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Changes in Free and Bound Carnitine in Muscle with Maximal Sprint Exercise in the Thoroughbred Horse

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Summary

To study the changes in carnitine in muscle and blood with sprint exercise, Thoroughbreds performed a 2-min treadmill test at 12 m/sec, and field gallops of 800 m and 2000 m. Biopsies of the middle gluteal and plasma samples taken before and after exercise and during recovery were analyzed for total free and acetylcarnitine, and other metabolites. Mean muscle total carnitine content at rest was 31.0 ± 2.0 mmol/kg d.m. Approximately 80% was free carnitine and 15% acetylcarnitine. Acylcarnitine was estimated at 5–10%.

Both the field and treadmill exercise resulted in the marked accumulation in muscle of lactate (100 mmol/kg d.m. or more) and glycerol-3-P. Accumulation of glycerol was much less, but as in other studies continued to rise during recovery. Sprint exercise (treadmill) did not affect total carnitine, but free carnitine fell from 24.3 ± 4.1 to 9.6 ± 3.2 mmol/kg d.m. (60% decrease, $P < 0.01$) with an almost equivalent rise in acetylcarnitine. Recovery of free and acetylcarnitine took approximately 30 min. After 70 min recovery muscle total carnitine was significantly higher than at rest indicating uptake from plasma. Similar findings were obtained in the field studies.

It is concluded that in sprint exercise above the lactate threshold in the Thoroughbred horse, carnitine assists in the regulation of the acetylCoA/CoA ratio by buffering excess acetylCoA production.

Index terms: CoA; acetylCoA; aerobic metabolism.

Introduction

Carnitine (γ -trimethylamino- β -hydroxybutyrate) is a low molecular weight, highly soluble compound with a structure similar to the amino acids. The occurrence of carnitine seems to be nearly ubiquitous in animals, many microorganisms and plants, although its concentration varies widely between species and tissues. The highest levels in mammals are found in heart and skeletal muscle tissue (Bremer 1983).

The primary role of carnitine is as a cofactor to the transport of fatty acids (FA), and in particular long chain FA, across the inner mitochondrial membrane (Fritz 1955). The

sequence of reaction-events involves initial formation of acylcarnitine from acylCoA in the cytoplasm, transport across the membrane, and regeneration of the carnitine with release of acylCoA into the inner matrix. The acylCoA is then available for use as an energy source via β -oxidation. Additional roles suggested for carnitine include the transport into the mitochondria of acetyl units produced by the peroxisomes during shortening of medium and long chain FA (Bremer 1983), and in the buffering of the inner-mitochondrial acetylCoA/CoA ratio (Pearson and Tubbs 1967; Alkonyi *et al.*, 1975; Brass and Hoppel 1980). On this basis carnitine has potential to play an important regulatory role not only in FA oxidation, but also in aerobic metabolism in general.

Because of the involvement of carnitine in lipid metabolism the emphasis of most investigations has been on correlating this with fat utilization during endurance exercise (Cederblad *et al.*, 1974; Costill *et al.* 1979).

Only a few studies (and none of these in the horse) have been undertaken to describe the actual changes in carnitine derivatives in muscle under different exercise conditions. Recently Lennon *et al.* (1983) reported an increase in acylcarnitine derivatives in the vastus lateralis in man in response to a 40 min bicycle exercise at 55% of maximal aerobic capacity. The increase was greater in well trained than moderately trained subjects. A rise in the concentration of acylcarnitine in plasma was also found in this study, which the authors suggested was due to a loss from muscle.

The present study into the fate of carnitine during sprint exercise was part of a broader investigation into the role of carnitine in the developing and competitive horse. Interest in this area was aroused by the finding that the concentration of glycerol in both muscle and blood in the horse are greatly increased during recovery from maximal sprint exercise (Snow *et al.*, 1985; Harris *et al.*, 1987) indicating possible breakdown of triglycerides. The amounts of FA liberated at this time, however, would almost certainly be far in excess of the cells energy needs, raising the possibility that they may be temporarily stored as esters of carnitine.

Materials and Methods

The study consisted of two parts, an investigation into the effects of treadmill exercise on carnitine levels and an earlier field study for comparison.

Treadmill study. The findings presented in this paper were obtained from five trained Thoroughbred geldings (SM, KJ, HR, JW, SL) used in the study of Marlin *et al.*, (1987) reported elsewhere in these Proceedings. Each horse performed a standard exercise on a treadmill (Sato, Sweden) consisting of 4 min walking (1.6 m/sec), 4 min trotting (3.2 m/sec) and a 2 min gallop (12 m/sec). Horses worked against an incline of 5°. The exercise was followed by a 70-min recovery period during which the horses remained stationary on the treadmill, the incline of this having been lowered to 0°. Muscle samples of the middle gluteal were taken as described in Marlin *et al.* (1987) before exercise, immediately at the end of the 2 min gallop, and after 10, 30 and 70 min recovery. Plasma was obtained by centrifugation of 10 ml blood samples collected into tubes containing lithium-heparin as anticoagulant.

Field study. Samples were obtained from five trained Thoroughbreds (MR, RO, LO, PE, JW) after maximal gallops of 800 and/or 2000 m. The animals were part of a study described in Harris *et al.* (1987). The horses were walked and trotted approximately 2500 m from the stables to the track where the exercise took place. Following

a short warm-up canter of 2000 m, four of the horses (MR, RO, LO, PE) were galloped maximally over a distance of 800 m on a wood chip track (mean time $56.1 \pm$ SD 2.4sec), and two (RO, JW) over a distance of 2000 m on an all-weather sand track (gallop times, 140 and 162sec). Following exercise the horses were walked during a 60 min recovery period, except when being sampled. Muscle samples of the middle gluteal were taken according to Harris *et al.* (1987), but only those obtained after the warm-up canter, within 2 min of the gallop, and at the end of 10 and 60 min of recovery were analyzed for carnitine. In two cases pre-gallop samples were taken before the warm-up canter due to excitement.

Biochemical analyses. Muscle biopsy samples were freeze-dried, dissected free of connective tissue and blood, and then powdered. Free carnitine and acetylcarnitine were assayed in 5–10 mg of muscle powder extracted with 0.5 mol/l perchloric acid (PCA) and neutralized with 2.1 mol/l KHCO_3 (Harris *et al.*, 1974). In the field studies, total carnitine was estimated as total acid soluble carnitine. For this carnitine derivatives in 50 μl of the neutralized extracts were hydrolyzed to free carnitine for 1 h at 60°C with 5 μl of 2 mol/l KOH and then neutralized with 10 μl 1 mol/l HCl. In the treadmill studies, sufficient muscle was available as to allow total carnitine to be determined by direct alkaline hydrolysis. For this muscle powder was digested in 0.5 mol/l KOH for 1 h at 60°C. Extracts were neutralized with 1 mol/l HCl to give a final dilution of 10 mg/ml. Other metabolites (phosphocreatine, creatine, glycerol-3-P, lactate and glycerol) were determined on the neutralized PCA extracts (Harris *et al.*, 1974).

For assay of free carnitine in plasma, 450 μl of plasma were extracted with 300 μl of 0.5 mol/l PCA. After centrifugation 500 μl of the supernatant were neutralized with 100 μl 2.1 mol/l KHCO_3 . Carnitine was assayed enzymatically using the DTNB method of Marquis and Fritz (1964). The volume of muscle extract used in the assays was 50 in 300 μl , whereas after treatment with KOH the volume was reduced to 25 in 300 μl to decrease interference by thiol groups in the extract. For assay of plasma free carnitine, 100 μl of extract in 300 μl was used.

Acetylcarnitine was assayed in neutralized extracts by the method of Pearson *et al.*, (1974), using acetylCoA as standard, a sample volume of 50 μl in 300 μl and a wavelength of 334 nm. Throughout results are presented as mean values \pm SD.

Results

The concentration of free carnitine in plasma was found to be approximately 1/300 of that in muscle. From this, the contribution of blood borne carnitine to the apparent muscle content was calculated to be less than 1% even in samples taken after exercise where the blood content is greatly increased. Muscle carnitine contents could, therefore, be adjusted to a constant value of total creatine to allow for variations in non-muscle constituents within the biopsies. At rest mean total carnitine of all horses in the treadmill and field studies was 32.3 ± 6.5 mmol/kg dry muscle (d.m.) ($n = 7$).

Treadmill study. At the start of the treadmill study free carnitine accounted for approximately 80%, acetylcarnitine for 15% and acylcarnitine for 5–10% of total carnitine (Table 1). The 2-min treadmill exercise did not affect total carnitine, but resulted in a significant fall in free carnitine ($P < 0.01$) from 24.3 ± 4.1 to 9.6 ± 3.2 mmol/kg d.m., a decrease of 60%. Almost all of the reduction in free carnitine with exercise could be attributed to formation of acetylcarnitine. Recovery of free carnitine to resting

TABLE 1. Mean muscle content (\pm SD) of carnitine and derivatives, glycerol-3-P, glycerol and lactate, and the concentration of free carnitine in plasma, before and after 2 min treadmill exercise at 12 m/sec.

	Rest	RECOVERY			
		0 min	10 min	30 min	70 min
<i>MUSCLE</i> mmol/kg d m					
Total Carnitine	31.0 \pm 2.0 (3)*	32.8 \pm 8.2 (3)	37.4 (2)	34.8 \pm 12.7 (3)	37.8 \pm 4.0 (3)
Free Carnitine	24.3 \pm 4.1 (4)	9.6 \pm 3.2 (5)	16.9 \pm 3.5 (5)	25.3 \pm 7.2 (5)	28.1 \pm 4.9 (5)
Acetylcarnitine	4.1 \pm 3.0 (4)	17.9 \pm 3.2 (4)	10.5 \pm 2.7 (4)	3.0 \pm 3.0 (3)	2.1 (2)
^a Acylcarnitine ²⁺	2.6	5.3	10.0	6.5	7.6
Glycerol-3-P	3.4 \pm 2.4 (5)	18.8 \pm 5.6 (5)	8.7 \pm 1.6 (7)	6.6 \pm 2.3 (5)	3.1 \pm 1.8 (5)
Glycerol	0.2 \pm 0.2 (5)	2.5 \pm 0.2 (5)	11.2 \pm 2.4 (5)	12.8 \pm 3.2 (5)	5.7 \pm 1.9 (5)
Lactate	10.0 \pm 5.8 (5)	112.1 \pm 27.5 (5)	86.2 \pm 17.7 (5)	43.1 \pm 17.6 (5)	14.9 \pm 2.2 (5)
<i>PLASMA</i> μ mol/l					
Free Carnitine	23.4 \pm 3.7 (4)	25.3 \pm 8.1 (4)	19.0 \pm 4.3 (4)	19.0 \pm 3.6 (4)	19.8 \pm 3.6 (4)

*Numbers of horses are given in parenthesis

^aAcylcarnitine was calculated from the difference between total carnitine and the sum of free and acetylcarnitine

levels took approximately 30 min, the changes in free carnitine being mirrored by an equivalent fall in acetylcarnitine. At the end of 70 min recovery both free carnitine and total carnitine were significantly ($P < 0.05$) higher than at rest. Mean changes were 4.4 ± 2.5 and 6.0 ± 2.0 mmol/kg d.m., respectively. No direct measurements of acylcarnitine were made in the present study. However, from differences between total carnitine and free carnitine plus acetylcarnitine there did not appear to be any major increase with exercise, although some small rise may have occurred during recovery. The concentration of plasma free carnitine was not affected immediately by the exercise, but there was a trend towards lower concentrations during the 70 min standing period.

Two min exercise resulted in the marked accumulation of the two anaerobic end products glycerol-3-P and lactate to 18.8 ± 5.6 and 112.1 ± 27.5 mmol/kg d.m., respectively. During recovery there was a rapid disappearance of both metabolites with normal values being attained after 70 min (Marlin *et al.*, 1987). In contrast, muscle glycerol showed only a small increase with exercise, but continued to rise during the first 30 min of recovery.

Field studies. Prior to the 800 m and 2000 m gallops, free carnitine in muscle accounted for approximately 85% of total acid soluble carnitine (Table 2), the remainder consisting principally of acetylcarnitine. Actual free carnitine concentrations following the 2000 m warm-up canter were generally lower than the resting concentrations observed in the treadmill study.

Immediately after both gallops, free carnitine was decreased by approximately 40%. The acetylcarnitine concentration increased markedly at this time, with recovery following the same trend as in the treadmill study. Combining data from the two field studies, total carnitine was again significantly increased ($P < 0.05$) at the end of the 60 min recovery compared to pre-exercise concentrations (mean change 5.47 ± 4.04).

Glycerol-3-P and lactate peaked at 14.4 ± 1.8 and 124.2 ± 42.6 mmol/kg d.m.

TABLE 2. Mean muscle content (\pm SD) of carnitine and derivatives, glycerol-3-P, glycerol and lactate before and after a maximal gallop of 800 or 2000 m in the field.

	800 m Gallop				200 m Gallop		
	Pre	RECOVERY		60 min	Pre	← RECOVERY →	
		0 min	10 min			0 min	10 min
<i>MUSCLE</i> mmol/kg d.m.							
Total Carnitine (acid soluble)	21.2 \pm 4.0 (4)*	22.6 \pm 7.0 (4)	25.3 \pm 3.5 (4)	26.9 \pm 2.2 (4)	21.6 (2)	16.1 (2)	21.5 (2)
Carnitine	17.8 \pm 2.7 (4)	13.9 \pm 2.2 (4)	21.7 \pm 2.6 (4)	24.9 \pm 2.2 (4)	18.6 (2)	10.8 (2)	16.7 (2)
Acetylcarnitine	3.2 \pm 2.8 (4)	13.0 \pm 7.6 (4)	7.1 \pm 3.6 (4)	2.1 \pm 1.8 (4)	7.8 (2)	15.9 (2)	4.1 (2)
Glycerol-3-P	2.7 \pm 0.7 (4)	14.4 \pm 1.8 (4)	10.7 \pm 0.5 (4)	3.2 \pm 0.8 (4)	3.5 (2)	19.4 (2)	9.8 (2)
Glycerol	0.2 \pm 0.2 (4)	1.7 \pm 0.9 (4)	8.7 \pm 2.9 (4)	5.7 \pm 3.8 (4)	0.4 (2)	1.8 (2)	8.0 (2)
Lactate	14.0 \pm 5.7 (4)	124.2 \pm 42.6 (4)	77.9 \pm 34.9 (4)	16.4 \pm 6.9 (4)	19.8 (2)	171.0 (2)	135.5 (2)

*Numbers of horses are given in parenthesis.

after the 800 m sprint. Even higher concentrations of these metabolites were measured at the end of the 2000 m gallop, with glycerol-3-P reaching 19.4 and lactate 171.0 mmol/kg d.m. In both cases glycerol concentrations continued to rise during recovery, returning to near resting levels by 60 min.

Discussion

As far as we are aware, the present findings on carnitine are the first to be reported in the horse. At rest the muscle content of total carnitine in the five Thoroughbreds presented in Table 1 was intermediate, between values of 18–24 mmol/kg d.m. for the vastus lateralis of man (Cederblad *et al.*, 1974; Lennon *et al.*, 1983) and 40+ mmol/kg d.m. in skeletal muscle of ruminants (Snoswell and Henderson 1980; Snoswell and Koundakjian 1972). Plasma concentrations, on the other hand, were lower than a mean of 33.4 μ mol/l reported in a group of 28 trained human volunteers (Lennon *et al.* 1983).

Clearly the major finding in this study was the decrease in free carnitine by the end of the sprint exercise, and the near stoichiometric increase in acetylcarnitine. In one horse (HR, treadmill study) the decrease in free carnitine amounted to 20.8 mmol/kg d.m. or 72% of the rest content, whilst acetylcarnitine accumulated was 20.2 mmol/kg d.m. These figures describe the changes in carnitine as seen in muscle one min after termination of the treadmill exercise and 1.5 to 2.5 min after the field exercise, which were the times taken for deceleration and sampling of the horses. Conceivably, the acetylcarnitine found is formed only during the deceleration phase. Alternatively, the acetylcarnitine is accumulated throughout the exercise, and, in view of the rapid fall in this during the first 10 min of recovery, immediate post-exercise contents could well be higher than those shown in Tables 1 and 2.

Judging from the increases in muscle lactate the exercise in both the field and treadmill studies was severe, with metabolic rates clearly in excess of the lactate threshold. Under these conditions carnitine appears to be functioning as a buffer to excess acetylCoA the majority of which, in view of the intensity and brevity of the exercise, was most probably derived from pyruvate decarboxylation. The ability of carnitine to act as an acetyl group buffer has been recognized for some years and has been demonstrated in rat heart (Pearson and Tubbs, 1967), blowfly flight muscle (Childress *et al.*, 1966) frog and rat skeletal muscle (Alkonyi *et al.*, 1975; Carter *et al.*, 1981). This role of carnitine is mediated by the action of the carnitine-acetyltransferase (CAT) (E.C.2.3.1.7) the activity of which in skeletal muscle is high (Marquis and Fritz 1964). This enzyme catalyzes the transfer of the acetyl unit from acetylCoA to acetylcarnitine. In blowfly muscle CAT is located mainly within the mitochondria and only about 10% in the cytoplasm (Childress *et al.*, 1966). In addition to enabling carnitine to act as an intramitochondrial acetyl group buffer, CAT is also believed to play a role in the transport of acetyl groups from the mitochondria into the cytoplasm for use in FA synthesis, and in the intramitochondrial translocation of acetyl groups between different acetylCoA pools.

The ability of carnitine to buffer acetyl groups confers a number of potential advantages upon cells functioning at or above their lactate threshold (Childress *et al.*, 1966). The possible benefits to the muscle cells of the maximally galloping racehorse are: 1)

By acting as an acceptor of acetyl units from acetylCoA, carnitine will help to maintain a viable pool of CoA under conditions where the rate of acetylCoA condensation with oxaloacetate is less than its rate of formation from pyruvate decarboxylation. This could be due to insufficient activity on the part of citrate synthase or to a lack of oxaloacetate. The regeneration of CoA and the maintenance of a free pool is essential if oxidation of oxoglutarate to succinate within the TCA is to continue. If this were stopped by lack of CoA the result would be a rapid fall in oxaloacetate which in the absence of a carnitine-acetyl-group buffer would further worsen the situation. 2) A marked lowering in the intramitochondrial pool of carnitine will limit the cells' ability to transport FA into the mitochondria and thus also the rate of FA oxidation (Bremer 1983). It is unlikely that the loss of this potential energy supply would affect performance under sprint conditions, but more importantly it will prevent the "flooding" of the mitochondrial matrix by acylCoA esters at a time when CoA is in short supply. 3) In addition to maintaining higher levels of CoA the accumulation of acetylcarnitine itself may be of benefit to the cells. It represents an additional "sink" for pyruvate and as an accumulation product of anaerobic metabolism is comparable to that of glycerol-3-P and alanine. The diversion of pyruvate to acetylcarnitine, when the cells are functioning above the lactate threshold, will offset its accumulation as lactate, an important consideration in the maximally galloping horse. As discussed by Childress *et al.* (1966) the acetylcarnitine itself provides a store of "active acetate" which is readily available for transacetylation back to acetylCoA. It is difficult to see how this could be of benefit to the sprinting horse where aerobic metabolism is already being used to the full, but if generated during an earlier warm-up exercise it could facilitate energy supply during the early stages of increased activity.

During the 70 min of recovery, muscle total carnitine showed a small but significant increase above the content at rest (Table 1). In fact most of this probably occurred during the first 10 min of recovery, since the site of carnitine synthesis is believed to be the liver rather than muscle, this clearly represents an uptake from plasma which showed a decrease in free carnitine at this time. These results are at variance with those of Lennon *et al.* (1983) who showed a decrease in the muscle total carnitine content, albeit with 40 min endurance exercise. At rest the concentration of free carnitine in muscle is approximately 300 times that in plasma indicating a highly active transport mechanism. We suggest that the fall in muscle in free carnitine with sprint exercise is the stimulus for a net influx of carnitine from plasma and that it is this that accounts for the rise in total. It remains to be seen how long the increase in total carnitine persists for after termination of the exercise.

As far as could be judged formation of acetylcarnitine accounted for virtually all of the decrease in free carnitine with exercise, and thus the formation of any additional pool of acylcarnitine must have been quantitatively much less. As in previous studies the small to moderate increase in muscle glycerol content with exercise was followed by a very much larger increase during recovery. It has earlier been suggested (Harris and Snow 1985; Harris *et al.* 1987) that muscle glycerol-3-P is the source of the rise in glycerol seen during recovery from sprint exercise rather than hydrolysis of triglyceride. The evidence for this rests on the equal and opposite changes seen in these two metabolites at this time. The failure to demonstrate in this study any comparable increase in acylcarnitine, which could act as a temporary store for fatty acids liberated in conjunction with the glycerol, would seem to further support this possibility.

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