17 Nimmo, M A and Snow, D H (1982) Time course of ultrastructural changes in skeletal muscle after two types of exercise J Appl Physiol 52, 910–913
18 Pexeider, T (1977) The role of buffer osmolarity in fixation for SEM and TEM Experientia 32, 806–814
19 Pierce, G N , Kutryk, M J B , Dhalla, K S , Beamish, R E and Dhalla, N S (1984) Biochemical alterations in heart after exhaustive swimming in rats J Appl Physiol 57, 326–331
20 Rose, R J , Hodgson, D R , Kelso, T B , McCutcheon, L J , Reid, T -A , Bayly, W M and Gollnick, P D (1988) Maximal O_2 uptake, O_2 debt and deficit, and muscle metabolites in Thoroughbred horses J Appl Physiol 64, 781–788
21 Schaper, J , Mulch, J , Winkler, B and Schaper, W (1979) Ultrastructural, functional, and biochemical criteria for estimation of reversibility of ischemic injury: a study on the effects of global ischemia on the isolated dog heart J Mol Cell Cardiol 11, 521–541
22 Terjung, R L , Klinkerfuss, G H , Baldwin, K M , Winder, W W and Holloszy, J O (1973) Effect of exercise on rat heart mitochondria Am J Physiol 225, 300–305
23 Thomas, D P and Marshall, K I (1988) Effects of repeated exhaustive exercise on myocardial subcellular structures Int J Sports Med 9, 257–260
24 Tomanek, R J and Banister, E W (1972) Myocardial ultrastructure after acute exercise stress with special reference to transverse tubules and intercalated discs Cardiovasc Res 6, 671–679
25 Warhol, E R , Siegel, A J , Evans, W J and Silverman, L M (1985) Skeletal muscle injury and repair in marathon runners after competition Am J Pathol 118, 331–339

Equine Exercise Physiology 3: 276–280, 1991

Carnosine Accounts for Most of the Variation in Physico-Chemical Buffering in Equine Muscle

D. A. SEWELL, R. C. HARRIS and M. DUNNETT

Department of Comparative Physiology, The Animal Health Trust, P O Box 5, Newmarket, Suffolk, CB8 7DW, England

ABSTRACT. The ability of muscle to buffer protons may be a key determinant of sprint performance. Buffering capacity determined by titration (Bm_{titr}, mmol H^+ kg^{-1} d.m.) between the pH limits of 7.1 to 6.5, carnosine content (carn, mmol kg^{-1} d.m.) and % fibre section area were measured in muscle taken from up to six different sites in the middle gluteal muscle of four Thoroughbred horses and one pony at post mortem. Bm_{titr} and carnosine were positively correlated to % type II fibre section area ($r=0.57$, $p<0.01$ and $r=0.75$, $p<0.001$ respectively). Multiple linear regression analysis was used to estimate the carnosine content of individual fibre types when present at 100%. Type I fibres appear to have a low carnosine content whilst type IIa and IIb fibres would appear to have a similar carnosine content. The variability in Bm_{titr} was accounted for almost exclusively through variability in the specific buffering of carnosine (Bm_{carn}) calculated from the Henderson-Hasselbach equation. Non-carnosine buffering ($Bm_{non\text{-}carn}$) was constant at all fibre compositions.

Key words Acidosis; horses; muscle fibres; middle gluteal.

INTRODUCTION

Sprinting mammals perform high intensity exercise of short duration resulting in the production and accumulation of large amounts of lactic acid. The energy providing pathways incur a rapid turnover of ATP, an accumulation of protons, and a decrease in intracellular pH. The buffering of protons occurs through the dynamic processes of bicarbonate exportation and proton loss into the blood, and within the muscle through phosphocreatine hydrolysis[8] and physico-chemical (static) buffering by proteins, dipeptides and inorganic phosphate. The ability of muscle to buffer protons may be a key determinant of sprint performance.[12]

The histidine dipeptides carnosine, anserine and ophidine have pKa values in the range 6.8 to 7.1, making them effective physico-chemical buffers over the normal physiological pH range. Based on their content in the muscle of various species, Davey[4] concluded that these dipeptides can contribute as much as 40% to the physico-chemical buffering in resting muscle. The higher content of histidine dipeptides in the muscles of animals exposed to prolonged periods of hypoxia, and those adapted for high speed running, suggests an adaptive response to the adverse effects of acidosis. In a comparison of the Thoroughbred horse, greyhound dog, and man, Harris et al.[7] concluded that the higher physico-chemical buffering (Bm_{titr}) in the horse and dog, compared with man, was due to the higher muscle content of histidine dipeptides.

In the present study, we have investigated the variability of carnosine with fibre type within the middle gluteal muscle of the horse and examined its contribution to muscle buffering capacity (Bm_{titr}).

MATERIALS AND METHODS

The left or right middle gluteal muscle of four Thoroughbred horses and one pony was

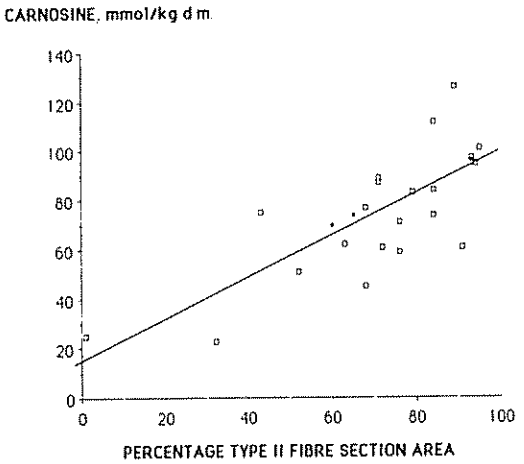

Fig. 1. A comparison of carnosine content with percentage type II muscle fibre section area in sample sites from Thoroughbred (⊡) and pony (♦). $r=0.75$, $p<0.001$.

removed at post-mortem. Animals had been referred following recent sudden death, usually in association with training. The Thoroughbreds (2 mares, 1 stallion, 1 gelding) were aged between 6 and 11 years.

A 1 cm transverse section from the middle of the whole muscle was taken and samples (approximately 5 mm^3) were obtained from up to six different sites. Portions from each sample were stored in liquid nitrogen until subsequent histochemical analysis. The remainder of the sample was freeze-dried, dissected free of blood and connective tissue and powdered for the analysis of carnosine and buffering capacity.

Histochemistry. Samples were mounted and cut into sections 10 μm thick in a cryostat at −20°C and stained for myosin ATPase[1] at pH 4.5. Analysis of the percentage type I, IIa and IIb muscle fibres and fibre area measurements were performed using a Joyce-Loebl 'Magiscan' image analysis system. The % type II fibre section area was calculated according to the formula:[9] 100·(type II fibre area·% type II fibres)/[(type I fibre area·% type I fibres) + (type II fibre area·% type II fibres)].

Measurement of buffering capacity. The method described by Marlin and Harris[11] was used. Measurements of pH were made using a MI-410 microelectrode attached to a Corning 150 pH/ion meter. Homogenisation reagent (Iodoacetic acid 5 mmol l^{-1}, NaCl 10 mmol l^{-1}, and KCl 145 mmol l^{-1}) was added to dry muscle powder (10 mg ml^{-1}) and the sample dispersed for 1 min using an Ultra-Turrax at 4°C. The tube containing the homogenate was subsequently placed in a water jacket at 37°C above a magnetic stirrer and allowed to equilibrate for 5 min. If required, an adjustment to pH 7.1 was made using 50 mmol l^{-1} NaOH. When once again stable (but no later than 5 min after adding NaOH), the homogenate was titrated from pH 7.1 to 6.5 using 20 μl aliquots of 10 mmol l^{-1} HCl. For verification, the homogenates were back titrated to pH 7.1 using 10 mmol l^{-1} NaOH. Buffering capacity determined by titration ($Bm_{titr.}$) was calculated by plotting acid/alkali added against pH. From this, the volume of acid/alkali added between pH 7.1 and 6.5 was found and a mean of the two values calculated. (As buffering curves are non-linear over the physiological pH range it is important to define the pH limits over which values are measured.)

Buffering due specifically to carnosine was calculated using a transformation of the Henderson-Hasselbach equation:

$$[\text{Acid}] = \frac{[\text{Total}]}{1+\text{antilog}_{10}(\text{pH}-\text{pKa})}$$

using a pKa value for carnosine of 6.83.

Assay of carnosine. Neutralised perchloric acid extracts of muscle were prepared according to Harris et al.[5] 20 μl was derivatised with *o*-phthalaldehyde and carnosine measured by HPLC.[10] Carnosine was eluted with a methanol gradient and identified using fluorescence detection.

RESULTS

The fibre composition over the different sites in the 5 horses showed a range in type II fibre section area of 1% to 95%. In keeping

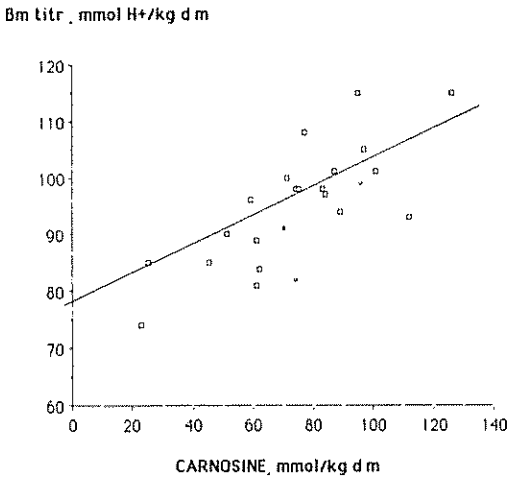

Fig. 2 A comparison of buffering capacity ($Bm_{titr.}$) with carnosine content in sample sites from Thoroughbred (⊡) and pony (♦) $r=0.76$, $p<0.001$.

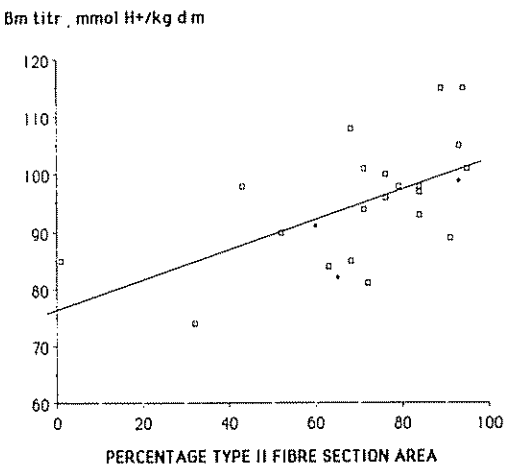

Fig. 3 A comparison of buffering capacity ($Bm_{titr.}$) with percentage type II muscle fibre section area in sample sites from Thoroughbred (⊡) and pony (♦). $r=0.57$, $p<0.01$.

with previous work of Bruce and Turek,[2] samples obtained from deeper parts of the muscle consistently contained a greater proportion of type I fibres. These samples showed the lowest content of carnosine (Table 1).

Carnosine content was positively correlated to type II fibre section area ($r=0.75$, $p<0.001$, Fig. 1) and in the samples assayed ranged from 23 to 126 mmol kg^{-1} d.m. A similar correlation ($r=0.78$, $p<0.001$) was obtained on multiple linear regression analysis of the carnosine content with percentage fibre section area (I and IIa, IIa and IIb, and, I and IIb). Using the intercept of each analysis the carnosine content of the different fibre types was estimated to range from 21 mmol kg^{-1} d.m. for type I fibres to 116

Table 1. *Carnosine content (carn) and buffering capacity (Bm_{titr}) compared with estimates of Bm_{carn}*

Bm_{carn} is the calculated contribution to buffering due specifically to carnosine in the pH range 6.5 to 7.1. $Bm_{non\text{-}carn} = Bm_{titr} - Bm_{carn}$, Bm = mmol H^+ kg^{-1} dry muscle pH 6.5–7.1, carnosine = mmol kg^{-1} dry muscle

Type II fibre section area (%)		0–60	61–70	71–80	81–90	91–100
Bm_{titr}	$\bar{x}$	88	90	95	101	102
	SD	9	12	7	10	9
Carnosine	$\bar{x}$	49	65	75	99	90
	SD	24	15	13	24	16
Bm_{carn}	$\bar{x}$	16	21	25	33	30
$Bm_{non\text{-}carn}$	$\bar{x}$	72	69	70	68	72
n		5	4	6	4	5

Table 2. *Carnosine content (carn) and buffering capacity (Bm_{titr}) of type I, II a, and II b muscle fibres estimated from multiple linear regression analysis*

Bm_{carn} is the calculated contribution to buffering due specifically to carnosine in the pH range 6.5 to 7.1. $Bm_{non\text{-}carn} = Bm_{titr.} - Bm_{carn}$, Bm = mmol H^+ kg^{-1} dry muscle pH 6.5–7.1, SE = standard error

Fibre type	I	II a	II b	SE
Carnosine	21	86	116	16
Bm_{titr}	78	98	109	9
Bm_{carn}	7	29	38	
$Bm_{non\text{-}carn}$	71	69	71	

mmol kg^{-1} d.m. for type II b fibres (Table 2). There appeared to be no difference between the Thoroughbred horses and the pony in a comparison of carnosine content with fibre composition.

Buffering capacity ranged from a mean of 88 mmol kg^{-1} d.m. in samples with the lowest type II fibre section area, to a mean of 102 mmol kg^{-1} d.m. in samples with the highest type II fibre section area (Table 1).

Buffering capacity was positively correlated to carnosine content ($r=0.76$, $p<0.001$, Fig. 2) and type II fibre section area ($r=0.57$, $p<0.01$, Fig. 3) with again no apparent difference between the horses and the pony in the dispersion of values.

Multiple linear regression analysis of Bm_{titr} against fibre section area gave a similar correlation to that in Fig. 2 ($r=0.60$, $p<0.01$). Results indicated a range in Bm_{titr} from 78 mmol kg^{-1} d.m. for type I fibres to 109 mmol kg^{-1} d.m. for type II b fibres.

In Tables 1 and 2, the specific buffering of carnosine (Bm_{carn}) has been calculated from the Henderson-Hasselbach equation. Values of Bm_{carn} have been deducted from Bm_{titr} to estimate non-carnosine physico-chemical buffering ($Bm_{non\text{-}carn}$). It can be seen that most of the variability in Bm_{titr} was accounted for through variability in Bm_{carn}. Non-carnosine buffering was constant at all fibre compositions showing minimal variation between the three fibre types.

DISCUSSION

Estimates of Bm_{titr} in Table 1 were measured in the pH range 7.1 to 6.5 as this appears typical of the change in muscle pH from rest to intense exercise.[10] Averaged over this pH range, carnosine accounted for approximately 30% of the measured physico-chemical buffering in muscle with a type II fibre section area ranging from 61% to 100%. A contribution to physico-chemical buffering of 10% to 15% is more typical for man.[8]

There is some concern that the homogenisation of samples will alter estimates of buffering capacity. Although major chemical changes do occur, the effect upon Bm_{titr} is relatively small due to the similarity of pKa's of the metabolites involved.[7] This is particularly true in the case of post-mortem and post-exercise samples.

Previous work by Bump et al.[3] has shown a higher carnosine content in the muscle of Quarterhorses (39.2 μmol g^{-1} wet muscle) than in Thoroughbreds (31.3 μmol g^{-1} wet muscle) and Standardbreds (27.6 μmol g^{-1} wet muscle). The higher content of carnosine in Quarterhorses is consistent with the greater percentage of fast twitch glycolytic fibres (38.8% compared to 24.0% and 23.8% respectively). These results are in agreement with the present study though they indicate that still higher carnosine contents must occur in the type II fibres of the Quarterhorse.

The inference of a low carnosine content in type I muscle fibres is consistent with their recruitment during low intensity activity when accumulation of lactic acid is minimal. The small difference between II a and II b fibres on the other hand is consistent with the similar glycolytic function in these fibres. A major finding from this investigation was that the variation in physico-chemical buffering within equine muscle can be accounted for almost exclusively by the carnosine content. This excludes any variation

in dynamic buffering due to bicarbonate loss or PCr hydrolysis during exercise. Physicochemical buffering by other sources measurable in freeze-dried muscle appeared constant over a wide range of fibre composition. Previous studies comparing human, greyhound dog and Thoroughbred horse have indicated that histidine dipeptides also account for the major variability in muscle buffering observed between these three species.[7]

ACKNOWLEDGEMENT

The authors gratefully acknowledge the financial support of the Horserace Betting Levy Board (HBLB), UK.

REFERENCES

1 Brook, M. H. and Kaiser, K. K. (1970) Muscle fibre types: How many and what kind? Arch. Neurol. 23, 369–379

2. Bruce, V. and Turek, R. J. (1985) Muscle fibre variation in the gluteus medius of the horse. Equine Vet. J. 17, 317–321

3 Bump, K. D., Lawrence, L. M., Moser, L. R., Miller-Graber, P. A. and Kurcz, E. V. (1989) Effect of breed type on muscle carnosine. Proc. 11th Eq. Nutr. Physiol. Symp., pp. 252–256.

4 Davey, C. L. (1960). The significance of carnosine and anserine in striated muscle. Arch. Biochem. Biophys. 89, 303–308

5 Harris, R. C., Hultman, E. and Nordesjö, L. O. (1974). Glycogen, glycolytic intermediates and high energy phosphates in biopsy samples of musculus quadriceps femoris of man at rest. Methods and variance of values. Scand. J. Clin. Lab. Invest. 33, 109–120.

6. Harris, R. C., Snow, D. H., Katz, A. and Sahlin, K. (1989). Effect of freeze-drying on measurements of pH in biopsy samples of the middle gluteal muscle of the horse: Comparison of muscle pH to the pyruvate and lactate content. Equine Vet. J. 21, 45–47

7 Harris, R. C., Marlin, D. J., Dunnett, M., Snow, D. H. and Hultman, E. (1990) Muscle buffering capacity and dipeptide content in the Thoroughbred horse, greyhound dog and man. Comp. Biochem. Physiol. 97A, 249–251

8 Hultman, E. and Sahlin, K. (1980). Acid–base balance during exercise. *In* Hulton, R. S. and Millar, D. I. (eds.): *Exercise and Sports Science Reviews*. Franklin Institute Press, USA, 8, 41–128.

9 Larsson, L. and Ansved, T. (1985) Effects of long term physical training and detraining on enzyme histochemical and functional skeletal muscle characteristics in man. Muscle Nerve 8, 714–722.

10. Marlin, D. J., Harris, R. C., Gash, S. P. and Snow, D. H. (1989). Carnosine content of the middle gluteal muscle in thoroughbred horses with relation to age, sex and training. Comp. Biochem. Physiol. 93A, 629–632.

11. Marlin, D. J. and Harris, R. C. (1991). Titrimetric determination of muscle buffering capacity (Bm_{titr}) in biopsy samples. Equine Vet. J. (In press.)

12 Parkhouse, W. S., McKenzie, D. C., Hochochka, P. W., Mommsen, T. P., Ovalle, W. K., Shinn, S. L. and Rhodes, E. C. (1983) The relationship between carnosine levels, buffering capacity, fibre type and anaerobic capacity in elite athletes. *In* Knuttgen, H., Vogel, J. and Poortmans, J. (eds.): *Biochemistry of Exercise. Human Kinetics*. 275–278