue samples were prepared and assayed as described in Table 1. Results are means ± S.E.M. for four animals.

	Concentrations (nmol/g wet wt.)					
Tissue	Acetylcarnitine	Free carnitine	Total acid-soluble carnitine	Acid-insoluble carnitine	Acetyl-CoA	Free CoA
Liver	35 ± 24	86 ± 18	153 ± 11	10 ± 1	46 ± 3	51 ± 4
Skeletal muscle (M. biceps femoris)	175 ± 43	3590 ± 585	4780 ± 456	33 ± 1	2.8 ± 0.2	3.3 ± 0.6

Table 3. *Subcellular distribution of carnitine, CoA, acetate thiokinase and carnitine acetyltransferase in sheep liver*

Homogenates and subcellular fractions of sheep liver were prepared and assayed as described in the text. The values are means ± S.E.M. of four experiments. N.D., non-detectable.

Metabolite or enzyme	Enzyme activity in homogenate (nmol/min per ml)	Metabolite concentration in homogenate (nmol/ml)	% of activity or concentration in homogenate		
			Cytosol	Mitochondria	Microsomal fraction
Free CoA + acetyl-CoA		8.6 ± 1.1	48 ± 3	49 ± 4	2 ± 1
Total acid-soluble carnitine		9.2 ± 2.4	96 ± 2	N.D.	N.D.
Acetate thiokinase	13 ± 3.1		90 ± 1	8 ± 1	N.D.
Carnitine acetyltransferase	122 ± 12		6 ± 5	96* ± 9	N.D.
Glutamate dehydrogenase	3790 ± 87		2 ± 1	96 ± 3	3 ± 1
Lactate dehydrogenase	5610 ± 892		98 ± 2	2 ± 1	2 ± 1

* Whole mitochondrial suspensions (i.e. not previously exposed to 0.1% Triton X-100 in hypo-osmotic sucrose) contained < 10% of this activity.

Table 4. *Subcellular distribution of carnitine, CoA, acetate thiokinase and carnitine acetyltransferase in sheep kidney cortex*

Homogenates and subcellular fractions of sheep kidney cortex were prepared and assayed as described in the text. The values are means ± S.E.M. of four experiments. N.D., non-detectable.

Metabolite or enzyme	Enzyme activity in homogenate (nmol/min per ml)	Metabolite concentration in homogenate (nmol/ml)	% of activity or concentration in homogenate		
			Cytosol	Mitochondria	Microsomal fraction
Free CoA + acetyl-CoA		4.5 ± 0.4	23 ± 4	56 ± 4	N.D.
Total acid-soluble carnitine		50 ± 12	101 ± 1	2 ± 1	N.D.
Acetate thiokinase	70 ± 5		32 ± 1	68 ± 1	3 ± 1
Carnitine acetyltransferase	241 ± 51		N.D.	97* ± 1	N.D.
Glutamate dehydrogenase	4220 ± 753		8 ± 1	87 ± 1	6 ± 1
Lactate dehydrogenase	24500 ± 3990		94 ± 1	5 ± 1	2 ± 1

* Whole mitochondrial suspensions (i.e. not previously exposed to 0.1% Triton X-100 in hypo-osmotic sucrose) contained < 10% of this activity.

Acid-soluble CoA (free CoA plus acetyl-CoA) was distributed equally between the mitochondrial and cytosol fractions (Table 3) whereas the carnitine was present solely in the cytosol, as it was in all tissues examined (see also Tables 4 and 5). In kidney cortex only approx. 25% of the acid-soluble CoA was present in the cytosol (Table 4).

Determination of the degree of acetylation of the CoA in the various subcellular fractions of liver and kidney cortex is meaningless, as this may change during isolation of the fractions. In the present work this change did occur, especially in the kidney cortex; thus only values for free CoA plus acetyl-CoA were used. It might even be argued that CoA may move from one subcellular compartment to another during the fractionation procedure. Skrede & Bremer (1970)

have shown there is considerable loss of CoA from rat liver mitochondria that were incubated at 30°C for 20min. However, at 0°C in homogenizing medium the mitochondrial CoA is apparently stable for several hours (J. Bremer, personal communication); thus any movement of CoA during fractionation would seem unlikely.

Carnitine acetyltransferase was mainly confined to the mitochondria in sheep liver (Table 3) and in kidney cortex (Table 4). Also, over 90% of the activity of this enzyme in the mitochondria was latent, as the activity measured in whole mitochondria was less than 10% of that in preparations disrupted in 0.025M-sucrose containing 0.1% Triton X-100.

About 90% of the acetate thiokinase was present in the cytosol in sheep liver (Table 3) but only 30% was present in this fraction in kidney cortex (Table 4).

Owing to the difficulties in preparing subcellular fractions from sheep heart and skeletal muscle, as outlined in the Experimental section, the amounts of metabolites and enzymes in 'press' fractions of these were related to those in whole homogenates. These 'press' fractions were considered to be relatively clean cytosol fractions as they contained very little citrate synthase, the mitochondrial matrix marker (Table 5). To express the results obtained with the 'press' fractions quantitatively and to compare them with those for homogenates all concentrations and activities were related to the amount of lactate dehydrogenase, the cytoplasmic marker enzyme. The results presented in Table 5 show that the amount of lactate dehydrogenase in the 'press' fractions was 10 times that in the corresponding 1-in-10 homogenates. On this basis the cytosol contained very little acid-soluble CoA, acetate thiokinase or carnitine acetyltransferase in either sheep heart or skeletal muscle (Table 5). It is assumed that these compounds and enzymes are found in the mitochondria of these tissues. In support of this assumption, mitochondrial fractions prepared from these tissues, although having lost some of their contents during homogenization, showed very much higher specific activities than those found in microsomal fractions from the corresponding tissues. Again approx. 90% of the carnitine acetyltransferase activity in such mitochondria prepared from heart and skeletal muscle was latent.

The loss of acetate thiokinase into the cytosol fraction during homogenization paralleled the release of citrate synthase. The latter enzyme is present in the matrix of mitochondria (Tubbs & Garland, 1968) and it is thus assumed that the acetate thiokinase is present in the matrix fraction of the sheep muscle tissues.

Free carnitine, carnitine esters, free CoA and acetyl-CoA in the tissues of alloxan-diabetic sheep

The amounts of acetylcarnitine in the liver, kidney cortex, heart and skeletal muscle of alloxan-diabetic sheep (Table 6) are considerably greater than in the same tissues of normal sheep (Table 1). Also, although the total acid-soluble carnitine concentrations of heart and skeletal muscle are the same in alloxan-diabetic sheep and normal sheep, the concentration of this carnitine fraction is approx. 7 times as great in the liver of alloxan-diabetic sheep ($P<0.001$) and nearly twice as much in the kidney ($P<0.05$).

An even more dramatic increase in the total acid-soluble carnitine fraction of liver was observed when insulin was withdrawn from an alloxan-diabetic

Table 5. *Carnitine, CoA, acetate thiokinase and carnitine acetyltransferase in homogenates and 'press' fractions of sheep heart and skeletal muscle, relative to lactate dehydrogenase activities*

Tissue homogenates and 'press' fractions were prepared and assayed as described in the text. The values shown are for 3 animals, and are in nmol/ml or nmol/min per ml divided by the appropriate lactate dehydrogenase activity. N.D., non-detectable. The values in parentheses refer to amounts in 'press' fractions expressed as percentages of those for the homogenates.

Fraction	Lactate dehydrogenase (μmol/min per ml)	Total acid-soluble carnitine	Acetyl-CoA plus free CoA	Acetate thiokinase	Carnitine acetyltransferase	Citrate synthase
Heart						
Homogenate	18.8 ± 1.96	7.28	0.095	3.78	64.5	568
'Press'	214 ± 20	7.32	0.00635	0.169	0.503	15.8
			(7)	(5)	(1)	(3)
Skeletal muscle						
Homogenate	39.7 ± 5.05	7.15	0.0031	0.52	3.27	32.7
'Press'	403 ± 36.1	7.13	N.D.	0.04	N.D.	0.084
				(7)		(<1)

Table 6. *Free carnitine, carnitine esters, free CoA and acetyl-CoA in tissues of alloxan-diabetic sheep*

Merino wethers were given an intravenous injection of alloxan (60mg/kg body wt.) 3 days before slaughter. Tissue samples and assays were as described in Table 1. Results are means ± S.E.M. for three animals.

	Concentrations (nmol/g wet wt.)					
Tissue	Acetylcarnitine	Free carnitine	Total acid-soluble carnitine	Acid-insoluble carnitine	Acetyl-CoA	CoA
Liver	461 ± 24	203 ± 15	886 ± 52	2 ± 2	81 ± 9	88 ± 18
Kidney cortex	509 ± 121	274 ± 73	993 ± 98	2 ± 1	49 ± 8	37 ± 6
Heart	1450 ± 168	1520 ± 361	3270 ± 440	96 ± 26	11 ± 2	9 ± 2
Skeletal muscle	4530 ± 471	7200 ± 574	12800 ± 820	34 ± 11	2.3 ± 0.6	1.7 ± 0.6

sheep that was previously stabilized by continuous intravenous infusion of insulin. Samples of liver were removed surgically. The first one contained 66nmol/g wet wt. of total acid-soluble carnitine, but 24h after the withdrawal of insulin this value, measured in a second sample of liver, was 2210nmol/g wet wt. Similar surgical removal of two liver samples from a normal control sheep showed that no variation in total acid-soluble carnitine was caused by the surgery alone.

As in the normal sheep, acid-insoluble carnitine is only a very minor fraction of the total carnitine in all four tissues of the alloxan-diabetic sheep (Table 6), although the increase in this fraction in the heart of the alloxan-diabetic sheep may be significant ($P=0.05$).

The concentrations of acetyl-CoA are approximately the same as the concentrations of free CoA in all four tissues of the alloxan-diabetic sheep (Table 6). The concentration of acid-soluble CoA in the liver of alloxan-diabetic sheep (Table 6) is approximately twice ($P<0.01$) that of the same tissue in the normal sheep (Table 1).

Discussion

The wide range in the concentration of total acid-soluble carnitine in the four tissues examined is very prominent. This range is much greater than that reported for other species, e.g. rat skeletal muscle has only about twice the total acid-soluble carnitine concentration of rat liver (Pearson & Tubbs, 1967). A similar range to that of the rat tissues is observed in the rabbit and a fivefold concentration difference between the liver and skeletal muscle was found in the dog (Fraenkel & Friedman, 1957). The total acid-soluble carnitine concentration of sheep skeletal muscle is about 20 times greater than that of rat skeletal muscle (Pearson & Tubbs, 1967). This difference is not due to the use of different assay procedures. We find the total acid-soluble carnitine content of rat liver and skeletal muscle is 232 and 634nmol/g wet wt. respectively (mean values), which is comparable with the figures of 296 and 627nmol/g wet wt. respectively (mean values) reported by Pearson & Tubbs (1967).

The acid-soluble CoA concentration is greatest in sheep liver and least in sheep skeletal muscle (of the tissues examined); this is in contrast with the variation observed for total acid-soluble carnitine concentrations. Thus in these sheep tissues there appears to be a reciprocal relationship between the concentration of total acid-soluble carnitine and of acid-soluble CoA. A similar gradation in CoA content of these four tissues has been observed in other species (Glock, 1961), but no obvious relationship between carnitine and CoA concentrations can yet be inferred for any other species. This reciprocal relationship between the concentration of total acid-soluble carnitine and acid-soluble CoA may be observed in a single sheep tissue: the skeletal muscle of lamb has only 40% of the total acid-soluble carnitine concentration of that of the adult sheep but 2.5 times the concentration of acid-soluble CoA (Table 2). This inverse relationship between the concentration of acid-soluble CoA and total acid-soluble carnitine was also seen when various muscles were compared and would suggest that the synthesis and/or degradation of carnitine and CoA in these tissues must be carefully integrated. This inverse relationship also emphasizes the role of carnitine acetyltransferase, particularly in muscle tissues of the sheep. The enzyme is localized in the mitochondria of all the four tissues examined and most of the activity is latent, i.e. only released after exposure of the mitochondria to hypo-osmotic solutions plus detergent. A similar situation occurs with the carnitine acetyltransferases of rat, guinea-pig, goat and sheep liver mitochondria (Barker *et al.*, 1968) and it was concluded that the enzyme is mainly membrane-bound and is not available to acetyl-CoA outside the mitochondria.

The high concentrations of carnitine in the sheep

muscle tissues examined suggest that the carnitine in these tissues, in conjunction with carnitine acetyltransferase, might be involved in acetate oxidation, since acetate is metabolized mainly in the extrahepatic tissues of the sheep (Mayfield *et al.*, 1966). However, the acetate thiokinase of sheep heart and skeletal muscle is predominantly present in the mitochondrial matrix, and the activity of this enzyme in sheep skeletal-muscle mitochondria is low (Cook *et al.*, 1969). Thus, it seems unlikely that carnitine acetyltransferase plays a significant role in acetate oxidation by the heart and skeletal muscle of sheep.

It would seem much more likely that the role of carnitine acetyltransferase and carnitine is in a buffer system, as suggested by Pearson & Tubbs (1967) after their studies with perfused rat hearts. In sheep muscle tissues the main reactions generating acetyl-CoA are localized in the mitochondria, i.e. the fatty acid oxidation system and the acetate thiokinase reaction. Also, the present studies show that CoA is virtually confined to the mitochondria and carnitine to the cytoplasm. Yates & Garland (1966) have shown, by using rat liver mitochondria, that the inner mitochondrial membrane is impermeable to carnitine. Thus the carnitine acetyltransferase located in the inner mitochondrial membrane would appear to act as a vectorial transferase that reacts with CoA and acetyl-CoA on the inside and carnitine and acetylcarnitine on the outside. A similar role in a vectorial transfer system has been suggested by Yates & Garland (1970) for the membrane-bound carnitine palmitoyltranferase. The net effect would be to remove acetyl groups from the mitochondria in times of increased 'acetyl pressure', i.e. during increased fatty acid oxidation, and transfer these out of the mitochondria to be 'stored' as acetylcarnitine, thereby relieving pressure on the CoA system. The amount of acetyl groups 'stored' in such a way is considerable, being about 6g in a 50kg sheep, if the total muscle mass is taken into account. In support of this concept the results presented here for alloxan-diabetic sheep indicate a marked increase in the acetylcarnitine concentration of skeletal muscle, but relatively little change in the ratio [acetyl-CoA]/[free CoA] compared with that of the normal animal.

For enzyme to function in a buffer system *in vivo* the reactants and products should be near or at equilibrium. Fritz *et al.* (1963) have calculated the apparent equilibrium constant for the reaction:

Acetyl-L-carnitine + CoA $\rightleftharpoons$ acetyl-CoA + L-carnitine

to be 0.6 at pH7.0 by using a partially purified enzyme from pig heart. From the results in Table 1, $K_{app.}$ = [acetyl-CoA][L-carnitine]/[acetyl-L-carnitine]-[CoA] = 1.9 for sheep heart and 4.2 for skeletal muscle. In alloxan-diabetes (Table 6) the values are 1.3 and 2.1 respectively. Thus the reaction in these sheep muscles *in vivo* appears to be near equilibrium, assuming that the carnitine acetyltransferase in these tissues is accessible to intramitochondrial CoA and acetyl-CoA and to cytoplasmic carnitine and acetylcarnitine. However, the situation in sheep liver and kidney cortex is considerably more complex, as in these tissues CoA is present both inside and outside the mitochondria.

The approximately sevenfold rise in total acid-soluble carnitine content of sheep liver in alloxan-diabetes is striking. Marked increases in the individual fractions of the total acid-soluble carnitine, i.e. free carnitine and acetylcarnitine also occur (compare Table 6 with Table 1). A significant but less pronounced (twofold) rise in acid-soluble CoA also occurs in sheep liver in alloxan-diabetes, in contrast with the rat, where there is little change (Tubbs & Garland, 1964). The [free CoA]/[acetyl-CoA] ratio in normal sheep liver is approx. 1:1 (Table 1), which is considerably lower than the ratio of 3–4:1 in rat liver (Allred & Guy, 1969; Bode *et al.*, 1970). This ratio remains at approx. 1:1 in the liver of alloxan-diabetic sheep (Table 3); thus, it seems that the CoA system in sheep liver has only a limited capacity to accommodate the increased 'acetyl pressure' presumably arising in alloxan-diabetes because of the increased fatty acid oxidation. This limitation in the CoA system of the liver appears to be compensated by the marked rise in carnitine concentration that occurs during alloxan-diabetes in sheep.

The other feature of the results obtained with alloxan-diabetic sheep is that the acid-insoluble carnitine fraction constituted only a very minor proportion of the total carnitine in any tissue examined. There was a significant increase in this fraction in the heart in alloxan-diabetic sheep, but even in this tissue it was only 3% of the total carnitine. This contrasts with the situation in the rat, where the amount of acid-insoluble carnitine fraction increases markedly in alloxan-diabetes and is a very significant proportion of the total carnitine (Bøhmer *et al.*, 1966; Pearson & Tubbs, 1967).

The very large amounts of carnitine in the skeletal muscle of sheep (over 2mg/g wet wt.) indicate an important quantitative role for carnitine in the overall metabolism of the sheep, as the muscles are the largest tissue mass of an animal. Fraenkel & Friedman (1957) reported very high concentrations of carnitine in commercial beef extracts. A high carnitine output in the milk of dairy cows, and particularly of acetylcarnitine in ketotic cows, has been observed (Erfle *et al.*, 1970). Thus it would appear that carnitine may have a particularly important role in the metabolism of ruminant animals, especially under conditions of metabolic stress.

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