CARNOSINE METABOLISM AND FUNCTION IN THE
THOROUGHBRED HORSE

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A thesis submitted in partial fulfilment of the requirements of the Open University
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Declaration

I hereby certify that the work contained within this thesis has not already been accepted for any other degree, nor is it currently submitted in candidature for any other degree. Furthermore, I hereby certify that the work contained within this thesis is the result of my own investigation except where reference is made to published literature and where assistance is acknowledged.

(Candidate)

16th November 1995
Publications

This thesis is in part based on the following published manuscripts:


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ABSTRACT

CARNOSINE METABOLISM AND FUNCTION IN THE THOROUGHBRED HORSE

Mark Dunnett

Thoroughbred horseracing involves high-intensity exercise characterized by the production and accumulation of hydrogen (H+) ions within the skeletal muscles. Without a system for maintaining acid-base balance the consequential accumulation of H+ ions within the working muscles would produce a rapid decline in intra-cellular pH with a concomitant impairment of the contractile process. Carnosine (ß-alanyl-L-histidine, pKₐ 6.83) occurs at high concentration in equine muscle where it functions as an effective H+ ion buffer at physiological pH.

High-performance liquid chromatography analytical methods were developed for carnosine and used to investigate its distribution and metabolism in equine fluids and tissues, with emphasis on type I, IIA and IIB muscle fibres. Foals and yearlings had significantly lower plasma carnosine concentrations than older horses. Plasma carnosine concentration showed little change during normal feeding and high-intensity exercise, however, episodes of equine exertional rhabdomyolysis produced large increases. Carnosine concentrations in tissues, such as the heart, liver and intestine were 10 to 100-fold lower than in skeletal muscle. Carnosine displayed a heterogeneous distribution within skeletal muscle. Its concentration in type IIA and IIB fibres was approximately 5-fold higher than in type I fibres.

Extensive, partly anaerobic training produced a 2-fold increase in the carnosine concentration in type IIA fibres, and an increase, although non-significant, in type I and IIB fibres. Thirty days of dietary ß-alanine and histidine supplementation produced an adaptive increase in ß-alanine and histidine bioavailability, and significant increases in the carnosine concentration in type IIA and IIB fibres.

A greater skeletal muscle carnosine concentration via training and/or ß-alanine and histidine supplementation would produce a corresponding increase in H+ ion buffering capacity, which may reduce the rate of metabolic acidosis during high-intensity exercise, and possibly delay the subsequent onset of localized muscle fatigue.
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CHAPTER 1

GENERAL INTRODUCTION AND A REVIEW OF THE LITERATURE ON CARNOSINE
1.1 GENERAL INTRODUCTION

1.1.1 The historical and contemporary role of the horse in society
Of the many animal species domesticated during the evolution of human civilization the horse has probably made the greatest contribution to Man's social development. The versatility of this species in terms of strength, speed, endurance and amenability to training has resulted in its use in many areas of human endeavour such as, hunting, transport, agriculture, warfare and sport. The decline in the use of the horse as a working animal has been accompanied by a corresponding increase in equine sporting activities both commercially and for leisure purposes. Equine sporting activities are probably as old as the overall relationship between man and horse, and most likely originated from the practise of skills necessary for the survival of the individual and the community. The number of equine sports throughout the World is considerable and reflects the diversity of cultures in which the horse is found. Some equine sports include; flat-racing, steeplechasing (including hurdling and point-to-point racing), harness racing (including trotting and pacing), endurance racing, three-day eventing, show-jumping, dressage, carriage driving, fox and stag hunting, polo and vaulting. World-wide sporting activities are dominated both in terms of numbers of animals involved and numbers of events staged by track-based racing which mostly comprises flat-racing, steeplechasing and harness racing.

1.1.2 An outline of thoroughbred racing and breeding
The breeding and racing of thoroughbred horses is a vast commercial enterprise both nationally and internationally. Recent figures estimate that there are some 50,000 thoroughbred horses in the USA, and 3,500 in France. In Britain alone horse racing and breeding is a multi-billion pound industry. In the tax year 1993/94 the leviable betting turnover alone was estimated at £4,441 million (HBLB 1993/94). During the 1994 British racing season, including both flat and jump racing, there were 11,202 Thoroughbreds in training owned by 8,778 (monthly average) active owners from a registered total of 18,888. In addition there were 559 licensed trainers and a further 286 permit-holding trainers, 743 professional jockeys and 748 amateurs, and 5,908 other racing stable employees (Jockey-Club 1994). The majority of these horses contributed to a total
of 7,089 races in 1,102 fixtures at 60 racecourses resulting in 71,217 runners competing for total prize money of £54.8 million (BHB 1994). In 1994 there were 11,594 foals bred for the purposes of racing from 22,072 registered mares and 1,061 active stallions in Britain and Ireland (Jockey-Club 1994). Other major centres for Thoroughbred breeding and racing include Ireland, Australia, Japan, South Africa, Dubai and Hong Kong.

Thoroughbred flat-race horses are required to exercise over distances ranging from 5 - 20 furlongs (1000 - 4000 m) for approximately 1 - 5 min. Average speeds for a 5 furlong race can often reach 18 m s⁻¹ and maximum speeds attained during racing over this distance can approach 22 m s⁻¹. Thoroughbred steeplechasers race over longer distances of 16 - 36 furlongs (3200 - 7200 m). Although average speeds are lower the horses are required to negotiate between 6 - 9 hurdles or fences per mile. Both forms of racing provide a severe test of horses strength, speed and stamina.

1.1.3 Metabolic acidosis and intra-cellular hydrogen ion buffering during exercise

Production and accumulation of large quantities of lactic acid, and lesser amounts of pyruvic, malic and other acids, occurs in the working skeletal muscles during periods of high-intensity exercise as a consequence anaerobic glycolysis. At physiological pH carboxylic acids dissociate into carboxylate anions (i.e. lactate) and hydrogen (H⁺) ions. It has been reported that during fatiguing exercise that lactic acid production is responsible for 94% of the total H⁺ ion load in human skeletal muscle (Hultman and Sahlin 1980). Lactate concentrations in the equine middle gluteal muscle in excess of 200 mmol kg⁻¹ dry weight (DW) following both racing (Valberg 1987) and repeated bouts of maximal treadmill exercise (Snow et al. 1985) have been observed. However, following both single and repeated bouts of maximal exercise, lactate concentrations in the middle gluteal muscle of the horse of 150 mmol kg⁻¹ DW are more common (Harris et al. 1989; Harris et al. 1987). Such a muscle lactate concentration is representative of a H⁺ ion production of 150 000 µmol kg⁻¹ DW which would theoretically cause a fall in the intra-cellular pH to a value of 1.0 - 2.0. In practice however, a reduction in intra-cellular pH from a resting value of 7.1 to a post-exercise value of 6.5 represents an increase in H⁺ ion concentration of only
0.71 μmol kg⁻¹ DW (Marlin and Harris 1991). Although a proportion of the H⁺ ions produced will be exported from the skeletal muscle to the blood there remains a strong correlation (r = 0.97) between muscle pH and the lactate and pyruvate concentration (Harris et al. 1989), and hence the majority of the H⁺ ions produced can be accounted for through buffering within the muscle.

Mammalian skeletal muscle is reliant upon several different buffering mechanisms which operate in concert to regulate intra-cellular pH homeostasis. Overall or total intra-cellular H⁺ ion buffering (B_{m\text{total}}) in skeletal muscle can be considered to have three major components: physico-chemical buffering, metabolic buffering and dynamic buffering. The physico-chemical component can be further divided into two components. First, intrinsic buffers which include; the weak acid-base moieties associated with proteins, the histidine and 3-methylhistidine residues of actin and myosin, the imidazole dipeptides carnosine, anserine and balenine, and free histidine. Second, extrinsic buffers, such as bicarbonate. The metabolic buffering component comprises the dephosphorylation of ATP to IMP, NH₄⁺ and inorganic phosphate, dephosphorylation of phosphocreatine, and NH₄⁺ ion production during amino acid catabolism. The dynamic buffering component regulates the intra-cellular H⁺ ion concentration via transmembrane H⁺ ion flux, for example from the cytosol to the blood plasma.

The imidazole dipeptides can account for a significant proportion of the intra-cellular physico-chemical buffering (Abe et al. 1985), although the exact proportion is highly variable between species (Okuma and Abe 1992).
1.2 REVIEW OF THE LITERATURE ON CARNOSINE

1.2.1 Discovery and chemical structures of carnosine and related compounds

The imidazole dipeptide carnosine (β-alanyl-L-histidine) is a polar, water soluble, small molecular weight compound (MW 226.2) which exists in the zwitterionic form at pH 7.0 (Figure 1.1). The molecule possesses three ionizable groups; a terminal amino group (pKₐ 9.66), a carboxylate group (pKₐ 2.77) and an imidazole ring (pKₐ 6.83) (Tanokura et al. 1976). Carnosine also possesses a chiral carbon atom and hence can exist as both the L- or the D-isomer. The L-isomer is the naturally occurring form of the compound.

Carnosine (β-alanyl-L-histidine) was first isolated from Liebig's meat extract (Gulewitsch and Amiradzhibi 1900a; Gulewitsch and Amiradzhibi 1900b). Five years later, a German biochemist isolated a nitrogenous organic compound from the same source which was named ignotine (Kutscher 1905). Subsequent comparative analysis established that carnosine and ignotine were identical (Gulewitsch 1906/7). Gulewitsch established the dipeptide nature of carnosine by demonstrating that it was hydrolysed to histidine (Gulewitsch 1906/7) and β-alanine (Gulewitsch 1911). The structure of carnosine was confirmed following its chemical synthesis (Barger and Tutin 1918; Baumen and Ingvaldsen 1918). Naturally occurring methylated analogues of carnosine were soon discovered. Anserine (β-alanyl-L-L-methylhistidine) was first isolated from skeletal muscle of the chicken and goose (Ackermann et al. 1929; Tolkachevskaya 1929) and its structure was confirmed following its chemical synthesis (Behrens and du-Vigneaud 1937). Balenine, also known as ophidine (β-alanyl-L-3-methylhistidine), was discovered in skeletal muscle of the Cobra (Imamura 1934), however, for many years it was thought to be β-alanyl-L-2-methylhistidine. The correct chemical structure for this dipeptide was later established using nuclear magnetic resonance spectroscopy (Wolff et al. 1968). Homocarnosine (γ-aminobutyryl-L-histidine), a dipeptide analogue of carnosine in which the β-alanine residue is replaced by γ-aminobutyric acid, was discovered in bovine brain (Pisano et al. 1961). N-α-acetyl-L-carnosine, a carnosine derivative in which one of the hydrogen atoms of the terminal amine group replaced by an acetyl group, was discovered in the central nervous system (CNS) and skeletal muscle of...
Figure 1.1 Empirical formula and chemical structure of carnosine. (* Denotes the chiral centre).
Table 1.1  Discovery of carnosine and related compounds. Adapted from Boldyrev and Severin (1990)
<table>
<thead>
<tr>
<th>Common Name</th>
<th>Name</th>
<th>Source</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Camosine</td>
<td>β-alanyl-L-histidine</td>
<td>Meat extract</td>
<td>Gulewitsch and Amiradzhibi (1900)</td>
</tr>
<tr>
<td>Anserine</td>
<td>β-alanyl-L-1-methylhistidine</td>
<td>Chicken muscle</td>
<td>Tolkachevskaya (1929)</td>
</tr>
<tr>
<td>Balenine</td>
<td>β-alanyl-L-3-methylhistidine</td>
<td>Goose muscle</td>
<td>Ackermann et al. (1929)</td>
</tr>
<tr>
<td>Homocarnosine</td>
<td>γ-aminobutyryl-L-histidine</td>
<td>Cobra muscle</td>
<td>Imamura (1934)</td>
</tr>
<tr>
<td>Homoanserine</td>
<td>γ-aminobutyryl-L-1-methylhistidine</td>
<td>Human CNS</td>
<td>Pisano et al. (1961)</td>
</tr>
<tr>
<td>Acetylcarnosine</td>
<td>N-α-acetyl-β-alanyl-L-histidine</td>
<td>CNS</td>
<td>Nakajima et al. (1969)</td>
</tr>
<tr>
<td>Acetylhomocarnosine</td>
<td>N-α-acetyl-γ-aminobutyryl-L-histidine</td>
<td>CNS</td>
<td>Sobue et al. (1975)</td>
</tr>
<tr>
<td>Acetylanserine</td>
<td>N-α-acetyl-β-alanyl-L-1-methylhistidine</td>
<td>Myocardium</td>
<td>O'Dowd et al. (1988)</td>
</tr>
<tr>
<td>Carcinine</td>
<td>β-alanylhistamine</td>
<td>Crab CNS</td>
<td>Arnould and Frentz (1975a)</td>
</tr>
</tbody>
</table>
Figure 1.2  Empirical formulae, chemical structures and molecular weights of histidine and other imidazole dipeptides.
HISTIDINE
C₆H₉N₃O₂
MW 155.2

ANSERINE
C₁₀H₁₆N₄O₃
MW 240.2

BALENINE
C₁₀H₁₆N₄O₃
MW 240.2

N-ACETYLCARNOSINE
C₁₁H₁₆N₄O₄
MW 268.2

HOMOCARNOSINE
C₁₀H₁₆N₄O₃
MW 240.3
the frog (Sobue et al. 1975). These and other carnosine related imidazole dipeptides which have since been discovered are listed in Table 1.1. The empirical formulae, chemical structures and MW of histidine and some imidazole dipeptides are shown in Figure 1.2.

### 1.2.2 Occurrence and distribution of carnosine and related compounds in nature

**Carnosine distribution in non-vertebrate species**

Carnosine and related compounds have a wide though heterogeneous phylogenetic distribution. To date the occurrence of the imidazole dipeptides has been investigated in more than 80 species. The literature appears to contain no reports of the presence of carnosine in the tissues of either plants or micro-organisms, although histidine is present in both plant and animal proteins. β-alanine is reported to be present at low concentrations in some plants, such as green coffee beans (2.7 - 5.7 mg kg\(^{-1}\) DW or 0.03 - 0.06 mmol kg\(^{-1}\) DW) (Arnold et al. 1994) and the cycad *Stangeria eriopus* (not quantified) (Osborne et al. 1994). Green coffee beans also contain free 1-methylhistidine (< 50 mg kg\(^{-1}\) DW or 0.3 mmol kg\(^{-1}\) DW), 3-methylhistidine (25.0 - 43.2 mg kg\(^{-1}\) DW or 0.16 - 0.28 mmol kg\(^{-1}\) DW) and γ-aminobutyric acid (not quantified) (Arnold et al. 1994). Carnosine is found in some arthropods such as, the lavae of the blowfly (not quantified) (Bodnaryk and Levenbrook 1968). Carnosine (and anserine) have been detected in some species of molluscs and crustaceans (Luckton and Olcott 1958), although they are reported to be absent in several others (Suyama et al. 1970). Some species of molluscs and crustaceans and many fish, however, appear to possess much greater amounts of the amino acid precursors of dipeptides, histidine and β-alanine, rather than the dipeptides themselves (Luckton and Olcott 1958). More recently a previously unknown imidazole dipeptide, carcinine (β-alanylhistamine), was detected in the CNS and muscle of the crustacean *Carcinus maenas* (Arnould and Frentz 1975a; Arnould and Frentz 1975b) and later in other crustacea (Arnould 1986). The typical concentrations of the imidazole dipeptides in the tissues of these species are shown in Table 1.2.
Table 1.2  Mean concentrations of carnosine and related compounds in the muscle tissue of invertebrates.
<table>
<thead>
<tr>
<th>Species</th>
<th>Camosine</th>
<th>Anserine</th>
<th>Balenine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blowfly (larvae)</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Crab</td>
<td>0.5</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Crab</td>
<td>13.3</td>
<td>ND</td>
<td>-</td>
</tr>
<tr>
<td>Crab</td>
<td>0.8</td>
<td>ND</td>
<td>-</td>
</tr>
<tr>
<td>Squid</td>
<td>2.0</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Shrimp</td>
<td>8.0</td>
<td>ND</td>
<td>-</td>
</tr>
<tr>
<td>Scallop</td>
<td>1.2</td>
<td>ND</td>
<td>-</td>
</tr>
<tr>
<td>Abalone</td>
<td>2.0</td>
<td>ND</td>
<td>6.4</td>
</tr>
<tr>
<td>Oyster</td>
<td>0.8</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>


*Camposine and balenie were not detected in the muscle tissue of shrimp, hard clam.

+ = Detected but not quantified.
ND = Not detected.

† = Gaper, top shell, abalone, squid, octopus, and sea cucumber.
Carnosine distribution in tissues

Skeletal muscles of vertebrates contain very high concentrations of carnosine and other imidazole dipeptides, often in excess of 50 mmol kg\(^{-1}\) DW. Early investigations by (Wood 1957) and (Davey 1960a), using colourimetric and ion-exchange assays, tentatively suggested the presence of carnosine in rat stomach but did not detect the peptide in other tissues, including heart, kidney and liver, nor in the heart of horses. Subsequent improvements in analytical techniques have shown that low millimolar concentrations of carnosine and the other imidazole dipeptides occur principally in heart and tissues of the CNS and gastro-intestinal tract (GIT) of several species including the rat, rabbit, guinea pig, frog, mouse, pigeon, chick and man. However, the distribution between different tissues of a given species and between species is highly variable.

Sobue et al. (1975) reported carnosine concentrations in rabbit heart, liver and kidney of 618, 441 and 39 nmol g\(^{-1}\) WW (2.47, 1.76 and 0.16 mmol kg\(^{-1}\) DW), respectively. Similar values in cardiac muscle were reported by O'Dowd et al. (1988) in rat, guinea pig and frog. The retina of pigeons and chickens contain 0.08 - 0.26 µmol g\(^{-1}\) WW (0.32 - 1.04 mmol kg\(^{-1}\) DW) of carnosine and anserine concentrations of 1.4 - 5.2 µmol g\(^{-1}\) WW (5.6 - 20.9 mmol kg\(^{-1}\) DW) (Margolis and Grillo 1984). In contrast, carnosine was almost invariably absent from mammalian (including human) retina, however, homocarnosine was present at concentrations of 0.05 - 0.10 µmol g\(^{-1}\) WW (0.2 - 0.4 mmol kg\(^{-1}\) DW). Retinal carnosine concentrations were highest in the frog (6.4 mmol kg\(^{-1}\) DW). Both carnosine and anserine were found at low concentrations in other regions of the avian CNS including chicken cerebellum, cerebral hemispheres and optic lobes (Margolis and Grillo 1984). Within the CNS of vertebrates studied, including the hamster, pig, dog, rabbit and rat, the highest concentrations of carnosine are localized within the olfactory bulb and epithelium (Ferriero and Margolis 1975; Neidle and Kandera 1974). The carnosine concentration in mouse olfactory bulb was 0.9 - 2.2 nmol mg\(^{-1}\) WW (3.6 - 8.8 mmol kg\(^{-1}\) DW) (Ferriero and Margolis 1975; Neidle and Kandera 1974; Wideman et al. 1978), and in the Geko a concentration of up to 3.9 µmol g\(^{-1}\) WW (15.6 mmol kg\(^{-1}\) DW) was reported (Margolis 1981). In other regions of the mammalian CNS, such as the cerebellum, cerebrum and brain stem,
homocarnosine, with tissue concentrations up to 52.4 μmol 100 g⁻¹ WW (2.1 mmol kg⁻¹ DW), is the major imidazole dipeptide present (Abraham et al. 1962). In human brain homocarnosine concentration was 2 to 4-fold higher in white matter than in grey matter (Abraham et al. 1962).

Flanbaum et al. (1990) reported the most comprehensive study of carnosine concentrations in tissues other than skeletal muscle when investigating a possible relationship between carnosine and histamine concentrations in the rat, mouse, guinea pig and man (see section 1.2.4 Histamine synthesis in response to shock and wound healing). These mean values of carnosine concentrations and those determined by other investigators are given in Table 1.3.

The total concentration of the acetylated forms of the imidazole dipeptides, N-α-acetylcarnosine, N-α-acetylanserine and N-α-acetylhomocarnosine, in cardiac muscle and tissues of the CNS (other than olfactory bulb) exceeds the concentrations of the non-acetylated forms. The estimated total concentration of the acetylated imidazole dipeptides in cardiac muscle is greater than 10 mmol kg⁻¹ WW (40 mmol kg⁻¹ DW) (O'Dowd et al. 1988).
Table 1.3 Mean carnosine concentrations in tissues of the rat, mouse, guinea pig and man. Adapted from Flancbaum et al. (1990).
<table>
<thead>
<tr>
<th>Tissue</th>
<th>Mean ± SD carnosine concentration (mmol kg(^{-1}) DW)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rat</td>
</tr>
<tr>
<td>Heart</td>
<td>0.44 ± 0.06</td>
</tr>
<tr>
<td>Kidney</td>
<td>0.29 ± 0.05</td>
</tr>
<tr>
<td>Stomach</td>
<td>0.19 ± 0.03</td>
</tr>
<tr>
<td>Liver</td>
<td>0.19 ± 0.03</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.14 ± 0.03</td>
</tr>
<tr>
<td>Lung</td>
<td>0.21 ± 0.10</td>
</tr>
<tr>
<td>Ileum</td>
<td>0.11 ± 0.03</td>
</tr>
<tr>
<td>Hypothalamus</td>
<td>0.07 ± 0.01</td>
</tr>
<tr>
<td>Pituitary</td>
<td>0.25 ± 0.06</td>
</tr>
<tr>
<td>Olfactory bulb</td>
<td>0.20 ± 0.09</td>
</tr>
<tr>
<td>Jejunum</td>
<td>-</td>
</tr>
</tbody>
</table>

† = Only one sample.

Table adapted from Flancbaum et al. (1990)
The concentrations of imidazole dipeptides found in vertebrate skeletal muscle are highly variable between species (Carnegie et al. 1983; Crush 1970; Plowman and Close 1988). Skeletal muscle of the Little Piked Whale contains the highest reported concentration of imidazole dipeptides with a total concentration of 2153 mg% or approximately 380 mmol kg\(^{-1}\) DW of which balenine contributed 350 mmol kg\(^{-1}\) DW (Suyama et al. 1970). Either carnosine, anserine or balenine, or a combination of these, occur at high concentrations in the skeletal muscle of almost all of the vertebrate species studied. The particular dipeptide, or combination of dipeptides present and their intra-muscular concentrations are extremely variable. From the very large number of species studied to date it is apparent that great similarities exist between the dipeptide ratios in individual species within the same zoological class. In general anserine predominates over carnosine in birds, balenine predominates over carnosine in reptiles and aquatic mammals whereas greater amounts of carnosine than anserine occur in many land based mammals. Balenine is absent from almost all land mammals (Crush 1970). The carnosine : anserine : balenine ratios in the skeletal muscles of different species have been studied in an attempt to establish a simple diagnostic test for the identification of the composition of consumer meat products (Carnegie et al. 1983; Plowman and Close 1988). Some examples of the concentrations of carnosine and related compounds in the skeletal muscles of different species are given in Table 1.4.

The incidence of a single dipeptide occurring in isolation in the skeletal muscle of a given species is comparatively rare. Carnosine alone appears to be present in only humans (Christman 1976) and equine skeletal muscle (Bump et al. 1989; Davey 1960a; Harris et al. 1990; Marlin et al. 1989). Christman (1976) reported carnosine concentrations in human psoas muscle in the range 1.0 - 7.7 \(\mu\)mol g\(^{-1}\) WW (4.0 - 30.8 mmol kg\(^{-1}\) DW). Anserine was not detected. These results have been confirmed by several subsequent investigations (Harris et al. 1990; Mannion et al. 1992; Parkhouse et al. 1985). The carnosine and anserine concentrations in human skeletal muscle in contrast to values from two other competitive athletic species, the greyhound and camel, are given in Table 1.5.
Table 1.4  Mean concentrations of carnosine and related compounds in the skeletal muscle of vertebrates.
<table>
<thead>
<tr>
<th>Class</th>
<th>Species</th>
<th>Muscle</th>
<th>Mean ± SD concentration (mmol kg⁻¹ DW)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Carnosine</td>
<td></td>
</tr>
<tr>
<td>Aves</td>
<td>Chicken</td>
<td>Pectoralis</td>
<td>70.7 ± 19.8</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Pigeon</td>
<td>Pectoralis</td>
<td>1.8†</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Goose</td>
<td>Pectoralis</td>
<td>16.7 ± 8.1</td>
<td>23.3†</td>
</tr>
<tr>
<td></td>
<td>Rook</td>
<td>NS</td>
<td>108.3 ± 16.1</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>58.3†</td>
<td>-</td>
</tr>
<tr>
<td>Amphibia</td>
<td>Toad</td>
<td>Sartorius</td>
<td>31.8†</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Frog</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reptilia</td>
<td>Sea snake</td>
<td>-</td>
<td>-</td>
<td>93.2†</td>
</tr>
<tr>
<td></td>
<td>King cobra</td>
<td>-</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>20.0†</td>
</tr>
<tr>
<td>Osteichthyes</td>
<td>Tuna</td>
<td>-</td>
<td>1.7†</td>
<td>181.5†</td>
</tr>
<tr>
<td></td>
<td>Salmon</td>
<td>-</td>
<td>ND</td>
<td>70.7†</td>
</tr>
<tr>
<td>Mammalia</td>
<td>Zebra</td>
<td>Gluteus medius</td>
<td>112.4 ± 11.3</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Donkey</td>
<td>NS</td>
<td>48.4 ± 24.0</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>Kangaroo</td>
<td>NS</td>
<td>9.2 ± 7.6</td>
<td>63.6 ± 15.2</td>
</tr>
<tr>
<td></td>
<td>Wallaby</td>
<td>NS</td>
<td>9.6†</td>
<td>123.6†</td>
</tr>
<tr>
<td></td>
<td>Hare</td>
<td>NS</td>
<td>20.0 ± 7.2</td>
<td>93.0 ± 10.8</td>
</tr>
<tr>
<td></td>
<td>Rabbit</td>
<td>NS</td>
<td>15.4 ± 7.7</td>
<td>77.6 ± 13.9</td>
</tr>
<tr>
<td></td>
<td>Pig</td>
<td>NS</td>
<td>48.4 ± 19.6</td>
<td>2.6 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>Deer</td>
<td>NS</td>
<td>34.2 ± 8.8</td>
<td>39.1 ± 11.0</td>
</tr>
<tr>
<td></td>
<td>Dolphin</td>
<td>NS</td>
<td>79.1†</td>
<td>15.4†</td>
</tr>
<tr>
<td></td>
<td>Whale</td>
<td>NS</td>
<td>40.1†</td>
<td>6.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

ND = Not detected.  NS = Not specified.  † = Single sample.  - = Not determined.
Table 1.5  Mean muscle carnosine and anserine concentrations in the skeletal muscles of man, greyhound and camel.
<table>
<thead>
<tr>
<th>Species</th>
<th>Muscle</th>
<th>Concentration (mmol kg⁻¹ DW)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Carnosine</td>
<td>Anserine</td>
</tr>
<tr>
<td>Man</td>
<td>Vastus lateralis</td>
<td>16.0 ± 7.2</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Quadriceps femoris</td>
<td>20.0 ± 4.7</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Quadriceps femoris</td>
<td>21.8 ± 4.1</td>
<td>ND</td>
</tr>
<tr>
<td>Greyhound</td>
<td>Various muscles†</td>
<td>33.0 ± 19.1</td>
<td>48.6 ± 18.4</td>
</tr>
<tr>
<td>Camel</td>
<td>Gluteus medius</td>
<td>28.7 ± 14.9</td>
<td>36.9 ± 12.4</td>
</tr>
</tbody>
</table>

ND = Not detected.
† = semitendinosus, biceps femoris, triceps, deltoid and longissimus dorsi.
The horse has been shown to possess one of the highest skeletal muscle imidazole dipeptide concentrations recorded amongst land based species. Early measurements of the carnosine concentration in equine skeletal muscle by Zapp and Wilson (1938) reported carnosine concentrations of 463.4, 436 and 161 mg/100 g WW (approximately 81.9, 77.1 and 28.5 mmol kg\(^{-1}\) DW) in the gluteus medius, gastrocnemius and soleus muscles, respectively. Anserine concentrations of 17 - 48 mg/100g WW (2.8 - 8.0 mmol kg\(^{-1}\) DW) were also reported, however this later finding has not been supported by subsequent work. A carnosine concentration of 25.5 \(\mu\)mol g\(^{-1}\) WW (102.0 mmol kg\(^{-1}\) DW) was detected in equine longissimus dorsi muscle (Davey 1960a). Slightly higher concentrations of between 108 and 134 mmol kg\(^{-1}\) DW have been reported more recently by several investigators (Bump et al. 1990; Harris et al. 1990; Marlin et al. 1989; Miller-Graber et al. 1990; Sewell et al. 1992). Furthermore, some differences in the skeletal muscle carnosine concentrations between various breeds of horses has been indicated. American Quarterhorses were shown to have higher concentrations than thoroughbred horses, which in turn had higher concentrations than standardbred horses (Bump et al. 1989; Bump et al. 1990). Carnosine concentrations in the middle gluteal muscle of Quarterhorses, Thoroughbreds and Standardbreds are given in Table 1.6.

**Sub-cellular distribution of carnosine in skeletal muscle**

It probable that carnosine exists predominantly in the free state in equine skeletal muscle with less than 10% present in a bound form (Bock 1958). A subsequent investigation which compared the dialysis rates of carnosine from aqueous standard solutions and fresh homogenates of frog skeletal muscle concurred with the earlier findings in equine muscle (Bock and Langley 1960). Evidence from the analysis of fractionated homogenates of rat gastrocnemius muscle indicates that 99.5% of the total imidazole dipeptide content is contained within the 15000g supernatant phase with only 0.3% present in the washed mitochondria and no detectable amounts of imidazole dipeptides in the residue. This suggests that the imidazole dipeptides are cytosolic in origin. Following intra-peritoneal administration of histidine-C\(^{14}\) or \(\beta\)-alanine-C\(^{14}\) to chicks and subsequent isolation of radio-labelled imidazole dipeptides, it was found that 99% of the radioactivity was present in the supernatant, 1% of the radioactivity was in the myofibrils and
Table 1.6  Mean muscle carnosine concentrations in different breeds of horses.
<table>
<thead>
<tr>
<th>Breed</th>
<th>Muscle</th>
<th>Carnosine mmol kg(^{-1}) DW</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thoroughbred</td>
<td>Gluteus medius</td>
<td>108.6 ± 15.2</td>
<td>Marlin et al. (1989)</td>
</tr>
<tr>
<td></td>
<td>Gluteus medius</td>
<td>108.3 ± 15.9</td>
<td>Harris et al. (1990)</td>
</tr>
<tr>
<td></td>
<td>Gluteus medius</td>
<td>125.3 ± 11.6</td>
<td>Bump et al. (1990)</td>
</tr>
<tr>
<td></td>
<td>Gluteus medius</td>
<td>133.6 ± 20.7</td>
<td>Miller-Graber et al. (1990)</td>
</tr>
<tr>
<td></td>
<td>Gluteus medius</td>
<td>125.0 ± 26.0</td>
<td>Sewell et al. (1992)</td>
</tr>
<tr>
<td></td>
<td>Gluteus medius</td>
<td>107.8 ± 12.9</td>
<td>Dunnett and Harris (1995b)</td>
</tr>
<tr>
<td>Standardbred</td>
<td>Gluteus medius</td>
<td>110.6 ± 2.4</td>
<td>Bump et al. (1990)</td>
</tr>
<tr>
<td>Quarterhorse</td>
<td>Gluteus medius</td>
<td>156.8 ± 7.1</td>
<td>Bump et al. (1990)</td>
</tr>
<tr>
<td></td>
<td>Glutius medius</td>
<td>159.1 ± 11.8</td>
<td>Bump et al. (1989)</td>
</tr>
</tbody>
</table>
less than 0.1% was present in mitochondria and microsomes (Winnick *et al.* 1963). Similarly, Harding and O'Fallon (1979) have reported that approximately 70% of the carnosine found in mouse olfactory bulb and epithelium was localized within the cytosol.

**Effect of training and exercise on muscle carnosine content**

Seven weeks of sub-maximal training resulted in a small but non-significant decrease in the carnosine concentration in equine middle gluteal muscle (Bump *et al.* 1989). Mean muscle carnosine concentrations, as described earlier, are higher in human subjects trained for high-intensity exercise such as sprinting and rowing in contrast to untrained individuals or those trained for sustained exercise of lower intensity, such as marathon running (Parkhouse *et al.* 1985). However, the higher muscle carnosine values evident in sprinters and rowers may well arise from a genetic predisposition rather than a training effect.

Bump *et al.* (1989) reported a significant reduction \( (p < 0.05) \) in the carnosine concentration in equine middle gluteal muscle from a pre-exercise value of approximately 140 mmol kg\(^{-1}\) DW to a value of 112 mmol kg\(^{-1}\) DW immediately following a sub-maximal exercise bout which resulted in a mean peak blood lactate concentration of 9.7 mM. However, this finding was refuted in a later study by Harris *et al.* (1992) in which the carnosine concentration in the middle gluteal muscle of the thoroughbred horse was unchanged from the pre-exercise value immediately following 4 x 700 m bouts of near maximal exercise which resulted in a mean peak blood lactate concentration of 26.8 mM. The changes previously reported by Bump *et al.* (1989) probably arose as a result of the use of wet tissue and could be attributed to an exercise induced increase in the blood content of muscle samples (Harris *et al.* 1992). No significant change in the muscle carnosine concentration of the gluteus medius of thoroughbred horses was found during fatiguing exercise (Miller-Graber *et al.* 1990).

1.2.3 Skeletal muscle carnosine and hydrogen ion buffering

Carnosine is an organic base. The imino nitrogen atom of the imidazole ring of carnosine and anserine have \( pK_a \) values of 6.83 and 7.04, respectively, and are capable of accepting (binding)
H⁺ ions (Tanokura et al. 1976). Compounds with a capacity for buffering H⁺ ions function most effectively when the pH of the environment is within one pH unit of the pKᵦ value. Carnosine will buffer most effectively between pH 5.83 and 7.83 and is therefore able to function as an efficient H⁺ ion buffer over the physiological pH range. The H⁺ ion buffering role of carnosine (and anserine) in skeletal muscle was first proposed as a significant component of the total intracellular physico-chemical muscle buffering capacity (Bm) by Bate-Smith (1938). This hypothesis was supported by the discovery that the pKᵦ values for carnosine and anserine were approximately 6.9 and 7.1, respectively (Bate-Smith 1938; Deutsch and Eggleton 1938). It was calculated that carnosine accounted for 20 - 25% of the post-rigour buffering in equine muscle between pH 5.5 and 7.5. Furthermore, it was estimated that it may have contributed approximately 40% of the total in pre-rigour muscle. Davey (1960a) reached a similar conclusion that carnosine and anserine accounted for the major proportion (up to 40%) of the total buffering of extracts of pre- and post-rigour muscle in several species between pH 7.5 and 6.5. The hypothesis that carnosine functioned as a H⁺ buffer in vivo was strengthened by the work of Severin et al. (1963) which demonstrated that electrically stimulated frog sartorius muscle immersed in Ringer's solution supplemented with carnosine or anserine (4 - 12 mM), in contrast to an almost identical muscle immersed in Ringer's solution only, showed a greater amplitude of contraction and a longer time to fatigue. In addition the accumulation of lactate was reportedly lower in the carnosine or anserine perfused muscle cited in (Severin et al. 1963). Whitaker and Louw (1984) reported similar findings when frog gastrocnemius muscle 'in situ' was electrically stimulated via the ipsilateral sciatic nerve (proximal blood and nerve supply was intact) whilst perfused with either Ringer's solution or Ringer's including 20 mM carnosine or histidine. In the presence of histidine or carnosine, the time to exhaustion was significantly longer (p < 0.01), and lactate accumulation and K⁺ ion efflux from the muscle were reduced but not significantly. They concluded that fatigue was reduced in the presence of histidine or carnosine, with histidine having a superior effect.

As early as 1938 it was reported that carnosine concentrations in skeletal muscle appeared to be higher in so-called 'white muscle' rather than 'red muscle' (Zapp and Wilson 1938). However,
(Davey 1960a) cautioned against a general acceptance of this across species after observing that although many red muscles which had a high myoglobin content had a low concentration of carnosine and anserine, the very red longissimus muscle of dolphins and whales contained very high dipeptide concentrations. A closer and inverse correlation appeared to exist between the oxidative capacity of the muscle, as indicated by its succinate dehydrogenase activity, and the carnosine and anserine content (Davey 1960a). Tamaki et al. (1976) dissected out both red and white muscle tissue from gastrocnemius, adductor magnus and latissimus dorsi muscles in rats. He found anserine contents in white muscle tissue of 12 - 17 μmol g⁻¹ WW (48 - 68 mmol kg⁻¹ DW) in contrast to values of 1 - 2 μmol g⁻¹ WW (4 - 8 mmol kg⁻¹ DW) in red muscle. Carnosine concentrations in white muscles were approximately two-fold higher than in red muscle (Tamaki et al. 1976). Similar findings were described by Suzuki et al. (1987b) who reported that the anserine content of tuna white muscle was higher than that of dark muscle. Turnisky and Long (1990) determined carnosine and anserine concentration in rat soleus and gastrocnemius muscles. Carnosine and anserine concentrations in gastrocnemius muscle (58% fast-twitch glycolytic fibres, 37% fast-twitch oxidative-glycolytic fibres) were 7.91 and 5.17 mmol l⁻¹ intra-cellular water (ICW), respectively, in contrast to values of 3.65 and 2.27 mmol l⁻¹ ICW, respectively in soleus muscle (> 80% slow-twitch oxidative fibres) (Turnisky and Long 1990).

A low but significant correlation (p < 0.05) was evident between muscle carnosine concentration and ßm (r = 0.69), and between muscle carnosine concentration and the percentage of fast-twitch fibres present (r = 0.46) in human vastus lateralis muscle from untrained subjects and sprint, rowing and marathon trained subjects (Parkhouse et al. 1985). However, Mannion et al. (1995) reported that there was only a moderate non-significant correlation between carnosine (r = 0.43, p > 0.05) or ßm (r = 0.42, p > 0.05) and percentage type II fibre area in the quadriceps femoris muscle of non-specifically trained subjects.

A similar investigation of gluteus medius muscle from Quarterhorses, Thoroughbreds and Standardbreds established a low but significant (p < 0.05) correlation between carnosine concentration and fast-twitch glycolytic fibre percentage (r = 0.53) and a low significant (p <
0.05) negative correlation between carnosine and slow-twitch oxidative fibre percentage \( (r = -0.51) \) (Bump et al. 1989). In a comparison of \( \beta m \) and total imidazole dipeptide concentration (carnosine + anserine) in skeletal muscle of Thoroughbreds. Greyhounds and humans, the higher \( \beta m \) of equine and canine muscle of 117.7 and 105.2 \( \mu \text{mol H}^+ \text{kg}^{-1} \text{DW} \) respectively, in contrast to the human \( \beta m \) of 79.5 \( \mu \text{mol H}^+ \text{kg}^{-1} \text{DW} \), was explained by the much greater concentration of the imidazole dipeptides in the former. Mean imidazole dipeptide concentrations in equine, canine and human muscle were 108.3, 81.6 and 16.0 mmol kg\(^{-1}\) DW. Subtraction of the specific buffering capacity arising from the imidazole dipeptides from \( \beta m \) resulted in almost identical residual values in equine, canine and human muscle of 81.8, 79.1 and 74.2 mmol H\(^+\) kg\(^{-1}\) DW, respectively. Therefore, the differences in \( \beta m \) between the three species appeared to arise predominantly from the differences in imidazole dipeptide contents (Harris et al. 1990). Similar results have been reported in several other species including, tuna, whale, pig and chicken, where differences in \( \beta m \) between species and muscles were mainly accounted for by differences in the concentrations of histidine and the imidazole dipeptides (Abe et al. 1985; Okuma and Abe 1992). Histidine and imidazole dipeptides were found to be major buffering constituents in these species accounting for 12 to 39% of \( \beta m \) (Okuma and Abe 1992).

Multiple linear regression analysis has been used to estimate the carnosine concentrations and \( \beta m \) of type I, IIA and IIB fibres from mixed muscle samples of the gluteus medius at post mortem of the untrained thoroughbred horses and one pony (Table 1.7A). Carnosine showed a strong significant \( (p < 0.001) \) correlation with \( \beta m \) in mixed muscle samples \( (r = 0.76) \) and a significant \( (p < 0.001) \) correlation with percentage type II fibre section area \( (r = 0.78) \). \( \beta m \) was also significantly \( (p < 0.01) \) correlated with percentage type II fibre section area \( (r = 0.60) \). Carnosine concentrations in type I, IIA and IIB fibres were estimated to be 21, 86 and 116 mmol kg\(^{-1}\) DW, respectively (Sewell et al. 1990). Comparative estimates of carnosine concentrations and \( \beta m \) by multiple linear regression analysis were later made in two-year-old Thoroughbreds actively engaged in training and racing (Table 1.7B). Carnosine concentrations were higher in these horses and were estimated to be 54, 85 and 180 mmol kg\(^{-1}\) DW in type I, IIA and IIB fibres, respectively (Sewell et al. 1992).
Table 1.7 A  Multiple linear regression analysis estimates of the carnosine concentration and βm of type I, IIA and IIB muscle fibres from untrained thoroughbred horses.
Adapted from Sewell et al. (1990).

Table 1.7 B  Multiple linear regression analysis estimates of the carnosine concentration and βm of type I, IIA and IIB muscle fibres from two-year-old thoroughbred horses engaged in training and racing.
Adapted from Sewell et al. (1992).
### A

<table>
<thead>
<tr>
<th>Fibre type</th>
<th>I</th>
<th>IIA</th>
<th>IIB</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carnosine (mmol kg(^{-1}) DW)</td>
<td>21</td>
<td>86</td>
<td>116</td>
<td>16</td>
</tr>
<tr>
<td>(\beta m) (mmol H(^+) kg(^{-1}) DW, pH 7.1 - 6.5)</td>
<td>78</td>
<td>98</td>
<td>109</td>
<td>9</td>
</tr>
<tr>
<td>(\beta m) (_{\text{carnosine}})</td>
<td>7</td>
<td>29</td>
<td>38</td>
<td></td>
</tr>
<tr>
<td>(\beta m) (_{\text{non-carnosine}})</td>
<td>71</td>
<td>69</td>
<td>71</td>
<td></td>
</tr>
</tbody>
</table>

SEM = Standard error of the mean.

### B

<table>
<thead>
<tr>
<th>Fibre type</th>
<th>I</th>
<th>IIA</th>
<th>IIB</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carnosine (mmol kg(^{-1}) DW)</td>
<td>54</td>
<td>85</td>
<td>180</td>
<td>15</td>
</tr>
<tr>
<td>(\beta m) (mmol H(^+) kg(^{-1}) DW, pH 7.1 - 6.5)</td>
<td>88</td>
<td>98</td>
<td>130</td>
<td>9</td>
</tr>
<tr>
<td>(\beta m) (_{\text{carnosine}})</td>
<td>18</td>
<td>28</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>(\beta m) (_{\text{non-carnosine}})</td>
<td>70</td>
<td>70</td>
<td>70</td>
<td></td>
</tr>
</tbody>
</table>

SEM = Standard error of the mean.
1.2.4 Other functions of carnosine

Antioxidant activity

The highest rates of oxidative metabolism in mammalian tissues are found in skeletal muscle and brain. Oxygen utilization in skeletal muscle is greatly increased during exercise and hence there is an increased potential for tissue damage via the generation of oxygen based free radicals and other reactive species (oxidative stress). It has been proposed that at the cellular level carnosine, and other imidazole dipeptides, may function as endogenous antioxidants which protect macromolecules, including DNA, RNA, proteins and membrane lipids, from oxidative stress induced damage caused by reactive oxygen species, such as singlet oxygen, hydroxyl radicals (\(\cdot\)OH), superoxide radicals (\(O_2^-\)), hydrogen peroxide (\(H_2O_2\)) and hypochlorite (\(\cdot\)OCl) (Boldyrev et al. 1988; Dahl et al. 1988; Dupin et al. 1984; Kohen et al. 1988). As a result of in vitro studies utilizing physiological concentrations of carnosine, several different mechanisms have been suggested by which carnosine may function as an antioxidant including free radical scavenging, chelation of divalent metal ions (Brown 1981; Kohen et al. 1988) and elimination of pre-formed lipid peroxidation products (Esterbauer et al. 1986).

Carnosine and anserine were reported to attenuate in vitro \(Fe^{2+}\) / ascorbate induced lipid peroxidation in rabbit sarcoplasmic reticulum membranes (Boldyrev et al. 1989; Boldyrev et al. 1988) and rat serum lipids (Chasovnikova et al. 1990) both by chelation of \(Fe^{2+}\) ions and by removal of pre-formed peroxidation products. A suppression of stress induced brain and serum lipid peroxidation in rats, following carnosine administration, has also been reported (Gulyaeva et al. 1989). Other studies however, have cast doubt on the ability of carnosine to significantly inhibit \(Fe^{2+}\) catalysed lipid peroxidation when it was shown that carnosine can interfere with the TBA assay used which can cause an overestimation of the degree of inhibition (Aruoma et al. 1989; Salim-Hanna et al. 1991). Furthermore, the ability of carnosine to effectively chelate \(Fe^{2+}\) ions has recently been refuted following nuclear magnetic resonance (NMR) studies which have shown that carnosine does not form a complex with iron (Decker et al. 1992). Carnosine is able to form chelates with \(Cu^{2+}\) ions and has been shown to be effective in preventing both \(Cu^{2+}\) catalyzed hydroxylation (Kohen et al. 1988) and oxidation of deoxyguanosine (Decker et al. 1992).
1992). Individually, physiological concentrations of carnosine, anserine and homocarnosine have been reported to be capable of scavenging *OH radicals but not O_2^••, H_2O_2 and -OCl (Aruoma et al. 1989), although (MacFarlane et al. 1991) have suggested that mixtures of carnosine, anserine, homocarnosine and 1-methylhistidine function synergistically to inhibit oxidation by O_2^•• in vitro. Carnosine has also been shown to be effective at quenching singlet oxygen (Shvachko et al. 1990). Conversely, in the presence of Cu^{2+} carnosine is reported to promote the formation of O_2^•• but also to catalyse its conversion to H_2O_2 (Hartman and Hartman 1992). It appears therefore, that carnosine can behave as an antioxidant towards some specific reactive oxygen species, but does not function as a broad-spectrum antioxidant.

It has been proposed that the putative antioxidant properties of carnosine, anserine and balenine may protect skeletal muscle tissue from free radical induced damage during oxidative stress (Kohen et al. 1988). This hypothesis may in part be supported by the presence of an estimated 21 - 54 mmol kg^{-1} DW of carnosine in equine type I muscle fibres (Sewell et al. 1990; Sewell et al. 1992), as they have a high oxidative capacity but negligible glycolytic capacity, minimal H^{+} production and hence very low buffering requirement.

Regulation of muscle phosphorylase activity
Carnosine has been shown to activate rabbit muscle phosphorylase a and b in vitro in the presence of AMP (Johnson et al. 1982). Phosphorylase a was also activated in the absence of AMP. The maximal degree of activation (approximately 100%) occurred at a carnosine concentration of 50 mM with a greater activation evident for phosphorylase b. Anserine activated both phosphorylase a and b to a lesser extent than carnosine, eliciting a maximal effect (approximately 40%) at 30 - 40 mM. However, anserine inhibits phosphorylase b at concentrations above 30 mM. The relationship between dipeptide concentration and relative phosphorylase activity displayed a biphasic relationship; increasing dipeptide concentrations beyond 50 mM for carnosine and 30 mM for anserine progressively reduced the relative activity. The inclusion of allosteric inhibitors including ATP and D-glucose in the assay medium attenuated but did not eliminate the activation effect of carnosine on phosphorylase a and b.
Some slight anserine induced activation of phosphorylase remained in the presence of allosteric inhibitors. Histidine inhibited a and b forms of the enzyme. β-alanine inhibited the a form and marginally activated phosphorylase b. 1-Methylhistidine slightly increased the activity of both forms of phosphorylase (Johnson et al. 1982). The activation of rabbit muscle phosphorylase a and b by carnosine and anserine was later replicated and contrasted with their effect on potato and yeast phosphorylase. Both carnosine and anserine inhibited the activity of potato and yeast phosphorylase at equivalent concentrations to those employed in the muscle enzyme assays. The dipeptides affected the enzyme $V_{\text{max}}$ values rather than the $K_M$ values for the substrates. It was surmised from kinetic data that both carnosine and anserine compete for the same binding site(s) on muscle phosphorylase. Furthermore, the contrast in the effects of the dipeptides between the muscle enzyme and those from the potato and yeast sources may be indicative of specific imidazole dipeptide binding sites on muscle phosphorylase (Johnson and Aldstadt 1984).

**Myosin ATPase activation**

Carnosine (2 - 10 mM) was reported to elicit a 30 - 60% activation of the in vitro hydrolysis of ATP in the presence of glycerinated rabbit skeletal muscle myofibrils at an ATP concentration of 5 mM (Yun and Parker 1965). In a later study in vitro, both carnosine and anserine were shown to promote ATP hydrolysis at physiological concentrations mediated by either myosin or glycerinated myofibrils in the presence of K⁺, Mg²⁺ and Ca²⁺ ions. Myosin ATPase activity of homogenized myofibrils was increased by 100% in the presence of 20 mM carnosine. Anserine produced greater activation than carnosine under the same conditions (Avena and Bowen 1969). Parker and Ring (1970) showed that carnosine had no effect on the myosin ATPase activity of invertebrate muscle and concluded that carnosine induced activation is confined to those muscles which naturally contain high concentrations of the imidazole dipeptides.

**Histamine synthesis in response to shock and wound healing**

Histamine induced vasodilation and its central role in the mediation of cardiovascular function and circulatory shock has been recognised for many years. More recently feline endotoxic and canine hemorrhagic shock have been associated with increases in plasma and tissue histamine
concentrations (Nagy et al. 1986; Parratt et al. 1986). Nagy offered no explanation for the source of the increased amount of histamine, however. Parratt observed an increase in histidine decarboxylase activity and postulated that greater histamine concentrations arose from increased histamine biosynthesis in non-mast cell sources.

Histamine is believed to be involved in the biochemical processes of wound healing, although the precise mechanism by which this occurs is unclear (Kahlson and Zenderfeldt 1972). Histamine production, stimulated by the administration of the mast-cell degranulation activator compound 48/80, enhances the healing response (Fitzpatrick and Fisher 1982b). Intra-peritoneal injections of carnosine have also been reported to restore at site wound breaking strength and collagen deposition in histidine deficient rats (Fitzpatrick and Fisher 1982a). Additionally, post-wounding reduction of tissue carnosine concentration was prevented by the intra-peritoneal administration of either histidine or histamine at the time of injury (Fitzpatrick and Fisher 1982b). On the basis of these results it was proposed that a possible metabolic inter-relationship between carnosine, histidine and histamine exists and that this provides a non-mast cell reservoir of histamine, via carnosine hydrolysis to histidine and subsequent decarboxylation, available which is available during periods of increased demand. This hypothesis was investigated in the rat by artificially stimulating mast-cell histamine release by administering compound 48/80. This procedure caused a rapid increase in muscle histamine concentration and histidine decarboxylase activity, which was followed by a sustained increase in carnosinase activity and a reduction in muscle carnosine concentration at 24 h post administration (Greene et al. 1984).

The involvement of carnosine in the biosynthesis of histamine and the subsequent effects on the wound healing processes was supported by the later work of Nagai et al. (1986) who demonstrated that in rats treated with hydrocortisone to suppress wound healing, subsequent treatment with carnosine lead to improved skin tensile strength at the incision site in contrast to controls. Similar effects were produced with the co-administration of histidine and β-alanine but not with β-alanine alone. Increases in both histidine decarboxylase activity and histamine content were found at the wound site following carnosine administration. No significant change
in carnosinase activity was found. It was proposed that the improvement in wound healing was partially attributable to the vasodilatory action of histamine which improved effusion from the wound site during the initial inflammation and also that free β-alanine arising from carnosine degradation was utilized for collagen synthesis (Nagai et al. 1986). The antioxidative properties of carnosine have also been used to explain the apparent efficacy of carnosine in promoting wound healing. Silaeva et al. (1990) reported that the topical application of carnosine to experimentally induced wounds in rats reduced the accumulation of lipid peroxidation products with conjugated diene and ketodiene groups and thiobarbituric acid-active products, in contrast to saline treated controls. The time to complete epithelization of the wounds was reduced in the carnosine treatment group in contrast to controls (Silaeva et al. 1990).

Possible correlations between the distributions of carnosine, histidine, histamine and 3-methylhistidine in different tissues of the rat, mouse, guinea pig and man have been investigated and higher concentrations of carnosine were often though not exclusively found in those tissues which had higher levels of histamine (Flancbaum et al. 1990).

Vasoconstriction

Carnosine was shown to exhibit stereospecific depressor action following intra-venous administration in anaesthetized cats (du-Vigneaud and Behrens 1939; du-Vigneaud and Hunt 1936). Melville et al. (1990) have reproduced this finding but also showed a pressor response in anaesthetized and pithed rats. Furthermore, they reported that carnosine exhibited both α-adrenoreceptor agonist and antagonist properties in isolated rabbit saphenous vein (Melville et al. 1990). More, recently it has been reported from in vitro studies that carnosine chelated with Zn²⁺ ions exhibits sustained vasoconstrictor activity in isolated rabbit saphenous vein and artery. Carnosine concentrations of up to 10 mM were necessary to induce contraction in the absence of Zn²⁺ ions, however, in the presence of 10 µM Zn²⁺ contracture was greatly potentiated, and the concentration of carnosine needed to elicit half of the maximum effect was reduced from 1.4 mM to 15 µM. The presence of zinc alone did not induce contraction. Certain zinc containing metalloproteins, such as angiotensin and Cu-Zn superoxide dismutase are significant in vascular
function and it was postulated that carnosine may potentiate the effects of these proteins by competitively binding zinc ions (O'Dowd et al. 1995).

**Neurotransmitter or neuromodulator**

There has been a steady accumulation of evidence which suggests that carnosine functions as either a neurotransmitter or neuromodulator in the olfactory bulb and epithelium of many vertebrates. It has been shown that carnosine is present in the primary olfactory pathway of the mouse and more specifically to be compartmentalized within the olfactory receptor neurons (Margolis 1974). Carnosine occurs at relatively high concentration in mouse olfactory bulb, 0.9 - 2.2 nmol mg\(^{-1}\) WW (3.6 - 8.8 mmol kg\(^{-1}\) DW), although these values are much lower than those associated with most mammalian skeletal muscles (Ferriero and Margolis 1975; Neidle and Kandera 1974; Wideman et al. 1978). It was also established that carnosine is synthesised in situ by carnosine synthetase (Harding and Marshall 1976; Horinishi et al. 1978; Ng and Marshall 1978) and that the carnosine synthetase activities in mouse olfactory bulb and epithelium were 92 and 495 pmol mg\(^{-1}\) protein h\(^{-1}\), respectively, in comparison with a skeletal muscle carnosine synthetase activity of 37 pmol mg\(^{-1}\) protein h\(^{-1}\) (Harding and Marshall 1976). Carnosine appears to be transported into regions of the olfactory bulb which contain the olfactory nerve axons and terminals (Burd et al. 1980). Carnosine is reported to be catabolised to its precursor amino acids by carnosinase within the olfactory pathway (Harding and Marshall 1976). Carnosinase activity within the epithelium, almost 15000 pmol mg\(^{-1}\) protein h\(^{-1}\), was only exceeded by its activity in the kidney (Harding and Marshall 1976). The suggestion that specific carnosine receptors are present in the olfactory pathway was supported by evidence showing that membrane fractions of olfactory bulbs displayed reversible, saturable and stereospecific carnosine binding (Hirsch et al. 1978; Hirsch and Margolis 1979). *In vitro* experiments have shown carnosine to be released from olfactory bulb synaptosomes by two processes, the first of which was gradual, spontaneous and non-reliant upon depolarization. The rate of this release was increased two-fold on the addition of 1 mM carnosine. The second mechanism was calcium-dependent and depolarization sensitive (Rochel and Margolis 1982). These results were considered to be consistent with the
proposed function of carnosine as a neurotransmitter in the olfactory pathway of the mouse (Rochel and Margolis 1982).

The presence of carnosine in other regions of the CNS including the retina of mammals has also been described (Margolis 1980; Margolis and Grillo 1984). The existence of anserine in the CNS and retina of birds has been reported (Fisher et al. 1977; Margolis and Grillo 1984). The cellular distributions of carnosine and anserine in the CNS of vertebrates have been investigated by the use of imidazole dipeptide specific antisera for immunocytochemical localization. In the rat, carnosine immunoreactivity was restricted to neurons, their axons and synaptic terminations in the glomerular layer of the olfactory bulb within the olfactory pathway, and non-olfactory carnosine distribution was confined to astrocytes and cerebellar Bergmann glia. In avian CNS widespread anserine immunoreactivity appeared to be solely associated with glial cells (Biffo et al. 1990). The presence of carnosine within the receptor neurons of human olfactory mucosa was also observed using immunohistochemical techniques (Sakai et al. 1990).

Activation of muscle calpains

It has been proposed that calpains, a group of calcium activated proteinases present in many types of eukaryotic cells, are involved in the degradation of contractile proteins (Dayton et al. 1976). Calpain I and calpain II exhibit maximum activity at micromolar and millimolar Ca²⁺ concentrations, respectively (Birkhold and Sams 1994; Murachi 1985; Suzuki et al. 1987a). It was recognised that calpain activity could be regulated by endogenous antagonists and agonists of Ca²⁺ ion binding such as calpastatin, a protein inhibitor of calpain, (Takano et al. 1986) and isovalerylcarnitine, an activator of human neutrophil calpain (Pontremoli et al. 1987). The high intra-cellular concentrations of carnosine and anserine in skeletal muscle prompted Johnson and Hammer (1989) to investigate a possible regulatory role for these and their constituent amino acids on the activity of calpain II isolated from hamster and chicken smooth muscle. During in vitro studies they found that 80 mM concentrations of carnosine and anserine increased the activity of chicken and hamster muscle calpain II by approximately 20 - 30% at a Ca²⁺ ion concentration of 2.5 mM. L-Methylhistidine increased the activity of chicken calpain II by about
the same amount and doubled the activity of hamster calpain II. The activation effects of these compounds were markedly attenuated at a Ca$^{2+}$ ion concentration of 5 $\mu$M. Calpain II activities were only 10 - 40% of those at 2.5 mM Ca$^{2+}$. It was also found that anserine and 1-methylhistidine reduced the inhibitory action of calpastatin on calpain II, whereas carnosine increased inhibition. Johnson and Hammer (1989) concluded that carnosine, anserine and 1-methylhistidine were not potent calpain II activators but that synergistically they may influence the interaction between calpain II and calpastatin. However, this effect is likely to be negligible in vivo where both imidazole dipeptide and Ca$^{2+}$ ion concentrations are much lower than the in vitro concentrations used.

In vitro studies have indicated that calpain II significantly reduces the activity of carnosine synthetase by as much as 60% at a calpain to tissue protein w/w ratio of 0.15 and that probably arises from extensive proteolysis of carnosine synthetase by calpain. It was proposed that such an effect might cause a reduction in muscle carnosine concentration in vivo and that this may explain reports of reduced muscle carnosine concentrations as a result of ageing, hypertension, muscular dystrophy and denervation (Johnson and Hammer 1994).

1.2.5 Carnosine metabolism

Biosynthesis and carnosine synthetase

Early experimental work involving the use of intact animals and isolated tissue slices provided evidence to support the direct biosynthesis of carnosine from histidine and $\beta$-alanine. Studies involving cell free extracts of chick skeletal muscle demonstrated the existence of an enzyme of broad specificity that catalyses not only the synthesis of carnosine, but also anserine, homocarnosine and several other dipeptides (Kalyanker and Meister 1959; Winnick and Winnick 1959). Similar enzyme regulated synthesis was evident in vivo and in vitro in frog brain (Yockey and Marshall 1969). Carnosine synthetase, or homocarnosine-carnosine synthetase, was subsequently isolated from rat brain, partially purified and characterized (Skaper et al. 1973). Carnosine synthetase (EC 6.3.2.11) which is cytosolic in origin (Ng and Marshall 1978) appears to be a dimer of MW 250,000, with a very high histidine content of 141 per 1000
residues (Rosario et al. 1981). The enzyme has an optimum activity at pH 7.4 and a requirement for ATP and Mg$^{2+}$ (Skaper et al. 1973). The apparent $K_M$ for histidine was approximately 16 $\mu$M (Horinishi et al. 1978), whereas that for $\beta$-alanine was 1.0 - 1.8 mM (Kish et al. 1978; Ng and Marshall 1978). The enzyme is also responsible for the synthesis of homocarnosine in the CNS and has a $K_M$ for $\gamma$-aminobutyric acid of approximately 8.8 mM (Kish et al. 1978). It does not catalyse the synthesis of anserine from $\beta$-alanine and 1-methylhistidine, rather anserine is formed via methylation of carnosine (Bauer and Schultz 1994). Carnosine synthetase is inhibited by several $\beta$-alanine analogues, but most effectively by 3-aminopropanesulphonic acid (Seely and Marshall 1982). Carnosine synthetase activities were measured in the 100000 g supernatant fractions of heart, brain, liver and muscle of rat, mouse, chick and frog and were found to be generally highest in skeletal muscle (chick pectoral muscle; 21.4 nmol g$^{-1}$ h$^{-1}$) and lowest in liver (rat liver 0.1 nmol g$^{-1}$ h$^{-1}$) (Ng and Marshall 1976a). A similar distribution of activities between tissues was found in all four species studied. High levels of carnosine synthetase activity have also been observed in chick erythrocytes (Ng and Marshall 1976b) and olfactory bulbs (Ng and Marshall 1978).

Intra-muscular carnosine biosynthesis has been demonstrated in vivo by the administration of radio-labelled precursor amino acids. Watanabe and Konosu (1979) demonstrated that following the intra-muscular injection of $^{14}$C-histidine in the eel, the radio-label was incorporated exclusively into carnosine within the muscle. Similar results using $^{14}$C-histidine and $^{14}$C-$\beta$-alanine were obtained in tuna (Abe et al. 1986), trout (Abe and Hochachka 1987) and rats (Tamaki et al. 1980). Furthermore, a greater rate of incorporation of the radio-label into trout muscle carnosine was observed following the administration of $^{14}$C-$\beta$-alanine rather than $^{14}$C-histidine, suggesting that $\beta$-alanine availability may be a limiting factor in carnosine biosynthesis (Abe and Hochachka 1987). This is supported by data from Tamaki et al. (1980) which showed a two-fold increase in the incorporation of $^{14}$C-histidine into carnosine following pre-administration of non-radioactive $\beta$-alanine.
Figure 1.3  Metabolic pathways of imidazole dipeptide biosynthesis and degradation.
Factors influencing biosynthesis and muscle content

The results from several studies in various species indicate that substrate availability, including dietary availability, may be a limiting factor to the regulation of carnosine biosynthesis and accumulation in vertebrate skeletal muscle. Reduced skeletal muscle carnosine concentrations were found in several species, including adult rats (Fuller et al. 1947; Quinn and Fisher 1977), eels (Abe and Ohmama 1987), salmon (Luckton and Olcott 1958) and adult roosters (Leveille et al. 1960), maintained on histidine-free or histidine deficient diets. Dietary histidine deficiency causes a more rapid decline in skeletal muscle carnosine concentration in young animals, such as chicks (Ousterhout 1960; Ousterhout and Luckton 1960) and rats (Barbaro et al. 1978). Conversely, supplementation of the diet with histidine increases in the skeletal muscle carnosine concentration once the histidine requirements for optimum growth have been fulfilled (Robbins et al. 1977). Rats fed a diet supplemented with histidine at a level of 5% w/w showed a two-fold greater carnosine concentration in the gastrocnemius muscle in contrast to rats on a control diet with a histidine content of 0.76% w/w (Tamaki et al. 1977). Dietary histidine supplementation over two weeks in the mature Quarterhorse, at 0.4% w/w in one study and up to 0.56% w/w twice per day in a further study, produced small but statistically non-significant increases in the carnosine concentration of the middle gluteal muscle (Powell et al. 1991; Miller-Graber and Seyers 1993).

No investigations of the influence of dietary β-alanine supplementation on muscle carnosine content appear to have made. However, twice-daily intra-peritoneal injections of very large doses of β-alanine (22 mmol kg⁻¹ BW, 2000 mg kg⁻¹ BW) in adult mice over a 5 day period produced a ten-fold increase in skeletal muscle carnosine concentration in contrast to controls (Margolis et al. 1985).

Increasing age appears to result in an initial increase in skeletal muscle imidazole dipeptide concentration during maturation and a subsequent decrease during senescence. The mean muscle imidazole dipeptide concentrations in rat longissimus dorsi increased from 2.49 to 2.99 µmol g⁻¹ WW from age 3 to 12 months followed by a reduction to 1.94 µmol g⁻¹ WW at 27 months. The
overall trend was due to changes in the anserine concentration as carnosine showed a continued
decline from 3 - 27 months. Similar results were evident in the quadriceps femoris (Johnson and
Hammer 1992). A similar trend appeared to evolve in human muscle with increasing age from
the analysis of 32 muscle samples from subjects aged between 3 months and 80 years. Muscle
carnosine concentrations in the younger age group (3 months to 3 years) were 1.5 - 2.5 μmol g⁻¹
ww in contrast to values in the teenage group of 4.9 - 8.3 μmol g⁻¹ ww. Subjects in the older
age group had carnosine values (1.0 - 1.5 μmol g⁻¹ ww) lower than the younger group.
However, the lower carnosine contents in the older age group were in patients undergoing
extended immobilization following hip fracture (Christman 1976).

In experimental rats unilateral ischemic denervation of gastrocnemius muscle caused a
significant decline (p < 0.01) in the carnosine concentration over a period of three weeks and a
slight but non-significant increase in the anserine concentration (Tamaki et al. 1976). Carnosine
and anserine concentrations in denervated and intact muscles are given in Table 1.8. Turnisky
and Long (1990) reported no effect of denervation on the anserine contents of rat soleus and
plantaris muscle, a non-significant decrease in the carnosine content of these two muscles, but a
significant increase in the muscle concentrations of carnosine (p < 0.05) and anserine (p <
0.005) in the gastrocnemius muscle. However, these muscles were analysed only three days after
denervation, in contrast to three weeks after in the study of Tamaki et al. (1976).
Table 1.8  Carnosine and anserine concentrations in denervated and intact rat gastrocnemius muscle. Adapted from Tamaki et al. (1976).
<table>
<thead>
<tr>
<th>Treatment</th>
<th>Weeks post-treatment</th>
<th>Mean concentration ± SEM (mmol kg⁻¹ DW)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Carnosine</td>
</tr>
<tr>
<td>Non treated rats</td>
<td>0</td>
<td>22.3 ± 3.9</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>28.9 ± 0.9</td>
</tr>
<tr>
<td>Treated rats</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Intact side</strong></td>
<td>1</td>
<td>29.6 ± 1.6</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>24.7 ± 1.1</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>24.4 ± 3.9</td>
</tr>
<tr>
<td><strong>Denervated side</strong></td>
<td>1</td>
<td>17.0 ± 3.8†</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>5.9 ± 0.8††</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>3.6 ± 0.7††</td>
</tr>
</tbody>
</table>

SEM = Standard error of the mean.

† = p < 0.05 compared to intact side.

†† = p < 0.01 compared to intact side.
Intestinal absorption and transport mechanisms

Experimental evidence has been provided for the existence of peptide specific transport mechanisms within the brush border membranes of the small intestine (Craft et al. 1968; Matthews 1975). Following absorption, carnosine, like many other peptides, was thought to be hydrolysed in the cytoplasm of the intestinal epithelium prior to transfer of the resulting amino acids into the portal blood. Carnosine administration to human subjects by ingestion or jejunal perfusion resulted in a rapid increase in circulatory concentrations of histidine and β-alanine, but no appearance of carnosine in the blood (Asatoor et al. 1970; Cook 1976). Carnosine and anserine were absorbed intact from rat intestine both in vitro and in vivo (Hama et al. 1976). Large increases in rat blood carnosine and anserine concentrations occurred two hours after forced feeding of large doses (up to 4500 mg kg⁻¹ BW) of the imidazole dipeptides (Hama et al. 1976). This finding was later reproduced although it was suggested that part of the orally administered carnosine was hydrolysed to histidine and β-alanine in the small intestine (Tamaki et al. 1985). However, more recent investigations have supported the hypothesis that a significant proportion of the intact peptides are transported into the circulation (Gardner and Wood 1989). Plasma carnosine (81.8, 44.4 and 4.6 μM) was detected in one subject 30, 60 and 90 min after administration of 4 g of carnosine in both isotonic and hypertonic test solutions containing lactulose, when sample collection and processing were performed at 4°C (Gardner et al. 1991).

*In vitro* studies provided evidence for the active transport of carnosine by hamster jejunum (Matthews et al. 1974). Nutzenadel and Scriver (1976) reported active transport of carnosine by a high $K_M$ system (5 - 10 mM) in rat intestine which discriminated it from β-alanine, α-amino acids and other dipeptides, and that intra-cellular hydrolysis prevented concentrative uptake despite the extent of hydrolysis being 10% only in enterocytes. Carnosine transport in rabbit intestinal brush-border membrane vesicles was optimized by an inward proton gradient with an external pH of 5.5 - 6.0 against a fixed internal pH of 7.5 (Ganapathy and Leibach 1983). *In vitro* carnosine transport in rabbit kidney and mouse and guinea pig intestine conformed to
Michaelis-Menten kinetics, and has been shown to be a carrier mediated Na⁺-independent process (Ganapathy and Leibach 1983; Himuki 1985; Rajendran et al. 1984).

**Catabolism and carnosinase**

Historically the investigation of enzyme catalysed hydrolysis of carnosine has been confused by the existence of at least two different forms of the enzyme and complicated by differences in the behaviour of each form in tissues from different species. (Hanson and Smith 1949) reported the existence in pig kidney of 'carnosinase', a specific enzyme responsible for catalysing the hydrolysis of the peptide bond in carnosine to yield its constituent amino acids, histidine and β-alanine. The enzyme was activated by the presence of Mn²⁺ ions. Carnosinase activity was detected in several tissues including kidney, liver and spleen of both pigs and rats, and a partially purified form of the enzyme was isolated from pig kidney and shown to be a metallo-protein activated by Mn²⁺ and Zn²⁺ ions (Rosenberg 1960; Wood 1957). Subsequently, two electrophoretically different forms of carnosinase were isolated from human liver, kidney and spleen. At pH 8.0 both exhibited the same substrate specificity and were approximately the same size (MW 90000) (Murphey et al. 1972). The existence of two carnosine-splitting enzymes was also described in hog kidney (Lenney 1976; Wolos et al. 1978). One of these two enzymes was later described as 'homocarnosinase' (Lenney et al. 1977). A Mn²⁺ - independent carnosinase and a Mn²⁺ - dependent carnosinase were isolated from mouse kidney (Margolis et al. 1979; Margolis et al. 1983). Rat brain was also shown to contain two carnosine hydrolysing enzymes; carnosinase and β-Ala-Arg hydrolase (Kunze et al. 1986). Studies in mouse olfactory tissues and rat liver to establish the subcellular distribution of carnosinase have indicated that the majority of the activity is localized in the cytoplasm (Harding and Marshall 1976; Kalra et al. 1988). Human tissue carnosinase (MW 90000) was isolated from kidney and characterized as a metallo-cysteine dipeptidase. The enzyme showed optimum activity at pH 9.5, had a pf of 5.6, and a $K_M$ (carnosine) of 10 mM. It was activated by dithiothreitol (DTT) and $p$-hydroxymercuribenzoate ($p$ HMB), was present in all tissues studied and had a wider specificity than hog kidney carnosinase. The enzyme appeared to be identical to prolinase, a non-specific peptidase (Lenney et al. 1985).
With more detailed analysis of the properties of hog kidney carnosinase Lenney (1990) attempted to rationalize characterization and nomenclature of carnosinase. He confirmed the presence of two dipeptidases in hog kidney capable of hydrolysing carnosine. He concluded that one was identical to the classical "carnosinase" described originally by (Hanson and Smith 1949), that it also fulfilled the requirements previously ascribed to "homocarnosinase" and "Mn\(^{2+}\) - independent carnosinase", and proposed that it be regarded as carnosinase. The second dipeptidase was functionally equivalent to "human tissue carnosinase", "Mn\(^{2+}\) - dependent carnosinase" and "ß-Ala-Arg hydrolase", and that it be considered a non-specific dipeptidase. Both carnosinase and non-specific dipeptidase were cytosolic (Lenney 1990).

The abnormal appearance of carnosine in the serum of two juvenile patients suffering from neurological disease and mental retardation prompted Perry et al. (1968) to investigate possible carnosinase activity in serum from these patients and in normal healthy subjects. The use of a previously published assay for tissue carnosinase activity indicated a mean enzyme activity of 0.71 and 1.22 \(\mu\)mol ml\(^{-1}\) h\(^{-1}\) in children and adults, respectively (Perry et al. 1968). Serum carnosinase from humans has been isolated (Lenney et al. 1982). A glycoprotein with a sub-unit MW of 75000 and a pI of 4.4, it was found to be structurally and functionally distinct from both carnosinase (tissue) and non-specific dipeptidase. Activities of serum carnosinase in tissues were measured and were proportional to the amount of trapped blood present. Brain however, contained 9 times more activity than could be accounted for by trapped blood (Jackson et al. 1991). It had previously been shown that CSF contained relatively high activities of serum carnosinase (Lenney et al. 1983). It was therefore proposed that serum carnosinase is synthesized in brain and transported to the blood via the CSF rather than through the blood-brain barrier. Serum carnosinase activity was also found in six higher primates but not in twelve non-primate mammals except for Golden hamster (Jackson et al. 1991). A comparison of the properties of carnosinase, non-specific dipeptidase and serum carnosinase is given in Table 1.9.
Table 1.9  Properties of rationalized carnosinase, non-specific dipeptidase and serum carnosinase. Adapted from Lenney (1990).
<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Enzyme</th>
<th>Carnosinase</th>
<th>Non-specific dipeptidase</th>
<th>Serum carnosinase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Specificity</td>
<td>carnosine</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>anserine</td>
<td>70</td>
<td>0</td>
<td>88</td>
</tr>
<tr>
<td></td>
<td>homocarnosine</td>
<td>47</td>
<td>0</td>
<td>11</td>
</tr>
<tr>
<td>pH optimum</td>
<td></td>
<td>8.8</td>
<td>8.8</td>
<td>8.3</td>
</tr>
<tr>
<td>$K_m$</td>
<td>carnosine</td>
<td>0.4 mM</td>
<td>5.0 mM</td>
<td></td>
</tr>
<tr>
<td>Activators</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>@ 50° C</td>
<td>Mn$^{2+}$, DTT</td>
<td></td>
<td></td>
<td>Mn$^{2+}$</td>
</tr>
<tr>
<td>Stabilizers</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% Inhibition</td>
<td>bestatin</td>
<td>0</td>
<td>50</td>
<td>39</td>
</tr>
<tr>
<td></td>
<td>pHMB</td>
<td>52</td>
<td>89</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>DTT</td>
<td>-</td>
<td>-</td>
<td>94</td>
</tr>
<tr>
<td>MW</td>
<td></td>
<td>57000</td>
<td>70000</td>
<td>75000</td>
</tr>
<tr>
<td>pI</td>
<td></td>
<td>5.5</td>
<td>5.0</td>
<td>4.4</td>
</tr>
<tr>
<td>Active</td>
<td>without Mn$^{2+}$</td>
<td>yes</td>
<td>no</td>
<td>yes</td>
</tr>
<tr>
<td></td>
<td>without DTT</td>
<td>yes</td>
<td>no</td>
<td></td>
</tr>
<tr>
<td>Location</td>
<td></td>
<td>kidney</td>
<td>kidney, liver, brain</td>
<td>Serum, brain, CSF</td>
</tr>
</tbody>
</table>

**Equivalent enzymes**

- "Carnosinase"
  Hanson and Smith (1949)
  Human tissue carnosinase
  Lenney et al. (1985)
- "Homocarnosinase"
  Lenney et al. (1977)
  Mn$^{2+}$ - dependent carnosinase
  Margolis et al. (1983)
- Mn$^{2+}$ - independent carnosinase
  β-ala-arg hydrolase
  Kunze et al. (1986)
The primary metabolites of carnosine, β-alanine and histidine, can be further metabolized. The deamination of β-alanine to malonate semialdehyde is catalyzed by β-alanine-α-ketoglutarate transaminase. Histidine can be further metabolized as shown in Figure 1.4.

**Excretion**

Normal urinary excretion of carnosine in humans is relatively high in the neonate but decreases during the first four years. Older children and adults excrete less than 4% of the amount excreted by young children. This urinary excretion pattern appears to be attributable to changes in serum carnosinase activity (Roesel et al. 1986).

Detection of ophidine (balenine) carnosine and anserine in human urine following meals comprising whale meat, other meats and tuna, respectively, has been reported (Abe et al. 1993; Undrum et al. 1982). Large amounts of carnosine were detected in the urine of fasted human subjects up to 3 - 5 h after oral administration of 4 g (approximately 18 mmol) of carnosine in both isotonic and hypertonic test solutions containing lactulose. As much as 14% of the dose was recovered in the urine during the subsequent 5 h. Urinary excretion of carnosine was lower following administration in hypertonic solution. Urinary excretion of carnosine was significantly correlated to plasma carnosinase activity (p < 0.005, r = -0.815) (Gardner et al. 1991).
Figure 1.4  Metabolic pathways of histidine catabolism.
1.2.6 Disorders of carnosine metabolism

**Hyper-β-alaninemia**

Hyper-β-alaninemia and β-aminoaciduria associated with somnolence and seizures was reported and was postulated to arise from a deficiency in the activity of β-alanine-α-ketoglutarate transaminase. Increased β-alanine concentrations in brain, liver, kidney and skeletal muscle and large increases in the concentration of carnosine in the brain and muscles, but not in the liver and kidneys were found. Deltoid and abdominal muscle carnosine concentrations, relative to leucine, were 45.0 and 36.1 µmol g⁻¹ WW (approximately 180.0 and 144.4 mmol kg⁻¹ DW), respectively, in contrast to a control subject where concentrations in the same muscles were 6.84 and 6.62 µmol g⁻¹ WW (approximately 27.4 and 26.5 mmol kg⁻¹ DW), respectively. These data suggested that increased substrate availability is the probable cause of the increased muscle carnosine content (Scriver et al. 1966).

**Carnosinemia**

Carnosinemia associated with carnosinuria, progressive neurologic disease, severe mental deficiency and myoclonic seizures was reported after excessive urinary excretion of carnosine was encountered in two juvenile patients (Perry et al. 1967). Carnosine was detected in fasting plasma from one patient after a normal diet (8 µM) and persisted for at least 72 h after the imposition of a meat-free diet (4 µM). Relatively large amounts of carnosine were excreted in the urine of both subjects (0.97 and 0.25 µmol mg⁻¹ creatinine) 72 h after the imposition of the meat-free diet. Carnosine was not detected in fasting plasma from 120 healthy control subjects. A metabolic challenge imposed on both subjects by the administration of a chicken breast diet resulted in an increased excretion of carnosine and the appearance of large quantities of anserine (2.27 and 8.59 µmol mg⁻¹ creatinine) in the urine. A greatly elevated carnosine concentration (45.3 µM) and the presence of large quantities of anserine (102 µM) were also detected in the plasma of the patient which had previously exhibited carnosinemia. Neither patient excreted 1-methylhistidine, the normal urinary metabolite of anserine. Carnosine loading tests were performed on both individuals by oral administration at 5 mg kg⁻¹ body weight (BW) (22 µmol kg⁻¹ BW). At 4 h post administration 15% of the dose had been excreted by the patient exhibiting
both carnosinemia and carnosinuria and 9% by the patient displaying only carnosinuria. The same dose given to a normal healthy subject resulted in the excretion of only 0.4% of the dose within 4 h. Perry et al. (1967) proposed the carnosinemia and carnosinuria occurred as a result of impaired degradation of imidazole dipeptides arising from a genetically derived deficiency in carnosinase activity.

The significance of plasma carnosinase activity in the aetiology of carnosinemia in these two patients was later investigated by Perry et al. (1968). Plasma carnosinase activities in the two patients were very low, 0.02 ± 0.02 and 0.04 μmol ml⁻¹ h⁻¹, with the lower activity present in the patient displaying both carnosinuria and carnosinemia. In contrast, the activities in normal healthy adults and children were 1.21 ± 0.52 and 0.71 ± 0.31 μmol ml⁻¹ h⁻¹, respectively. Significant tissue carnosinase activity was found in post mortem extracts of liver, kidney and heart of one patient (Perry et al. 1968).

Similar findings to these were later reported, for a family in which two boys with progressive neurological disorders and their physically and mentally normal sister exhibited carnosinuria, by Murphey et al. (1973). Carnosinemia was evident in both boys with serum carnosine concentrations of 200 - 300 μM, but not in the sister. Carnosinuria was evident in all three children with carnosine excretion higher in the sister (312 μmol d⁻¹) than in the brothers (63 and 204 μmol d⁻¹). Serum carnosinase activities were < 0.03 μmol ml⁻¹ h⁻¹. All three subjects excreted 1-methylhistidine when fed a meal comprising chicken breast, which indicated an unimpaired ability to catabolise anserine (Murphey et al. 1973). Electrophoresis of extracts of post mortem liver, kidney and spleen tissue from one of the brothers indicated that one of the two electrophoretic forms of carnosinase was absent and that the one present corresponded to the slower form of the enzyme and exhibited normal activity. Murphey et al. (1973) postulated that the absence of serum carnosinase activity in the physically and mentally normal sister precluded a causal relationship between serum carnosinase deficiency and neurological disease. Reduced serum carnosinase activity has also been reported in urea cycle defects which in some cases correlated with carnosinemia (Burgess et al. 1975), liver cirrhosis, hepatoma and chronic
hepatitis (Bando et al. 1986), Parkinson's disease and multiple sclerosis (Wassif et al. 1994), Duchenne muscular dystrophy (Bando et al. 1984) and alcoholic chronic skeletal muscle myopathy (Duane and Peters 1988).

**Homocarnosinosis**

Homocarnosinosis characterized by an increased homocarnosine concentration in cerebrospinal fluid (CSF) and brain, serum carnosinase deficiency and carnosinuria often associated with progressive mental retardation, spastic paraplegia and retinal pigmentation was first described in a 37 year-old woman (Gjessing and Sjaastad 1974). Her two brothers displayed increased CSF homocarnosine concentrations and similar clinical symptoms, her mother showed no neurological abnormalities but had elevated CSF homocarnosine concentrations, and the father, other sibling and two maternal aunts were physiologically, biochemically and mentally normal (Sjaastad et al. 1976). CSF homocarnosine concentrations in the mother, two brothers and daughter were 48-75 μM in contrast to the unaffected father and second daughter (0.5 - 2.0 μM). The normal range was 0.9 ± 0.1 to 1.8 ± 1.8 μM (Perry et al. 1975; Perry et al. 1982). Homocarnosine concentration in the brain of one brother was four-fold higher than in controls. The mother and three children all showed a marked elevation of carnosine excretion on a meat-free diet and after oral carnosine administration, minimal excretion of 1-methylhistidine following a chicken meal and oral anserine administration. These findings were consistent with a deficiency in serum carnosinase activity. Subsequent analysis of carnosinase activity in the four homocarnosinosis patients indicated zero or minimal activity present in both serum and CSF, in contrast to both controls and the unaffected father and daughter (Lenney 1985; Lenney et al. 1983). These findings were identical with those associated with carnosinemia and a deficiency in serum carnosinase activity was probably responsible for the increased homocarnosine concentrations evident in homocarnosinosis patients (Lenney et al. 1983). A causal relationship between homocarnosinosis, impaired serum carnosinase activity and neurological disorder was considered unlikely (Gjessing et al. 1990). No homocarnosinosis was found in twenty-five patients afflicted with various forms of spastic paraplegia (Sjaastad et al. 1977).
Other disorders

Increased urinary excretion of carnosine, has been reported in Juvenile Amaurotic idiocy (Stengel/Batten/Vogt/Spielmeyer disease) (Bessman and Baldwin 1962; Levenson et al. 1964). Raised homocarnosine concentrations were reported in some untreated cases of Phenylketonuria (Føllings disease) (Sande et al. 1970). Reduced carnosine concentration in human lens was correlated with the severity of senile cataracts (Boldyrev et al. 1987).

1.3 Objectives

The principal objectives of this research were as follows:

i To develop and validate sensitive, selective and accurate analytical methods for the determination of the concentrations carnosine and other associated biogenic imidazoles in equine tissues and fluids. (Chapter 3)

ii To determine the normal concentration range of carnosine and associated compounds in equine plasma, and to investigate the effects of normal feeding and exercise. (Chapter 4)

iii To determine by direct measurement carnosine concentrations in type I, IIA and IIB fibres from normal and abnormal equine skeletal muscle, and to investigate the effect of training. (Chapter 5)

iv To measure carnosine concentrations in other equine tissues. (Chapter 5)

v To study aspects of carnosine metabolism, transport and excretion in vivo. (Chapter 6)

vi To examine the effectiveness of oral carnosine or histidine and β-alanine administration in increasing endogenous skeletal muscle carnosine concentrations. (Chapter 7)
CHAPTER 2

GENERAL METHODOLOGY
2.1 SAMPLE COLLECTION AND PREPARATION PROCEDURES

2.1.1 Blood collection

Single blood samples were obtained by venepuncture using disposable sterile (1.2 mm i.d. x 40 mm) 'Monoject' needles and 10 ml plastic syringes (Sherwood Medical, Balmoney, N. Ireland).

Serial blood samples were collected via an indwelling catheter. A 2 cm² area over the left jugular vein was scrubbed and shaved. Following local anaesthesia with Xylocaine (0.5 ml 2%) administered subcutaneously, an 'Intraflon 2' (2.4 mm i.d. x 120 mm) Teflon trocart catheter (Vygon UK Ltd., Cirencester, UK.) was inserted, closed with a three-way tap and sutured in place. The catheter was flushed with physiological saline (10 ml) containing 5000 IU 1⁻¹ heparin (CP Pharmaceuticals Ltd., Wrexham, UK.) as anticoagulant. Blood (5 ml) was drawn through the catheter and discarded immediately prior to collecting each 10 ml blood sample. The catheter was flushed with saline (5 ml) after each sample withdrawal. Blood was collected into 5 ml lithium heparin tubes (LIP Ltd., Shipley, UK.) and stored in ice prior to centrifugation (2000 g for 4 min at 4°C). Plasma was aspirated and stored at -20°C prior to analysis.

2.1.2 Urine collection

Twelve-hour urine collections from both fillies and geldings were performed by the free-flow technique using a collection harness (Harris and Snow 1988). Individual samples were retained in polythene bags suspended under the harness. Samples were mixed thoroughly, the volume recorded, and a 20 ml aliquot taken and stored at -20°C prior to analysis.

2.1.3 Muscle sampling

Muscle samples were collected both from live experimental animals using the percutaneous biopsy technique and by dissection at post mortem from euthanased horses which had failed to respond to treatment for a variety of chronic orthopaedic conditions. All animals, with one exception, (see Chapter 4) had no clinical history of muscle disorders or significant weight loss.
Biopsy sampling

Percutaneous muscle biopsies were collected from the right gluteus medius muscle at a point one third of the distance along a line running from the tuber coxae to the head of the tail using a 5 mm Bergström-Stille biopsy needle (Bergström 1962; Snow and Guy 1976). The biopsy site was shaved and disinfected prior to local anaesthesia with 2% Xylocaine™ (1 - 2 ml). The biopsy needle was introduced through a small stab incision made through the skin and muscle fascia with a scalpel blade. Biopsies were taken at depths of 6 cm. Biopsy samples were blotted on filter paper to remove excess blood, placed in a screw-cap tube, frozen in liquid nitrogen (-196°C) and subsequently freeze-dried. Freeze-dried samples were stored in liquid nitrogen.

Post mortem sampling

Tissue samples were collected as quickly as possible, and always within 90 min of euthanasia. The entire left or right middle gluteal muscle was removed and a 1 cm-thick transverse section was dissected from the intact muscle at the mid-point and at points one quarter of the muscle length from each end. Triplicate tissue samples approximately 5 mm² by 20 mm were dissected from 9 pre-determined sites (Figure 2.1) such that the fibre orientation was parallel with the long edge. Samples were blotted on filter paper to remove excess blood, stretched slightly to straighten the fibres, frozen in liquid nitrogen (-196°C) and subsequently freeze-dried. Freeze-dried samples were stored in liquid nitrogen.

Muscle powdering

Freeze-dried muscle was allowed to reach ambient temperature in sealed tubes before powdering. The outer surfaces of the samples were removed in order to minimize the contamination from dried blood. The remaining muscle was coarsely powdered with an agate pestle and mortar and finely powdered by drawing the tissue between the serrated tips of sharp curved forceps. Contaminating connective tissue and blood were removed as far as possible. Powdered muscle was stored in liquid nitrogen.
2.1.4 Sampling of other tissues

Other tissue samples comprising liver, kidney, lung, spleen, diaphragm, heart (myocardium), brain (medulla and cerebellum) and gastro-intestinal tract (small intestine, colon, stomach and rectum) were prepared and stored using the procedures described previously for muscle.

2.2 SAMPLE EXTRACTION PROCEDURES

2.2.1 Plasma extraction

Several plasma extraction procedures were employed; the exact procedure used being dependent on the particular analytes to be determined. Samples for carnosine, histidine, β-alanine and taurine determination were deproteinized and extracted with sulphosalicylic acid, as described in detail in Chapter 3. Samples for N-α-acetylcarnosine determination were deproteinized and extracted with perchloric acid/phosphoric acid, as described in detail in Chapter 3.

2.2.3 Urine extraction

Frozen urine samples were thawed at 37°C and mixed thoroughly. Samples were vortex-mixed for one minute to re-suspend any particulate material immediately prior to taking a 1 ml aliquot for extraction.

2.2.4 Muscle extraction

Powdered muscle (8 - 10 mg) was extracted with aqueous 0.5 M perchloric acid containing 1 mM EDTA (100 μl mg⁻¹), as described previously (Harris et al. 1974). Extracts were vortex-mixed at 2 min intervals over a total period of 15 min, or until the floating muscle particles sink. During mixing intervals samples were stored on ice. Extracts were centrifuged for 5 min at 12000 g at ambient temperature. Aliquots of the acid extracts, for the determination of low concentrations, were stored at -20°C prior to further extraction and analysis. The remaining volumes of the extracts were neutralized with aqueous 2.1 M potassium hydrogen carbonate and centrifuged. The supernatants were stored at -20°C prior to analysis. Neutralized supernatant fluid (1 ml) was equivalent to 10 mg of freeze-dried muscle.
2.3.5 Extraction of other tissues
Powdered tissue samples were extracted with perchloric acid as described previously (Harris et al. 1974). Acid extracts were stored at -20°C until analysis.

2.3 INDIVIDUAL MUSCLE FIBRE PREPARATION, WEIGHING AND EXTRACTION PROCEDURE

2.3.1 Dissection
A thin longitudinal slice was cut from the freeze-dried muscle sample whilst ensuring that the long edge was parallel with the fibre bed orientation. The thin muscle slice was placed in an agate mortar and compressed gently with the pestle. Gentle rocking of the pestle helped to partially separate the muscle fasciculi. Further separation of the fasciculi was obtained using a pair of dissection needles. One fasciculus was transferred to a pre-cleaned (IMS followed by acetone) microscope slide. With the aid of a stereo dissection microscope and a pair of very fine dissection needles (0.1 mm sharp-point ophthalmic suture needles), individual muscle fibres were separated. Between 30 and 50 fibres per sample were dissected. Separated fibres were stored overnight at -85°C.

2.3.2 Mounting and weighing
Each of three pre-cleaned microscope slides were engraved with a small central grid of 25 squares and labelled with the identity of the horse, the sample No. (i.e. MG3, MG4 etc.), the horse colour code label and the slide number (Figure 2.2).

One individual fibre was removed from the flip-top Eppendorf tube and the tube was re-capped. Three small fragments were cut from one end of the fibre. A small drop of water was placed in the first square of the grid on each slide. One fragment was placed in the first grid-square on each slide. The remaining larger fibre portion was transferred on a microscope slide to a pre-calibrated quartz-fibre fish-pole balance for weighing (Lowry and Passoneau 1972). The cover glass was removed from the glass barrel of the balance and the a dissection needle was used to
transfer the muscle fibre to the end of the quartz fibre. The muscle fibre is held by static attraction. The cover glass was replaced and the deflection recorded using a graticule eyepiece. The muscle fibre was removed from the balance and placed in a 200 µl glass micro-vial which was immediately capped. The above procedures were repeated until 25 fibres were mounted in triplicate and weighed. Completed slides were stored at -20°C prior to histochemical staining. Fibres for extraction were stored at -85°C.

2.3.3 Metabolite extraction

Individual muscle fibres were extracted as described in detail in Chapter 3.

2.4 HISTOCHEMICAL STAINING OF INDIVIDUAL MUSCLE FIBRES.

Freeze-dried individual muscle fibre fragments and frozen muscle sections were characterized as type I, type IIA or type IIB by histochemical staining for myosin ATPase activity by a modification of a published method (Brook and Kaiser 1970).

2.4.1 Preparation of reagents

Sodium acetate solution (0.2 M): Sodium acetate trihydrate (27.22 g) was dissolved in distilled de-ionised water (1000 ml).

Acetic acid solution (50% v/v): Glacial acetic acid (50 ml) was diluted with distilled de-ionised water (50 ml).

Glycine/calcium chloride buffer: Glycine (3.00 g) and calcium chloride dihydrate (2.94 g) were dissolved in distilled de-ionised water (1000 ml).

Potassium hydroxide solution (0.1 M): Potassium hydroxide (5.60 g) was dissolved in distilled de-ionised water (1000 ml).

Calcium chloride solution (2% w/v): Calcium chloride dihydrate (10.00 g) was dissolved in distilled de-ionised water (500 ml).
Cobalt chloride solution (1% w/v): Cobalt chloride hexahydrate (5.00 g) was dissolved in distilled de-ionised water (500 ml).

Ammonium sulphide solution (1% w/w): 20% w/w ammonium sulphide solution (1 ml) was diluted with distilled de-ionised water (19 ml).

2.4.2 Staining procedure

The pH meter (Corning pH/ion meter, CIBA-Corning) was calibrated between pH 7.0 and 10.0 using a two-point calibration. Glycine/calcium chloride solution (~ 45 ml) was added to two Coplin jars which were incubated at 20°C. One solution was subsequently titrated to pH 9.6 and the other to pH 9.8 using 0.1M Potassium hydroxide. ATP disodium salt (75 mg) was added to the glycine/calcium chloride buffer at pH 9.8 and the solution re-titrated to pH 9.6. The pH meter was re-calibrated between pH 4.000 and 7.000 using a two-point calibration. Sodium acetate solution was incubated, in a Coplin jar at 20°C and subsequently titrated to precisely pH 4.500 with acetic acid (50% v/v). Solutions of calcium chloride (2% w/v) and cobalt chloride (1% w/v) were incubated at 20°C. Two slides of mounted fibres were incubated in the sodium acetate /acetic acid buffer for exactly 5 min, rinsed briefly four times in distilled water and once in the glycine/calcium chloride solution for 30 s prior to incubation in the ATP/glycine/calcium chloride solution for 30 min. After rinsing the slides were incubated in the calcium chloride solution for 90 s, rinsed, incubated in cobalt chloride for 3 min, rinsed and flooded with the ammonium polysulphide solution for 2 min. The slides were rinsed, coverslips were mounted on the slides using UV free aqueous mountant and the edges of the coverslips sealed using clear nail varnish. The stained fibres were viewed using low power microscopy using and characterized as type I, IIA or IIB according to stain intensity; type I fibres stained black, type IIA fibres white and type IIB fibres grey.
Figure 2.1  Muscle sampling sites within the middle gluteal muscle.
Figure 2.2  Individual muscle fibre mounting procedure.
2.5 DETERMINATION OF β-ALANINE AND TAURINE CONCENTRATIONS IN PLASMA, URINE AND MUSCLE

Neutralized perchloric acid extracts of muscle and sulphosalicylic acid extracts of plasma were prepared as described in Section 2.2. Extracts (20 µl) were buffered to pH 9.65 with aqueous 400 mM borate buffer (100 µl) in 300 µl glass micro-vials. Buffered extracts were analysed by the high-performance liquid chromatography (HPLC) method developed for the determination of muscle imidazoles, as described in Chapter 3. Extracts were not subjected to solid-phase extraction for the analysis of taurine and β-alanine. Chromatograms of a mixed standard solution of taurine and β-alanine, and typical plasma and urine extracts are shown in Figures 2.3, 2.4 and 2.5, respectively. Extract concentrations were calculated from a range of known external standards (10 - 1000 µM) buffered as per samples.

2.6 DETERMINATION OF PLASMA CARNOSINASE ACTIVITY

Plasma carnosinase activity was determined using a modification of a previously published method (Bando et al. 1984).

2.6.1 Preparation of reagents

Buffer, 50 mM TRIS.HCl: \( \text{Tris} -(\text{hydroxymethyl})\)-methylamine (3.028 g) was dissolved in distilled water (500 ml) and titrated to pH 8.40 with 1.0 M and 0.1 M hydrochloric acid.

Substrate, 50 mM carnosine: Carnosine (1.131 g) was dissolved in 50 mM TRIS.HCl buffer, pH 8.40 (100 ml).

2.6.2 Assay

Plasma (50 µl) was diluted with aqueous 50 mM TRIS.HCl buffer, pH 8.40 (150 µl), vortex-mixed and pre-incubated for 5 min at 37°C. Aqueous 50 mM carnosine (50 µl) was added. The solution was vortex-mixed briefly and incubated for 120 min at 37°C. The reaction was stopped
and protein precipitated by the addition of ice-cold perchloric acid (750 \(\mu l\)). The extract was vortex-mixed, cooled on ice for 5 min and subsequently centrifuged for 5 min at 12000 g at ambient temperature. The supernatant fluid (500 \(\mu l\)) was neutralized with aqueous 2.1 \(M\) potassium hydrogen carbonate (200 \(\mu l\)). The neutralized extract was mixed, cooled on ice and centrifuged as described previously. The supernatant fluid was collected and analysed for histidine concentration by HPLC. A linear relationship between incubation time and histidine produced was evident for incubation times up to 120 min. The plasma carnosinase assay was established and validated using human plasma.

### 2.7 DETERMINATION OF TISSUE CARNOSINASE ACTIVITY

Tissue carnosinase activity was determined by a modification of a previously published method (Lenney 1990).

#### 2.7.1 Preparation of reagents

**Buffer, 50 mM TRIS.HCl:**

Tris-(hydroxymethyl)-methylamine (3.028 g) was dissolved in distilled water (500 ml) and titrated to pH 8.8 with 1.0 \(M\) and 0.1 \(M\) hydrochloric acid.

**Substrate, 100 mM carnosine:**

Carnosine (2.262 g) was dissolved in 50 mM TRIS.HCl buffer, pH 8.8 (100 ml).

**Co-factors, 0.5 mM Mn\(^{2+}\): and 20 mM dithiothreitol**

Manganese dichloride tetrahydrate (9.90 mg) and DL-dithiothreitol (0.308 g) were dissolved in distilled water (100 ml).

#### 2.7.2 Assay

Freeze-dried powdered tissue (5 mg) was homogenized (Ultra-Turax motorized homogenizer) for 15 sec in ice-cold 50 mM TRIS.HCl buffer, pH 8.8 (700 \(\mu l\)). Further TRIS.HCl buffer (100 \(\mu l\)) or co-factor solution containing 0.5 mM Mn\(^{2+}\) and 20 mM dithiothreitol was added. The homogenate was vortex-mixed briefly and pre-incubated for 5 min at 37°C. Aqueous 100 mM carnosine (200 \(\mu l\)) was added, and the homogenate was vortex-mixed and incubated for 120 min.
at 37°C. The reaction was stopped by the addition of ice-cold 0.5 M perchloric acid (1000 µl). The extract was vortex-mixed, cooled on ice for 5 min and subsequently centrifuged for 5 min at 12000 g at ambient temperature. The supernatant fluid (500 µl) was neutralized with 2.1 M potassium hydrogen carbonate (200 µl), mixed, cooled on ice and centrifuged as described previously. The supernatant fluid was collected and analysed for histidine concentration by HPLC. A linear relationship between incubation time and histidine produced was evident for incubation times up to 120 min.

Values for the coefficients of variation for the enzyme assays are given in Table 2.1.

2.8 STATISTICAL METHODS

Unless specified otherwise all data are presented as the mean and standard deviation (mean ± SD). Coefficient of variation (CV) was calculated as follows:

\[ CV = \frac{SD \times 100}{X} \]

Pooled standard deviation (Sp) was calculated according to the following formula:

\[ Sp = \sqrt{\frac{\sum (n_i - 1 \cdot SD_i^2)}{\sum (n - 1)}} \]

Where differences in measured parameters between groups were to be compared initial statistical analysis was performed using 1-factor analysis of variance (ANOVA). Where differences were detected significance was determined by the use of a multiple comparison test; Fisher's Protected Least Significant Difference (PLSD). Significance was declared at \( p < 0.05 \). Simple linear regression analysis where performed was by the least squares method.
Table 2.1  Coefficients of variation for the HPLC analyses and enzyme assays.
<table>
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<tr>
<th>Metabolite</th>
<th>Sample</th>
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<tr>
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<td>Tissue</td>
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<td>10</td>
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Figure 2.3  Chromatogram of a mixed standard solution containing taurine and β-alanine at 100 μM.
Figure 2.4 Chromatogram of a typical sulphosalicylic acid extract of equine plasma.

(* Indicates the position where β-alanine would appear if it was present in normal equine plasma.)
Figure 2.5 Chromatogram of a typical sulphosalicylic acid extract of equine urine.

(* Indicates the position where β-alanine would appear if it was present in normal equine urine.)
2.9 REAGENTS (Ordering information)

Glacial acetic acid 99-101% w/w Fisons A0400/PB17
Acetonitrile 190 (Far UV) super purity solvent Romil Chemicals H718
Acetone super purity solvent Romil Chemicals H031
N-α-acetyl-L-carnosine Dr. E. Hultman (gift) -
β-Alanine Sigma A7752
Ammonium dihydrogen phosphate Aldrich 21,600-3
Ammonium sulphide 20% w/w Aldrich 30,941-9
L-Balenine Dr. H. Abe (gift) -
L-Anserine nitrate Sigma A1131
ATP disodium salt: From equine muscle. Sigma A5394
Orthoboric Acid AnalaR BDH 10058
Calcium chloride dihydrate AnalaR BDH 10070
L-Carnosine Sigma C9625
Cobalt (II) chloride hexahydrate AnalaR Sigma C2644
Diammonium hydrogen phosphate Aldrich 33,879-6
DL-Dithiothreitol Sigma D5545
Glycine Sigma G7126
L-Histidine free base Sigma H8000
L-Homocarnosine Sigma H4885
Hydrochloric acid 34.5% v/v AnalaR BDH 10125
Manganese(II) chloride tetrahydrate Aldrich 22,127-9
3-Mercaptopropionic acid Sigma M6750
Methanol (205) super purity solvent Romil Chemicals H410
Methanol (215) super purity solvent Romil Chemicals H409
L-1-Methylhistidine Sigma M9005
L-3-Methylhistidine Sigma M3879
p-Nitrophenol Sigma 104-8
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<td>Phosphoric acid 85% w/w</td>
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<tr>
<td>Potassium hydrogen carbonate AnalR</td>
<td>BDH</td>
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<td>Sigma</td>
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<td>Taurine</td>
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<tr>
<td>Tetrahydrofuran super purity solvent</td>
<td>Romil Chemicals</td>
<td>H718</td>
</tr>
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<tr>
<td>Tris-(hydroxymethyl)-methylamine</td>
<td>Sigma</td>
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CHAPTER 3

DEVELOPMENT OF HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC) METHODS FOR THE ANALYSIS OF CARNOSINE, ITS ANALOGUES, AND THEIR METABOLITES IN EQUINE BODY FLUIDS AND TISSUES.
3.1 GENERAL INTRODUCTION

The quantification of the concentrations of carnosine, its analogues anserine, balenine, homocarnosine and N-α-acetylcarnosine, and their metabolites histidine, 1-methylhistidine, 3-methylhistidine in equine plasma, urine and tissues (skeletal and smooth muscle, diaphragm, myocardium, liver, kidney, lung, spleen, cerebellum and medulla), required the development of selective and sensitive analytical methodologies.

Thin-layer chromatography, electrophoresis, and ion-exchange chromatography have previously been used for the measurement of amino acid and imidazole concentrations in biological fluids and tissues (Efron 1969; Smith 1969; Wadman et al. 1971; Wadman and de-Bree 1976). However, limitations associated with these techniques including limited selectivity, poor sensitivity and long analysis times, have resulted in their replacement by high-performance liquid chromatography (HPLC). HPLC has proved to be a highly versatile and adaptable analytical technique for the determination of the concentrations of very many low molecular mass compounds in physiological samples including; lipids, vitamins, steroid hormones, carbohydrates, nucleotides, nucleosides and nucleo-bases, organic acids, catecholamines, imidazoles, amines, amino acids and small peptides. Probably the greatest advantage of HPLC over the more traditional techniques is its capacity to resolve and quantify many, often 30 or more, compounds simultaneously with high accuracy, precision and sensitivity; although the rate of sample throughput may be compromised somewhat.

3.2 DETERMINATION OF CARNOSINE AND OTHER BIOGENIC IMIDAZOLES IN EQUINE PLASMA BY ISOCRATIC REVERSED-PHASE ION-PAIR HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

Dunnett and Harris (1992).

3.2.1 Introduction

Difficulties were encountered in the adaptation of previously published methods for the measurement of tissue carnosine contents, to the determination of carnosine in equine plasma
owing to the low plasma content and tendency for co-elution with amino acids or other small peptides. Analysis times were typically 30 - 60 min and the complex chromatograms produced contain superfluous information. An HPLC method for the quantification of carnosine and anserine in rabbit serum (Kurisaki and Hiraiwa 1988) proved to be grossly inaccurate when applied to equine plasma. An new analytical method was developed which combined isocratic reversed-phase ion-pair HPLC with sorbent extraction to provide rapid, selective and sensitive detection of carnosine, histidine, anserine, and 1-methylhistidine in plasma with previously unobtained specificity.

3.2.2 Experimental

Instrumentation and reagents

The HPLC system comprised a Constametric 3000 pump (LDC Analytical, Stone, Staffs., UK), a Rheodyne 7125 injector with a 200 µl sample loop (Cotati, CA., USA.), a LC-UV variable wavelength ultra-violet spectrophotometric detector (Unicam, Cambridge, UK) and a LKB 6500 flat-bed potentiometric recorder (LKB Biochrom, South Croydon, UK). A Sepralyte ODS 40 µm sacrificial column (50 x 4.6 mm I.D.) (Analytichem International, Harbor City, CA., USA.) and a Rheodyne in-line filter (0.5 µm pore size) (Cotati, CA, USA.) were inserted between the pump and the injector. Ultra-violet absorption spectra were recorded on a Unicam SP800 ultra-violet spectrophotometer (Unicam, Cambridge, UK.).

All reagents were analytical reagent grade unless specified otherwise (see Chapter 2). Water was purified by reverse osmosis and de-ionization (Elgastat Spectrum RO1, Elga, High Wycombe, UK.).

Sample deproteinization and solid-phase extraction

Heparinized plasma (1ml) was deproteinized with 200 µl 30% (w/v) 5-sulphosalicylic acid (SSA), and centrifuged. Plasma SSA extracts (500 µl) were loaded onto 100 mg / 1 ml Bond Elut PRS (propylsulphonyl) solid-phase extraction (SPE) cartridges (International Sorbent Technology, Hengoed, UK) previously conditioned with methanol (4 ml) and 1.0 M phosphoric
acid (4 ml). Interfering substances were washed from the cartridges with 1.0 $M$ phosphoric acid (2 x 500 $\mu$l) and the eluate discarded. The sorbent was air dried and the isolates (carnosine and other imidazoles) were eluted with HPLC mobile phase (2 x 500 $\mu$l). Solvents at all stages were drawn through the cartridges at a flow-rate of 1 ml min$^{-1}$. A 200 $\mu$l aliquot was injected onto the HPLC column.

**Chromatography**

Chromatography was performed on a Hypersil ODS 3 $\mu$m (150 x 4.6 mm I.D.) analytical column (Shandon Scientific, Runcorn, UK.) protected by a Hypersil ODS 5 $\mu$m (20 x 4.6 mm I.D.) guard column.

The compounds of interest were eluted isocratically at ambient temperature using a mobile phase comprising an aqueous solution of 200 mM ammonium dihydrogen phosphate ($\text{NH}_4\text{H}_2\text{PO}_4$) and 100 mM sodium pentanesulphonate adjusted to pH 2.0 with concentrated phosphoric acid, and containing 4% (v/v) acetonitrile. The mobile phase was filtered through 0.45 $\mu$m (HVLP 047 PTFE filters, Millipore UK Ltd., Watford, Herts., UK.) and degassed by helium sparging prior to use and periodically throughout the day. The mobile phase was freshly prepared each day. The flow-rate was 0.8 ml min$^{-1}$. UV absorption was measured at 220 nm with the detector sensitivity set for full-scale deflection (FSD) at 320 milliabsorbance units (mAU). Plasma concentrations were determined by comparing sample peak heights to those of external standards.

**Standard preparation**

Individual 10 mM stock standard solutions of carnosine, anserine, histidine and 1-methylhistidine were prepared by dissolving the required weight of each compound in HPLC grade water (10 ml). Working standard solutions for each compound were prepared over the concentration range 5 - 30 $\mu$M by dilution of stock standards with mobile phase. Both stock standard solutions and working standard solutions were stored at -20°C when not used.
Recovery study

Pooled equine plasma was spiked at 25 μM with a mixed standard containing 1-methylhistidine, histidine, anserine and carnosine. Overall extraction recoveries (n = 5) for the combined deproteinization and sorbent extraction stages were determined by comparing the chromatograms obtained from the spiked plasma extracts with those obtained from a mixed standard solution containing 25 μM 1-methylhistidine, histidine, anserine and carnosine. The concentration of these compounds in the plasma prior to spiking was determined and taken into account when calculating overall recoveries.

Reproducibility study

Standards for the determination of the precision and accuracy of the analysis were prepared from pooled equine plasma extract which was spiked at 25 μM with 1-methylhistidine, histidine, anserine and carnosine. Repeated injections of the spiked extract (n = 5) were made on day 1, day 5 and day 10 to assess the inter-assay variation. Intra-assay variation was determined by repeated injections (n = 5) of the spiked extract on three occasions within day 1.

Lower detection limits

The lower limits of detection for the compounds of interest were determined by injecting individual standards of 1-methylhistidine, histidine, anserine and carnosine sufficient to elicit FSD on the recorder at a detector sensitivity setting of 10 mAU. The height of the smallest detectable peak was 5mm which was equivalent to a signal : noise ratio of 2:1.

3.2.3 Results and discussion

Chromatography

Spectra over the UV range 190 - 450 nm were recorded for the imidazole compounds of interest. Their mean λ\text{max} values occurred at a wavelength of 220 ± 5 nm (carnosine, λ\text{max} = 223 nm) in 0.1M (NH\text{4})H\text{2}PO\text{4} (pH 2.0). The reference cell contained 0.1M (NH\text{4})H\text{2}PO\text{4}. The UV spectra of several other amino acids were also recorded and exhibited λ\text{max} values at slightly lower wavelengths (β-alanine, λ\text{max} = 205 nm). At 220 nm the absorbance ratio of β-alanine:
carnosine was 1.00 : 5.88 for equimolar solutions. A detection wavelength of 220 nm was selected.

Inevitably there is the potential for significant interference when utilizing UV detection at such a non-specific wavelength. In addition to the amino acids, many other polar low molecular mass compounds (Mr < 2000), endogenous to plasma, display strong UV absorption at 220 nm. Some such compounds are; creatine, creatinine, purine nucleotides, nucleosides and bases, and carboxylates (lactate and pyruvate). However, it has been demonstrated previously that for a non-polar stationary phase and a polar mobile phase, retention of the biogenic imidazoles is maximised at low pH (O'Dowd et al. 1988), and furthermore that creatine, creatinine and the purine based compounds are minimally retained (Sellevold et al. 1986).

A variety of columns and mobile phase parameters were evaluated in order to optimize the resolution of the various imidazole compounds and to further minimize any potential interference. Aqueous potassium, or sodium, dihydrogen phosphate buffers have generally formed the basis of previous eluents (O'Dowd et al. 1988) for the HPLC of carnosine and the other imidazoles (O'Dowd et al. 1988; Kurisaki and Hiraiwa 1988). The use of alkali metal phosphates during the present method development caused considerable peak tailing of these basic compounds and was apparent in a previous method (O'Dowd et al. 1988). The alternative use of (NH₄)H₂PO₄ resulted in a marked improvement in peak symmetry. The effect of a range of ion-pairing agents (sodium pentanesulphonate, sodium heptanesulphonate, sodium octanesulphonate and sodium dodecyl sulphate) and acetonitrile as the organic modifier, on the retention and resolution of the compounds of interest was investigated. Sodium pentanesulphonate (100 mM ) and 4% acetonitrile produced the best resolution within the optimum capacity factor (K' ) range , 1< K' >10. Of the different analytical columns evaluated; Apex ODS1 5 µm, Apex Phenyl 5 µm, Spherisorb ODS2 5 µm, Hypersil ODS 5 µm and Hypersil ODS 3 µm, superior resolution was obtained with the latter. The resultant chromatography conditions produced a good separation of 1-methylhistidine, histidine, anserine and carnosine as shown in Figure 3.1. The retention times for 1-methylhistidine, histidine,
anserine and carnosine were 4.6, 5.8, 7.4 and 9.2 min, respectively. The efficiency of the separation is indicated by a resolution factor, $R_s > 4.2$ for all adjacent peaks.

Sample deproteinization and solid-phase extraction

Deproteinization of plasma using traditional reagents such as 1.0 $M$ perchloric acid, 0.1% trifluoroacetic acid or methanol (3 - 4 equivalent volumes) produce an unwanted dilution. This is particularly undesirable in the analysis of carnosine where the plasma concentration is known to be generally of the order of 10 $\mu M$. The addition of a small volume (100 $\mu l$) of a concentrated solution of SSA efficiently precipitates plasma protein, minimises the dilution effect and is more reproducible than adding SSA in solid form. Chromatography of SSA deproteinized plasma was inadequate for the determination of carnosine as the carnosine peak eluted within the tailing side of a large front peak, thus making quantification impossible at low levels. In addition, the peaks arising from 1-methylhistidine, histidine and anserine were entirely obscured by this large front peak. Furthermore, large peaks evolving from well retained compounds continued to appear in the chromatogram up to 22 min after sample injection and only after this time did the base line absorbance begin a significant reduction towards the pre-injection value. SPE was employed to provide pre-chromatography sample clean-up. A variety of methodologies were investigated to determine the effectiveness of the technique. At low pH values, carnosine (and the other imidazole molecules) exist as cationic species. The degree of ionization of the imidazole ring and the terminal amine group approaches 100% at pH 2.0. This property was exploited by use of a cation-exchange mechanism for sorbent extraction. The requirement for an acid environment throughout the extraction procedure was simplified by the inherent acidic nature of the SSA deproteinized plasma and by the requirement for an acidic final extract to provide good chromatography in the pH 2.0 mobile phase. Using the SPE method described, an excellent purification of the deproteinized plasma was achieved, and few, if any, interference peaks were present in the final extract. The contrast in sample quality between pre- and post-SPE is demonstrated for a typical equine plasma extract in Figure 3.2.
Figure 3.1  HPLC separation of a mixed standard containing 1-methylhistidine, histidine, anserine and carnosine.
Figure 3.2  
HPLC separation of a SSA extract of equine plasma: A) Pre-SPE. B) Post-SPE.
Spiking of a final equine plasma extract with a mixed standard, prepared from authentic compounds, produced only single peaks for putative carnosine and histidine endogenous to plasma, as shown in Figure 3.3. Peaks for anserine and 1-methylhistidine in the spiked extract were absent in the normal final extract. Spiked canine plasma extracts displayed single peaks for each of the imidazoles.

*Standards*

Standard curves for carnosine and the other imidazoles showed a linear relationship between peak height and concentration in the range 5 - 30 µM. The linear regression equations for each compound were 1-methylhistidine, \( y = 6.21x + 0.61 \) (r > 0.99); histidine, \( y = 5.91x + 0.14 \) (r > 0.99); anserine, \( y = 5.48x + 0.54 \) (r > 0.99); carnosine, \( y = 4.65x + 0.11 \) (r > 0.99) where \( y \) = peak height (mm) and \( x \) = concentration (µM).

*Recovery and reproducibility studies*

Overall recoveries (mean ± CV, %) from spiked plasma were 1-methylhistidine, 103.7 ± 9.4 %; histidine, 105.4 ± 9.9 %; anserine, 97.0 ± 4.4 %; carnosine, 101.8 ± 9.4 %. This deproteinization and extraction technique provided a highly selective determination of the biogenic plasma imidazoles.

Values for typical intra-assay and inter-assay variation are given in Tables 3.1 and 3.2, respectively. In these tables, imidazole concentrations above 25 µM are a consequence of the endogenous plasma content. The mean intra-assay coefficients of variation (CV) were 7.9% or less for the compounds of interest in spiked plasma. Inter-assay mean CV were 6.4% or less for the compounds of interest.

*Lower detection Limits*

Lower detection limits for 1-methylhistidine, histidine, anserine and carnosine were 58.3, 65.9, 71.5 and 80.1 nM, respectively.
Figure 3.3. HPLC separation of a SSA extract of plasma spiked with a mixed standard containing 1-methylhistidine, histidine, anserine and carnosine.
Histidine
1-Methylhistidine
Anserine
Carnosine

Absorbance @ 220 nm

Retention time (min)
0 4 8 12
Histidine
1-Methylhistidine
Carnosine
Anserine
Table 3.1  Intra-assay precision and accuracy for the determination of the biogenic imidazoles in equine plasma.
<table>
<thead>
<tr>
<th>Session</th>
<th>1-Methylhistidine</th>
<th>Histidine</th>
<th>Anserine</th>
<th>Carnosine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Concentration ($\mu M$)</td>
<td>CV (%)</td>
<td>Concentration ($\mu M$)</td>
<td>CV (%)</td>
</tr>
<tr>
<td>1</td>
<td>29.11 ± 1.80</td>
<td>6.2</td>
<td>67.96 ± 1.29</td>
<td>1.9</td>
</tr>
<tr>
<td>2</td>
<td>31.10 ± 1.49</td>
<td>4.8</td>
<td>71.49 ± 1.34</td>
<td>1.9</td>
</tr>
<tr>
<td>3</td>
<td>29.96 ± 1.70</td>
<td>5.7</td>
<td>70.42 ± 1.64</td>
<td>2.3</td>
</tr>
<tr>
<td>Mean</td>
<td>30.06 ± 1.67†</td>
<td>5.6</td>
<td>69.96 ± 1.43†</td>
<td>2.0</td>
</tr>
</tbody>
</table>

† = Pooled intra-assay SD.
Table 3.2  Inter-assay precision and accuracy for the determination of the biogenic imidazoles in equine plasma.
<table>
<thead>
<tr>
<th>Day</th>
<th>1-Methylhistidine</th>
<th>Histidine</th>
<th>Anserine</th>
<th>Carnosine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Concentration (µM)</td>
<td>CV (%)</td>
<td>Concentration (µM)</td>
<td>CV (%)</td>
</tr>
<tr>
<td>Day 1</td>
<td>29.33 ± 2.41</td>
<td>8.2</td>
<td>74.43 ± 2.63</td>
<td>3.5</td>
</tr>
<tr>
<td>Day 5</td>
<td>31.12 ± 1.47</td>
<td>4.7</td>
<td>71.49 ± 1.34</td>
<td>1.9</td>
</tr>
<tr>
<td>Day 10</td>
<td>30.93 ± 1.83</td>
<td>5.3</td>
<td>66.95 ± 5.79</td>
<td>8.7</td>
</tr>
<tr>
<td>Mean</td>
<td>30.46 ± 1.94†</td>
<td>6.4</td>
<td>70.96 ± 3.75†</td>
<td>5.3</td>
</tr>
</tbody>
</table>

† = Pooled inter-assay SD.
Equine plasma extracts were also injected and analysed at 254 nm and 280 nm, however, no peaks were present in the chromatograms. No detectable peaks at these two wavelengths is clear evidence for the absence of interfering compounds such as: purine nucleotides, nucleosides and bases, tyrosine, phenylalanine, tryptophan and their metabolites, and other aromatic species.

The HPLC method developed provided a rapid, selective, sensitive and reproducible analysis of carnosine, histidine, anserine and 1-methylhistidine in plasma.

3.3 HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF IMIDAZOLE DIPEPTIDES, HISTIDINE, 1-METHYLHISTIDINE AND 3-METHYLHISTIDINE IN EQUINE TISSUES AND INDIVIDUAL MUSCLE FIBRES

Dunnett and Harris (1995b)

3.3.1 Introduction

Several HPLC methods have been developed to determine imidazole compounds in tissue samples. The techniques employed include ion-exchange elution with UV detection (Abe and Ohmama 1987) or with derivatization and fluorescence detection (Carnegie et al. 1983), isocratic reversed-phase elution with UV detection (O'Dowd et al. 1988; O'Dowd et al. 1992), iso-cratic reversed-phase elution with pre-column derivatization and fluorescence detection (Kasziba et al. 1988), combined reversed-phase and ion-exchange gradient elution with pre-column derivatization and fluorescence detection (Teahon and Rideout 1992), and reversed-phase gradient elution with pre-column derivatization and fluorescence detection (Wideman et al. 1978; Godel et al. 1984; Qureshi et al. 1984; Boon et al. 1989).

Since reversed-phase HPLC of o-phthaldialdehyde / 2-mercaptoethanol pre-column derivatized amino acids was introduced (Lindroth and Mopper 1979), various modifications have been incorporated into the technique. Initially the only imidazole measured was histidine (Jones et al. 1981; Godel et al. 1984), although subsequent alterations made possible the additional measurement of 1-methylhistidine and 3-methylhistidine (Jones and Gilligan 1983). Later
modifications to the separation parameters and the derivatization chemistry (substituting 3-mercaptopropionic acid for 2-mercaptoethanol) enabled the resolution of histidine, 1-methylhistidine and 3-methylhistidine and carnosine in extracts of plasma, tissues and urine (Qureshi et al. 1984; Jones and Gilligan 1983; Qureshi et al. 1986; Furst et al. 1990). These methods, however, were not developed for the specific analysis of imidazole-derived amino acids and small peptides. In an attempt to provide specificity in the analysis of carnosine, anserine and 3-methylhistidine, reversed-phase HPLC of o-phthaldialdehyde / 3-mercaptopropionic acid (OPT) derivatives has been adapted by incorporating column-switching (Teahon and Rideout 1992).

None of the previous methods encompassed all of the imidazoles. A new method was developed which combined solid-phase extraction, reversed-phase HPLC with binary gradient elution, automated pre-column derivatization with o-phthaldialdehyde / 3-mercaptopropionic acid, and fluorescence detection to enable the selective determination of carnosine, anserine, balenine, homocarnosine, histidine, 1-methylhistidine and 3-methylhistidine in tissue extracts within a single chromatographic run. In conjunction with a micro-extraction technique the method allowed the quantification of the compounds of interest in individual skeletal muscle fibres, where sample weights can be as low as 0.8 µg.

3.3.2 Experimental

Instrumentation and reagents

The HPLC system comprised two Constametric 3000 pumps (LDC Analytical, Stone, Staffs., UK.), a LDC high pressure solvent mixer, a Waters WISP 712 autosampler with auto-addition facility (Waters Chromatography, Watford, Herts., UK.), a Spectrovision FD 300 dual monochromator fluorescence detector (Severn Analytical Ltd., Shefford, Beds., UK.), a LDC MP 3000 chromatography workstation - gradient controller, and a LDC printer. A Seprealyte ODS pellicular 40 µm sacrificial column (20 x 2.1 mm I.D.) (Analytichem International, Harbor City, CA., USA.) and a Rheodyne 0.5 µm in-line filter (Cotati, CA., USA.) were connected between the high pressure mixer and the autosampler.
All reagents were analytical reagent grade unless specified otherwise (see Chapter 2). Water was purified by reverse osmosis and de-ionization (Elgastat Spectrum RO1, Elga, High Wycombe, UK.).

**Tissue deproteination and solid-phase extraction**

Powdered freeze-dried tissue (10.00 ± 2.00 mg) was extracted in 0.5 M perchloric acid (100 µl / mg tissue) on ice for 15 min with regular vortex mixing. The extraction mixture was centrifuged at 12,000 g for 5 min at ambient temperature. Tissue perchloric acid extracts (500 µl) were loaded onto 100 mg / 1 ml Isolute PRS (propylsulphonyl) SPE columns previously conditioned with methanol (4 ml) followed by 1.0 M phosphoric acid (4 ml). Interfering compounds were washed from the columns with 1.0 M phosphoric acid (2 x 500 µl), water (500 µl) and 0.4 M disodium tetraborate buffer, pH 9.65 with sodium hydroxide (250 µl). The sorbent was dried under vacuum for two minutes. The isolates were eluted with 0.4 M disodium tetraborate buffer, pH 9.65 (3 x 250 µl). A flow-rate of 2 ml min⁻¹ was used at all stages.

**Individual muscle fibre extraction**

Individual muscle fibres were extracted with 75% methanol / 25% 0.4 M disodium tetraborate buffer pH 9.65 (100 µl). Extractions were performed on ice with regular vortex mixing for 15 min whilst ensuring that the muscle fibres were permanently submerged. Extracts were frozen in liquid nitrogen, freeze-dried, re-dissolved in 0.4 M disodium tetraborate buffer pH 9.65 (50 µl) and stored at -85°C until analysis.

**Chromatography**

Chromatography was performed on a Hypersil ODS (3 μm, 150 x 4.6 mm I.D.) analytical column protected by a Hypersil ODS (5 μm, 20 x 4.6 mm I.D.) guard column, at ambient temperature, utilizing a binary gradient formed from Solvent A; 12.5 mM sodium acetate, pH 7.2 (995 ml) and tetrahydrofuran (5 ml), and Solvent B; 12.5 mM sodium acetate, pH 7.2 (500 ml), methanol (350 ml) and acetonitrile (150 ml). The 12.5 mM pH 7.2 acetate buffer was prepared by mixing isomolar solutions of sodium acetate and acetic acid. The solvents were
filtered to 0.45 μm and degassed by helium sparging prior to and throughout the analytical run. The mobile phase was freshly prepared for each new sample batch. The composition of the gradient was: 0 - 3 min., 0% solvent B; 20 min., 35% B; 36 min., 60% B; 40 min., 100% B; 43 min., 100% B; 45 - 55 min., 0% B. The flow-rate was 1.0 ml min⁻¹ and chromatographic run-time was 55 min. per sample. The excitation wavelength of the detector was 330 nm and the emission wavelength was 450 nm. The derivatization reagent was prepared by mixing 3-mercaptopropionic acid (80 μl) with o-phthaldialdehyde reagent solution (4 ml). The derivatization reagent was stored in the dark at 2°C for 24 hours before use. During the automated derivatization, 25 μl of the extract was mixed with 25 μl of reagent. The reaction proceeded for 90 s before injection onto the column. Fresh reagent was used with each new sample batch. Tissue concentrations were calculated by comparison of the integrated peak areas with those from a range of external standards.

Standard preparation

A mixed stock standard (10 mM) solution containing histidine, 1-methylhistidine, 3-methylhistidine, anserine, carnosine, balenine and homocarnosine was prepared by dissolving the required weight of each compound in HPLC grade water (10 ml). Working standard solutions were prepared over the concentration range 0.010 - 1.000 mM by dilution of the stock standard with borate buffer. The stock standard solution was stored at -20°C when not used.

Recovery study

Aliquots of pooled perchloric acid extracts of freeze-dried powdered diaphragm muscle were spiked with a mixed standard containing imidazole dipeptides, histidine, 1-methylhistidine and 3-methylhistidine. These samples were spiked to simulate tissue concentrations of 100 mmol kg⁻¹ DW (n = 5), 50 mmol kg⁻¹ DW (n = 5), 5 mmol kg⁻¹ DW (n = 5) and 1 mmol kg⁻¹ DW (n = 5). Recoveries following SPE were calculated by comparison with equivalent standards. The concentrations of the endogenous compounds prior to spiking were determined and subtracted when calculating the recoveries.
Reproducibility study

A perchloric acid extract of pooled diaphragm muscle was spiked with a mixed standard of imidazole dipeptides, histidine, 1-methylhistidine and 3-methylhistidine to simulate individual muscle concentrations of 100 mmol kg\(^{-1}\) DW. The spiked extract was divided into 25 aliquots. Intra-assay variation of the combined SPE and HPLC analysis was determined by repeated injections \((n = 5)\) of the SPE eluates on three occasions within day 1. Inter-assay variation was determined by repeated injections \((n = 5)\) of the SPE eluates on day 1, day 5 and day 10.

Lower detection limits

The lowest levels of detection for the imidazole dipeptides, histidine, 1-methylhistidine and 3-methylhistidine were quantified by injecting of 100 \(\mu l\) of a mixed standard which resulted in FSD at a detector sensitivity setting of 20 nA. The minimum area reliably measurable was 3000 area units which approximated to a 5 mm peak at a signal to noise ratio of 3:1. The concentration of the minimum peak was interpolated from the mixed standard.

3.3.3 Results and discussion

Chromatography

Use of pre-column derivatization with OPT for the HPLC analysis of amino acids and primary amines in physiological samples can produce detailed chromatograms containing as many as 30 - 40 peaks (Turnell and Cooper 1982). Such complexity may be advantageous, for example in the clinical diagnosis of aminoacidemias (Moretti et al. 1990) and muscle amino acid profiles during uraemia (Qureshi et al. 1989), however, poor resolution between certain amino acid pairs makes it difficult to accurately quantify the area of either peak.

Previously published chromatographic methods applied to the determination of imidazole dipeptides, histidine, 1-methylhistidine and 3-methylhistidine in equine tissue extracts failed to resolve all the compounds of interest. Various combinations of columns (Apex Phenyl 5 \(\mu m\), Apex ODS 5 \(\mu m\), Spherisorb ODS 2.5 \(\mu m\) and 3 \(\mu m\), Hypersil ODS 3 \(\mu m\), Hypersil ODS HC 3 \(\mu m\) and Primesphere ODS 3 \(\mu m\)) and mobile phases (10 mM, 12.5 mM and 25 mM acetate,
phosphate and mixed acetate / phosphate buffers) were evaluated to optimize the resolution of the imidazole compounds from interfering amino acids. An acceptable resolution of the seven imidazole standards alone could not be achieved using the 5 µm packed columns. The optimal separation of the imidazole dipeptides, histidine, 1-methylhistidine and 3-methylhistidine from 22 amino acids (including taurine, ß-alanine and GABA) was achieved using the Hypersil ODS 3 µm column in conjunction with the 12.5 mM acetate buffer binary gradient. As shown in Figure 3.4, the final chromatographic parameters resulted in a good separation of the imidazole dipeptides, histidine, 1-methylhistidine and 3-methylhistidine, although base-line separation was not quite achieved between 1-methylhistidine and 3-methylhistidine. Retention times for the individual compounds were: histidine, 20.19 min.; 1-methylhistidine, 21.82 min.; 3-methylhistidine, 22.23 min.; anserine, 26.43 min.; carnosine, 27.45 min.; balenine, 30.08 min.; homocarnosine, 31.71 min.

Solid-phase extraction

During the analysis of physiological samples for low levels of the compounds of interest there is the possibility of impaired resolution from citrulline, alanine, taurine and arginine when these amino acids are present at relatively much higher concentrations. SPE employing a benzene propylsulphonyl bonded phase had been used for the extraction of amino acids from urine (Moodie et al. 1989), however, this procedure did not discriminate between different classes of amino acids. The presence of the benzene ring in the bonded phase may also induce secondary non-polar interactions thus complicating the adaptation of this method. A propylsulphonyl strong cation-exchange bonded phase was previously shown to be effective in retaining biogenic imidazole compounds from acidic plasma extracts (Dunnett and Harris 1992). SPE was adopted to provide a selective extraction of the imidazole dipeptides, histidine, 1-methylhistidine and 3-methylhistidine from the other amino acids and small peptides present in tissues. The present SPE method proved highly selective for the imidazole compounds. Most amino acids, including citrulline, alanine and taurine, were absent from the final extract, however arginine (26.01 min.) was fully recovered. Chromatograms of perchloric acid extracts of myocardium pre- and post-SPE are shown in Figure 3.5 and gluteus medius muscle post-SPE in Figure 3.6.
The SPE resulted in an effective concentration of the analytes as separate neutralization and buffering (pH 9.65) of the perchloric acid extracts was avoided. Post SPE extracts of equine tissues were spiked with a mixed standard of the authentic compounds. Subsequent HPLC produced only single peaks at the retention times for the putative compounds.

**Standards**

The standard curves demonstrated a linear relationship between integrated peak area and concentration in the range 0.010 - 1.000 mM. Linear regression equations for each compound were: histidine, $y = 65510x + 103$ ($r = 0.998$); 1-methylhistidine, $y = 103497x + 1164$ ($r = 0.997$); 3-methylhistidine, $y = 100599x + 3682$ ($r = 0.996$); anserine, $y = 257649x + 2977$ ($r = 0.997$); carnosine, $y = 247476x + 833$ ($r = 0.997$); balenine, $y = 211263x + 5845$ ($r = 0.995$), and homocarnosine, $y = 294440x + 2597$ ($r = 0.995$). ($y =$ integrated peak area in arbitrary units, $x =$ concentration, mM).

**Recovery and reproducibility studies**

The overall recoveries (mean ± CV, %) of the imidazole compounds from spiked tissue samples following SPE are shown in Table 3.3. Excellent recoveries were obtained for all the compounds of interest. The intra-assay and inter-assay reproducibility (mean ± SD and CV, %) of the combined SPE-HPLC analysis of the compounds of interest are given in Tables 3.4 and 3.5, respectively. The intra-assay mean CV for all the compounds of interest in spiked muscle extracts ranged from 1.2 - 6.3%. The inter-assay mean CV ranged from 1.7 - 2.9%.

**Lower detection limits**

Lower limits of detectability determined for 100 µl injected at a detector sensitivity setting of 20 nA FSD were 0.010 mmol kg⁻¹ DW (1.6 pmoles on column) for histidine, 1-methylhistidine and 3-methylhistidine, and 0.005 mmol kg⁻¹ DW (0.8 pmoles on column) for carnosine, anserine, balenine and homocarnosine. Detector sensitivity settings at 10 nA FSD or less resulted in unacceptable signal-to-noise ratios. Injection volumes greater than 100 µl resulted in peak broadening and reduced resolution between 1-methylhistidine and 3-methylhistidine.
**Individual fibre extraction validation**

Owing to the extremely low sample weights, typically 0.8 - 8.0 μg, it was necessary to minimize the sample extract volume by maximizing detector response. The sample extracts were therefore concentrated by freeze-drying and re-dissolution. As perchloric acid cannot be removed by freeze-drying an alternative solvent for tissue extraction had to be found. Comparative extractions of freeze-dried powdered middle gluteal muscle (1 mg ml⁻¹) between perchloric acid and 3 alternative solvents were performed (n = 4 for each solvent). The relative recoveries of carnosine in three alternative solvents compared to perchloric acid were: 100.9% (75% methanol / 25% 0.4 M borate buffer pH 9.65), 87.3% (75% acetonitrile / 25% 0.4 M borate buffer pH 9.65) and 101.0% (4% sulphosalicylic acid). Comparative extractions between perchloric acid and 75% methanol / 25% 0.4 M borate buffer were made in 12 individual equine muscle fibres. Each fibre was halved; one half was extracted with perchloric acid the other with methanol-borate buffer. The mean (± SD) carnosine in the perchloric acid extracted fibres and the methanol / borate buffer extracted fibres, 46.94 ± 9.66 mmol kg⁻¹ DW and 47.21 ± 11.44 mmol kg⁻¹ DW were not significantly different (p > 0.05).

The present SPE-HPLC method developed and validated for the isolation and quantification of carnosine, anserine, balenine, homocarnosine, histidine, 1-methylhistidine and 3-methylhistidine in equine tissues and individual muscle fibres across a broad concentration range in tissue samples weighing from < 1 μg to 10 mg proved to be selective, sensitive and reproducible. This method avoids the interference from other physiological amino acids and small peptides associated with earlier techniques and for the first time enables the resolution of all seven compounds within a single chromatographic run.
Figure 3.4  HPLC separation of a mixed standard containing the imidazole dipeptides, histidine, 1-methylhistidine and 3-methylhistidine.
Figure 3.5  HPLC separation of a perchloric acid extract of myocardium: A) Pre-SPE. B) Post-SPE.
Figure 3.6  HPLC separation of a perchloric acid extract of equine middle gluteal muscle post-SPE.
Table 3.3  Mean recoveries ± CV(%) of the imidazoles from muscle.
<table>
<thead>
<tr>
<th>Compound</th>
<th>100 mmol kg⁻¹ DW Recovery ± CV (%)</th>
<th>50 mmol kg⁻¹ DW Recovery ± CV (%)</th>
<th>1 mmol kg⁻¹ DW Recovery ± CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histidine</td>
<td>106.0 ± 5.5</td>
<td>105.1 ± 5.0</td>
<td>96.0 ± 8.3</td>
</tr>
<tr>
<td>1-Methylhistidine</td>
<td>105.1 ± 5.0</td>
<td>109.1 ± 10.8</td>
<td>96.9 ± 3.9</td>
</tr>
<tr>
<td>3-Methylhistidine</td>
<td>101.7 ± 4.1</td>
<td>103.6 ± 11.1</td>
<td>100.8 ± 9.5</td>
</tr>
<tr>
<td>Anserine</td>
<td>99.5 ± 10.2</td>
<td>111.1 ± 6.8</td>
<td>101.4 ± 6.7</td>
</tr>
<tr>
<td>Camosine</td>
<td>91.1 ± 10.2</td>
<td>109.5 ± 9.2</td>
<td>100.8 ± 6.7</td>
</tr>
<tr>
<td>Balenine</td>
<td>99.1 ± 8.8</td>
<td>105.1 ± 7.0</td>
<td>103.1 ± 6.6</td>
</tr>
<tr>
<td>Homocarnosine</td>
<td>100.8 ± 8.1</td>
<td>112.7 ± 9.5</td>
<td>115.6 ± 9.6</td>
</tr>
</tbody>
</table>
Table 3.4  Intra-assay precision and accuracy for the determination of imidazoles in muscle.
<table>
<thead>
<tr>
<th>Compound</th>
<th>Session 1</th>
<th>Session 2</th>
<th>Session 3</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histidine</td>
<td>Concentration</td>
<td>99.75 ± 0.95</td>
<td>97.88 ± 2.80</td>
<td>96.63 ± 1.19</td>
</tr>
<tr>
<td></td>
<td>CV</td>
<td>1.0</td>
<td>2.9</td>
<td>1.2</td>
</tr>
<tr>
<td>1-Methylhistidine</td>
<td>Concentration</td>
<td>90.79 ± 6.03</td>
<td>100.29 ± 3.78</td>
<td>103.96 ± 8.88</td>
</tr>
<tr>
<td></td>
<td>CV</td>
<td>6.7</td>
<td>3.8</td>
<td>8.5</td>
</tr>
<tr>
<td>3-Methylhistidine</td>
<td>Concentration</td>
<td>104.20 ± 2.52</td>
<td>99.84 ± 1.67</td>
<td>98.26 ± 1.55</td>
</tr>
<tr>
<td></td>
<td>CV</td>
<td>2.4</td>
<td>1.7</td>
<td>1.6</td>
</tr>
<tr>
<td>Anserine</td>
<td>Concentration</td>
<td>103.31 ± 2.01</td>
<td>99.90 ± 1.96</td>
<td>100.65 ± 2.61</td>
</tr>
<tr>
<td></td>
<td>CV</td>
<td>1.9</td>
<td>2.0</td>
<td>2.6</td>
</tr>
<tr>
<td>Carnosine</td>
<td>Concentration</td>
<td>103.41 ± 1.43</td>
<td>102.72 ± 0.84</td>
<td>102.61 ± 1.62</td>
</tr>
<tr>
<td></td>
<td>CV</td>
<td>1.4</td>
<td>0.8</td>
<td>1.6</td>
</tr>
<tr>
<td>Balanine</td>
<td>Concentration</td>
<td>100.33 ± 1.47</td>
<td>97.36 ± 0.92</td>
<td>96.55 ± 1.26</td>
</tr>
<tr>
<td></td>
<td>CV</td>
<td>1.5</td>
<td>0.9</td>
<td>1.3</td>
</tr>
<tr>
<td>Homocarnosine</td>
<td>Concentration</td>
<td>98.08 ± 1.88</td>
<td>96.75 ± 0.97</td>
<td>97.57 ± 2.53</td>
</tr>
<tr>
<td></td>
<td>CV</td>
<td>1.9</td>
<td>1.0</td>
<td>2.6</td>
</tr>
</tbody>
</table>

† = Pooled intra-assay SD.
Table 3.5  Inter-assay precision and accuracy for the determination of imidazoles in muscle.
<table>
<thead>
<tr>
<th>Compound</th>
<th>Day 1</th>
<th>Day 5</th>
<th>Day 10</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histidine</td>
<td>Concentration</td>
<td>97.88 ± 2.80</td>
<td>101.04 ± 2.07</td>
<td>101.17 ± 2.36</td>
</tr>
<tr>
<td></td>
<td>CV</td>
<td>2.9</td>
<td>2.1</td>
<td>2.3</td>
</tr>
<tr>
<td>1-Methylhistidine</td>
<td>Concentration</td>
<td>100.29 ± 3.78</td>
<td>105.44 ± 2.93</td>
<td>98.35 ± 2.00</td>
</tr>
<tr>
<td></td>
<td>CV</td>
<td>3.8</td>
<td>2.8</td>
<td>2.0</td>
</tr>
<tr>
<td>3-Methylhistidine</td>
<td>Concentration</td>
<td>99.84 ± 1.67</td>
<td>101.54 ± 1.90</td>
<td>100.58 ± 2.43</td>
</tr>
<tr>
<td></td>
<td>CV</td>
<td>1.7</td>
<td>1.9</td>
<td>2.4</td>
</tr>
<tr>
<td>Anserine</td>
<td>Concentration</td>
<td>99.90 ± 1.96</td>
<td>98.98 ± 1.39</td>
<td>100.72 ± 2.14</td>
</tr>
<tr>
<td></td>
<td>CV</td>
<td>2.0</td>
<td>1.4</td>
<td>2.1</td>
</tr>
<tr>
<td>Carnosine</td>
<td>Concentration</td>
<td>102.72 ± 0.84</td>
<td>95.58 ± 2.14</td>
<td>103.36 ± 1.91</td>
</tr>
<tr>
<td></td>
<td>CV</td>
<td>0.8</td>
<td>2.2</td>
<td>1.9</td>
</tr>
<tr>
<td>Balenine</td>
<td>Concentration</td>
<td>97.36 ± 0.92</td>
<td>97.00 ± 2.32</td>
<td>102.44 ± 2.71</td>
</tr>
<tr>
<td></td>
<td>CV</td>
<td>0.9</td>
<td>2.4</td>
<td>2.7</td>
</tr>
<tr>
<td>Homocarnosine</td>
<td>Concentration</td>
<td>96.75 ± 0.97</td>
<td>90.16 ± 3.75</td>
<td>108.36 ± 2.62</td>
</tr>
<tr>
<td></td>
<td>CV</td>
<td>1.0</td>
<td>4.2</td>
<td>2.4</td>
</tr>
</tbody>
</table>

† = Pooled inter-assay SD.
3.4 HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF N-α-ACETYLCARNOSINE IN EQUINE PLASMA

3.4.1. Introduction
Owing to the absence of a free primary amine group in N-α-acetylcarnosine plasma concentrations could not be determined by high-performance liquid chromatography (HPLC) with fluorescence detection following derivatization with o-phthalaldehyde/3-mercaptopropionic acid or fluorescamine. Concentrations of N-α-acetylcarnosine and acetylamino acids have been determined in skeletal muscle, myocardium and brain of several species, including the rat, frog and rabbit. The analytical techniques employed for the analysis included thin layer chromatography (TLC) (Sobue et al. 1975), HPLC (O'Dowd et al. 1992; O'Dowd et al. 1990; O'Dowd et al. 1988) and more recently liquid chromatography-mass spectrometry (LC-MS) (Sugahara et al. 1994). However, these methods were not developed for the analysis of plasma and with the exception of the latter technique the earlier methods lacked the necessary sensitivity to quantify low concentrations of the compounds of interest in plasma. A new analytical method which combined isocratic reversed-phase HPLC with SPE to provide rapid, selective and sensitive detection of N-α-acetylcarnosine in equine plasma was developed.

3.4.2. Experimental

Instrumentation and reagents
The HPLC system comprised a Hewlett-Packard HP1050 quaternary-gradient solvent delivery system, a Rheodyne 7125 injector (with 200 µl volume sample loop), a HP1040 diode array detector, a Hewlett-Packard Chemstation and Thinkjet printer. A Seprlyte ODS 40 µm (30 x 4.6 mm) sacrificial guard column (Analytichem International, Habor City, CA, USA) and a Rheodyne in-line filter (0.5 µm pore size) were inserted between the pump and the injector.

All reagents were analytical reagent grade unless specified otherwise (see Chapter 2). Water was purified by reverse osmosis and de-ionization (Elgastat Spectrum RO1, Elga, High Wycombe, UK.).
Deproteinization and solid-phase extraction

Plasma (250 µl) was deproteinized and extracted with 1.2 M perchloric acid in 1.0 M phosphoric acid (350 µl), vortex-mixed, centrifuged and the supernatant collected. The precipitate was re-extracted with 1.0 M phosphoric acid (400 µl). The supernatants were combined. N-α-acetylcarnosine was isolated from interfering substances by SPE. Plasma extracts (250 µl) were loaded onto 1 ml / 100 mg Isolute PRS (propylsulphonyl) strong cation-exchange columns previously conditioned with methanol (3 x 1000 µl.) and 1.0 M phosphoric acid (3 x 1000 µl.). Columns were washed with 50 mM potassium dihydrogen phosphate (KH₂PO₄), pH 2.5 (2 x 250 µl) and air-dried for 2 min. The analyte was eluted with 100 mM KH₂PO₄, pH 2.5 (2 x 250 µl). A flow-rate of 0.5 ml min⁻¹ was used at all times.

Chromatography

HPLC of N-α-acetylcarnosine was performed on a Hypersil ODS (3 µm, 150 x 4.6 mm) analytical column protected by a Hypersil ODS (5 µm, 20 x 4.6 mm) guard column. N-α-acetylcarnosine was eluted using a mobile phase comprising 5 mM phosphoric acid and 1 mM triethylamine, pH 2.5 in HPLC grade water. The mobile phase was filtered to 0.45 µm and degassed by helium sparging prior to and continually during use. The mobile phase was freshly prepared each day. The flow-rate was 0.8 ml min⁻¹ and the injection volume was 200 µl. Detection was by UV absorbance at 220 nm.

Standards

A 1 mM stock standard of N-α-acetylcarnosine was prepared by dissolving the required weight in HPLC grade water (10 ml). Working standards over the concentration range 0.1 - 100.0 µM were prepared daily by dilution of the stock standard with 100 mM KH₂PO₄ buffer, pH 2.5.

Recoveries study

Plasma was spiked at 800 µM and 0.8 µM with a standard containing N-α-acetylcarnosine, to produce nominal concentrations in the final eluate of 100 and 0.1 µM, assuming recoveries of 100%. Extraction recoveries (n = 5), at each concentration, for the combined deproteinization
and SPE were determined by comparing concentrations found in the final eluates with standards at the nominal concentrations.

Reproducibility study

The reproducibility of the method was assessed by calculation of the intra- and inter-assay accuracy and precision. Plasma was spiked with N-α-acetylcarnosine at 800 µM and 0.8 µM concentrations. The precision of the method was derived from intra- and inter-assay coefficients of variation (CV) of replicate analyses (n = 5) at each concentration. The accuracy of the assay was expressed as: (measured concentration / nominal concentration)\cdot 100. Inter-assay CV were determined by analysis on days 1, 5 and 10 over a 10-day period.

Lower detection limit

The lower limit of detection for N-α-acetylcarnosine was quantified by injecting 200 µl of a 10 µM standard which resulted in FSD at a detector sensitivity setting of 10 mAU. The minimum area reliably measurable was 30 area units which approximated to a 5 mm peak at a signal to noise ratio of 3:1. The concentration of the minimum peak was interpolated from the standard curve.

3.4.3. Results and discussion

Chromatography and solid-phase extraction

A method developed for the determination of N-α-acetylcarnosine in tissue samples (O'Dowd et al. 1988) which utilized 100 mM sodium dihydrogen phosphate, pH 2.0 buffer as the mobile phase, was applied to the measurement of these analytes in equine plasma. However, this proved unsuccessful. Spiked plasma samples indicated an interfering peak co-eluting with N-α-acetylcarnosine. Furthermore, N-α-acetylcarnosine exhibited poor peak symmetry; the asymmetry factor was 2.56. The degree of interference was reduced and retention time increased by using a mobile phase containing only 5 mM phosphoric acid. The inclusion of triethylamine at 20% of the phosphate concentration further improved peak symmetry; asymmetry factor was 1.07. However, triethylamine concentrations > 1 mM had an adverse effect. The combination
of 5 mM phosphoric acid and 1 mM triethylamine resulted in the pH 2.5 solution. No further mobile phase pH adjustment was made and the batch-batch reproducibility of pH was excellent. Analytical columns with several different ODS 3 μm stationary phases were assessed during method development: including Spherisorb, Apex, Primesphere and Hypersil. The latter provided superior performance in terms of resolution and peak symmetry. The retention time for N-α-acetylcarnosine was 5.25 min.

Complete resolution of the N-α-acetylcarnosine peak from the interfering peak was not achieved by column selection alone, and it proved necessary to employ SPE to achieve the sample purity required for accurate quantification of the N-α-acetylcarnosine peak. Despite the absence of a charged terminal amine group on N-α-acetylcarnosine unlike carnosine, the presence of a positive charge on the imidazole ring allowed sufficient retention on cation-exchange sorbents. Optimum retention was obtained on PRS sorbent. The use of 50 and 100 mM phosphate buffers for washing the SPE column resulted in a significant reduction in the number of interfering compounds in the final eluate. A comparison of plasma extracts pre- and post SPE is shown in Figure 3.8.

**Standards**

The standard curve demonstrated a linear relationship between concentration and integrated peak area. The linear regression equations for the N-α-acetylcarnosine standard curve was \( y = 11.713x - 0.028 \) \( (r = 0.999) \), where \( y \) = peak height (mm) and \( x \) = concentration (μM).

**Lower detection limit**

The lower limit of detection in plasma was 0.1 μM.

**Recovery and reproducibility studies**

Mean (± CV) recoveries of N-α-acetylcarnosine from equine plasma at 800 μM and 0.8 μM concentrations were 93.9 ± 5.0% and 99.7 ± 0.5%, respectively. The intra- and inter-assay
accuracy and precision of the combined SPE and HPLC method at high and low concentrations are given in Table 3.6.

The combined SPE procedure and isocratic reversed-phase HPLC method developed provided a selective, sensitive and reproducible analysis of N-α-acetylcarnosine concentrations in equine plasma.
Figure 3.7.  HPLC separation of a N-α-acetylcarnosine standard.
N-α-acetylcarnosine
Figure 3.8. HPLC separation of a perchloric acid extract of plasma; A) Pre-SPE.
B) Post-SPE.
Table 3.6  Intra- and inter-assay accuracy and precision of the N-α-acetylcarnosine analysis.
<table>
<thead>
<tr>
<th>Concentration</th>
<th>Intra-assay (n = 5)</th>
<th>Inter-assay (n = 15)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SD</td>
<td>CV</td>
</tr>
<tr>
<td>100 µM</td>
<td>98.3 ± 3.3†</td>
<td>3.4</td>
</tr>
<tr>
<td>0.1 µM</td>
<td>100.1 ± 0.1†</td>
<td>0.1</td>
</tr>
</tbody>
</table>

† = Pooled SD.
CHAPTER 4.

CARNOSINE DISTRIBUTION IN TYPE I, IIA AND IIB FIBRES IN THE MIDDLE GLUTEAL MUSCLE, AND OTHER TISSUES OF THE THOROUGHBRED HORSE.
4.1 INTRODUCTION

Carnosine and the other imidazole dipeptides, with the exception of homocarnosine, occur at high concentrations, often in excess of 100 mmol kg\(^{-1}\) DW, within the skeletal muscles of many mammalian species (Crush 1970; Carnegie et al. 1983; Plowman and Close 1988). Carnosine also occurs at low millimolar concentrations in other organs such as, the heart, and liver, and tissues of the CNS and gastro-intestinal tract (GIT). Accordingly additional roles, other than acid-base regulation, have been ascribed to carnosine. For example, millimolar concentrations of both carnosine and homocarnosine have been detected in various regions of the central nervous systems of several species (Margolis 1974; Margolis and Grillo 1984). Carnosine is particularly abundant in the olfactory bulb and epithelium of mice and rats (Wideman et al. 1978). It has been fairly well established that both carnosine and homocarnosine function as neurotransmitters/neuromodulators in the CNS, and that this role for carnosine is particularly significant within the olfactory pathway (Margolis 1980; Margolis 1981). Owing to the presence of significant amounts of carnosine in certain tissues it has been proposed that carnosine may represent a reservoir of histamine since its catabolism involves hydrolysis to histidine and subsequent decarboxylation to histamine (Flancbaum et al. 1990). Species which possess high intra-muscular concentrations of these dipeptides are generally characterized by an adaptation to either high speed running, such as the horse, or to prolonged periods of hypoxia, such as the whale, where muscle contraction is reliant upon a rapid anaerobic turnover of ATP. Without an integrated system for the maintenance of acid-base balance, the consequential production and accumulation of H\(^+\) ions within the working muscle cells would result in a rapid reduction in intra-cellular pH with a concomitant impairment of the contractile process. The imidazole dipeptides have p\(K_a\) values in the range 6.8 to 7.1 enabling them to function as effective proton buffers over the physiological pH range (Bate-Smith 1938; Davey 1960a; Harris et al. 1990; Sewell et al. 1990). Maintenance of intra-muscular acid-base status during exercise may be as important to athletic performance as the ability to transport oxygen via the cardio-vascular system.
It has been shown that within a species the highest carnosine concentrations are found in skeletal muscles which have the greatest proportion of type II fibres (Tamaki et al. 1976; Castelini and Somero 1981). Sewell et al. (1990) used multiple linear regression analysis to estimate the carnosine concentrations in types I, IIA and IIB fibres from mixed fibre samples taken from the middle gluteal muscles of horses of different ages and training status. The estimated carnosine concentrations were 21, 86 and 116 mmol kg\(^{-1}\) DW in type I, IIA and IIB fibres, respectively. The same approach was used in a comparative study, where muscle samples were obtained by percutaneous biopsy exclusively from young highly trained horses. Estimated carnosine concentrations in this study were found to be higher in type I and IIB fibres, than in the previous study. The estimated carnosine concentrations in type I, IIA and IIB fibres were 54, 85 and 180 mmol kg\(^{-1}\) DW, respectively (Sewell et al. 1992).

Taurine, a β-amino acid (2-aminoethanesulphonic acid), is found in most vertebrate tissues and at millimolar concentrations in excitable tissues such as skeletal muscle, heart and CNS (Jacobson and Smith 1968). Skeletal muscle accounts for an estimated 75% of the total body taurine pool (Stern and Stim 1959). Significant variation in taurine concentration exists between different muscles within and between species. Human muscle taurine concentrations range 8.0 - 21.6 mmol kg\(^{-1}\) DW (Zachmann et al. 1966) and in the rat values are higher and the range greater; 28.0 - 83.2 mmol kg\(^{-1}\) DW (Airaksinen et al. 1990). The soleus muscle of the rat, a predominantly slow-twitch muscle, has a two-fold higher taurine content than the extensor digitorum longus, a predominantly fast-twitch glycolytic muscle (Iwata et al. 1986). This suggested that an inverse relationship existed between the carnosine and taurine contents of a given muscle which may have been related to the relative proportions of type I (slow-twitch) and type II (fast-twitch) fibres within a given muscle. Subsequent studies in both young highly trained horses and older horses of variable training status indicated that taurine concentrations in equine skeletal muscle were strongly correlated to type I % FSA (\(p < 0.001, r = 0.94\)) and that type I fibres were estimated to contain 35.4 - 45.4 mmol kg\(^{-1}\) DW of taurine, whereas type IIA fibres contained only 4.5 - 7.9 mmol kg\(^{-1}\) DW, and taurine appeared to be absent from type IIB fibres (Dunnett et al. 1992).
4.2 STUDY A: CARNOSINE CONCENTRATIONS AND CARNOSINASE ACTIVITIES IN OTHER EQUINE TISSUES

4.2.1 Objectives
The aims of this study were to determine the carnosine concentrations and carnosinase activities in various equine skeletal muscles and other tissues.

4.2.2 Experimental methodology

Sampling procedure
Samples from tissues were collected at post mortem, as described in Chapter 2, from eighteen thoroughbred horses aged from 7 months to 4 years and from two foetuses.

Analytical methods
Tissue extracts were analysed for carnosine and other imidazole concentrations by high-performance liquid chromatography as described in Chapter 3 (Dunnett and Harris 1995). Tissue carnosinase activity was determined by a modification of the method of Lenney (1990), as described in Chapter 2.

4.2.3 Results
Samples were collected from the following tissues; myocardium, kidney, liver, spleen, cerebellum, medulla, lung, stomach, small intestine, colon and rectum. Mean (± SD) tissue concentrations of carnosine and histidine, and tissue carnosinase activities are presented in Table 4.1. Mean values for carnosine concentrations determined in middle gluteal and internal intercostal muscle samples are included for comparative purposes. Carnosine was present at much lower concentrations in myocardium, kidney, liver, spleen, lung and tissues from the CNS and GIT, than those found in typical locomotory skeletal muscles such as the middle gluteal. The carnosine concentration in the diaphragm is two to three-fold lower than the concentration typically found in mixed fibre samples from the middle gluteal muscle. However, the diaphragm carnosine concentration is similar to the value found in type I skeletal muscle fibres. Carnosine
concentrations measured in cardiac muscle (myocardium) and smooth muscle (small intestine, colon, stomach and rectum) were twenty-fold or more lower than those found in diaphragm.

Anserine and balenine were also detected in equine skeletal muscle and diaphragm at concentrations two to three orders of magnitude lower than for carnosine. Anserine was present at $0.66 \pm 0.27$ and $0.66 \pm 0.13 \text{ mmol kg}^{-1} \text{ DW}$ in the middle gluteal and intercostal muscles, respectively, and at $0.82 \pm 0.10 \text{ mmol kg}^{-1} \text{ DW}$ in the diaphragm. Balenine was present at $0.20 \pm 0.06$ and $0.10 \pm 0.05 \text{ mmol kg}^{-1} \text{ DW}$ in the middle gluteal and intercostal muscles, respectively, and at $0.08 \pm 0.03 \text{ mmol kg}^{-1} \text{ DW}$ in the diaphragm. Anserine and balenine were not detected in other tissues. Homocarnosine was detected only in tissues from the CNS. It was present at concentrations of $0.09 \pm 0.09$ and $0.04 \text{ mmol kg}^{-1} \text{ DW}$ in cerebellum and medulla, respectively. Trace amounts only of 1-methylhistidine were present in skeletal muscle and it was not detected in other tissues. No free 3-methylhistidine was detected in muscle samples although it is a constituent of the contractile proteins, actin and myosin.

Tissue carnosinase activities were highest in the kidney, and other tissues exhibiting relatively high activities were, the small intestine, spleen and lung. Carnosinase activity in the middle gluteal muscle, internal intercostal muscle and the diaphragm was below the lower limit of quantification of the assay ($1.4 \mu\text{mol g}^{-1} \text{ h}^{-1} \text{ DW}$).
Table 4.1  Carnosine and histidine concentrations, and carnosinase activities in various tissues of the thoroughbred horse.
<table>
<thead>
<tr>
<th>Tissue</th>
<th>n</th>
<th>Carnosine (mmol kg(^{-1}) DW)</th>
<th>Histidine (mmol kg(^{-1}) DW)</th>
<th>Carnosinase (µmol g(^{-1}) h(^{-1}) DW)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Middle gluteal</td>
<td>7</td>
<td>107.81 ± 12.87</td>
<td>0.25 ± 0.05</td>
<td>ND</td>
</tr>
<tr>
<td>Internal intercostal</td>
<td>3</td>
<td>63.70 ± 16.16</td>
<td>0.39 ± 0.01</td>
<td>ND</td>
</tr>
<tr>
<td>Diaphragm</td>
<td>5</td>
<td>38.62 ± 5.09</td>
<td>0.60 ± 0.36</td>
<td>ND</td>
</tr>
<tr>
<td>Stomach</td>
<td>2</td>
<td>2.43 ± 0.80</td>
<td>1.30 ± 0.06</td>
<td>4.2 ± 3.3</td>
</tr>
<tr>
<td>Myocardium</td>
<td>6</td>
<td>2.22 ± 1.21</td>
<td>0.56 ± 0.06</td>
<td>3.0 ± 2.5</td>
</tr>
<tr>
<td>Rectum</td>
<td>1</td>
<td>1.27</td>
<td>1.57</td>
<td>4.4</td>
</tr>
<tr>
<td>Small intestine</td>
<td>11</td>
<td>0.93 ± 0.47</td>
<td>1.44 ± 0.41</td>
<td>22.9 ± 17.7</td>
</tr>
<tr>
<td>Colon</td>
<td>1</td>
<td>0.58</td>
<td>1.46</td>
<td>3.6</td>
</tr>
<tr>
<td>Lung</td>
<td>2</td>
<td>0.43 ± 0.05</td>
<td>0.86 ± 0.52</td>
<td>13.7 ± 15.1</td>
</tr>
<tr>
<td>Kidney</td>
<td>7</td>
<td>0.13 ± 0.04</td>
<td>0.33 ± 0.10</td>
<td>604.0 ± 24.2</td>
</tr>
<tr>
<td>Spleen</td>
<td>3</td>
<td>0.12 ± 0.07</td>
<td>0.61 ± 0.14</td>
<td>23.7 ± 7.3</td>
</tr>
<tr>
<td>Liver</td>
<td>8</td>
<td>0.11 ± 0.05</td>
<td>0.90 ± 0.43</td>
<td>5.6 ± 2.0</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>9</td>
<td>0.04 ± 0.03</td>
<td>0.25 ± 0.10</td>
<td>1.8 ± 1.4</td>
</tr>
<tr>
<td>Medulla</td>
<td>1</td>
<td>0.04</td>
<td>0.40</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND = Not detected
4.3 STUDY B: CARNOSINE AND TAURINE CONTENTS IN TYPE I, IIA AND IIB FIBRES IN THE MIDDLE GLUTEAL MUSCLE OF THE NORMAL THOROUGHBRED HORSE.

Dunnett and Harris (1995a)

4.3.1 Objectives

The aims of the present study was to determine by direct measurement the carnosine and taurine concentrations in type I, type IIA and type IIB fibres from the middle gluteal muscle of the thoroughbred horse, and to assess whether taurine : carnosine ratios could be used as a biochemical means for determining fibre type in individual muscle fibres.

4.3.2 Experimental methodology

Protocol and sampling procedure

Muscle samples from the left or right middle gluteal muscle were collected at post mortem from 5 thoroughbred horses (3 colts, 2 geldings) which had no history of muscle disorders. The horses were aged between 2 and 13 years and had a variety of training backgrounds. However, all the horses had been out of training for at least 6 months. All of the horses were euthanased having failed to respond to treatment for a variety of chronic conditions. One entire middle gluteal muscle was removed from each horse and samples were collected, as described in detail in Chapter 2. Owing to the relatively low abundance of type I fibres within equine middle gluteal muscle, the number of individual type I fibres retrieved was maximized by selecting some of the darker (more red) samples for dissection. Individual muscle fibres were dissected from freeze-dried muscle samples as described in Chapter 2.

Histochemistry

Fragments of individual muscle were characterized as either type I, IIA or IIB by histochemical staining for myosin ATPase activity at pH 9.6 following pre-incubation at pH 4.50 as described previously (Chapter 2).
Individual muscle fibre analysis

Weighed individual muscle fibres were extracted and analysed for carnosine and taurine content simultaneously by reversed-phase high-performance liquid chromatography, as described in Chapter 3 (Dunnett and Harris 1995b).

Statistics

Data on the mean carnosine and taurine concentrations of each fibre type are given together with a pooled estimate of the within-horse standard deviation (SD_p). SD_p was calculated using the equation

$$SD_p = \sqrt{\frac{\sum SSQ_h}{\sum n_h - 1}}$$

where SSQ_h is the sum of squares calculated within each horse and n_h - 1 is the degrees of freedom upon which this value is based. Analysis of carnosine and taurine distribution in type I, IIA and IIB fibres was performed by normalization of individual values which enabled data from all horses to be combined. Individual values were divided by the mean value for that horse and the results were normalized by multiplying by the overall mean value for all horses. Type I, IIA and IIB fibres were treated independently. One-factor ANOVA was used to identify significant differences in mean (± SD) values for carnosine and taurine concentrations, and taurine : carnosine ratios between type I, IIA and IIB fibres. In the instance where significance was detected a multiple comparison test was applied, Fisher's Protected Least Significant Difference (PLSD) was applied. Significance was declared at p < 0.05.

4.3.3 Results

Carnosine and taurine concentrations were determined in a total of 324 individual muscle fibres taken from the middle gluteal muscle of 5 thoroughbred horses (FO, TI, DP, GI, EX) at post mortem. The number of fibres and mean (± SD) carnosine and taurine concentrations in type I, IIA and IIB muscle fibres for individual horses, and mean values (± SD_p) for all horses combined are given in Tables 4.2 and 4.3, respectively. No carnosine was detected in the type I fibres of one horse (EX), however, this may have been due to an unrepresentative population as only 2 type I fibres were found in the samples from this horse. Distribution plots of carnosine and
taurine concentrations in type I, IIA and IIB fibres from the middle gluteal muscle of the individual horses are given in Figures 4.1 and 4.2, respectively. Carnosine concentrations were significantly different between the three fibre types (p < 0.001). The overall mean (± SDp) carnosine concentrations in type I, type IIA and type IIB fibres were 24.9 ± 6.4, 94.8 ± 6.8 and 104.3 ± 11.9 mmol kg⁻¹ DW, respectively.

There was a significant difference in taurine concentration between type I, and type IIA and IIB fibres (p < 0.001), but not between type IIA and type IIB fibres (p > 0.05). The overall mean (± SDp) taurine concentrations in type I, type IIA and type IIB fibres were 54.3 ± 8.3, 2.8 ± 2.1 and 1.8 ± 1.9 mmol kg⁻¹ DW, respectively. Taurine: carnosine ratios for individual horses and overall mean (± SDp) values are given in Table 4.4. Taurine: carnosine ratios between type I fibres, and type IIA and IIB fibres were significantly different (p < 0.001). There was however, no significant difference between type IIA and type IIB fibres (p > 0.05). It was therefore not possible to discriminate between type IIA and IIB fibres by use of the taurine: carnosine ratio. Frequency distribution plots of normalized carnosine and taurine concentrations for all fibres with respect to fibre type are shown in Figures 4.3 and 4.4, respectively.
Table 4.2 Individual mean (± SD) carnosine concentrations in type I, IIA and IIB fibres from the middle gluteal muscle of 5 thoroughbred horses (FO, TI, DP, GI, EX) and overall mean (± SDp) concentrations for all horses.
<table>
<thead>
<tr>
<th>Horse</th>
<th>Carnosine, mmol kg(^{-1}) DW (number of fibres)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Type I</td>
</tr>
<tr>
<td>FO</td>
<td>25.5 ± 6.5 (22)</td>
</tr>
<tr>
<td>TI</td>
<td>12.2 ± 6.7 (53)</td>
</tr>
<tr>
<td>DP</td>
<td>27.3 ± 5.9 (50)</td>
</tr>
<tr>
<td>GI</td>
<td>22.2 ± 4.0 (27)</td>
</tr>
<tr>
<td>EX</td>
<td>0.0 ± 0.0 (2)</td>
</tr>
<tr>
<td>Mean ± SD(_p)</td>
<td>20.6 ± 6.0 (154)</td>
</tr>
</tbody>
</table>

SD\(_p\) Pooled within-horse standard deviation
Table 4.3  Individual mean (± SD) taurine concentrations in type I, IIA and IIB fibres from the middle gluteal muscle of 5 thoroughbred horses (FO, TI, DP, GI, EX) and overall mean (± SD_p) concentrations for all horses.
<table>
<thead>
<tr>
<th>Horse</th>
<th>Taurine, mmol kg$^{-1}$ DW (number of fibres)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Type I</td>
</tr>
<tr>
<td>FO</td>
<td>$62.6 \pm 9.0$ (22)</td>
</tr>
<tr>
<td>TI</td>
<td>$54.9 \pm 15.7$ (53)</td>
</tr>
<tr>
<td>DP</td>
<td>$62.7 \pm 11.8$ (50)</td>
</tr>
<tr>
<td>GI</td>
<td>$69.8 \pm 8.3$ (27)</td>
</tr>
<tr>
<td>EX</td>
<td>$58.4 \pm 3.5$ (2)</td>
</tr>
<tr>
<td>Mean $\pm SD_p$</td>
<td>$61.2 \pm 12.5$ (154)</td>
</tr>
</tbody>
</table>

$SD_p$ Pooled within-horse standard deviation.
Table 4.4 Individual mean (± SD) taurine : carnosine ratios in type I, IIA and IIB fibres from the middle gluteal muscle of 5 thoroughbred horses (FO, TI, DP, GI, EX) and overall mean (± SD_p) concentrations for all horses.
Horse Taurine : Carnosine ratio

<table>
<thead>
<tr>
<th></th>
<th>Type I</th>
<th>Type IIA</th>
<th>Type IIB</th>
</tr>
</thead>
<tbody>
<tr>
<td>FO</td>
<td>2.573 ± 0.564</td>
<td>0.057 ± 0.121</td>
<td>0.045 ± 0.093</td>
</tr>
<tr>
<td>TI</td>
<td>5.559 ± 2.872</td>
<td>0.017 ± 0.031</td>
<td>0.005 ± 0.019</td>
</tr>
<tr>
<td>DP</td>
<td>2.331 ± 0.331</td>
<td>0.030 ± 0.050</td>
<td>0.034 ± 0.043</td>
</tr>
<tr>
<td>GI</td>
<td>3.202 ± 0.494</td>
<td>0.022 ± 0.019</td>
<td>0.002 ± 0.007</td>
</tr>
<tr>
<td>EX</td>
<td>LARGE†</td>
<td>0.037 ± 0.057</td>
<td>0.024 ± 0.027</td>
</tr>
<tr>
<td>Mean ± SDp</td>
<td>3.646 ± 2.241†</td>
<td>0.033 ± 0.029</td>
<td>0.022 ± 0.026</td>
</tr>
</tbody>
</table>

SDp Pooled within-horse standard deviation

† Carnosine was not detected in EX type I fibres. Mean calculated from 4 horses (EX excluded).
Figure 4.1  Individual distribution plots of carnosine concentrations in type I, IIA and IIB fibres from the middle gluteal muscle of 5 thoroughbred horses (FO, TI, DP, GI, EX).
Figure 4.2  Individual distribution plots of taurine concentrations in type I, IIA and IIB fibres from the middle gluteal muscle of 5 thoroughbred horses (FO, TI, DP, GI, EX).
Figure 4.3 Frequency distribution plots of normalized carnosine concentrations in individual type I, IIA and IIB fibres from the middle gluteal muscle of 5 normal thoroughbred horses (n = 324).
Figure 4.4 Frequency distribution plots of normalized taurine concentrations in individual type I, IIA and IIB fibres from the middle gluteal muscle of 5 normal thoroughbred horses (n = 324).
The figure shows a histogram of taurine content (mmol kg\(^{-1}\) DW) for different fiber types in muscle tissue. The x-axis represents the taurine content, while the y-axis shows the number of fibers. The graph includes three categories: Type I, Type IIA, and Type IIB fibers. Each fiber type is represented by a different color: black for Type I, white for Type IIA, and gray for Type IIB.
4.4 STUDY C: CARNOSINE AND TAURINE CONTENTS OF TYPE I, IIA AND IIB FIBRES IN THE MIDDLE GLUTEAL MUSCLE OF THE UNTRAINED AND TRAINED THOROUGHBRED HORSE.

4.4.1 Objectives
The aim of the present study was to investigate whether carnosine and taurine concentrations in type I, type IIA and type IIB fibres from the middle gluteal muscle of the thoroughbred horse were affected by training.

4.4.2 Experimental methodology

Protocol and sampling procedure
Muscle samples from the left middle gluteal muscle were collected at post mortem from 6 three-year-old Thoroughbred fillies. Three of the horses, commencing the study as yearlings, underwent extensive treadmill based training for a period of 15 months. Each horse underwent an identical weekly training protocol which was kept constant over the 15 month period. Details of the weekly training protocol are given in Table 4.5. The remaining 3 horses were turned-out in paddocks for the 15 month period and received no formal training. The 6 horses were humanely euthanased. One entire middle gluteal muscle was removed from each horse and samples were collected as described in detail in Chapter 2. Owing to the relatively low abundance of type I fibres within equine middle gluteal muscle, the number of individual type I fibres retrieved was maximized by selecting some of the darker (more red) samples for dissection. Individual muscle fibres were dissected from freeze-dried muscle as described in Chapter 2.

Histochemistry
Fragments of individual muscle were characterized as either type I, IIA or IIB by histochemical staining for myosin ATPase activity at pH 9.6 following pre-incubation at pH 4.50 as described previously (Chapter 2).
Table 4.5  Weekly training protocol
<table>
<thead>
<tr>
<th>Day</th>
<th>Walk</th>
<th>Trot</th>
<th>Canter / Gallop (treadmill)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>40 min</td>
<td>-</td>
<td>5 min @ 12 m s(^{-1}) (3% incline)</td>
</tr>
<tr>
<td>2</td>
<td>40 min</td>
<td>10 min</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>40 min</td>
<td>-</td>
<td>2 x 90 s @ 13 &amp; 14 m s(^{-1}) (3% incline)</td>
</tr>
<tr>
<td>4</td>
<td>40 min</td>
<td>10 min</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>40 min</td>
<td>-</td>
<td>3 x 70 s @ 12, 13 &amp; 14 m s(^{-1}) (3% incline)</td>
</tr>
<tr>
<td>6</td>
<td>40 min</td>
<td>10 min</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>40 min</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
Individual muscle fibre analysis

Weighed individual muscle fibres were extracted and analysed for carnosine and taurine content simultaneously by reversed-phase high-performance liquid chromatography, as described in Chapter 3 (Dunnett and Harris 1995b).

Statistics

Analysis of carnosine and taurine distribution in type I, IIA and IIB fibres was performed by normalization of individual values which enabled data from all horses to be combined. Individual values were divided by the mean value for that horse and the results were normalized by multiplying by the overall mean value for all untrained or trained horses. Type I, IIA and IIB fibres were treated independently. One-factor ANOVA was used to identify significant differences in mean ($\pm$ SD$_p$) values for carnosine and taurine concentrations in type I, IIA and IIB fibres between untrained and trained horses. In the instance where significance was detected a multiple comparison test was applied, Fisher's Protected Least Significant Difference (PLSD) was applied. Significance was declared at $p < 0.05$.

4.4.3 Results

Carnosine and taurine concentrations were determined in a total of 468 individual muscle fibres (untrained, $n = 239$; trained, $n = 229$) taken from the middle gluteal muscle of 6 thoroughbred horses at post mortem. The number of fibres and mean ($\pm$ SD) carnosine and taurine concentrations in type I, IIA and IIB muscle fibres for individual horses, and overall mean values ($\pm$ SD$_p$) for untrained and trained horses are given in Tables 4.6 and 4.7, respectively. Carnosine concentrations were higher in both type I ($p < 0.05$) and type IIA fibres ($p < 0.001$) in trained horses. There was no significant difference in the carnosine concentration in type IIB fibres between untrained and trained horses ($p > 0.05$). Taurine concentration were higher in type I fibres ($p < 0.001$), and type IIA and IIB fibres ($p < 0.05$) in trained horses. Frequency distribution plots of normalized carnosine and taurine concentrations for all fibres with respect to fibre type are shown in Figures 4.5 and 4.6, respectively.
Table 4.6  Individual mean (± SD) carnosine concentrations in type I, IIA and IIB fibres of the middle gluteal muscle of 3 untrained and 3 trained thoroughbred horses, and overall mean (± SDp) concentrations for untrained and trained horses.
<table>
<thead>
<tr>
<th>Training status</th>
<th>Horse</th>
<th>Carnosine, mmol kg(^{-1}) DW (number of fibres)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Type I</td>
</tr>
<tr>
<td>Untrained</td>
<td>B</td>
<td>32.2 ± 19.2 (25)</td>
</tr>
<tr>
<td></td>
<td>D</td>
<td>11.0 ± 15.4 (26)</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>26.9 ± 2.1 (2)</td>
</tr>
<tr>
<td></td>
<td>Mean ± SD(_p)</td>
<td>23.4 ± 17.2 (53)</td>
</tr>
<tr>
<td>Trained</td>
<td>A</td>
<td>25.0 ± 10.4 (41)</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>30.9 ± 11.4 (30)</td>
</tr>
<tr>
<td></td>
<td>E</td>
<td>35.3 ± 26.6 (8)</td>
</tr>
<tr>
<td></td>
<td>Mean ± SD(_p)</td>
<td>30.4 ± 13.1 (79)</td>
</tr>
</tbody>
</table>

SD\(_p\)  Pooled standard deviation
\(\dagger\)  Significantly different to untrained \(p < 0.001\)
Table 4.7 Individual mean (± SD) taurine concentrations in type I, IIA and IIB fibres of the middle gluteal muscle of 3 untrained and 3 trained thoroughbred horses, and overall mean (± SDp) concentrations for untrained and trained horses.
<table>
<thead>
<tr>
<th>Training status</th>
<th>Horse</th>
<th>Taurine, mmol kg(^{-1}) DW (number of fibres)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Type I</td>
</tr>
<tr>
<td>Untrained</td>
<td>B</td>
<td>36.4 ± 11.0 (25)</td>
</tr>
<tr>
<td></td>
<td>D</td>
<td>33.6 ± 17.6 (26)</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>49.9 ± 15.2 (2)</td>
</tr>
<tr>
<td>Mean ± SD(_p)</td>
<td></td>
<td>40.0 ± 14.8 (53)</td>
</tr>
<tr>
<td>Trained</td>
<td>A</td>
<td>59.6 ± 7.6 (41)</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>53.2 ± 15.4 (30)</td>
</tr>
<tr>
<td></td>
<td>E</td>
<td>74.6 ± 26.4 (8)</td>
</tr>
<tr>
<td>Mean ± SD(_p)</td>
<td></td>
<td>62.5 ± 13.6 (79)</td>
</tr>
</tbody>
</table>

SD\(_p\)  Pooled standard deviation

† Significantly different to untrained *p < 0.05
‡ Significantly different to untrained *p < 0.001
Figure 4.5  Frequency distribution plots of normalized carnosine concentrations in individual type I, IIA and IIB fibres from the middle gluteal muscle of 3 untrained (n = 239) and 3 trained (n = 229) thoroughbred horses.
Figure 1. Frequency distribution plots of untrained (UP) and trained (TP) CARNOSINE (mmol kg⁻¹ DW) concentrations in individual Type I, IIA, and IIB fibres from an active muscle of a trained horse.

UNTRAINED

CARNOSINE (mmol kg⁻¹ DW)

NUMBER OF FIBRES

40 80 120 160 200 240 280 320 360 400

30 25 20 15 10 5 0

Type I
Type IIA
Type IIB

TRAINED

CARNOSINE (mmol kg⁻¹ DW)

NUMBER OF FIBRES

40 80 120 160 200 240 280 320 360 400

30 25 20 15 10 5 0

Type I
Type IIA
Type IIB
Figure 4.6 Frequency distribution plots of normalized taurine concentrations in individual type I, IIA and IIB fibres from the middle gluteal muscle of 3 untrained (n = 239) and 3 trained (n = 229) thoroughbred horses.
4.5 STUDY D: COMPARISON OF THE CARNOSINE AND TAURINE CONTENTS IN TYPE I, IIA AND IIB FIBRES FROM AFFECTED AND UNAFFECTED MIDDLE GLUTEAL MUSCLE FROM A HORSE WITH UNILATERAL NEUROPATHY OF THE HIND-LIMB MUSCLES.

4.5.1 Objectives
The aim of the present study was to compare carnosine and taurine concentrations in individual type I, IIA and IIB fibres from normal and abnormal middle gluteal muscle from the same horse.

4.5.2 Experimental methodology

Protocol and sampling procedure
Muscle samples from the left middle gluteal muscle were collected at post mortem from a 6 year-old Thoroughbred-X pony mare. The horse was diagnosed as having a unilateral neuropathy of the left hind limb muscles arising from the development of bony cysts on the 5th and 6th sacral vertebra and was euthanased six months after the initial diagnosis. Muscle atrophy was pronounced on the affected side. The entire middle gluteal muscle was removed and samples were collected at post mortem as described in detail in Chapter 2. The number of individual type I fibres retrieved was maximized as described earlier in this chapter. Individual muscle fibres were dissected from freeze-dried muscle, as described in Chapter 2.

Histochemistry
Fragments of individual muscle were characterized as either type I, IIA or IIB by histochemical staining for myosin ATPase activity at pH 9.6 following pre-incubation at pH 4.50 as described previously in Chapter 2.

Individual muscle fibre analysis
Weighed individual muscle fibres were extracted and analysed for carnosine and taurine content simultaneously by reversed-phase high-performance liquid chromatography, as described in Chapter 3 (Dunnett and Harris 1995b).
Statistics

Mean (± SD) values for carnosine and taurine concentrations in type I, IIA and IIB fibres, were calculated. One-factor ANOVA was used to identify significant differences in mean (± SD) values for carnosine and taurine concentrations in type I, IIA and IIB fibres between the normal and affected (neuropathic) middle gluteal muscles. In the instance where significance was detected a multiple comparison test was applied, Fisher's Protected Least Significant Difference (PLSD) was applied. Significance was declared at $p < 0.05$.

4.5.3 Results

Carnosine and taurine concentrations, and taurine : carnosine ratios were determined in a total of 68 individual fibres from both normal and neuropathic middle gluteal muscles. Mean (± SD) carnosine and taurine concentrations in type I, IIA and IIB fibres are given in Table 4.8.

Carnosine and taurine concentrations in fibres from the unaffected middle gluteal muscle were similar to those found previously in normal horses. Taurine concentrations were slightly higher, particularly in type IIA and IIB fibres. This horse however, was not pure Thoroughbred and hence the higher taurine contents may been the result of inter-breed differences. As found previously in normal horses, carnosine concentrations were significantly different between type I fibres and both type IIA and type IIB fibres ($p < 0.001$). There was no significant difference between type IIA and IIB fibres ($p > 0.05$). Taurine contents were significantly different between type I fibres and both type IIA and IIB fibres ($p < 0.001$). There was no significant difference between type IIA and IIB fibres ($p > 0.05$).

The taurine concentration in type I fibres from the neuropathic middle gluteal muscle was significantly lower than in fibres from the unaffected middle gluteal muscle ($p < 0.001$). Taurine concentrations in type IIA and IIB fibres were not significantly different between the neuropathic and normal middle gluteal muscles ($p < 0.05$). The carnosine concentration in type I fibres from the neuropathic middle gluteal muscle was not significantly different to the unaffected muscle ($p < 0.05$). The carnosine concentration in type IIA fibres from the
neuropathic middle gluteal muscle was lower than the corresponding value in the normal middle gluteal. The difference however, was not significant ($p > 0.05$). The carnosine concentration in type IIB fibres from the neuropathic middle gluteal was significantly lower than in type IIB fibres from the unaffected middle gluteal ($p < 0.01$).

Frequency distribution plots of carnosine and taurine concentrations for all fibres with respect to fibre type are shown in Figures 4.7 and 4.8, respectively.
Table 4.8  Carnosine and taurine concentrations in unaffected and affected (neuropathic) muscle fibres.
<table>
<thead>
<tr>
<th>Fibre type</th>
<th>Muscle</th>
<th>n</th>
<th>Carnosine mmol kg(^{-1}) DW</th>
<th>Taurine mmol kg(^{-1}) DW</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type I</td>
<td>Unaffected</td>
<td>13</td>
<td>24.5 ± 5.4</td>
<td>73.2 ± 9.4</td>
</tr>
<tr>
<td></td>
<td>Affected</td>
<td>14</td>
<td>27.6 ± 3.1</td>
<td>39.9 ± 4.3</td>
</tr>
<tr>
<td>Type IIA</td>
<td>Unaffected</td>
<td>9</td>
<td>100.8 ± 23.8</td>
<td>9.7 ± 2.2</td>
</tr>
<tr>
<td></td>
<td>Affected</td>
<td>5</td>
<td>96.9 ± 16.0</td>
<td>11.0 ± 1.2</td>
</tr>
<tr>
<td>Type IIB</td>
<td>Unaffected</td>
<td>12</td>
<td>122.6 ± 30.0</td>
<td>11.9 ± 2.7</td>
</tr>
<tr>
<td></td>
<td>Affected</td>
<td>15</td>
<td>93.4 ± 20.1(\dagger)</td>
<td>10.1 ± 2.1</td>
</tr>
</tbody>
</table>

\(n = \text{Number of fibres}\)

\(\dagger\) Significantly different to unaffected fibres \(p < 0.01\)

\(\ddagger\) Significantly different to unaffected fibres \(p < 0.001\)
Figure 4.7 Carnosine distribution in unaffected and affected (neuropathic) type I, IIA and IIB muscle fibres.
Figure 4.8  Taurine distribution in unaffected and affected (neuropathic) type I, IIA and IIB muscle fibres.
4.6 DISCUSSION

The mean carnosine concentration in the middle gluteal muscle measured in Study A is consistent with previous measurements. The low concentration of anserine in the skeletal muscle of the thoroughbred horse is comparable with values of 0.80 ± 0.16 and 0.37 ± 0.44 mmol kg⁻¹ DW reported previously for equine muscle (Carnegie et al. 1983; Plowman and Close 1988). Although low concentrations of anserine and balenine are found in equine skeletal muscle they are negligible in contrast to the carnosine concentration. Therefore, there is essentially a single imidazole dipeptide present in the skeletal muscle of the equine. The occurrence of a single imidazole dipeptide in the skeletal muscle of a species is uncommon, although carnosine alone is also found in the muscles of some primates, including humans. The reasons for this situation in certain species, including the horse, is unclear. However, (Crush 1970) proposed that the differences between the occurrence and hence relative ratios of the imidazole dipeptides present in the skeletal muscle of different species, result from differences between zoological families rather than between species per se. It has been demonstrated in several species, that the biosynthesis of carnosine occurs directly from its constituent amino acids, histidine and β-alanine, and is catalysed by the enzyme carnosine synthetase (Kalyanker and Meister 1959; Bauer and Schultz 1994). However, it was also shown that carnosine synthetase has a broad substrate specificity both in vivo and in vitro and that it is capable of catalysing the synthesis of other β-alanyl-dipeptides, such as anserine, and γ-aminobutyryl-dipeptides, such as homocarnosine (Kalyanker and Meister 1959; Bauer and Schultz 1994). In addition to the direct formation of anserine by carnosine synthetase it has also been reported that the biosynthesis of anserine can arise through the subsequent methylation of pre-formed carnosine which involves the methyl donor S-adenosyl-methionine and a specific N-methyl transferase (McManus 1962). Differences in the occurrence and relative ratios of the imidazole dipeptides between zoological families may therefore arise from differences in substrate or methyl donor availability, and the activity/specificity of N-methyl transferase.

The lower concentrations of carnosine in the internal intercostal muscle and diaphragm in contrast to the middle gluteal muscle probably reflect the different functions of the former. The
middle gluteal muscle is essentially a locomotory skeletal muscle which although involved in maintaining posture and locomotion during low-intensity exercise, its function is of greater importance during high-intensity exercise, such as sprinting and jumping. Consequently there is a preponderance of fast-contracting type II fibres in this muscle. In contrast, the internal intercostal muscles and the diaphragm are involved in ventilation, a process which requires a lower rate of muscle contraction. As such they are likely to have a lesser proportion of type II fibres resulting in a lower overall glycolytic capacity, therefore lower lactic acid production and consequently a reduced need for H+ ion buffering. The two to three-fold lower carnosine concentrations in these muscles relative to the middle gluteal is consistent with their reduced demand for H+ ion buffering. The carnosine concentration in diaphragm is similar to the estimated value for type I skeletal muscle fibres which suggests a predominance of these fibres in this muscle, although differences in the carnosine concentration within a given fibre type between different muscles is also possible.

Carnosine concentrations measured in cardiac muscle (myocardium), smooth muscle (small intestine, colon, stomach and rectum) and other non-muscle tissues were fifty-fold or more lower than those found in the middle gluteal muscle. Such low concentrations of carnosine provide a minimal H+ ion buffering capacity, therefore its presence in these tissues may be explained by some of the other ascribed physiological functions. Carnosine concentrations in equine myocardium are similar to values reported in the cardiac muscle of other vertebrate species (Sobue et al. 1975; O'Dowd et al. 1988). Total concentrations of acetylated imidazole dipeptides in cardiac muscle from rat, guinea pig and frog were greater than 40 mmol kg⁻¹ DW (O'Dowd et al. 1988) and it is therefore possible that the presence of the non-acetylated forms of the dipeptides, including carnosine, arise from metabolism of the acetylated forms, and that carnosine per se has no physiological role in cardiac muscle.

The specific activity attributable to tissue carnosinase was not discriminated from the activity of non-specific dipeptidase in the assay employed. However, the total activity for carnosine degradation is of greater physiological relevance. The mean total carnosinase and non-specific
dipeptidase activity was higher in the small intestine, kidney, spleen and lung of the thoroughbred horse than in the other tissues examined. The activity in the small intestine was less than the value of 0.36 \( \mu \text{mol g}^{-1} \text{min}^{-1} \text{WW} \) (86 \( \mu \text{mol g}^{-1} \text{h}^{-1} \text{DW} \)) reported in the rat (Tamaki et al. 1985), and considerably lower than the value of 8.8 \( \mu \text{mol g}^{-1} \text{min}^{-1} \text{WW} \) (2112 \( \mu \text{mol g}^{-1} \text{h}^{-1} \text{DW} \)) reported in the human (Sadikali et al. 1975). As the site of tissue sampling was not standardized in the equine small intestine the large standard deviation associated with the tissue carnosinase and non-specific dipeptidase activity is probably due to a decrease in the activity of these enzymes towards the ileocaecal valve. Such a distribution has previously been described in the small intestine of the rat (Tamaki et al. 1985). The lower activity in equine small intestine probably reflects the dietary absence of carnosine in the equine, in contrast to the carnosine containing diets of the other two species. The mean total carnosinase and non-specific dipeptidase activity in the equine kidney was lower than that reported in the rat 4.76 \( \mu \text{mol g}^{-1} \text{min}^{-1} \text{WW} \) (1142 \( \mu \text{mol g}^{-1} \text{h}^{-1} \text{DW} \)) (Tamaki et al. 1985). The relatively high values found in the spleen and lung of the horse are also consistent with the tissue distribution in the rat.

Re-determination of tissue carnosinase and non-specific dipeptidase activities in some tissues was performed in the presence of 0.2 \( \mu \text{M} \) bestatin, an inhibitor of non-specific dipeptidase but not tissue carnosinase. Although this does not completely inhibit non-specific dipeptidase, it enabled an estimate to be made of the activity attributable to tissue carnosinase alone. This indicated that the true tissue carnosinase activity in the kidney, spleen and small intestine was only 36.7, 15.0 and 14.6\% of the total activity, respectively. Enzyme activities measured \textit{in vitro} do not necessarily represent accurately the true \textit{in vivo} value. However, the apparent low overall tissue carnosinase activities, and absence of carnosinase activity in skeletal muscle, in the thoroughbred horse are probably indicative of a need to limit carnosine catabolism in order to maintain the high muscle carnosine concentrations required for optimum \( \text{H}^+ \) ion buffering.

The studies described in this chapter are the first to measure the concentrations of carnosine and taurine directly in individual skeletal muscle fibres. Earlier studies relied upon indirect estimates derived from multiple linear regression analysis of data relating muscle carnosine and taurine.
concentrations to type I, IIA and IIB % FSA in 'large' mixed fibre muscle samples (Sewell et al. 1990; Sewell et al. 1992; Dunnett et al. 1992). The present values for carnosine concentrations in type I, IIA and IIB fibres are in close agreement with the estimates of (Sewell et al. 1990). These earlier studies determined carnosine concentrations in muscle obtained from horses of differing ages and states of training. When the present measurements are compared with these previous estimates the agreement is not as close with respect to type I and type IIB fibres although the same trend is apparent, i.e. the highest carnosine concentration occurring in type IIB fibres and the lowest in type I fibres, is apparent. This disparity may be the result of deficiencies in the earlier mathematical model, or more probably arose from the exclusive use of young (two year-old) highly trained horses. These horses underwent high-intensity training and racing and hence would have an increased requirement for intra-muscular buffering capacity.

The values for taurine concentrations in type I, IIA and IIB fibres in study B are similar to previous estimates for older horses sampled at post mortem (Dunnett et al. 1992), and the general finding is identical, i.e. taurine is almost exclusively localized within type I fibres. A poorer agreement with estimates derived from percutaneous muscle biopsy samples may arise for those reasons mentioned previously; namely the exclusive sampling of young intensely trained animals (Dunnett et al. 1992). A similar distribution of carnosine and taurine has also been observed in type I, IIA and IIB fibres in the camel (Dunnett and Harris 1995c).

Previous experimental models have predicted differences between the mean carnosine concentrations of type IIA and IIB fibres of 20 and 95 mmol kg\(^{-1}\) DW, respectively (Sewell et al. 1990; Sewell et al. 1992). The current data however, shows a difference of only 9.6 mmol kg\(^{-1}\) DW. This discrepancy may arise from limitations within the fibre characterization procedure where uncertainty may arise owing to variations in fibre thickness. In contrast fewer problems of classification are encountered when staining sections of uniform thickness and where the assessor is presented with a field of mixed fibres to be viewed simultaneously. The difference in carnosine concentrations between type I and type II muscle fibres is consistent with their individual metabolic characteristics (Snow 1982). During low intensity exercise type I fibres are
predominantly recruited (Lindholm et al. 1974; Snow et al. 1982), H⁺ ion production is minimal and a lower buffering capacity is required (Sewell et al. 1990; Sewell et al. 1992). During high intensity exercise type II fibres are recruited (Lindholm et al. 1974; Snow et al. 1982) and the high rate of glycolysis requires a large in situ H⁺ ion buffering capacity (Sewell et al. 1990; Sewell et al. 1992). Some buffering capacity from carnosine within type I fibres may be necessary owing to probable diffusion of H⁺ ions into these fibres during periods of anaerobic exercise. It has also been proposed that carnosine functions as an antioxidant in vivo and that it is able to protect membrane lipids against damage from reactive oxygen species (Esterbauer et al. 1986; Boldyrev et al. 1988; Kohen et al. 1988). This provides an alternative explanation for the presence carnosine in the oxidative type I muscle fibres.

Without a clearer understanding of the functions of taurine within skeletal muscle per se it is difficult to establish a rationale for the much higher taurine concentrations in type I fibres. The presence of such high concentrations of taurine within these fibres suggest some significant physiological or biochemical role. Taurine may possibly compensate for the lower concentrations of carnosine by maintaining the osmolarity within type I muscle cells. It has also been proposed that taurine functions as an antioxidant and exerts a protective effect against reactive oxygen species in vivo (Franconi et al. 1985) and through reaction with malondialdehyde (Ogasawara et al. 1993). This may be a further explanation for the presence of high a taurine concentration in type I muscle fibres, a highly oxidative tissue.

Changes in the carnosine and taurine concentrations within the different fibre types, as a consequence of training, as described in Study C, are likely to be influenced by the relative contributions of aerobic and anaerobic pathways to energy production during exercise as part of the training programme. Bump et al. (1989) reported a decrease in muscle carnosine concentration in Quarterhorses during training. However, the 7 week training protocol was only of moderate intensity, as shown by the relatively low peak mean plasma lactate concentration of 87.6 mg 100 ml⁻¹ (9.7 mM). A training protocol with a high anaerobic component will result in large increases in muscle lactate and associated H⁺ ion concentrations. As a result of exposure
of the musculature to repeated periods of acidosis the significant increase in the carnosine concentration in IIA muscle fibres in the middle gluteal muscle suggests an adaptive response within the integrated system for the maintenance of intra-muscular pH homeostasis. A previously reported increase in $B_m$ in the middle gluteal muscle of the horse during high-intensity training (McCutcheon et al. 1987; Sinha et al. 1991), could be explained by an increase in the muscle carnosine concentration, such as that observed in Study C. It is possible that the observed increase in the carnosine concentration in type IIA fibres only, is indicative of greater 'trainability' in these mixed oxidative-glycolytic fibres.

Without a clear understanding of the precise function(s) of taurine in skeletal muscle it is difficult to explain the observed increase in taurine concentration in all three fibre types as a result of training. It has been proposed that taurine functions as an antioxidant in vivo (Franconi et al. 1985; Ogasawara et al. 1993), hence the increase in muscle taurine concentration may be a consequence of the aerobic component of the training protocol.

Unilateral ischemic denervation of gastrocnemius muscle in experimental rats has been shown to cause a significant decline ($p < 0.01$) in the muscle carnosine concentration over a period of three weeks (Tamaki et al. 1976). A similar study involving the denervation of the soleus, plantaris and gastrocnemius muscles in experimental rats, showed a non-significant decrease in the carnosine content of the soleus and plantaris muscles and a significant increase in the carnosine concentration ($p < 0.05$) in the gastrocnemius muscle (Turnisky and Long 1990). However, these muscles were analysed only three days after denervation, in contrast to three weeks after in the study of Tamaki et al (1976). The present results for changes in muscle carnosine concentration during denervation are consistent with the evidence from these two earlier studies on whole muscles.
CHAPTER 5

EQUINE PLASMA CARNOSINE CONCENTRATION AND CARNOSINASE ACTIVITY: NORMAL VALUES, DAILY VARIATION, AND THE EFFECTS OF EXERCISE AND MUSCLE DAMAGE
5.1 INTRODUCTION

Plasma carnosine concentrations appear to have been measured in very few species. Not surprisingly most measurements have been made in human plasma. Carnosine is present in the plasma of human neonates at concentrations of less than 1.2 mg l\(^{-1}\) (5.3 \(\mu M\)) in pre-term infants on a low protein diet and 2.0 ± 1.6 mg l\(^{-1}\) (8.8 ± 7.1 \(\mu M\)) in pre-term infants on a high protein diet. Lower concentrations of 0.7 ± 1.7 mg l\(^{-1}\) (3.1 ± 7.5 \(\mu M\)) were found in older full-term infants (Valman et al. 1971). Carnosine is however, absent from the plasma of normal adults. This age related disappearance of carnosine appears to be correlated with an increase in serum carnosinase activity. Serum carnosinase activity in young children (less than 1 year old) is 0.14 ± 0.07 and 0.22 ± 0.15 \(\mu\)mol ml\(^{-1}\) h\(^{-1}\) for males and females, respectively, in contrast to adult values of 1.85 ± 0.59 - 9.66 ± 0.42 \(\mu\)mol ml\(^{-1}\) h\(^{-1}\) (Bando et al. 1986; Bando et al. 1984; Wassif et al. 1994). Relatively high plasma carnosine concentrations (hypercarnosinemia) have been reported in cases of human serum carnosinase deficiency (Perry et al. 1968; Perry et al. 1967).

Carnosine has been detected in the plasma and other blood constituents of chicks, rats and rabbits. Chick plasma and rabbit serum were reported to contain 27 ± 3 \(\mu M\) and 1.9 - 7.7 \(\mu M\) carnosine, whereas only trace amounts were found in rat plasma (Seely and Marshall 1981; Kurisaki and Hiraiwa 1988). However, chick erythrocytes contained large amounts of carnosine (2510 ± 70 nmol g\(^{-1}\) cells), a value comparable with chick skeletal muscle. Lower quantities were present in rabbit reticulocytes (105 ± 11 nmol g\(^{-1}\) cells) and erythrocytes (18 ± 2 nmol g\(^{-1}\) cells), and only trace amounts in rat erythrocytes (< 5 nmol g\(^{-1}\) cells) (Seely and Marshall 1981). The occurrence of carnosine in frog erythrocytes has also been reported (Balgooy et al. 1974). The high concentration of carnosine in chick erythrocytes was associated with the presence of carnosine synthetase activity (Ng and Marshall 1976b). Carnosine has also been detected in the blood of some species of fish, such as trace amounts in Rainbow trout blood (Abe 1991) and a concentration of 0.67 ± 0.17 \(\mu\)mol g\(^{-1}\) in tuna blood (Abe et al. 1986). The only previously reported measurement of carnosine concentration in equine plasma recorded values of between 1.7 and 5.2 \(\mu M\) (McLean et al. 1987).
With the exception of diet (i.e. the consumption of meat and associated foodstuffs) no previous investigations appeared to consider other possible factors, such as diurnal variation and exercise, which may influence the concentration of carnosine in plasma. Owing to the extremely high concentration of carnosine in skeletal muscle relative to plasma it is possible that significant increases in the plasma concentration may arise as a consequence of muscle damage. Large increases in both plasma carnosine and anserine concentrations in the rat following traumatic shock induced muscle damage have been reported (Kurisaki and Hiraiwa 1988).

5.2 STUDY A: NORMAL PLASMA CARNOSINE CONCENTRATION AND CARNOSINASE ACTIVITY IN THE THOROUGHBRED HORSE, AND THE INFLUENCE OF AGE AND GENDER.

5.2.1 Objectives
The objectives of this study were to establish the normal range in plasma carnosine concentration and plasma carnosinase activity in normal thoroughbred horses and to investigate the possible influence of age, gender, and plasma histidine and ß-alanine concentrations.

5.2.2 Experimental methodology
Protocol and sampling procedure
Single heparinized venous blood samples (5 ml) were obtained from 112 thoroughbred horses in training (26 males, 30 females, 25 geldings), yearlings (13 males, 13 females) and foals (2 males, 3 females) from four racing yards and one stud farm in the Newmarket area. All blood samples were collected between 15.00 and 17.00 h.

Analytical methods
Plasma carnosine and histidine concentrations were determined by the method described in Chapter 3 (Dunnett and Harris 1992). Plasma ß-alanine concentrations were determined as described in Chapter 2. Plasma carnosinase activity was determined using a modification of the method of Bando et al. (1984), as described in Chapter 2.
Statistical analysis

A two factor analysis of variance (ANOVA) was used to identify significant effects of age and gender. Significance was declared at \( p < 0.05 \). In the instance where significance was detected a multiple comparison test, Fisher's Protected Least Significant Different (PLSD), was applied.

5.2.3 Results

Preliminary measurements of plasma carnosine, histidine, anserine and 1-methylhistidine concentrations in a small group of experimental horses (\( n = 8 \)) have been reported (Dunnett and Harris 1992). Anserine was absent from equine plasma and 1-methylhistidine was present at \( 4.64 \pm 0.97 \mu M \). No further determinations of 1-methylhistidine concentrations were made. Carnosine, histidine and \( \beta \)-alanine concentrations in plasma were measured in a further 104 Thoroughbreds. Anserine and \( \beta \)-alanine were absent from the plasma of all horses (\( n = 112 \)). The ranges in plasma carnosine and histidine concentrations in thoroughbred horses of different ages and gender are shown in Figures 5.1 and 5.2, respectively. Mean plasma carnosine and histidine concentrations for these horses are given in Tables 5.1 and 5.2, respectively. The mean (\( \pm SD \)) carnosine and histidine concentrations in all horses were 10.2 \( \pm 3.8 \mu M \) and 51.4 \( \pm 10.1 \mu M \), respectively. There was no significant difference in either plasma carnosine or histidine concentrations between males, females and geldings at any age (\( p > 0.05 \)), with the exception of a significant difference in plasma histidine concentration between 3 year-old geldings and males (\( p < 0.05 \)). There was a significant effect of age on both plasma carnosine and histidine concentrations which was more marked with respect to the former. Plasma carnosine concentrations in both foals and yearlings were approximately half those found in older horses (\( p < 0.001 \)). Plasma histidine concentrations were significantly lower in foals and yearlings compared to older horses (\( p < 0.05 \)). No significant differences in plasma carnosine and histidine concentrations were evident between older horses. The range in plasma carnosine concentrations in yearlings (3.9 - 8.7 \( \mu M \)) was also much narrower than in older horses (e.g. 6.5 - 17.0 and 8.2 - 21.0 \( \mu M \) in 2 and 3 year-olds, respectively). For horses of 2 years and older there was no significant correlation between plasma carnosine and histidine concentrations (r =
0.173, \( p > 0.05 \), however, a weak but significant correlation was found in foals and yearlings (\( r = 0.498, \ p < 0.005 \)), as shown in Figure 5.3.

Plasma carnosinase activity was absent in samples from all horses. The plasma carnosinase assay was validated by making comparative measurements on human plasma (\( n = 5 \) in duplicate). The mean (\( \pm \) SD) activity obtained (2.08 \( \pm \) 1.48 \( \mu \text{mol ml}^{-1} \text{h}^{-1} \) ) was consistent with serum values reported in the literature (1.85 \( \pm \) 0.59 \( \mu \text{mol ml}^{-1} \text{h}^{-1} \)) (Bando et al. 1984).
Figure 5.1 Range in plasma carnosine concentrations at different ages in male, female and gelded thoroughbred horses (n = 112).
Figure 5.2: Rotation in plasma histidine concentrations at different ages in male, female and gelding thoroughbred horses (n = 112).
Figure 5.2  Range in plasma histidine concentrations at different ages in male, female and gelded thoroughbred horses (n = 112).
Table 3.1  Mean (± SD) plasma vanosine concentrations in male, female and gelding

male horses of different ages (n = 112).
Table 5.1  
Mean (± SD) plasma carnosine concentrations in male, female and gelded thoroughbred horses of different ages (n = 112).
<table>
<thead>
<tr>
<th>Gender</th>
<th>Plasma carnosine concentration (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Foals (n)</td>
</tr>
<tr>
<td>Males</td>
<td>6.8 ± 1.6 (2)</td>
</tr>
<tr>
<td>Females</td>
<td>5.1 ± 0.5 (3)</td>
</tr>
<tr>
<td>Geldings</td>
<td>-</td>
</tr>
<tr>
<td>Total</td>
<td>5.8 ± 1.3 (5)</td>
</tr>
</tbody>
</table>

† significantly different from foals and yearlings (p < 0.001)
Table 5.2  Mean (± SD) plasma histidine concentrations in male, female and gelded thoroughbred horses of different ages (n = 112).
<table>
<thead>
<tr>
<th>Gender</th>
<th>Plasma histidine concentration (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Foals (n)</td>
</tr>
<tr>
<td>Males</td>
<td>35.6 ± 17.2 (2)</td>
</tr>
<tr>
<td>Females</td>
<td>38.1 ± 11.4 (3)</td>
</tr>
<tr>
<td>Geldings</td>
<td>-</td>
</tr>
<tr>
<td>Total</td>
<td>37.1 ± 11.8 (5)</td>
</tr>
</tbody>
</table>

† significantly different from foals and yearlings (p < 0.05)
Figure 5.3  Correlation between plasma carnosine and histidine concentrations in thoroughbred horses (n = 112).
PLASMA HISTIDINE (μM)

- **FOALS & YEARLINGS**
  \[ y = 0.057x + 3.422 \quad r = 0.498 \]

- **2 YEAR-OLDS & OLDER**
  \[ y = 0.062x + 8.456 \quad r = 0.173 \]
5.3 STUDY B: VARIATION IN PLASMA CARNOSINE AND HISTIDINE CONCENTRATIONS OVER 24 HOURS IN FED AND FASTED HORSES

5.3.1 Objectives
The objective of this study was to investigate the within-day variation in the plasma carnosine and histidine concentrations in resting thoroughbred horses during a normal dietary regimen and following a 12 h overnight fast. A knowledge of the normal within-day variation in plasma carnosine and histidine concentration was also necessary for later pharmacokinetic investigations following oral and intra-venous carnosine administration.

5.3.2 Experimental methodology

Protocol and sampling procedure
Heparinized venous blood samples (5 ml) were collected at 2 hourly intervals via an indwelling catheter from six resting thoroughbred horses, aged 4 - 7 years (5 geldings and 1 filly), over a 24 h period on two separate days. On the first day the horses underwent a normal feeding regimen. Five of the horses were fed a normal diet comprising Spillers Stud Cubes and the sixth was fed Spillers Racehorse Cubes. Feed (approximately 2 kg) was provided three times per day at 07.00, 12.30 and 16.30 h. Hay (2 - 3 kg) was provided with both the morning and evening feeds. Water was provided ad libitum throughout the day. On the second day the horses were fasted following the evening feed. The two experimental sessions were separated by a period of two days.

Analytical methods
Plasma carnosine and histidine concentrations were determined by the method described in Chapter 3 (Dunnett and Harris 1992).

Statistical Analysis
Plasma carnosine and histidine concentrations at different times in fed and fasted horses were compared using Student's t-test for unpaired data. Plasma carnosine and histidine concentrations
between fed and fasted horses at different times were compared using Student's \( t \)-test for paired data.

### 5.3.3 Results

Large between-horse variations in plasma carnosine and histidine concentrations were evident. However, values for all six horses were within the normal ranges established in the preceding study. The ranges in mean plasma carnosine and histidine concentrations over 24 h in resting horses during the normal feeding regime were 8.8 - 11.7 and 56.9 - 68.5 \( \mu M \), respectively. No significant changes in mean plasma carnosine or histidine concentrations were found over 24 h in resting horses during normal feeding (\( p > 0.05 \)). The mean within-horse variances in plasma carnosine and histidine concentrations during normal feeding were \( \pm 1.6 \) and \( \pm 9.0 \) \( \mu M \), respectively. Mean (\( \pm SD \)) plasma carnosine and histidine concentrations over 24 h are shown in Figures 5.4 and 5.5, respectively. The ranges in mean plasma carnosine and histidine concentrations over 24 h in resting horses during fasting were 10.0 - 11.7 and 55.8 - 65.6 \( \mu M \), respectively. No significant changes in mean plasma carnosine and histidine concentrations were found over 24 h in resting horses during fasting (\( p > 0.05 \)). The mean within-horse variance in plasma carnosine and histidine concentrations were \( \pm 1.5 \) and \( \pm 6.4 \) \( \mu M \), respectively. Mean plasma carnosine and histidine concentrations were not significantly different between fed and fasted horses (\( p > 0.05 \)). Comparisons of mean (\( \pm SD \)) plasma carnosine and histidine concentrations between fed and fasted horses over the period 12 - 24 h are shown in Figure 5.6.
Figure 5.4  Variation in mean (± SD) plasma carnosine and histidine concentrations in resting thoroughbred horses over 24 h during a normal feeding regimen (n = 6).
(Feeding times are indicated by vertical dotted lines)
Figure 5.5  Variation in mean (± SD) plasma carnosine and histidine concentrations in resting thoroughbred horses during 24 h fasting (n = 6).

(Feeding times are indicated by vertical dotted lines)
Figure 5.6  Comparison of mean (± SD) plasma carnosine and histidine concentrations over 24 h in fed and fasted resting thoroughbred horses (n = 6).

(Feeding times are indicated by vertical dotted lines)
5.4 STUDY C: VARIATION IN PLASMA CARNOSINE CONCENTRATION AS A RESULT OF HIGH-INTENSITY EXERCISE.

5.4.1 Objectives
The aim of the study was to evaluate whether high-intensity exercise in the thoroughbred horse elicited a change in plasma carnosine concentration.

5.4.2 Experimental methodology

Protocol and sampling procedure
Six thoroughbred horses, NV, NS, OJ, RI, HO and EL (4 geldings, 2 fillies), aged 5 - 13 years, were trained for 4 weeks prior to undertaking a treadmill based standardized exercise test (SET). During the SET the horses were exercised continuously and at an intensity which was increased in a step-wise fashion to a point of near-maximal performance during the exercise protocol. Horses were walked at 1.6 m s\(^{-1}\) for 10 min on a level surface followed by a succession of canters of 1 min duration on a 5\(^\circ\) incline at speeds of 6, 8, 9, 10, 11 and 12 m s\(^{-1}\), or until the onset of fatigue, as defined by the point at which they were no longer able to match the speed of the treadmill despite humane encouragement. Heparinized venous blood samples (5 ml) were collected immediately prior to exercise and 5 min, 30 min, 2 h and 24 h after exercise.

Analytical methods
Plasma carnosine and histidine concentrations were determined by the method described in Chapter 3. Plasma aspartate transaminase (AST: EC 2.6.1.1) and creatine kinase (CK: EC 2.7.3.2) activities were determined by kinetic methods using commercial diagnostic reagents (AST, Randox Laboratories Ltd., Co. Antrim, N. Ireland.; CK, Kone\textsuperscript{TM} Diagnostics, San Diego, USA.) on a Kone\textsuperscript{TM} Specific Autoanalyser.

Statistical analysis
Pre- and post-exercise plasma carnosine concentrations were compared using Student's \(t\)-test for paired data.
5.4.3 Results

The mean (± SD) pre-exercise plasma carnosine concentration (10.3 ± 1.0 \( \mu M \)) was within the normal range established in Study A. Plasma carnosine concentration increased in 5 out of 6 horses following high-intensity exercise. The peak mean plasma carnosine concentration of 12.4 ± 4.4 \( \mu M \) which occurred at 30 min post-exercise was significantly greater than the pre-exercise concentration (\( p < 0.05 \)). However, the significance of this increase was due to the concentration change in one horse (NV). The mean peak concentration only marginally exceeded the upper limit of the normal within-day variation in plasma concentration of thoroughbred horses at rest (8.8 - 11.7 \( \mu M \)), as established in Study B, and was within the normal range for older horses. The greater increase in post-exercise plasma carnosine concentration in horse NV correlated with a greater increase in plasma AST activity in this horse. However, for all horses the mean pre- and post-exercise plasma AST and CK activities were within the normal clinical reference range (AST, 105 - 230 IU l\(^{-1}\); CK, 16 - 49 IU l\(^{-1}\)) and were not indicative of the occurrence of skeletal muscle damage. Mean pre- and post-exercise AST and CK activities were not significantly different (\( p > 0.05 \)).
Figure 5.7  Individual plasma carnosine concentrations in thoroughbred horses following high-intensity exercise (n = 6).
Changes in plasma carnosine concentration were evaluated in this study to assess whether changes in skeletal muscle occurring during the onset of exertional rhabdomyolysis syndrome (ERS) caused a significant increase in plasma carnosine concentration. Results were obtained by jugular venous blood samples from three horses at time 0, 5, 30, 60, and 240 minutes after the occurrence of episodes ERS. The occurrence of ERS in each horse was determined by the owner and confirmed by the equine veterinarian. Established criteria were used to assess the clinical condition and the severity of the episode in each case (Harris et al., 1999). These criteria included swelling, pulse rate, respiratory rate, sweating, pulse rhythm, and urinalysis.

The plasma carnosine concentration was determined using the method described in Chapter 3 (Dunnett et al., 1998). Plasma carnosine concentration was determined as described in Chapter 2. The activities of CK and AST and the result of muscle biopsies were determined by methods using commercially available kits (CarnoPro kit, Bellevue, WA).

The figure shows the plasma carnosine concentration over time for different horses (NS, NV, OJ, RI, HO, EL). The x-axis represents time points: pre-exercise, 5 min post, 30 min post, 120 min post, and 24 hr post, while the y-axis represents plasma carnosine concentration in μM. The color lines indicate different horses, with NS, NV, OJ, RI, HO, and EL representing different horses.
5.5 STUDY D: CHANGES IN PLASMA CARNOSINE CONCENTRATION FOLLOWING THE ONSET OF EQUINE EXERTIONAL RHABDOMYOLYSIS SYNDROME

5.5.1 Objectives
The aim of this study was to evaluate whether damage to skeletal muscle occurring during episodes of equine exertional rhabdomyolysis syndrome (ERS) caused a significant increase in plasma carnosine (and taurine) concentration(s).

5.5.2 Experimental methodology

Protocol and sampling procedure
Heparinized plasma samples (5 ml each) were obtained by jugular venepuncture from three horses after the occurrence of episodes ERS. The occurrence of ERS in each horse was diagnosed by the same veterinary surgeon. Established criteria were used to assess the clinical severity of the episode in each case (Harris 1989). These criteria included mobility, rigidity and swelling of the skeletal muscles, sweating, pulse and respiration rates, and urine colouration. Severity was graded on a scale from I to V, with a grading of V signifying the greatest severity. The clinical criteria associated with episodes of differing severity are summarized in Table 5.3. Initial plasma samples were obtained within 90 min of the onset of the episode and subsequent samples were obtained at varying intervals during recovery.

Analytical methods
Plasma carnosine concentration was determined by the method described in Chapter 3 (Dunnett and Harris 1992). Plasma taurine concentration was determined as described in Chapter 2. Plasma AST and CK activities were determined by kinetic methods using commercially available reagents on a Kone Specific Autoanalyzer, as described in Study C.
Table 5.3  Grading of the severity of ERS episodes and the associated clinical criteria.
Adapted from Harris (1989).
<table>
<thead>
<tr>
<th>Grade of severity</th>
<th>Mobility</th>
<th>Muscle condition</th>
<th>Excessive sweating</th>
<th>Elevated pulse resp.</th>
<th>Evidence of GIT disturbance</th>
<th>Discoloured urine</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Slight stiffness. Shortened stride.</td>
<td>Appears normal.</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>II</td>
<td>Reluctant to move.</td>
<td>Often appears normal.</td>
<td>±</td>
<td>+</td>
<td>±</td>
<td>±</td>
</tr>
<tr>
<td>IV</td>
<td>Unable to move. May become transiently recumbent.</td>
<td>Firm and swollen. May not resent palpation.</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>V</td>
<td>Rapidly become recumbent.</td>
<td>Firm with possible wasting (atrophy) subsequent to the episode.</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
</tbody>
</table>
5.5.3 Results

Three cases of ERS were investigated during the course of this study. The clinical details of each case are summarised below.

*Case 1* A 17 year-old Thoroughbred X Arab mare (AB) experienced an episode of ERS following approximately 10 - 20 min of moderate exercise comprising walking, trotting and cantering over a series of 6 cross-country fences. The horse showed a reluctance to move, firm and swollen gluteal muscles, and a slight discolouration of the urine. The clinical severity of the episode in this horse was graded II - III.

*Case 2* An 11 year-old Thoroughbred mare (EL) experienced an episode of ERS following moderate exercise comprising approximately 40 min walking, 15 min trotting and 5 min half-speed up-hill cantering. The horse lay down immediately on returning to the stables after exercise but could be encouraged to stand and move. The clinical severity of the episode in this horse was graded II.

*Case 3* A 10 year-old Thoroughbred X gelding (PD). Details of the exercise undertaken immediately prior to the occurrence of the ERS episode are not known. Some slight stiffness was evident but no muscle abnormality was apparent. All other clinical signs appeared normal. The clinical severity of the episode in this horse was graded I.

The peak increases in plasma carnosine and taurine concentrations, and AST and CK activities following the onset of ERS in each of the three cases are detailed in Table 5.4. Changes in plasma carnosine and taurine concentrations, and AST and CK activities with respect to time following the episodes of ERS in the individual horses are shown in Figure 5.8. Peak plasma carnosine concentrations were significantly elevated (5 - 70-fold) above the normal range in all three horses. The increases in plasma carnosine and taurine concentrations, and AST and CK activities varied considerably between the individual horses and did not necessarily correlate with the severity of the clinical signs in each case. A large increase in plasma taurine...
concentration was evident in only one of the horses (PD). The significance of the much smaller increases in the other two horses was difficult to assess in the absence of sufficient data relating to the normal range and possible within-day variation in equine plasma taurine concentration. Plasma AST and CK activities, which are often used as indices of muscle damage, were significantly increased above the normal range (see previous study). The highest values for all parameters were evident in horse PD. Although only three horses were studied when the data were combined there were close correlations between peak plasma carnosine concentration and both peak plasma AST and CK activities. Similar correlations were found between peak plasma taurine concentration and both peak plasma AST and CK activities. The peak plasma carnosine concentration occurred approximately 1 - 2 h after the onset of ERS as did peak plasma CK activity in the two horses with the lower increases in CK (AB and EL). In contrast, peak plasma AST activities occurred approximately 24 - 48 h after the ERS episode. There was a rapid initial clearance of carnosine from the plasma, although concentrations did not return to values close to the normal range for several days.
Table 5.4  Peak values in plasma carnosine and taurine concentrations, and AST and CK activities following the onset of ERS in three cases.
<table>
<thead>
<tr>
<th>Case</th>
<th>Grade</th>
<th>Severity</th>
<th>Peak values measured (time after onset)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Carnosine, $\mu$M</td>
</tr>
<tr>
<td>AB</td>
<td>II-III</td>
<td>Moderate</td>
<td>57.6 (2 h)</td>
</tr>
<tr>
<td>EL</td>
<td>II</td>
<td>Mild</td>
<td>143.0 (1 h)</td>
</tr>
<tr>
<td>PD</td>
<td>I</td>
<td>Mild</td>
<td>702.3 (1 h)</td>
</tr>
</tbody>
</table>
Figure 5.8 Changes in plasma carnosine and taurine concentrations, and AST and CK activities with respect to time following the onset of ERS in the individual horses.
Measurements of plasma carnosine concentrations have been performed in very few species. The concentrations of carnosine in plasma from standardbred horses found during this study (1.5-2.5 µM) (McKearn et al. 1987) were approximately similar to values reported for human plasma (Seyf and Marshall 1983). The histidine concentration in ethanolized plasma is independent of age, consistent with earlier reported values of histidine (3.7 ± 1.0 µM) (McKearn et al. 1987) and post-EMA (49.0 ± 57.2 µM) in plasma, but a slightly higher than these values. The plasma concentration in placenta, however, has not been previously reported. However, a similar age-related increase in plasma carnosine concentration has been reported (Fessler et al. 1989). Carnosine has been detected in 90% of the tissues within the skeletal muscle of some vertebrates. Assuming a similar condition in vivo in the horse, the age-related increase in plasma carnosine concentration may reflect changes in muscle carnosine concentration. Another factor to consider is the concentration of plasma amino acids, particularly the amino acids that are known to be precursors of carnosine synthesis. However, this factor does not appear to be a significant factor in the production of carnosine in blood. In summary, the absence of a significant age-related increase in plasma carnosine concentration may be due to the presence or absence of co-factors and inhibitors, including Mg²⁺, Ca²⁺, and Cu²⁺, which may have significant effects on the synthesis of plasma carnosine. The absence of a significant age-related increase in plasma AST (UY·L⁻¹) concentration in young horses during later infancy. The lack of correlation with the gradual increase in plasma AST activity (Bando et al. 1989) in contrast, plasma AST activity was not significantly correlated. The differences in the characteristics of plasma AST activity in young horses are consistent with earlier reports that a significant increase in plasma AST activity occurs in young horses during later infancy.
5.6 DISCUSSION

Measurements of plasma carnosine concentrations have been performed in very few species. Micromolar concentrations of carnosine in plasma from thoroughbred horses found during this study are similar to the only previously reported value for equine plasma in standardbred horses (1.7 ± 1.7 to 5.2 ± 2.8 μM) (McLean et al. 1987), and are also similar to values reported for chick and rabbit plasma (Seely and Marshall 1981; Kurisaki and Hiraiwa 1988). The histidine concentration in Thoroughbred plasma is in close agreement with earlier reported values in Thoroughbred plasma (53.7 ± 3.0 μM) (McKeever et al. 1986) and pony sera (48.0 - 57.2 μM) (Reitnour et al. 1970), but is slightly higher than that reported for Standardbreds (35.6 ± 5.6 to 40.9 ± 9.4 μM) (McLean et al. 1987). The age related increase in equine plasma carnosine concentration has not previously been reported. However, a similar age related increase in equine plasma carnitine concentration has been reported (Foster et al. 1989). Carnosine has been reported to be synthesized within the skeletal muscle of some vertebrates. Assuming a similar situation exists in the horse, the age-related increase plasma carnosine concentration may arise from an increased carnosine bio-synthetic capacity and thus greater 'leakage' from skeletal muscle. This is opposite to the trend evident in humans where carnosine is initially present in the blood of premature infants, at comparable concentrations to those in foals and yearlings, but subsequently declines during later infancy. This reduction coincides with a gradual increase in plasma carnosinase activity (Bando et al. 1984). In contrast, plasma carnosinase activity was absent from all horses sampled.

Possible inter-species differences in the characteristics of the enzyme, such as $K_M$ value, requisite co-factors and optimum pH which may have impaired the effectiveness of the assay, were investigated. However, changes in pH between 7.0 and 9.0, substrate concentrations from 0.5 - 50 mM, and the presence or absence of co-factors and stabilizers, including Mn$^{2+}$, Co$^{2+}$ and Cd$^{2+}$ (2 mM), and DTT (1 mM), failed to indicate the existence of plasma carnosinase activity. The absence of plasma carnosinase activity in the thoroughbred horse is consistent with earlier data from other non-primates including equine serum from an undisclosed breed (Jackson et al.
1991). The presence of carnosine in equine plasma is consistent with the absence of plasma carnosinase activity.

In both humans and horses it has been established that the plasma concentrations of certain metabolites, such as glucose, triiodothyronine, free fatty acids, cortisol and growth hormone exhibit regular fluctuations during the course of the day (Gibson et al. 1985; Orme et al. 1994; Youket et al. 1985; Zilva and Parnall 1984). Such changes can be related to feeding. Increases in plasma concentrations of proteinaceous amino acids occur as a result of normal food intake, as has been demonstrated in lambs and poultry (Featherston 1972; Nimrick et al. 1971), and consumption of diets containing an excess of a given amino acid cause produce an increase in the plasma concentration of that amino acid (Zimmerman and Scott 1975). The higher mean within-horse variance in plasma histidine concentration in normally fed horses (9.0 μM) compared with fasted horses (6.4 μM) suggests that normal feeding does to some extent induce alterations in circulating concentrations of histidine. This is consistent with data from previous studies in horses which showed post-feeding increases in the plasma concentrations of amino acids in general (Johnson and Hart 1974; Russell et al. 1986) and histidine in particular (Johnson and Hart 1974). Furthermore, Johnson and Hart (1974) reported a significant reduction in plasma histidine concentration fasting horses. However, no reduction in mean plasma histidine concentration during fasting was observed in the present investigation. This apparent discrepancy may be due to the use of resting horses in the present study, unlike the former, in which the horses exercised for 2 - 4 h at low to moderate intensity during the experimental period. Comparison of the within-horse variance in plasma carnosine concentration between fed and fasted horses (1.6 μM fed, 1.5 μM fasted) suggests that normal feeding has very little if any influence on circulating concentrations of carnosine.

In conclusion, there appeared to be little influence of feeding on plasma carnosine concentrations. The within-day fluctuations in plasma carnosine concentration were random and differences in concentration at different times were not statistically significant (p > 0.05). The within-day variation in plasma histidine concentration was random and differences in
concentration at different times were not statistically significant (p > 0.05). However, normal feeding appeared to increase the within-day variation in plasma histidine concentration.

Post-exercise increases in carnosine concentrations in equine plasma were reported briefly in a previous investigation of changes in plasma amino acid concentrations in two groups of five standardbred racehorses before and after racing. In one group where post-exercise samples were collected between 2 and 5 min after racing, the pre- and post-exercise plasma carnosine concentrations were 5.2 ± 2.8 and 11.0 ± 9.1 μM, respectively. In the second group where post-exercise samples were collected between 2 and 3 h after racing, the pre- and post-exercise concentrations were 1.7 ± 2.7 and 8.3 ± 3.3 μM, respectively (McLean et al. 1987).

It is possible that increases in plasma carnosine concentrations after high-intensity exercise may arise from muscle cell membrane damage and the subsequent transfer of the cellular contents into the circulation. Owing to the presence of very high concentrations of carnosine within equine skeletal muscle significant increases in plasma carnosine concentrations may arise as a consequence of even minor muscle fibre damage. Although no previous investigations appear to have addressed this point in association with exercise, 10 to 20-fold increases in plasma carnosine and anserine concentrations have been reported following traumatic shock induced skeletal muscle damage in rats (Kurisaki and Hiraiwa 1988). Study C indicates that high-intensity exercise per se does not result in large increases in plasma carnosine concentration. Evidence from this study however, suggests that large increases in plasma carnosine concentration may occur as a result of skeletal muscle damage associated with ERS, and that the peak concentration is probably proportional to the severity or extent of the damage. The use of pharmacokinetic parameters determined after intra-venous injection of carnosine could enable an estimate to be made of the mass of muscle damaged. However, it would not be possible to discriminate between mild but widespread damage, and severe localized damage.

Owing to the almost exclusive localization of taurine in type I muscle fibres and the much greater concentration of carnosine in type II fibres, the appearance of significantly elevated plasma

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carnosine or taurine concentrations (assuming other causes of plasma taurine elevation are eliminated) may be indicative of selective type I or type II fibre damage, respectively. In the one horse (PD) large increases in both plasma AST and CK activities are indicative of extensive skeletal muscle damage. The large increases also seen in plasma carnosine and taurine concentrations suggest the involvement of both type I and type II fibre damage. This contradicts the earlier suggestion that muscle damage associated with episodes of ERS is confined to the type II muscle fibres (Valberg et al. 1993). However, considerable previous investigations have attested to the ubiquitous nature of taurine in mammalian tissues in many species (Jacobson and Smith 1968) and although no such studies appear to have been conducted in the horse, it is reasonable to assume that a similar widespread distribution of taurine in equine tissues exists. In view of this it may be difficult to attribute increases in plasma taurine concentrations solely to skeletal muscle damage.

Plasma carnosine concentrations may offer a more reliable estimation of the degree of muscle damage incurred than plasma AST and CK activities. Neither plasma AST or CK activities are indicative of damage specifically to the skeletal muscles. Although CK activities are relatively high in skeletal muscle, significant activities are also present in the brain and heart. AST activity is not specific to skeletal muscle but also occurs at significant levels within the heart and liver. Hence, increases in these parameters are also likely with various forms of soft tissue damage. Owing to the relatively low concentration of carnosine in tissues other than skeletal muscle, damage to these other tissues is unlikely to result in increases in plasma carnosine concentrations which could be misinterpreted as skeletal muscle damage. For example, from the data given in Chapter 4, it can be estimated that complete destruction of the myocardium would result in a peak plasma carnosine concentration of only 30 - 40 μM.
CHAPTER 6

METABOLISM OF CARNOSINE AND N-α-ACETYLCARNOSINE FOLLOWING ORAL AND INTRA-VENOUS ADMINISTRATION IN THE THOROUGHBRED HORSE
6.1 INTRODUCTION

Carnosine metabolism has been investigated using both *in vitro* and *in vivo* techniques. *In vivo* metabolic studies, predominantly in rats and humans, but also in trout, have involved the administration of carnosine by intra-muscular injection or orally via naso-gastric intubation or as a supplement to the normal feed. There appears to be no previous studies describing the metabolism of carnosine *in vivo* following administration via the intra-venous route. *In vitro* studies, focusing mostly on carnosine transport, have been conducted utilizing isolated kidney and intestinal tissue from several species including rats, mice, guinea pigs and rabbits. The transport, metabolism and excretion of carnosine and the other imidazole dipeptides varies markedly between species.

Carnosine and other imidazole dipeptides are absent from fasting human plasma and are present at only very low concentrations in the urine of normal subjects. They are however, detectable following ingestion of meals with a high meat content, although plasma concentrations are still low. Perry *et al.* (1967) reported plasma carnosine concentrations of between 6 and 18 \( \mu M \) in 2 normal subjects, 2 h after ingestion of between 16 and 20 g d\(^{-1}\) kg\(^{-1}\) BW of chicken, a rich dietary source of carnosine. Approximately 3 - 4 mmol d\(^{-1}\) of carnosine was also excreted in the urine, in addition to smaller quantities of the primary metabolites histidine and \( \beta \)-alanine (Perry *et al.* 1967). Similar results describing the urinary excretion of balenine and anserine have also been reported following the ingestion of various other meat sources of these imidazole dipeptides, including whale, tuna and pork (Undrum *et al.* 1982; Abe *et al.* 1993). Oral administration of the carnosine in normal human subjects resulted in large increases in plasma histidine and \( \beta \)-alanine concentrations (Asatoor *et al.* 1970; Sadikali *et al.* 1975; Cook 1976; Gardner *et al.* 1991). No plasma carnosine was detected however, unless cooling procedures were employed during and following sample collection to inhibit plasma carnosinase activity (Gardner *et al.* 1991). Urinary carnosine excretion was rapid and highly variable with 2 - 14% of the administered dose appearing as carnosine and up to a further 2% appearing as \( \beta \)-alanine in the urine within 5 h of administration (Gardner *et al.* 1991). Both carnosine and anserine are absorbed intact from the GIT in rats following force feeding of large doses, up to 4500 mg kg\(^{-1}\) BW (Hama *et al.* 1976),
although at lower doses of approximately 3500 and 350 mg kg\(^{-1}\) BW partial hydrolysis of carnosine to its constituent amino acids occurred within the small intestine of the rat with greater hydrolysis being found at the lower dose (Tamaki et al. 1985).

*In vitro* studies have shown that active trans-membrane carnosine transport mechanisms exist in the GIT of several species including the hamster (Matthews et al. 1974), rat (Nutzenadel and Scriver 1976), rabbit (Ganapathy and Leibach 1983), mouse (Rajendran et al. 1984) and guinea pig (Himuki 1985). Active carnosine transport has also been demonstrated in kidney of rabbits (Ganapathy and Leibach 1982; Ganapathy and Leibach 1983) and rats (Nutzenadel and Scriver 1976). Large doses of carnosine or anserine (approximately 1500 mg kg\(^{-1}\) BW) administered by injection into trout white muscle were rapidly removed from this tissue and transferred predominantly to the blood and kidney, with lesser amounts appearing in red muscle and liver. Following carnosine and anserine injection increases in the histidine and 1-methylhistidine concentrations, respectively, were observed in all tissues (Abe 1991).

No previous studies to investigate carnosine transport or metabolism have been conducted in the horse either *in vivo* or *in vitro*. Furthermore, data on the bioavailability of carnosine from the GIT and its metabolic fate are necessary in order to predict the effectiveness of long-term dietary carnosine supplementation as a method to enhance the endogenous skeletal muscle concentration.

### 6.2 STUDY A: DETERMINATION OF CARNOSINE PHARMACOKINETIC PARAMETERS FOLLOWING INTRA-VENOUS ADMINISTRATION IN THE THOROUGHBRED HORSE.

#### 6.2.1 Objectives

The aims of this study were to measure changes in plasma and urine carnosine concentrations following bolus injection of carnosine in the thoroughbred horse, and subsequently to determine the pharmacokinetic parameters of carnosine clearance from the plasma and to examine aspects
of carnosine metabolism \textit{in vivo} by measuring changes in the plasma and urine concentrations of its metabolites, histidine and $\beta$-alanine.

### 6.2.2 Experimental methodology

\textit{Protocol and sampling procedures}

Six experimental thoroughbred horses (5 geldings, 1 filly) aged from 4 - 9 years were used. All horses were fasted overnight, for a minimum period of 12 h, prior to undertaking the study and received no feed during the course of the experiment. Water was, however, provided \textit{ad libitum}. Furthermore, the horses undertook no exercise on the day of the study. Horses were weighed on the morning of the study to enable doses of carnosine to be administered on a body weight basis. Carnosine dissolved in physiological saline (50 ml) was sterilized and administered by intravenous bolus injection through a Millex\textsuperscript{TM} GS 0.22 $\mu$m sterile Teflon\textsuperscript{TM} filter (Millipore UK Ltd., Watford, UK) and 16 gauge catheter inserted into the right jugular vein. Heparinized blood samples (5 ml) were collected via a 14 gauge catheter inserted into the left jugular vein. Catheterization was performed at least 1 hr prior to administration. Carnosine was administered at a dose of 20 mg kg\textsuperscript{-1} BW (approximately 10 g total dose). Pre-administration blood samples were collected immediately prior to the carnosine injection, and subsequent blood samples were collected at 5, 10, 20, 40, 60, 120, 240, 360 and 480 min. Urine samples were collected from geldings only over a 12 h period and a 20 ml aliquot retained, as described in Chapter 2 (Harris and Snow 1988).

\textit{Analytical methods}

Plasma and urine carnosine and histidine concentrations were determined by the HPLC method described in Chapter 3 (Dunnett and Harris 1992). Plasma and urine $\beta$-alanine concentrations were determined as described in Chapter 2.

\textit{Pharmacokinetic and statistical analysis}

The disposition kinetics of drugs and other substances administered to animals and humans are in general adequately described by a two compartment model, in which the administered dose
initially enters the central compartment comprising the blood and the extra-cellular fluids of high blood-flow tissues, such as the heart, lungs, liver and kidneys. From here distribution to the peripheral compartment occurs, which comprises low blood-flow tissues, such as the muscles and the skin. Elimination subsequently occurs from the central compartment. Drug movement between these compartments generally conforms to first-order kinetics, hence the rate of drug removal from a given compartment is proportional to the drug concentration within that compartment, and the concentration decreases exponentially with time. Following bolus intravenous injection the change in plasma concentration ($C_p$) with time ($t$) can be described mathematically by the general biexponential equation:

$$C_p = Ae^{-\alpha t} + Be^{-\beta t}$$

where $A$ and $B$ are the zero time concentrations for the component distribution and elimination phases, and $\alpha$ and $\beta$ are the overall distribution and elimination rate constants, respectively. The empirical values for these four constants ($A$, $B$, $\alpha$, $\beta$) can be obtained by curve stripping of a semi-logarithmic plot ($\ln C_p$ vs. $t$) from the experimental data. Estimates of the following common pharmacokinetic parameters can then be calculated from the empirical constants:

$$C_0 = A + B$$  \hspace{1cm} \text{(Plasma concentration at } t = 0)$$

$$V_c = \frac{\text{Dose}}{A+B}$$  \hspace{1cm} \text{(Volume of the central compartment)}$$

$$\text{AUC} = \frac{A}{\alpha} + \frac{b}{\beta}$$  \hspace{1cm} \text{(Area under } C_p \text{ vs. } t \text{ curve)}$$

$$k_{21} = \frac{(A\alpha + B\beta)}{(A+B)}$$  \hspace{1cm} \text{(Micro-rate constant of distribution from peripheral to central compartment)}$$

$$k_{10} = \frac{\alpha\beta}{k_{21}}$$  \hspace{1cm} \text{(Micro-rate constant of elimination)}$$

$$k_{12} = a + \beta - k_{21} - k_{10}$$  \hspace{1cm} \text{(Micro-rate constant of distribution from central to peripheral compartment)}$$

$$V_{d(ss)} = V_c \left(1 + \frac{k_{12}}{k_{21}}\right)$$  \hspace{1cm} \text{(Volume of distribution at steady state)}$$

$$V_{d(area)} = \frac{\text{Dose}}{\text{AUC}} \cdot B$$  \hspace{1cm} \text{(Volume of distribution)}$$

$$\text{CL}_B = k_{10} \cdot V_c$$  \hspace{1cm} \text{(Total body clearance)}$$

$$\text{MRT} = \frac{V_{d(ss)}}{\text{CL}_B}$$  \hspace{1cm} \text{(Mean residence time)}$$
Pre-administration plasma carnosine and histidine concentrations were subtracted from post-administration concentrations prior to the determination of the pharmacokinetic parameters. Mean (± SD) pharmacokinetic parameters were calculated from parameters estimated in individual horses.

6.2.3 Results

The validity of a two compartment model to describe the change in plasma carnosine concentration with respect to time, in the thoroughbred horse following administration by bolus intra-venous injection, was tested on the mean data for all horses. A plot of ln \( C_p \) vs. \( t \) produced a straight line for the terminal (elimination phase), i.e. \( C_p \) decreases exponentially with time. Interpolation of this line to \( t = 0 \) allowed estimates of \( B \) (y-axis intercept) and \( \beta \) (gradient) to be made. Stripping of this line from the original ln \( C_p \) vs. \( t \) curve produced a straight line representing the initial (distribution phase). Interpolation of this line to \( t = 0 \) allowed estimates of \( A \) (y-axis intercept) and \( \alpha \) (gradient) to be made, as shown in Figure 6.1. Superposition of these two linear components resulted in a theoretical model curve which was a close approximation of the actual plasma clearance curve (Figure 6.2).

The mean plasma carnosine concentration vs. time curve following bolus intra-venous carnosine injection in the thoroughbred horse was described mathematically by the biexponential equation:

\[
C_p = 629.5e^{-0.0251t} + 131.1e^{-0.0050t}
\]

The mean (± SD) measured plasma carnosine concentration 5 min after intra-venous injection (671.8 ± 80.1 \( \mu M \)) was very close to the value estimated from the pharmacokinetic data (683.1 \( \mu M \)). The mean (± SD) pharmacokinetic parameters are given in Table 6.1. There was only a
small variation in the plasma carnosine clearance curves between individual horses, as shown in Figure 6.3.

Changes in plasma histidine concentrations with time in the individual horses following intra-venous carnosine administration are shown Figure 6.4. There was a large between-horse variation in plasma histidine concentration. There was no detectable β-alanine found in the plasma of any horse following intra-venous carnosine administration.

Large concentrations of carnosine, up to 17 mM, were detected in urine from individual thoroughbred horses following intra-venous carnosine injection. Urine samples collected from 4 of the horses (all geldings) over the 12 hr period indicated that up to 36% of the administered carnosine dose was excreted unmetabolized in the urine, although the percentage recovered was highly variable between horses, as shown in Figure 6.5. Only low concentrations of histidine were detected in urine from the individual horses following intra-venous carnosine injection, as shown in Figure 6.9.
Figure 6.1 Interpolated distribution and elimination components of the ln plasma carnosine concentration vs. time curve in the thoroughbred horse following intra-venous bolus injection at dose of 20 mg kg$^{-1}$ BW.
8.000
A-
6.000
B-
a
4.000
2.000
0.000

+ MEASURED CONCENTRATION
" ELIMINATION COMPONENT
In C_p = -0.0050t + 4.8762
" DISTRIBUTION COMPONENT
In C_p = -0.0251t + 6.4449

ß = 0.0050 min^{-1}
α = 0.0251 min^{-1}

0 60 120 180 240 300 360 420 480
TIME (min)

MEASURED CONCENTRATION

ELIMINATION COMPONENT
\ln C_p = -0.0050t + 4.8762

DISTRIBUTION COMPONENT
\ln C_p = -0.0251t + 6.4449
Figure 6.2 Comparison of the actual measured and theoretical model plasma carnosine concentration vs. time curves in the thoroughbred horse following intra-venous bolus injection at dose of 20 mg kg$^{-1}$ BW.
PLASMA CARNOSONE, $C_p$ (µM)

TIME (min)

MEASURED CONCENTRATION

PREDICTED CONCENTRATION

\[ C_p = 629.5e^{-0.025t} + 131.1e^{-0.0050t} \]
Figure 6.3  Measured plasma carnosine concentration ($C_p$) vs. time curves for individual horses ($n = 6$) following bolus intra-venous carnosine injection.
Figure 6.4 Changes in plasma histidine concentration in individual horses ($n = 6$) following bolus intra-venous carnosine injection.
Table 6.1 Pharmacokinetic parameters for somatostatin in the thorough bioassay involving a single intra-venous bolus dose at 20 mg kg⁻¹ bw (n = 6).
Table 6.1  Mean pharmacokinetic parameters for carnosine in the thoroughbred horse following a single intra-venous bolus dose at 20 mg kg\(^{-1}\) BW (n = 6).
<table>
<thead>
<tr>
<th></th>
<th>Mean</th>
<th>SD</th>
<th>CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>$C_0 (\mu M)$</td>
<td>688.2</td>
<td>96.4</td>
<td>14.0</td>
</tr>
<tr>
<td>$A (\mu M)$</td>
<td>567.4</td>
<td>125.7</td>
<td>22.2</td>
</tr>
<tr>
<td>$B (\mu M)$</td>
<td>120.8</td>
<td>41.1</td>
<td>34.0</td>
</tr>
<tr>
<td>$\alpha (\text{min}^{-1})$</td>
<td>0.0205</td>
<td>0.0021</td>
<td>10.0</td>
</tr>
<tr>
<td>$\beta (\text{min}^{-1})$</td>
<td>0.0045</td>
<td>0.0010</td>
<td>22.4</td>
</tr>
<tr>
<td>$k_{21} (\text{min}^{-1})$</td>
<td>0.0073</td>
<td>0.0019</td>
<td>26.0</td>
</tr>
<tr>
<td>$k_{10} (\text{min}^{-1})$</td>
<td>0.0128</td>
<td>0.0016</td>
<td>12.9</td>
</tr>
<tr>
<td>$k_{12} (\text{min}^{-1})$</td>
<td>0.0050</td>
<td>0.0008</td>
<td>16.0</td>
</tr>
<tr>
<td>$V_c (\text{l kg}^{-1})$</td>
<td>0.1308</td>
<td>0.0205</td>
<td>15.6</td>
</tr>
<tr>
<td>$V_{dss} (\text{l kg}^{-1})$</td>
<td>0.2235</td>
<td>0.0234</td>
<td>10.5</td>
</tr>
<tr>
<td>$V_{d(area)} (\text{l kg}^{-1})$</td>
<td>0.3819</td>
<td>0.0858</td>
<td>22.5</td>
</tr>
<tr>
<td>$CL (\text{l min}^{-1} \text{kg}^{-1})$</td>
<td>0.0017</td>
<td>0.0001</td>
<td>5.2</td>
</tr>
<tr>
<td>AUC ($\mu\text{mol min}^{-1}$)</td>
<td>53797</td>
<td>2850</td>
<td>5.3</td>
</tr>
<tr>
<td>AUMC ($\mu\text{mol min}^2 \text{l}^{-1}$)</td>
<td>7369167</td>
<td>1426627</td>
<td>19.4</td>
</tr>
<tr>
<td>MRT (min)</td>
<td>136.3</td>
<td>19.6</td>
<td>14.4</td>
</tr>
<tr>
<td>$t_{1/2(\alpha)} (\text{min})$</td>
<td>34.0</td>
<td>3.6</td>
<td>10.7</td>
</tr>
<tr>
<td>$t_{1/2(\beta)} (\text{min})$</td>
<td>161.9</td>
<td>43.3</td>
<td>26.7</td>
</tr>
</tbody>
</table>
Figure 6.5 Cumulative percentage recovery of the administered carnosine dose in the urine of individual thoroughbred horses (n = 4) following a single intra-venous bolus injection at 20 mg kg\(^{-1}\) BW.
6.3 STUDY B: DETERMINATION OF CHANGES IN PLASMA CARNOSINE CONCENTRATION FOLLOWING ORAL ADMINISTRATION BY NASO-GASTRIC INTUBATION IN THE THOROUGHBRED HORSE.

6.3.1 Objectives
The aims of this study were to measure changes in plasma and urine carnosine concentrations following oral administration of carnosine by naso-gastric intubation in the thoroughbred horse. This was done in order to examine aspects of carnosine metabolism in vivo by measuring changes in the plasma and urine concentrations of its metabolites, histidine and β-alanine, and to calculate the bio-availability of carnosine from the GIT. Furthermore, it was intended to assess the potential efficacy of carnosine for use as a supplement by which endogenous muscle carnosine concentration may be increased.

6.3.2 Experimental methodology
Protocol and sampling procedures
Nine experimental thoroughbred horses (6 geldings, 3 fillies) aged from 4 - 9 years were used in two groups of 5. All horses were fasted overnight, for a minimum period of 12 h, prior to undertaking the study and received no feed during the course of the experiment. Water was, however, provided ad libitum. Furthermore, the horses undertook no exercise on the day of the study. Horses were weighed on the morning of the study to enable doses of carnosine to be administered on a body weight basis. Carnosine was dissolved in distilled water (500 ml) and administered by naso-gastric intubation. The container and naso-gastric tube was then flushed through with a further 250 ml of distilled water. Heparinized blood samples (5 ml) were collected via a 14 gauge catheter inserted into the left jugular vein. Catheterization was performed at least 1 h prior to administration.

Carnosine was administered at three different doses. On the first occasion it was administered at 50 mg kg⁻¹ BW (approximately 25 g total dose) to six horses, on the second occasion at 100 mg kg⁻¹ BW (approximately 50 g total dose) to five horses of which 4 were the same as those used in
the first session, and on the final session at 200 mg kg$^{-1}$ BW (approximately 100 g total dose) to six horses. Pre-administration blood samples were collected immediately prior to the carnosine administration, and subsequent blood samples were collected at 30, 60, 90, 120, 180, 240, 360 and 480 min during the first two sessions, and additionally at 15, 45, 75, 105 and 300 min on the final (higher dose) session. Urine samples were collected from geldings only over a 12 h period and a 20 ml aliquot retained, as described in Chapter 2 (Harris and Snow 1988).

**Analytical methods**

Plasma and urine carnosine and histidine concentrations were determined by the HPLC method described in Chapter 3 (Dunnett and Harris 1992). Plasma and urine β-alanine concentrations were determined as described in Chapter 2.

**Pharmacokinetic and statistical analysis**

Pre-administration plasma concentrations were subtracted from all post-administration concentrations prior to calculating pharmacokinetic parameters. The area under the plasma carnosine concentration, Cp vs. time curve (AUC) from 0 - 480 min, following oral carnosine administration, was estimated using the Trapezoidal rule:

$$\text{AUC}_{0-480\text{ min}} = \sum \left[ \frac{1}{2}(C_0 + C_{15})t_{0-15}, \left[ \frac{1}{2}(C_{15} + C_{30}) \right] t_{15-30}, \ldots, \left[ \frac{1}{2}(C_{360} + C_{480}) \right] t_{360-480} \right]$$

The bioavailability (F) of carnosine following oral administration, i.e. the percentage of the total administered dose which is absorbed and which enters the central compartment, was calculated according to:

$$F = 100\left( \frac{\text{AUC}_{\text{oral}} \cdot \text{Dose}_{\text{oral}}}{\text{AUC}_{\text{iv}} \cdot \text{Dose}_{\text{iv}}} \right)$$

where AUC$_{\text{iv}}$ and Dose$_{\text{iv}}$ values were taken from the preceding study. Both AUC and F were estimated for each individual horse using its corresponding AUC$_{\text{iv}}$, as determined in the preceding study, prior to calculating the mean value (± SD).
6.3.3 Results

Oral administration of carnosine by naso-gastric intubation, following overnight fasting, at doses of 50, 100 and 200 mg kg\(^{-1}\) BW resulted in significant increases in plasma carnosine concentrations, with mean (± SD) peak concentrations (C\(_{\text{max}}\)) of 20.6 ± 10.6, 54.7 ± 14.6 and 133.2 ± 40.1 \(\mu\text{M}\), respectively. Peak concentrations occurred between 60 and 120 min after administration. Following administration at the highest dose, the plasma carnosine concentration at 480 min was still higher than the pre-administration value. Changes in plasma carnosine concentrations in individual horses following oral carnosine administration at doses of 50, 100 and 200 mg kg\(^{-1}\) BW are shown in Figure 6.6. Mean peak plasma carnosine concentrations increased proportionally with the increase in dose. A comparison of the changes in the mean (± SD) plasma carnosine concentrations following oral carnosine administration at the three doses are shown in Figure 6.7.

Increases in plasma histidine concentrations were also observed following oral carnosine administration. Peak plasma histidine concentration occurred at a similar time to the peak plasma carnosine concentration. Mean (± SD) plasma carnosine and histidine concentrations following oral administration at a dose of 200 mg kg\(^{-1}\) BW are shown in Figure 6.8. The mean (± SD) plasma C\(_{\text{max}}\), t\(_{\text{max}}\), AUC and F for carnosine and histidine following oral carnosine administration at the 3 doses are given in Table 6.2. There was a decrease in the proportion of the administered carnosine which was metabolized to histidine as the size of the dose was increased. \(\beta\)-alanine was not detected in plasma or urine either before or after carnosine administration at any dose. No significant increases in urinary carnosine or histidine excretion were observed over 12 h following oral carnosine administration. The changes in urine plasma carnosine and histidine concentrations are shown in Figure 6.9.
Figure 6.6 Changes in plasma carnosine concentrations in individual horses following oral carnosine administration at doses of 50, 100 and 200 mg kg\(^{-1}\) BW.

\((n = 5\) at 100 mg kg\(^{-1}\) BW, \(n = 6\) at the other doses)
Figure 6.7: Demonstration of the changes in mean (± SD) plasma carnosine concentrations over time for various doses of carnosine administration at 50, 100 and 200 mg kg⁻¹ BW.
Figure 6.7  Comparison of the changes in mean (± SD) plasma carnosine concentrations with time following oral carnosine administration at 50, 100 and 200 mg kg⁻¹ BW.
Figure 6.8  Comparison of the changes in mean (± SD) plasma carnosine and histidine concentrations following oral carnosine administration at 200 mg kg⁻¹ BW. (n = 6)
Table 6.2  Plasma $C_{\text{max}}$, $t_{\text{max}}$, AUC and bioavailability (F) of carnosine and histidine in the thoroughbred horse following oral carnosine administration at doses of 50, 100 and 200 mg kg$^{-1}$ BW.
<table>
<thead>
<tr>
<th>Carnosine dose</th>
<th>Metabolite</th>
<th>C&lt;sub&gt;max&lt;/sub&gt;(Mean ± SD)</th>
<th>t&lt;sub&gt;max&lt;/sub&gt;</th>
<th>AUC</th>
<th>Metabolism</th>
<th>F</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 mg kg&lt;sup&gt;-1&lt;/sup&gt; BW</td>
<td>Carnosine</td>
<td>20.6 ± 10.3</td>
<td>60</td>
<td>3047 ± 1291</td>
<td>39</td>
<td>2.3 ± 1.0</td>
<td></td>
</tr>
<tr>
<td>50 mg kg&lt;sup&gt;-1&lt;/sup&gt; BW</td>
<td>Histidine (Total)</td>
<td>20.1 ± 9.3</td>
<td>90</td>
<td>4764 ± 3839</td>
<td>61</td>
<td>3.0 ± 1.0</td>
<td></td>
</tr>
<tr>
<td>100 mg kg&lt;sup&gt;-1&lt;/sup&gt; BW</td>
<td>Carnosine</td>
<td>54.7 ± 14.6</td>
<td>90</td>
<td>9374 ± 3381</td>
<td>58</td>
<td>3.5 ± 1.3</td>
<td></td>
</tr>
<tr>
<td>100 mg kg&lt;sup&gt;-1&lt;/sup&gt; BW</td>
<td>Histidine (Total)</td>
<td>29.0 ± 17.1</td>
<td>120</td>
<td>6717 ± 4880</td>
<td>42</td>
<td>4.4 ± 1.4</td>
<td></td>
</tr>
<tr>
<td>200 mg kg&lt;sup&gt;-1&lt;/sup&gt; BW</td>
<td>Carnosine</td>
<td>133.2 ± 40.1</td>
<td>105</td>
<td>23638 ± 7554</td>
<td>60</td>
<td>4.4 ± 1.5</td>
<td></td>
</tr>
<tr>
<td>200 mg kg&lt;sup&gt;-1&lt;/sup&gt; BW</td>
<td>Histidine (Total)</td>
<td>60.5 ± 13.1</td>
<td>105</td>
<td>15464 ± 6350</td>
<td>40</td>
<td>(39102)</td>
<td></td>
</tr>
</tbody>
</table>

μM
Figure 6.9  Comparison of histidine excretion in the urine of the thoroughbred horse
following intra-venous and oral carnosine administration at 20 mg kg\(^{-1}\) BW and
200 mg kg\(^{-1}\) BW, respectively
6.4 STUDY C: COMPARATIVE MEASUREMENTS OF CHANGES IN PLASMA N-α-ACETYLCARNOSINE CONCENTRATIONS IN THE THOROUGHBRED HORSE FOLLOWING BOTH ORAL AND INTRA-VENOUS ADMINISTRATION.

6.4.1 Objectives
The aim of this study was to determine the effect of N-α-acetylation of carnosine on its bioavailability from the GIT of the thoroughbred horse.

Experimental methodology
The experimental thoroughbred horses were used owing to the limited number of horses available for the study. The horses were fasted for 12 h prior to undertaking the study and were housed in individual pens on each experimental day. Water was, however, available ad lib.

Further experimental details are described in Chapter 2. The horses were weighed on the morning of the study and were given a 34-gauge catheter inserted into the right jugular vein. Hyperalized blood samples were taken every 60 min from a 34-gauge catheter inserted into the left jugular vein. Catheters were inserted at least 1 h prior to the N-α-acetylcarnosine injection, and subsequent blood samples were collected at 3, 10, 20, 40, 60, 120, 180, 300, 360 and 480 min. Urine samples were collected immediately prior to the N-α-acetylcarnosine injection and every 30 min over a 2 hr period and a 20 ml sample was stored, as described in Chapter 2. N-α-acetylcarnosine was administered as a dose of 10 mg.kg⁻¹ i.v. (topper).

URINE HISTIDINE (mmol)

PRE 1 2 3 4 5 6 7 8

URINE SAMPLE

- BO IV
- RI IV
- DS IV
- MO IV
- BO ORAL
- RI ORAL
- DS ORAL
6.4 STUDY C: COMPARATIVE MEASUREMENTS OF CHANGES IN PLASMA N-α-ACETYLCARNOSINE CONCENTRATIONS IN THE THOROUGHBRED HORSE FOLLOWING BOTH ORAL AND INTRA-VENOUS ADMINISTRATION.

6.4.1 Objectives
The aim of this study was to determine the effect of N-α-acetylation of carnosine on bioavailability from the GIT of the thoroughbred horse.

6.4.2 Experimental methodology

Protocol and sampling procedures

Only one experimental thoroughbred horse (DS), aged 9 years, was used owing to the limited availability of N-α-acetylcarnosine. The horse was fasted overnight for 12 h prior to undertaking each part of the study and received no feed on each experimental day. Water was, however, provided ad libitum. Furthermore, the horse undertook no exercise on the day of the study. The horse was weighed on the morning of the study to enable doses to be administered on a body weight basis.

During the first session N-α-acetylcarnosine dissolved in physiological saline (50 ml) was sterilized and administered by intra-venous bolus injection through a Millex™ GS 0.22 μm sterile Teflon™ filter (Millipore UK Ltd., Watford, UK) and 16 gauge catheter inserted into the right jugular vein. Heparinized blood samples (5 ml) were collected via a 14 gauge catheter inserted into the left jugular vein. Catheterization was performed at least 1 h prior to administration. Pre-administration blood samples were collected immediately prior to the N-α-acetylcarnosine injection, and subsequent blood samples were collected at 5, 10, 20, 40, 60, 120, 180, 240, 300, 360 and 480 min. Urine samples were collected over a 12 hr period and a 20 ml aliquot retained, as described in Chapter 2 (Harris and Snow 1988). N-α-acetylcarnosine was administered at a dose of 10 mg kg⁻¹ BW (approximately 5 g total dose).
During the second session N-α-acetylcarnosine was dissolved in distilled water (500 ml) and administered by naso-gastric intubation. The container and naso-gastric tube were flushed through with a further 250 ml of distilled water. Heparinized blood samples (5 ml) were collected via a 14 gauge catheter inserted into the left jugular vein. Catheterization was performed at least 1 h prior to administration. Pre-administration blood samples were collected immediately prior to the N-α-acetylcarnosine administration, and subsequent blood samples were collected at 30, 60, 90, 120, 180, 240, 300, 360 and 480 min. Urine samples were collected over a 12 h period and a 20 ml aliquot retained, as described in Chapter 2 (Harris and Snow 1988). N-α-acetylcarnosine was administered at a dose of 50 mg kg⁻¹ BW (approximately 30 g total dose).

**Analytical methods**

Plasma N-α-acetylcarnosine concentration was determined by the HPLC method described in Chapter 3. Plasma and urine carnosine and histidine concentrations were determined by the HPLC method described in Chapter 3 (Dunnett and Harris 1992). Plasma and urine β-alanine concentrations were determined, as described in Chapter 2.

**Pharmacokinetic analysis**

The determination of the mathematical equation describing the plasma N-α-acetylcarnosine concentration vs. time curve and the subsequent estimation of the conventional pharmacokinetic parameters were performed as described in Study A.

**6.4.3 Results**

Intra-venous bolus injection of N-α-acetylcarnosine produced a rapid increase in plasma N-α-acetylcarnosine to 296 µM 5 min after administration. This was followed by a rapid decline in the plasma N-α-acetylcarnosine concentration which reached the pre-administration value (1.9 µM) within 360 min. There was subsequently, only a very small increase in plasma carnosine concentration from a pre-administration value of 13.8 µM to a peak value of 20.4 µM at 20 min, but no increase in plasma histidine concentration. No β-alanine was detected in the plasma following the injection of N-α-acetylcarnosine. The clearance of N-α-acetylcarnosine from
equine plasma following bolus intra-venous injection was described mathematically by the biexponential equation:

\[ C_p = 165.4e^{-0.0426t} + 170.5e^{-0.0092t} \]

Pharmacokinetic parameters associated with a single bolus intra-venous injection of N-\(\alpha\)-acetylcarnosine in the thoroughbred horse are given in Table 6.3.

Following oral administration of N-\(\alpha\)-acetylcarnosine by naso-gastric intubation, no increase in plasma N-\(\alpha\)-acetylcarnosine was observed, nor were there any increases in either plasma carnosine, histidine or \(\beta\)-alanine concentrations over the subsequent 480 min. The urinary excretion of N-\(\alpha\)-acetylcarnosine was not determined.
Table 6.3  Pharmacokinetic parameters for N-α-acetylcarnosine following a single intra-venous bolus injection at a dose 20 mg kg\(^{-1}\) BW.
<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$C_0$ ($\mu M$)</td>
<td>335.9</td>
</tr>
<tr>
<td>$A$ ($\mu M$)</td>
<td>165.4</td>
</tr>
<tr>
<td>$B$ ($\mu M$)</td>
<td>170.5</td>
</tr>
<tr>
<td>$\alpha$ (min$^{-1}$)</td>
<td>0.0426</td>
</tr>
<tr>
<td>$\beta$ (min$^{-1}$)</td>
<td>0.0092</td>
</tr>
<tr>
<td>$k_{21}$ (min$^{-1}$)</td>
<td>0.0261</td>
</tr>
<tr>
<td>$k_{10}$ (min$^{-1}$)</td>
<td>0.0149</td>
</tr>
<tr>
<td>$k_{12}$ (min$^{-1}$)</td>
<td>0.0107</td>
</tr>
<tr>
<td>$V_C$ (l kg$^{-1}$)</td>
<td>0.2220</td>
</tr>
<tr>
<td>$V_{d(\text{ss})}$ (l kg$^{-1}$)</td>
<td>0.3127</td>
</tr>
<tr>
<td>$V_{d(\text{area})}$ (l kg$^{-1}$)</td>
<td>0.3617</td>
</tr>
<tr>
<td>$\text{CL}$ (l min$^{-1}$ kg$^{-1}$)</td>
<td>0.0033</td>
</tr>
<tr>
<td>AUC ($\mu$mol min$^{-1}$)</td>
<td>22485</td>
</tr>
<tr>
<td>AUMC ($\mu$mol min$^2$ l$^{-1}$)</td>
<td>2119844</td>
</tr>
<tr>
<td>MRT (min)</td>
<td>94.3</td>
</tr>
<tr>
<td>$t_{1/2(\alpha)}$ (min)</td>
<td>16.3</td>
</tr>
<tr>
<td>$t_{1/2(\beta)}$ (min)</td>
<td>75.6</td>
</tr>
</tbody>
</table>
6.5 DISCUSSION

No previous studies of carnosine transport and metabolism have been conducted following carnosine administration by intra-venous injection in any species. Following bolus intra-venous injection the change in plasma carnosine concentration \( C_p \) with time \( t \) in the thoroughbred horse was described adequately by the general biexponential equation:

\[
C_p = Ae^{-\alpha t} + Be^{-\beta t}
\]

where \( A \) and \( B \) are the zero time concentrations for the component distribution and elimination phases, and \( \alpha \) and \( \beta \) are the distribution and elimination rate constants, respectively. Therefore, data describing the change in plasma concentration following intra-venous bolus injection conformed to a two compartment model.

The mean \( V_{d(ss)} \) value of 0.2235 l kg\(^{-1}\) for carnosine determined in Study A is equivalent to a total volume of approximately 112 l in a typical 500 kg horse, in contrast to an approximate total blood volume of 45 l. If an administered substance is extensively bound within the tissues then the \( V_{d(ss)} \) will be greater than the volume of the total body water which in man is approximately 42 l or 0.6 l kg\(^{-1}\) (Hladky 1990). Assuming a similar volume of total body, water expressed per kg body weight in the horse then a \( V_{d(ss)} \) of approximately 0.2 l kg\(^{-1}\) suggests that a significant amount of the administered carnosine is distributed to the extra-cellular fluid, but that little if any is bound within the tissues.

The fate of carnosine following intra-venous administration therefore appears to involve both direct excretion of the unmetabolized compound in the urine, which accounts for a mean of 11.76 ± 4.48 mmol or 24.4 ± 10.0% of the total administered dose, and catabolism to histidine catalysed by carnosinase and non-specific dipeptidase. There was however, a large between-horse variability in the amount of carnosine excreted in the urine. The proportion of the administered carnosine dose which is catabolized to histidine is difficult to quantify accurately in the absence of pharmacokinetic data describing both the distribution of histidine to the tissues and the elimination of histidine from the central compartment (including the plasma) following
intra-venous administration. If it is assumed that the overall distribution and elimination rate constants, α and β, for histidine are very similar to those of carnosine, then for a given dose the AUC for histidine and carnosine would be the same. Significant increases in plasma histidine concentration following the intra-venous administration of carnosine at a dose of 20 mg kg⁻¹ BW were seen, although there was considerable between-horse variation. Using the trapezoidal rule it was estimated that the mean AUC₀⁻₄₈₀ min for histidine was 17950 μmol min ml⁻¹ which would be equivalent to approximately 14.7 mmol, or 33% of the total carnosine dose administered. Over the same time period the mean total amount of histidine excreted in the urine was negligible, although it is possible that some of the histidine may have been deaminated via histidase and excreted in the urine as urocanic acid, or metabolized by other pathways (see Figures 1.3 and 1.4). In total urinary carnosine excretion and measured plasma histidine up to 480 min accounted for approximately 57% of the administered dose. The remaining portion of the administered dose was probably accounted for partly by elevated plasma histidine concentration beyond 480 min and histidine uptake by tissues. This data when considered together with the moderate value for the apparent Vₐ(σσ) suggests therefore, that there is minimal uptake, either by passive diffusion or active transport, of carnosine by low blood-flow equine tissues, such as skeletal muscle, in vivo. Nutzenadel et al. (1984) reported that there was no active uptake of carnosine in rabbit skeletal muscle in vitro, although the opposite was found in kidney. The rate of in vivo metabolism of carnosine to histidine in the thoroughbred horse following intra-venous administration was also highly variable between horses, which suggests a large between-horse variability in the activities of tissue carnosinase and non-specific dipeptidase, as described earlier in Chapter 4.

Although the bioavailability of carnosine following oral administration increased proportionally with increasing dose it was still very low, with only 4.5% of the total administered dose being absorbed at the highest dosage. The low bioavailability is probably due to the zwitterionic nature of carnosine, and hence an inability to penetrate lipid membranes. The fact that some increase in plasma carnosine occurs may be evidence for the existence of a specific, but low capacity, carnosine transporter in the equine GIT, as reported in other mammalian species, such as the
hamster (Matthews et al. 1974), rat (Nutzenadel and Scriver 1976), rabbit (Ganapathy and Leibach 1983), mouse (Rajendran et al. 1984) and guinea pig (Himuki 1985). This would however, seem to be unlikely given the absence of carnosine from the normal equine diet. More probably, some carnosine may be taken up via another amino acid or peptide transporter.

A portion of the carnosine dose is catabolized and appears in the plasma as histidine. At the highest dose level, where blood sampling was more frequent, the peak plasma carnosine and histidine concentrations occurred at the same time. This suggests that there is an initial phase of carnosine degradation in the GIT. Although a first pass effect through the liver cannot be excluded, data in Chapter 4 indicates a much higher carnosinase and non-specific dipeptidase activity in the GIT in comparison with the liver. From a comparison of the AUC for plasma carnosine and histidine it is probable that histidine accounts for a further 2 - 3% of the total dose absorbed at the highest dosage. The ratio of histidine to carnosine in the plasma decreases as the size of the dose increases, suggesting saturation of carnosinase and non-specific dipeptidase. This is consistent with the results from oral carnosine administration in the rat (Tamaki et al. 1985). Negligible excretion of carnosine and histidine was observed following oral carnosine administration in the horse, unlike that seen in humans (Gardner et al. 1991). This probably reflects the small quantities which enter the systemic circulation and the longer interval over which this takes place, in contrast to intra-venous administration.

The absence of N-α-acetylcarnosine in plasma following its oral administration suggests that the free terminal amine moiety of carnosine is essential for its uptake from the GIT, although carnosine uptake is low normally.

Increased plasma activities of cytosolic muscle enzymes, such as AST and CK, are associated with changes in skeletal muscle cell membrane permeability, or cell damage (Janssen et al. 1989). This can occur as a result of intra-muscular injection in humans (Metzler et al. 1970) and dogs (Aktas et al. 1995), exercise in humans (Noakes 1987; Janssen et al. 1989) and horses (Anderson 1975; Rose et al. 1983) and ERS in horses (Cardinet et al. 1963; Harris 1989). The
increase in plasma AST and CK activity has been used to estimate the mass of muscle damaged during trauma (Lefebvre et al. 1994) or following exercise in humans (Appel and Rhodes 1988; Janssen et al. 1989) and horses (Volfinger et al. 1994). However, AST and CK are not specific to skeletal muscle. Significant amounts of AST are present in the liver and heart, and CK in the heart and brain. Other factors cause an increase in plasma CK activity, such as cardiac muscle damage associated with rhabdomyolysis (Fujii et al. 1983), myocardial infarction (Klein et al. 1973), and incorrect venepuncture technique (Fayolle et al. 1992) can lead to the erroneous diagnosis of skeletal muscle damage, or to an over-estimation of the mass of muscle damaged. Furthermore, examination of skeletal muscle by electron microscopy indicates less extensive damage than is suggested by parallel measurement of plasma enzyme activity (van-der-Meulen et al. 1991). Carnosine is almost exclusively distributed within the skeletal muscles, with concentrations in other tissues, such as the heart and GIT being 50 to 100-fold lower than that in skeletal muscle. Consequently, a large increase in plasma carnosine concentration would provide a much more specific indicator of skeletal muscle damage than AST or CK.

The controlled intra-venous bolus injection of carnosine in the horse could be used as a simple model to represent the changes in plasma carnosine concentrations following the onset of muscle damage during episodes of ERS, as described in the preceding chapter. The area under the concentration-time curve (AUC) following an intra-venous bolus injection is proportional to the total dose administered. Therefore the AUC calculated from the change in plasma carnosine concentration following release from damaged muscle would be proportional to the total amount released. Hence, the AUC from a known intra-venous dose could be used to calculate the mass of carnosine released into the circulation from damaged skeletal muscle, and hence an estimate of the mass of muscle damaged.
CHAPTER 7

EFFECT OF DIETARY SUPPLEMENTATION WITH L-HISTIDINE AND β-ALANINE ON CARNOSINE CONCENTRATIONS IN TYPE I, IIA AND IIB MUSCLE FIBRES OF THE MIDDLE GLUTEAL OF THE THOROUGHBRED HORSE
7.1 INTRODUCTION

Dietary availability of histidine plays an important role in the regulation of the biosynthesis and accumulation of carnosine in vertebrate skeletal muscle. The effects of both histidine deficient diets and histidine supplemented diets have been investigated in several species. Reduced skeletal muscle carnosine concentrations were found in experimental adult rats (Fuller et al. 1947; Quinn and Fisher 1977), eels (Abe and Ohmama 1987) and salmon (Luckton 1958) maintained on a histidine deficient diet in contrast to controls. A similar reduction in the concentration of carnosine and anserine in the breast and leg muscles of adult roosters maintained on a histidine-free diet for a two week period has also been reported (Leveille et al. 1960). Amend et al. (1979) reported that pectoral and leg muscle carnosine and anserine concentrations in adult cockerels, depleted by 12 weeks maintenance on histidine deficient diets, were subsequently restored to normal levels after 4 weeks on a histidine supplemented diet. Dietary histidine deficiency causes a more rapid decline in muscle carnosine concentration in younger animals. A decline in chick pectoral muscle carnosine concentration to 4% of its initial concentration following 5 weeks of histidine deprivation has been reported (Ousterhout 1960; Ousterhout and Luckton 1960). Histidine deprivation also causes a more rapid decline in skeletal muscle carnosine concentration in younger rats (Barbaro et al. 1978) than in older rats (Barbaro et al. 1977; Tamaki et al. 1977). Furthermore, other studies indicate that histidine is an essential dietary amino acid during the growing phase in other species including the human infant (Snyderman et al. 1963; Strecker 1970), although 24 days of histidine deprivation in young pregnant sows caused no significant reduction in muscle carnosine concentrations (Easter and Baker 1977).

It has been shown by providing increasing levels of histidine in the diet, that increases in the skeletal muscle carnosine concentration occur once the histidine requirements for optimum growth have been fulfilled (Robbins et al. 1977). Rats fed a diet supplemented with histidine at a level of 5% w/w showed a two-fold greater carnosine concentration in the gastrocnemius muscle in contrast to rats on a control diet with a histidine content of 0.76% w/w (Tamaki et al. 1977). In the mature Quarterhorse, supplementation of a basal diet with histidine, at levels of 0.4% w/w
over a period of two weeks in one study and up to 0.56% w/w twice per day in a further study, produced small but statistically non-significant increases in the carnosine concentration of the middle gluteal muscle (Powell et al. 1991; Miller-Graber and Seyers 1993).

In contrast to the extensive investigations of the influence of dietary histidine on skeletal muscle carnosine concentration, the effect of dietary β-alanine supplementation on muscle carnosine content has not been investigated. However, Margolis (1981) demonstrated an increase in olfactory bulb carnosine concentration in adult mice following β-alanine administration. Furthermore, twice-daily intra-peritoneal injections of very large doses of β-alanine (22 mmol kg⁻¹ BW, 2000 mg kg⁻¹ BW) in adult mice over a 5 day period produced a ten-fold increase in skeletal muscle carnosine concentration in contrast to controls (Margolis et al. 1985).

The results of earlier investigations in other species suggest that substrate availability may be a limiting factor to the regulation of carnosine biosynthesis and accumulation within skeletal muscle. Thus increasing the endogenous concentrations of both histidine and β-alanine may result in an increase in muscle carnosine content. Histidine and β-alanine required for carnosine synthesis in the skeletal muscles are probably obtained via active transport from the blood. Earlier results show a normal plasma histidine concentration in the mature horse of approximately 50 - 60 μM (Chapter 5). β-alanine was however, not detected in normal plasma indicating a concentration of less than 3 μM, which was the lower limit of quantification for the analysis (Chapter 5). The low plasma β-alanine concentration may be due to the absence of significant quantities of β-alanine from the normal equine diet and/or inadequate β-amino acid membrane transport mechanisms in the gastro-intestinal tract (GIT). It is therefore possible that a large and sustained increase in plasma β-alanine concentration in conjunction with a moderate increase in plasma histidine concentration in the horse, via dietary supplementation, may have a more significant effect on muscle carnosine biosynthesis than that previously achieved via histidine alone (Powell et al. 1991; Miller-Graber and Seyers 1993). This approach does not appear to have been adopted in earlier supplementation studies.
Increasing the carnosine concentration within the skeletal muscles of the horse will produce an increase in the total intra-cellular physico-chemical buffering capacity of the muscles and thus may attenuate the rate of decline in intra-muscular pH during anaerobic exercise such as sprinting and prolong the time to fatigue.

7.2 EFFECT OF DIETARY SUPPLEMENTATION WITH L-HISTIDINE AND β-ALANINE ON CARNOSINE CONCENTRATIONS IN TYPE I, IIA AND IIB MUSCLE FIBRES OF THE MIDDLE GLUTEAL OF THE THOROUGHBRED HORSE.

7.2.1 Objectives
The aim of the study was to investigate whether chronic supplementation of the normal diet with multiple daily doses of L-histidine and β-alanine would increase the carnosine content of type I, IIA and IIB skeletal muscle fibres.

7.2.2 Experimental methodology
Protocol and sampling procedure
Three experimental thoroughbred horses (DS, JS, GT) (2 fillies and one gelding) aged 4, 5 and 9 years, respectively, underwent one month of dietary conditioning (pre-supplementation period) prior to the commencement of the supplementation period. During the dietary conditioning phase each horse was fed a diet comprising 1 kg of pelleted feed (Spillers racehorse cubes) and 1 kg of soaked sugar beet pulp, three times per day, at 08.30 h, 12.30 h and 16.30 h, respectively. Soaked hay (3 kg dry weight) was also provided twice daily at 09.00 h and 17.00 h. Water was provided ad libitum.

During the supplementation period an identical feeding regime was implemented. However, each hard feed meal was supplemented with L-histidine (free base) and β-alanine. Histidine and β-alanine were administered as dry powders mixed directly into the normal feed. Individual doses of histidine and β-alanine were calculated according to body weight. β-alanine was administered at 100 mg kg⁻¹ BW and histidine at 12.5 mg kg⁻¹ BW. Dietary supplementation was
begun on day 1 of the protocol and discontinued at the end of day 30. From day 31 to day 60 the horses were returned to the non-supplemented diet used during the conditioning phase. Heparinized blood samples (5 ml) were collected on days 1, 6, 12, 18, 24 and 30. On days 1 and 30 blood samples were collected prior to the first feed and at hourly intervals thereafter up to 12 h. On the four intervening sampling days blood was collected prior to the first feed and 2 h after each subsequent feed. On day 0, pre-supplementation muscle samples were collected from the right middle gluteal muscle of each horse using the percutaneous needle biopsy technique described in Chapter 2. Subsequent muscle biopsies were collected immediately after the end of the supplementation period (day 31) and thirty days after returning to the non-supplemented diet (day 60). A diagrammatic representation of the experimental protocol and sampling intervals is shown in Figure 7.1. Clinical monitoring of the horses was performed daily over all three experimental periods. Clinical monitoring comprised a visual examination and measurement of body weight on a daily basis, twice-daily measurement of rectal temperature, and weekly blood sampling for clinical biochemistry and haematology. During the course of the study the horses received no formal training or exercise, although they were allowed one hour of free exercise each day.

**Histochemistry**

Fragments of individual muscle fibres were characterized as either type I, IIA or IIB by histochemical staining for myosin ATPase activity at pH 9.6 following pre-incubation at pH 4.50 as described in Chapter 2.

**Plasma and individual muscle fibre analysis**

Heparinized plasma samples were extracted and analysed for carnosine and histidine, and β-alanine concentrations by the HPLC methods described in Chapter 3 (Dunnett and Harris 1992) and Chapter 2, respectively. Weighed individual muscle fibres were extracted and analysed for carnosine and taurine concentrations by the method described in Chapter 3 (Dunnett and Harris 1995b).
Statistical analysis

Owing to the small number of horses used in this study no between-horse statistical comparisons were made. Differences in carnosine and taurine concentrations within fibre types before and after supplementation were established within horses using 1-way ANOVA. In instances where differences were detected, significance was determined using a multiple comparison test (Fisher's PLSD).
Figure 7.1  Diagrammatic representation of the experimental protocol and sampling intervals.
7.3 Results

All three horses were clinically normal prior to commencing the supplementation. No palatability problems with the histidine and β-alanine supplemented diet were encountered and complete feeds were consumed on every occasion. No adverse physiological or behavioural effects of the supplemented diet were observed in any of the horses either during the thirty days of supplementation or during the subsequent withdrawal period. No significant changes in body weight were recorded and rectal temperatures remained within the normal range. No acute or chronic changes in clinical biochemistry or haematology were observed.

Plasma concentrations

Plasma histidine concentrations in DS, JS and GT prior to supplementation on day 1 of the study were 43.8, 37.1 and 37.9 µM, respectively. The values were within the lower end of the normal range (see Chapter 5). β-alanine was not detected in the plasma of any of the horses prior to the beginning of supplementation. The lower limit of quantitation for β-alanine in plasma by the method used was 3 µM. Changes in plasma histidine and β-alanine concentrations for all horses prior to the first feed of each day and 2 h after each subsequent feed on every sixth day are shown in Figures 7.2 and 7.3, respectively. As the supplementation period progressed there was a trend towards an increase in both plasma histidine and β-alanine concentrations prior to the first feed of each day. Over the 30 day supplementation period large increases in the within-day peak plasma histidine and β-alanine concentrations were also evident. No sustained increase in plasma carnosine concentration over the thirty day supplementation period was evident in any of the horses and both within-day and between-day changes appeared to be random. Comparisons of changes in plasma histidine and β-alanine concentrations prior to the first feed of the day and hourly thereafter between the first and final days of the supplementation period, for the individual horses, are shown in Figures 7.4 - 7.6. Plasma histidine, β-alanine and carnosine concentrations prior to the first feed of the day and within-day maximum values on day 1 and day 30 of the supplementation period, for the individual horses, are given in Table 7.1.
Figure 7.2 Changes in pre-feeding and 2 h post-feeding plasma histidine concentrations for the individual horses.
Figure 7.3 Changes in pre-feeding and 2 h post-feeding plasma β-alanine concentrations for the individual horses.
Figure 7.4 Comparison of within-day changes in plasma histidine and β-alanine concentrations between day 1 and day 30 for horse DS.
(Feeding times are indicated by vertical dotted lines.)
Figure 7.5  Comparison of within-day changes in plasma histidine and β-alanine concentrations between day 1 and day 30 for horse JS.

(Feeding times are indicated by vertical dotted lines.)
HISTIDINE

PLASMA CONCENTRATION (μM)

TIME (hr)

DAY 1

DAY 30

β-ALANINE

PLASMA CONCENTRATION (μM)

TIME (hr)
Figure 7.6  Comparison of within-day changes in plasma histidine and β-alanine concentrations between day 1 and day 30 for horse GT.

(Feeding times are indicated by vertical dotted lines.)
Table 7.1: Values for pre-feeding and within-day maximum plasma histidine, β-alanine and carnosine concentrations on day 1 and day 30 in individual horses.
<table>
<thead>
<tr>
<th>Horse</th>
<th>Value</th>
<th>Plasma histidine ($\mu M$)</th>
<th>Plasma $\beta$-alanine ($\mu M$)</th>
<th>Plasma carnosine ($\mu M$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Day 1</td>
<td>Day 30</td>
<td>Day 1</td>
</tr>
<tr>
<td>DS</td>
<td>Pre-feeding</td>
<td>43.8</td>
<td>96.2</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>Within-day max.</td>
<td>221.2</td>
<td>243.6</td>
<td>64.5</td>
</tr>
<tr>
<td>JS</td>
<td>Pre-feeding</td>
<td>37.1</td>
<td>73.5</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>Within-day max.</td>
<td>258.7</td>
<td>338.6</td>
<td>84.8</td>
</tr>
<tr>
<td>GT</td>
<td>Pre-feeding</td>
<td>37.9</td>
<td>109.8</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>Within-day max.</td>
<td>124.2</td>
<td>300.4</td>
<td>241.0</td>
</tr>
</tbody>
</table>
Individual muscle fibre concentrations

A total of 207 individual muscle fibres (101 pre-supplementation; 106 post-supplementation) from the three horses were dissected, characterized and analysed for carnosine and taurine concentrations. Mean (± SD) carnosine and taurine concentrations in pre- and post-supplementation type I, IIA and IIB muscle fibres from the individual horses are given in Tables 7.2 and 7.3, respectively. Free histidine and ß-alanine were not detected in pre- or post-supplementation individual muscle fibres. The lower limits of quantitation for histidine and ß-alanine in individual muscle fibres by the method used were 1.0 and 0.4 mmol kg⁻¹ DW. Mean carnosine and taurine concentrations in pre-supplementation type I, IIA and IIB individual muscle fibres from the three horses were in good agreement with values reported previously in Chapter 4. Following 30 days of histidine and ß-alanine supplementation the mean carnosine concentration increased in type IIA and IIB fibres in all three horses compared with pre-supplementation values. These increases reached statistical significance in three instances. In type IIA and IIB fibres from horse GT mean carnosine concentrations increased significantly by 31.2 and 43.8 mmol kg⁻¹ DW (p < 0.01 and p < 0.05) or by 49% and 40% above the pre-supplementation contents, respectively. Mean carnosine concentration in type IIB fibres from horse JS also increased significantly by 22.1 mmol kg⁻¹ DW (p < 0.01) or 17% above the pre-supplementation value. Owing to the presence of so few type I fibres it was difficult to make a realistic assessment of the significance of changes in carnosine concentration in type I muscle fibres. Distribution plots of carnosine concentrations in pre- and post-supplementation type I, IIA and IIB fibres for the individual horses are shown in Figures 7.7 - 7.9.

There was no statistically significant difference in the taurine concentrations of type I, IIA and IIB before and after supplementation in any of the horses. However, for type I fibres the previously mentioned proviso applies.
Table 7.2  Mean (± SD) carnosine concentrations in pre- and post-supplementation type I, IIA and IIB fibres from individual horses.
<table>
<thead>
<tr>
<th>Horse</th>
<th>Treatment</th>
<th>Carnosine, mmol kg(^{-1}) DW (number of fibres)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Type I</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Pre</td>
</tr>
<tr>
<td>DS</td>
<td>Pre</td>
<td>32.3 ± 14.5 (3)</td>
</tr>
<tr>
<td></td>
<td>Post</td>
<td>-</td>
</tr>
<tr>
<td>JS</td>
<td>Pre</td>
<td>59.5 ± 3.9 (2)</td>
</tr>
<tr>
<td></td>
<td>Post</td>
<td>55.5    (1)</td>
</tr>
<tr>
<td>GT</td>
<td>Pre</td>
<td>44.8 ± 6.6 (4)</td>
</tr>
<tr>
<td></td>
<td>Post</td>
<td>37.0 ± 9.3 (2)</td>
</tr>
</tbody>
</table>

‡ = significantly different to pre, \( p < 0.05 \)

‡ = significantly different to pre, \( p < 0.01 \)
Table 7.3  Mean (± SD) taurine concentrations in pre- and post-supplementation type I, IIA and IIB fibres from individual horses.
<table>
<thead>
<tr>
<th>Horse</th>
<th>Treatment</th>
<th>Taurine, mmol kg(^{-1}) DW (number of fibres)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Type I</td>
</tr>
<tr>
<td>DS</td>
<td>Pre</td>
<td>23.7 ± 4.2 (3)</td>
</tr>
<tr>
<td></td>
<td>Post</td>
<td>1.8 ± 1.6 (17)</td>
</tr>
<tr>
<td>JS</td>
<td>Pre</td>
<td>63.1 ± 16.1 (2)</td>
</tr>
<tr>
<td></td>
<td>Post</td>
<td>54.0 (1)</td>
</tr>
<tr>
<td>GT</td>
<td>Pre</td>
<td>55.1 ± 52.7 (4)</td>
</tr>
<tr>
<td></td>
<td>Post</td>
<td>23.0 ± 16.7 (2)</td>
</tr>
</tbody>
</table>
Figure 7.7  Distribution plots of carnosine concentrations in pre- and post-supplementation type I, IIA and IIB fibres for horse DS.
Figure 7.8  Distribution plots of carnosine concentrations in pre- and post-supplementation type I, IIA and IIB fibres for horse JS.
PRE-SUPPLEMENTATION

CARNOSINE (mmol kg\(^{-1}\) DW)

POST-SUPPLEMENTATION

CARNOSINE (mmol kg\(^{-1}\) DW)
Figure 7.9  Distribution plots of carnosine concentrations in pre- and post-supplementation type I, IIA and IIB fibres for horse GT.
PRE-SUPPLEMENTATION

NUMBER OF FIBRES

CARNOSINE (mmol kg\(^{-1}\) DW)

POST-SUPPLEMENTATION

NUMBER OF FIBRES

CARNOSINE (mmol kg\(^{-1}\) DW)
7.4 DISCUSSION

The horse has evolved as a herbivore. As such, carnosine is absent from its natural diet and must therefore be obtained via endogenous biosynthesis from its constituent amino acids, histidine and β-alanine. Histidine is readily available in the equine diet as it is a common component of many plant proteins. β-alanine is however, a non-protein amino acid which has a very low abundance in plant tissues. In the horse, as evident in other species, β-alanine may be predominantly synthesised in the liver, as a final metabolite in the biosynthesis of uracil and thymine (Fink et al. 1953; Fink et al. 1956). Skeletal muscle lacks the necessary mechanisms for β-alanine synthesis (Matthews and Traut 1987). The normal skeletal muscle concentration of histidine in the horse is between 0.25 ± 0.05 mmol kg⁻¹ DW (Chapter 4) and 0.85 ± 0.05 mmol kg⁻¹ DW (Miller-Graber et al. 1990), or approximately 80 - 280 μmol l⁻¹ intra-cellular water (ICW). Carnosine synthetase is reported to have an apparent $K_M$ value for histidine of 16.8 μM (Horinishi et al. 1978) and therefore, normal muscle histidine concentrations are sufficient to saturate the enzyme. However, the normal physiological concentration of free β-alanine in equine skeletal muscle is 0.58 ± 0.17 mmol kg⁻¹ DW or approximately 190 μmol l⁻¹ ICW (Miller-Graber et al. 1990). This concentration is much lower than the $K_M$ value of 1.0 - 2.3 mM β-alanine previously reported for carnosine synthetase (Kish et al. 1978; Ng and Marshall 1978; Skaper et al. 1973). It is therefore possible that β-alanine availability may be a limiting factor in the regulation of carnosine biosynthesis and skeletal muscle carnosine concentration.

Preliminary single dose oral administrations of histidine and β-alanine indicated that the bioavailabilities were approximately 100% and 25%, respectively. This information was used in conjunction with data on the normal endogenous plasma concentration of histidine and β-alanine to calculate the doses which were used for the supplementation. The resulting doses of 12.5 and 100 mg kg⁻¹ BW were intended to produce approximately equal plasma concentrations of these two amino acids during supplementation. Added to the feed the doses of histidine and β-alanine represented 0.31% and 2.44% of the total diet on a weight per weight basis, respectively. This histidine dose was lower than that employed previously in the horse, 0.40% and up to 0.56%, (Powell et al. 1991; Miller-Graber and Seyers 1993), and much lower than those employed in
other species, and the β-alanine dose was twenty-fold lower that employed previously in mice (Margolis et al. 1985).

Plasma histidine concentrations on day 1 of the supplementation period were similar between horses DS and JS. The response in horse GT was somewhat lower. In contrast, horse GT displayed the greatest increase in plasma β-alanine concentration during supplementation on day 1 than the other two horses. During the course of the 30 day supplementation period there was a progressive increase in both pre- and post-feeding histidine concentrations in all three horses and a convergence in the values between horses. By day 30 plasma histidine concentrations before and after feeding in horse GT were similar to the other horses. Post-feeding peak plasma histidine concentrations increased by up to 140%. Miller-Graber and Seyers (1993) reported an 80% increase in mean plasma histidine concentration in Quarterhorses during dietary histidine supplementation. A similar change in plasma histidine concentration has also been described in pigs during histidine supplementation (Izquierdo et al. 1988). Plasma β-alanine concentrations showed much greater changes over the supplementation period. By day 30 β-alanine was detectable in pre-feeding plasma and post-feeding peak plasma concentrations had increased by 300 - 400%. These changes in plasma histidine and β-alanine concentrations throughout the supplementation period indicate an adaptive response to the prolonged administration of these amino acids which is more pronounced for β-alanine than for histidine. Differences in the magnitude of the adaptive responses for histidine and β-alanine probably arose as a consequence of the existence of different membrane transport mechanisms for α-amino acids and β-amino acids. Distinct highly specific but low capacity transport mechanisms for the β-amino acids, β-alanine, taurine and γ-aminobutyric acid, have been demonstrated in the GIT of several species (Munck and Munck 1992; Munck and Munck 1994; Navab et al. 1984) and in chick pectoral muscle cells in primary culture (Bakardjiev and Bauer 1994).

Increases in the carnosine concentrations in type I, IIA and IIB fibres as a result of histidine and β-alanine supplementation differed markedly between horses and on visual inspection of the data appeared to be correlated to adaptive increases in plasma β-alanine concentration over the 30 day
supplementation period rather than to changes in plasma histidine concentration. This is consistent with the results of Powell et al. (1991) and Miller-Graber and Seyers (1993) where despite larger histidine doses no significant increase in middle gluteal muscle carnosine concentration was detected during histidine supplementation alone. The greatest increase, which was evident in both type IIA and IIB fibres, was found in horse GT. This correlates with the greater adaptive response to β-alanine supplementation found in this horse as shown by changes in plasma β-alanine concentration. Horse JS also showed a significant increase in the carnosine concentration in type IIB fibres which correlates with the lesser though sustained increase in plasma β-alanine concentration. No significant change in the carnosine concentrations in type IIA and IIB fibres in horse DS is supported by the fact that a significant increase in plasma β-alanine concentration was only recorded on day 30, which suggests a slower adaptation to β-alanine supplementation.

The increase in the carnosine content of equine skeletal muscle, resulting from increased biosynthesis during dietary supplementation would therefore result in a corresponding increase in the total intra-cellular physico-chemical buffering capacity of the muscle ($\beta_{\text{m_{total}}}$). An increase in $\beta_{\text{m_{total}}}$ arising from an increase in the muscle carnosine concentration ($\beta_{\text{m_{carnosine}}}$) can be determined by using the Henderson-Hasselbach equation to calculate the increase in the protonated form of carnosine ($pK_a 6.83$) over the pH range 7.1 - 6.5, which represents the typical change in intra-cellular pH during high-intensity (fatiguing) exercise:

$$\text{pH} = pK_a + \log_{10} \left( \frac{[\text{Salt}]}{[\text{Acid}]} \right)$$

Hence, for a muscle carnosine concentration of 100 mmol kg$^{-1}$ DW the quantities of carnosine in the protonated form at pH 7.1 and 6.5, respectively are:

- pH 7.1 $[\text{Acid}] = \frac{100}{1 + \text{antilog}_{10}(7.1 - 6.83)}$
  
  $= 34.9 \text{ mmol kg}^{-1} \text{ DW}$

- pH 6.5 $[\text{Acid}] = \frac{100}{1 + \text{antilog}_{10}(6.5 - 6.83)}$
  
  $= 68.1 \text{ mmol kg}^{-1} \text{ DW}$
\[ \Delta [\text{Acid}] \text{ pH 7.1 - 6.5} = 33.2 \text{ mmol H}^+ \text{ kg}^{-1} \text{ DW} \]

The increase in the protonated form of carnosine represents the amount of H\(^+\) ions buffered by an intra-muscular carnosine concentration of 100 mmol kg\(^{-1}\) DW. For example, in horse GT the mean carnosine concentrations in the type IIB fibres before and after supplementation were 108 and 152 mmol kg\(^{-1}\) DW, respectively.

For the pre-supplementation type IIB fibre carnosine concentration of 108 mmol kg\(^{-1}\) DW:

\[ \Delta [\text{Acid}] = \frac{108}{1 + \text{antilog}(6.5 - 6.83)} - \frac{108}{1 + \text{antilog}(7.1 - 6.83)} \]

\[ \beta_{mcarnosine} = 35.9 \text{ mmol H}^+ \text{ kg}^{-1} \text{ DW} \]

For the post-supplementation type IIB fibre carnosine concentration of 152 mmol kg\(^{-1}\) DW:

\[ \Delta [\text{Acid}] = \frac{152}{1 + \text{antilog}(6.5 - 6.83)} - \frac{152}{1 + \text{antilog}(7.1 - 6.83)} \]

\[ \beta_{mcarnosine} = 50.5 \text{ mmol H}^+ \text{ kg}^{-1} \text{ DW} \]

The increase in the mean carnosine concentration in type IIB fibres as a result of supplementation would produce an increase in \(\beta_{mcarnosine}\) of 14.6 mmol H\(^+\) kg\(^{-1}\) DW or 41%.

The same calculation performed using the carnosine concentration in type IIA fibres from horse GT before and after supplementation gives an increase in \(\beta_{mcarnosine}\) of 10.4 mmol H\(^+\) kg\(^{-1}\) DW, or 49% as a result of supplementation.

Titremetric measurements previously used have estimated the total intra-cellular physico-chemical buffering capacity of skeletal muscle (\(\beta_{m\text{total}}\)) in the thoroughbred horse to be 117 ± 8.5 mmol H\(^+\) kg\(^{-1}\) DW over the pH range 7.1 - 6.5 (Harris et al. 1990). Subtraction of the buffering capacity directly attributable to carnosine (\(\beta_{mcarnosine}\)), as calculated from the carnosine concentration using the Henderson-Hasselbach equation, indicated the residual buffering capacity from the effects of protein and phosphates (\(\beta_{m\text{residual}}\)) to be 81.8 ± 9.7 mmol.
H+ kg⁻¹ DW (Harris et al. 1990). The use of multiple linear regression analysis enabled estimates to be made of the residual buffering capacity to be made in type I, IIA and IIB fibres. $\beta m_{\text{residual}}$ was shown to be relatively constant in the three fibre types with a value between 68 and 72 mmol H+ kg⁻¹ DW (Sewell et al. 1990). Assuming a mean value of 70 mmol H+ kg⁻¹ DW for residual buffering capacity in type IIB fibres it can be calculated from the example of the horse (GT) above that $\beta m_{\text{total}}$ in type IIB fibres as a result of supplementation would have increased by 14%. The same approach indicates that $\beta m_{\text{total}}$ in type IIA fibres from this horse as a result of supplementation would have increased by 11%. Estimated values of $\beta m_{\text{total}}$ and $\beta m_{\text{carnosine}}$ before and after supplementation and the increase in $\beta m_{\text{total}}$ (%) in type I, IIA and IIB fibres for the individual horses are given in Table 7.4.

During high-intensity exercise, such as that encountered during Thoroughbred flat-racing, a high rate of ATP production is maintained through anaerobic glycolysis. However, this incurs a rapid production and accumulation of lactic acid within the skeletal muscles. Dissociation of the lactic acid into lactate ions and H⁺ ions results in a progressive increase in H⁺ ion concentration and subsequent fall in intra-cellular pH. In turn, this reduction in intra-cellular pH can impair the integrity of the various mechanisms involved in muscle contraction, such as ATP resynthesis, which may result in localized muscle fatigue. An increase in the intra-cellular physico-chemical buffering capacity of equine skeletal muscle, through an increase in skeletal muscle carnosine concentration, should attenuate the rate of decline in intra-cellular pH during high-intensity exercise thus enabling the maximal speed attained to be sustained for a longer period prior to the onset of fatigue.
Table 7.4 Estimates of $\beta_{m_{\text{total}}}$ and $\beta_{m_{\text{carnosine}}}$ before and after supplementation and the increase in $\beta_{m_{\text{total}}}$ (%) in type I, IIA and IIB fibres for the individual horses.
<table>
<thead>
<tr>
<th>Fibre type</th>
<th>Buffering capacity</th>
<th>DS</th>
<th>JS</th>
<th>GT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Pre</td>
<td>Post</td>
<td>Pre</td>
</tr>
<tr>
<td>Type I</td>
<td>$B_m$&lt;sub&gt;carnosine&lt;/sub&gt;</td>
<td>10.7</td>
<td>-</td>
<td>19.8</td>
</tr>
<tr>
<td></td>
<td>$B_m$&lt;sub&gt;total&lt;/sub&gt;</td>
<td>80.7</td>
<td>-</td>
<td>89.8</td>
</tr>
<tr>
<td></td>
<td>$\Delta B_m$&lt;sub&gt;total&lt;/sub&gt; (%)</td>
<td>-1.6</td>
<td>-</td>
<td>-1.6</td>
</tr>
<tr>
<td>Type IIA</td>
<td>$B_m$&lt;sub&gt;carnosine&lt;/sub&gt;</td>
<td>23.9</td>
<td>25.3</td>
<td>34.1</td>
</tr>
<tr>
<td></td>
<td>$B_m$&lt;sub&gt;total&lt;/sub&gt;</td>
<td>93.9</td>
<td>95.3</td>
<td>104.1</td>
</tr>
<tr>
<td></td>
<td>$\Delta B_m$&lt;sub&gt;total&lt;/sub&gt; (%)</td>
<td>1.5</td>
<td>1.5</td>
<td>3.0</td>
</tr>
<tr>
<td>Type IIB</td>
<td>$B_m$&lt;sub&gt;carnosine&lt;/sub&gt;</td>
<td>37.1</td>
<td>39.1</td>
<td>43.6</td>
</tr>
<tr>
<td></td>
<td>$B_m$&lt;sub&gt;total&lt;/sub&gt;</td>
<td>107.1</td>
<td>109.1</td>
<td>113.6</td>
</tr>
<tr>
<td></td>
<td>$\Delta B_m$&lt;sub&gt;total&lt;/sub&gt; (%)</td>
<td>1.9</td>
<td>1.9</td>
<td>6.4</td>
</tr>
</tbody>
</table>
CHAPTER 8

GENERAL DISCUSSION
The imidazole dipeptide carnosine (ß-alanyl-L-histidine) and its methylated derivatives anserine (ß-alanyl-L-1-methylhistidine) and balenine (ß-alanyl-L-3-methylhistidine), are found predominantly in vertebrates and occur almost exclusively in the skeletal muscles (Crush 1970; Suyama et al. 1970; Carnegie et al. 1983; Plowman and Close 1988). Although they can all be found in varying proportions in a given species carnosine is usually dominant in terrestrial mammals, anserine in birds and balenine in aquatic mammals and reptiles. The carnosine concentration in the skeletal muscle of the horse is the highest found in any terrestrial mammal. In the eighty or so other species of animals studied only a very few such as the Tuna, sea snakes and some deep-diving whales have greater skeletal muscle imidazole dipeptide contents, predominantly balenine (Crush 1970; Suyama et al. 1970). The typical carnosine concentration in equine skeletal muscle is generally in excess of 100 mmol kg⁻¹ DW, a concentration which easily exceeds that exhibited by other small molecules including phosphocreatine, creatine, ATP and taurine. This probably makes carnosine the most abundant low molecular mass compound present in mammalian skeletal muscle. The occurrence of such high intra-muscular carnosine concentrations in the horse is inextricably linked to its evolutionary adaptation to high speed running wherein muscle contraction is reliant upon anaerobic glycolysis to provide a rapid turnover of ATP. As a consequence of this energy producing pathway, there is a high rate of production and subsequent accumulation of lactic acid which dissociates into lactate and H⁺ ions within the working muscles. Muscle lactate concentrations as high as 200 mmol kg⁻¹ DW have been observed in the horse following racing (Valberg 1987) and experimental exercise studies (Snow et al. 1985). In the absence of an effective mechanism to counter this large accumulation of H⁺ ions (acidosis) the intra-muscular pH would theoretically fall to a value of approximately pH 1.0; a value well below that which is physiologically tolerable. In practice, however, during high-intensity exercise intra-cellular pH can decline from a normal resting level of pH 7.1 to a post-exercise value of pH 6.5 or slightly less (Harris et al. 1987). Even such a relatively high post-exercise pH can impair the contractile process and result in localized muscle fatigue and loss of performance. Therefore the maintenance of intra-muscular pH homeostasis may be as important to athletic performance as the ability to transport oxygen via the cardio-vascular system.
The horse, like many species, has evolved a system for the intra-cellular physico-chemical buffering of H+ ions in skeletal muscle comprising several different components, such as protein histidine residues, inorganic and organic phosphates, and the imidazole dipeptides including carnosine, which act in concert to provide the overall buffering capacity. Carnosine has a $pK_a$ value of 6.83 (Tanokura et al. 1976) which enables it to function as an effective H+ ion buffer over the physiological pH range and it has been calculated that carnosine accounts for approximately 30% of the total intra-cellular physico-chemical buffering capacity in equine skeletal muscle (Sewell et al. 1991). The concentrations in skeletal muscle of the aforementioned buffering constituents, protein histidine residues, organic and inorganic phosphate are constrained within narrow physiological limits which allows limited scope for an increase in their muscle content in response to evolutionary demands for greater H+ buffering capacity. Much greater flexibility exists for increases to the imidazole dipeptide concentration in skeletal muscle in order to enhance total buffering. This adaptive response to the need for greater buffering is exemplified by differences between three athletic species, namely horses, dogs and humans. The total physico-chemical buffering capacity is higher in horses than in dogs and lowest in humans (Harris et al. 1990). However, the residual buffering capacity attributable to the histidine residues in the muscle structural proteins actin and myosin, and also phosphates is almost identical in each species. The differences between total buffering and residual buffering between species is attributable to the different concentrations of the imidazole dipeptides (Harris et al. 1990). This potential is visibly exploited in equine skeletal muscle where its high anaerobic capacity and therefore considerable capacity for H+ ion production, is compensated for by an equally large carnosine concentration.

There is considerable experimental evidence which clearly demonstrates the importance of carnosine towards intra-cellular physico-chemical H+ ion buffering capacity. The middle gluteal muscle is a principal locomotory muscle in the horse. The mean carnosine concentration in this muscle (107.8 mmol kg$^{-1}$ DW), as determined in Chapter 4, is in agreement with previously established values (Marlin et al. 1989; Harris et al. 1990), but is significantly higher than concentrations found in 'non-locomotory' skeletal muscles, such as the internal intercostal (63.7
mmol kg\(^{-1}\) DW), and in the diaphragm (38.6 mmol kg\(^{-1}\) DW) both of which can be considered to be 'non-skeletal' muscle. These latter two muscles have no direct role in the mechanics of locomotion, in contrast to the middle gluteal. As such they are likely to have a low glycolytic capacity and low potential for H\(^+\) ion production, and consequently a lower buffering requirement. The same argument can be used to explain the very low carnosine concentrations found in cardiac muscle (2.2 mmol kg\(^{-1}\) DW) and the smooth muscle of the GIT (0.6 - 2.4 mmol kg\(^{-1}\) DW) in the horse. Furthermore, carnosine concentrations in non-muscle tissues including the liver and kidney are approximately twenty-fold lower than the cardiac muscle values.

Comparative measurements of carnosine concentrations and the relative proportions of type I, IIA and IIB fibres present, in muscle samples from different regions of the middle gluteal muscle, indicated that the carnosine content was positively correlated to type II % FSA and negatively correlated with type I % FSA (Sewell et al. 1991). From this it was estimated that the carnosine concentrations in type I, IIA and IIB fibres from horses of various ages and states of training were 21.86 and 116 mmol kg\(^{-1}\) DW, respectively (Sewell et al. 1991). The development of the analytical methodology described in Chapter 3 (Dunnett and Harris 1995b) enabled the direct measurement of carnosine concentrations in individual type I, IIA and IIB muscle fibres from the middle gluteal of the thoroughbred horse, as described in Chapter 4. The measured carnosine concentrations in type I, IIA and IIB fibres were 21, 95 and 104 mmol kg\(^{-1}\) DW, respectively (Dunnett and Harris 1995a). The higher concentration in type II fibres in contrast to type I fibres is consistent with the physiological and metabolic differences between them (Snow 1983) and the recruitment of type II fibres during high-intensity exercise (Lindholm et al. 1974; Snow et al. 1982). This provides further evidence for the importance of carnosine to H\(^+\) buffering in the horse. The lower concentration of carnosine found in type I fibres is, however, relatively high in comparison with other muscle metabolites, and may be explained partly through the necessity to buffer H\(^+\) ions diffusing from adjacent type II fibres, and partly through its proposed antioxidant function which provides protection to membrane lipids against damage from reactive oxygen species, such as OH\(^-\) radicals, HOCl, H\(_2\)O\(_2\) and singlet oxygen, (Boldyrev et al. 1988; Kohen et al. 1988) which can be generated in this highly oxidative tissue.
Higher muscle carnosine contents have been observed in horses selectively bred for higher intensity exercise. American Quarterhorses have mean middle gluteal muscle carnosine concentrations of approximately 159 mmol kg\(^{-1}\) DW, in comparison to values of 125 mmol kg\(^{-1}\) DW in Thoroughbreds and 111 mmol kg\(^{-1}\) DW in Standardbreds (Bump et al. 1990). Although carnosine undoubtedly makes an important contribution to total intra-cellular H\(^+\) ion buffering in skeletal muscle and, therefore in all probability, to the capacity for sprint exercise in the horse, there has been no previous direct experimental evidence demonstrating an increase in muscle carnosine concentration as a consequence of training. However, estimates of the carnosine contents in the different muscle fibre types were generally higher in exclusively highly trained thoroughbred horses in contrast to horses of mixed training (Sewell et al. 1992). Data from Chapter 4 suggests that long-term intensive training with a significant sprint exercise component can result in higher carnosine concentrations in all three fibre types in the middle gluteal muscle of young thoroughbred horses in contrast to non-trained horses of the same age. Although the difference in carnosine concentration in the type I and type IIB fibres between the trained and untrained groups did not reach statistical significance, a large increase was observed in the carnosine content of the type IIA fibres. The mean carnosine concentration in type IIA fibres from the trained horses was 92 mmol kg\(^{-1}\) DW in contrast to a value of only 68 mmol kg\(^{-1}\) DW in the untrained horses. It is possible that this increase in the type IIA fibres rather than the type IIB fibres reflects a greater 'trainability' of the oxidative-glycolytic type IIA fibres in the horse.

Carnosine is present in the plasma of the normal resting thoroughbred horse at concentrations ranging from approximately 5 to 20 \(\mu M\). Furthermore, the plasma concentration in a given horse is relatively constant during a 24 h period and is unaffected by fasting. The constancy of the plasma carnosine concentration may in part be due to the minimal influence of the diet. The horse is an herbivorous animal and as such carnosine is not a normal constituent of the diet. The mean within-horse variance in plasma carnosine concentration (\(\pm 1.6 \mu M\)) over of the second half of a 24 h period during a triple feed regime was almost identical to that found during the same period during a 24 h fast (\(\pm 1.5 \mu M\)). Other factors however, such as age, exercise and muscle damage may exert a variable influence on the plasma carnosine concentration both in the
short and the long term. In foals and yearlings, the concentration of carnosine in the plasma is significantly lower than that found in older horses. In other mammals carnosine is synthesized almost exclusively in skeletal muscle. As the animal matures the muscle carnosine content increases (Christman 1976; Johnson and Hammer 1992), probably due to an increased biosynthetic capacity. Therefore, the age dependent increase in equine plasma carnosine concentration most probably arises from increased 'leakage' of carnosine from skeletal muscle associated with a higher rate of carnosine turnover in mature horses. Furthermore, plasma carnosinase activity present in humans and other primates is completely absent in equine plasma. Short duration high-intensity exercise can also cause a small, up to two-fold, elevation in plasma carnosine concentration, possibly due to minor muscle damage. Still larger increases in circulating levels of carnosine of as much as 700 μM, can occur during episodes of ERS and probably arise from the occurrence of extensive muscle damage or changes in sarcolemmal permeability. This is suggested also by correspondingly large increases in the plasma activities of the muscle enzymes AST and CK.

Carnosine administration to the horse via the intra-venous route produces a large but short term increase in the plasma carnosine concentration. Pharmacokinetic calculations indicate that the elimination half-life of carnosine from the central compartment, which includes the systemic circulation, is approximately 2 h, and that plasma carnosine concentrations have almost returned to normal within 8 h. This data in conjunction with a low value for the apparent volume of distribution at steady-state suggests that carnosine uptake into the peripheral compartment, which includes the skeletal muscle, is minimal. Although urinary excretion of unmetabolized carnosine can account for as much as 36% of the administered dose following intra-venous injection, there is a large between-horse variance in the proportion of the dose eliminated from the body in this manner. The fate of the remainder of the administered carnosine dose can probably be accounted for through carnosinase and non-specific dipeptidase catalysed degradation to histidine, with subsequent re-uptake of the amino acid by various tissues as evinced by a delayed and sustained increase in the plasma histidine concentration observed after intra-venous carnosine administration and the absence of increased urinary histidine excretion.
Orally administered carnosine is absorbed from the GIT of the thoroughbred horse and subsequently produces an increase in the plasma carnosine concentration. A large part of the dose is metabolized to histidine at an initial rate similar to the rate of absorption of carnosine resulting in an increase in the plasma histidine concentration. The coincidence of the peak plasma histidine and carnosine concentrations suggests that the majority of the carnosine hydrolysis occurs within the GIT, although a first-pass effect in the liver may also contribute. The greater carnosinase and non-specific dipeptidase activity observed in the equine GIT compared with the liver supports this. The proportion of the absorbed carnosine dose which appears as histidine in the plasma decreases as the size of the dose is increased suggesting that the enzymatic hydrolysis is becoming saturated. The bioavailability of carnosine via the oral route increases proportionally with the size of the dose. However, the bioavailability is very low with only 4% of the total administered dose entering the systemic circulation at the highest dose used. The low bioavailability of carnosine from the GIT is probably due in part to its extremely lipophobic nature which prevents diffusion across the membranes, and to the absence or low capacity of a specific membrane transporter. Owing to its low bioavailability, oral carnosine supplementation as a means to enhance the endogenous muscle concentration in the thoroughbred horse would be ineffective. Furthermore, the low \( V_d(s) \) of 0.22 l kg\(^{-1}\) and short \( t_{1/2} \) of 162 min suggest that carnosine does not penetrate extensively into the intra-cellular space of low blood-flow tissues, such as skeletal muscle.

Dietary supplementation with the amino acid precursors of carnosine, histidine and \( \beta \)-alanine, has the potential to increase the endogenous muscle carnosine concentration. Studies in other mammalian species have indicated that carnosine biosynthesis occurs within the skeletal muscles. However, the endogenous \( \beta \)-alanine concentration of 0.58 mmol kg\(^{-1}\) DW in equine skeletal muscle (Miller-Graber et al. 1990) is lower than its reported \( K_M \) for carnosine synthetase (Skaper et al. 1973; Kish et al. 1978; Ng and Marshall 1978), and therefore \( \beta \)-alanine availability may be a limiting factor to carnosine biosynthesis \textit{in vivo}. In Chapter 7, sustained dietary supplementation with histidine and \( \beta \)-alanine in the thoroughbred horse produced increases in skeletal muscle carnosine concentrations in all horses. However, there was considerable
variability in the extent of this increase both between horses and between the different fibre types. Statistically significant increases were observed in only two of the three horses. Both of these demonstrated a significant increase in the type IIB fibre carnosine concentration, whereas only one showed a significant increase in the type IIA fibre carnosine concentration. It was not possible to assess the effect of supplementation on the carnosine concentration in type I fibres owing to the small number of these fibres obtained from the biopsy samples.

In general, sustained β-alanine supplementation resulted in a progressive day-to-day increase in plasma β-alanine concentration, although this parameter also showed a large between-horse variability. This progressive response to continued β-alanine supplementation was probably due to an adaptive increase in the gastro-intestinal bioavailability of β-alanine. Changes in plasma histidine concentrations over the same period were much less marked. The extent of the increase in skeletal muscle carnosine content in a given individual appeared to be related to the magnitude of the adaptive increase in plasma β-alanine concentration within that individual. This supports the earlier hypothesis that β-alanine availability is a limiting factor to in vivo carnosine biosynthesis.

The higher carnosine concentrations present in type IIA and IIB fibres in contrast to type I fibres suggests a greater capacity for carnosine biosynthesis in the former. This could be established by an investigation of the distribution of carnosine synthetase activity in the different fibre types. Following the observation of an increase in the carnosine concentration in type IIA fibres with training, it would be of interest to determine whether this was accompanied by an adaptive increase in the activity of carnosine synthetase in these fibres, and whether previously observed increases in muscle buffering capacity during high-intensity training (McCutcheon et al. 1987; Sinha et al. 1991) can be accounted for by increases in the muscle carnosine content. Further work could investigate whether an increased skeletal muscle carnosine concentration, following long-term dietary supplementation with β-alanine and histidine, influences the metabolic responses to maximal exercise, such as a delayed onset of metabolic acidosis, and a reduction in
muscle ATP depletion and IMP accumulation at the single fibre level, and plasma ammonia production.

Several practical applications of the information gained from the current research may be envisaged. The release of carnosine into the systemic circulation can occur under various conditions, such as during training and racing, episodes of ERS, or as a result of trauma, and is indicative of muscle damage. The principal advantage of using equine plasma carnosine concentration as a clinical indicator of skeletal muscle damage is that owing to the extremely low carnosine concentrations in other tissues it is essentially a specific indicator of skeletal muscle damage. This is in contrast to AST and CK. If sufficient serial blood samples were collected the total amount of carnosine released and hence the mass of tissue affected could be estimated from comparison of the AUC data with that calculated from a known intra-venous bolus dose, or more accurately from an intra-muscular bolus injection.

It has been reported that during episodes of ERS there appears to be selective damage to type II skeletal muscle fibres (Valberg et al. 1993). Then on this basis, since the distribution of carnosine is compartmentalized mostly into type IIA and IIB fibres, and taurine into type I fibres, during an episode of ERS a significant increase in plasma carnosine concentration only should be observed. However, this is contradicted by the plasma data in Chapter 4 from two of the horses which were presented with episodes of ERS. A significant increase in plasma taurine concentration was also observed in each case. Because of their heterogeneous distribution increases in the plasma concentration of carnosine or taurine may in theory be used as indicators of selective type II or type I muscle fibre damage, respectively.

The compartmentalization of carnosine into type II fibres and taurine into type I fibres may enable them to be used as physico-chemical markers of the respective fibre types. This could be exploited by nuclear magnetic resonance imaging techniques to map the regional distribution of type I and type II fibres in the skeletal muscles and the relative proportions of each fibre type in the whole tissue. In principal it would be possible to extrapolate the technique to estimate these
relative proportions in all the locomotory muscles, and therefore to make an assessment of the athletic potential of an individual for endurance or sprint type exercise.

Experimentally, sustained oral administration of β-alanine and histidine appears to be an effective method for increasing the endogenous skeletal muscle carnosine concentration in the thoroughbred horse. An increased skeletal muscle carnosine concentration would result in a greater H+ ion buffering capacity which is likely to reduce the rate of metabolic acidosis during high-intensity exercise and subsequently may delay the onset of local muscle fatigue. It is therefore possible that a dietary supplement comprising these two amino acids could be used as an ergogenic aid to enhanced performance during sprint exercise.
9. **BIBLIOGRAPHY**


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