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**CARBOHYDRATE-PROTEIN INGESTION
DURING RECOVERY FROM PROLONGED
EXERCISE IN MAN**

by

James A. Betts

A Doctoral Thesis

Submitted in partial fulfilment of the requirements for the award of
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Abstract

Evidence supports that the ingestion of carbohydrate solutions in the post-exercise period can facilitate the restoration of exercise capacity both through providing the fluid necessary for rehydration and through stimulating carbohydrate storage. The accrual of this evidence has now established many components of the optimal carbohydrate feeding strategy during recovery and further progress has been sought through investigating the potential influence of other macronutrients. Specifically, combined ingestion of protein and carbohydrate may promote a more rapid resynthesis of endogenous glycogen stores than when either nutrient is ingested in isolation. This possibility has led to speculation that ingestion of a mixed carbohydrate-protein solution (CHO-PRO) might restore the capacity for physical exercise more completely during a short-term recovery than when a matched quantity of carbohydrate alone is ingested. However, evidence in support of this hypothesis is not yet available and the present series of studies will therefore attempt to directly examine the effects of CHO-PRO ingestion on recovery of exercise capacity. It was established in the first of these studies that restoration of exercise capacity at 85% $\dot{V}O_2$ max was not enhanced when protein was added to a recovery solution providing quantities of carbohydrate likely to optimise carbohydrate storage when ingested alone (i.e. 1.1 g CHO·kg⁻¹·h⁻¹). The second study then repeated this protocol using a lower quantity of carbohydrate which would not be expected to maximise the rate of carbohydrate storage during recovery (i.e. 0.8 g CHO·kg⁻¹·h⁻¹). Nonetheless, despite confirming that the addition of protein produces a more conducive hormonal milieu for the resynthesis of muscle glycogen, exercise capacity following recovery remained unaffected. In addition, the metabolic data taken from these initial studies indicated that a lower intensity exercise capacity test might provide more valid reflection of changes in carbohydrate status during recovery. It was subsequently demonstrated in Study 3 that the addition of 0.3 g·kg⁻¹·h⁻¹ of either protein or carbohydrate to a solution providing 0.8 g CHO·kg⁻¹·h⁻¹ can improve the recovery of exercise capacity when assessed at 70% $\dot{V}O_2$ max. The precise mechanism underlying the ergogenic benefit of CHO-PRO ingestion remains unclear, although the data gathered in the final study of this thesis negates the possibility that this effect was related to an increased availability of muscle glycogen.

Keywords: EXERCISE CAPACITY, GLYCOGEN, CARBOHYDRATE, AMINO ACIDS.

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CHAPTER 1

Introduction

Many athletes are required to train or compete on multiple occasions within a single day and therefore strive to maximise their recovery in the relatively short interval between each exercise session. Similarly, individuals undertaking less intensive training regimens might also benefit from an effective and rapid recovery as this would be likely to increase the frequency of their participation while also improving adherence to the exercise program. Taking part in prolonged physical exercise of moderate to high intensity places a high demand on the body's limited carbohydrate stores to provide energy for continued muscular contraction (Gollnick *et al.*, 1972; Hermansen *et al.*, 1967). Endogenous sources of carbohydrate, such as muscle glycogen, are therefore progressively depleted during exercise of this intensity and fatigue has been found to occur when the availability of such sources reaches critically low levels (Ahlborg *et al.*, 1967). A logical extension of this finding consequently dictates that the capacity to perform physical exercise for extended periods will be determined by the availability of muscle glycogen at the onset of exercise (Bergstrom *et al.*, 1967).

Given the relationship described above, it is reasonable to suggest that the replenishment of depleted carbohydrate stores will constitute a crucial component of an effective recovery. The resynthesis of muscle glycogen is perhaps most important and will be initiated immediately following exercise as a direct result of prior muscular contraction (Bergstrom and Hultman, 1966; Ivy and Holloszy, 1981). However, a sustained rate of muscle glycogen accumulation over a more extended period will require nutritional intervention to maintain high concentrations of circulating glucose and insulin (Costill *et al.*, 1981; Piehl, 1974). When recovery time is not restricted, it appears that simply ingesting carbohydrate in quantities sufficient to replace losses can completely restore the capacity for physical exercise within 24 h (Nicholas *et al.*, 1997). Conversely, neither muscle glycogen concentrations nor exercise capacity are likely to be entirely restored within 8 h of prior exercise, which has placed great emphasis on identifying the optimal nutritional strategy for a short-term recovery.

In contrast to more prolonged recovery periods, it has become increasingly evident that subtle differences in post-exercise nutrition can have a profound influence on the efficacy of a given supplement during a more short-term recovery. The rate of muscle glycogen resynthesis immediately following exercise is mediated to a large extent by the precise feeding schedule which is employed along with considerations regarding the specific type of carbohydrate that is ingested (Ivy, 2001, 1998; Jentjens and Jeukendrup, 2003). In fact, it is likely that variations in the frequency and form of carbohydrate intake across different studies has been primarily responsible for the difficulty in determining the optimal amount of carbohydrate that should be ingested during a short-term recovery. Nonetheless, when all available evidence is considered, a dose-response relationship does appear to exist between carbohydrate intake and the rate of muscle glycogen resynthesis over the first 6 h following exercise. More important, however, is the finding that muscle glycogen resynthesis with carbohydrate feeding appears to plateau at a rate of ≈ 45 mmol glucosyl units \cdot kg dry mass $^{-1}\cdot$ h $^{-1}$, a rate that can be achieved through ingestion of 1.2 g \cdot kg $^{-1}\cdot$ h $^{-1}$ of carbohydrate at 30 min intervals during a 5 h recovery (van Loon *et al.*, 2000a).

A large amount of research has attempted to elucidate the precise factors which might limit the rate of muscle glycogen storage following exercise. Of particular relevance have been the findings that intravenous infusion of glucose and insulin but not glucose alone can increase the rate of muscle glycogen resynthesis above that attainable through oral carbohydrate intake (Hansen *et al.*, 1999; Reed *et al.*, 1989). These observations have led to the suggestion that insulin concentration may limit the rate of carbohydrate storage when glucose availability is adequate, with the implication that augmentation of the insulinaemic response to a standard carbohydrate solution may further enhance the rate of muscle glycogen storage during recovery (Ivy, 2001). It is therefore noteworthy that the combined ingestion of protein with carbohydrate can stimulate pancreatic insulin secretion to a greater extent than ingestion of either macronutrient alone (Rabinowitz *et al.*, 1966), a finding which introduces the interesting possibility that muscle glycogen storage might also be facilitated when a mixed carbohydrate-protein solution is ingested during recovery. Indeed, early evidence appeared to support this hypothesis as Zawadzki *et al.* (1992) observed a 38% increase in muscle glycogen storage over a 4 h recovery when ≈ 0.3

$\text{g}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$ of protein was added to a solution already providing $\approx 0.8 \text{ g}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$ of carbohydrate. However, subsequent attempts to exceed the proposed maximal rate of muscle glycogen storage through adding a similar amount of protein to $1.2 \text{ g}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$ of carbohydrate have not been successful, despite the synergistic effect of carbohydrate and protein on insulin release (Jentjens *et al.*, 2001; Van Hall *et al.*, 2000b). The authors of these latter studies postulated that insulin stimulated glycogen resynthesis may be maximal following ingestion of $1.2 \text{ g}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$ of carbohydrate alone, which would explain why further increases in insulin release following the addition of protein provided no additional benefit. It might therefore be deduced from this evidence that the presence of protein in a carbohydrate recovery solution may only be of benefit in terms of muscle glycogen storage when added to more moderate quantities of carbohydrate.

In terms of recovery of exercise capacity, however, current understanding is far less developed regarding the effect of nutritional intervention. Even without the additional variable of added protein to consider, it remains uncertain whether exercise capacity will be enhanced in a dose-response relationship with increasing carbohydrate intake. The study of Fallowfield *et al.* (1995) certainly supports that exercise capacity can be restored more effectively when carbohydrate rather than placebo is ingested during a short-term recovery, yet manipulating the carbohydrate dose has not been found to produce any graded effect on exercise capacity concomitant with the expected rate of muscle glycogen resynthesis (Fallowfield and Williams, 1997; Wong and Williams, 2000). Contrary to these findings, a more recent study from Williams *et al.* (2003) examined the influence of simultaneously increasing carbohydrate intake and providing additional protein on the resynthesis of muscle glycogen and restoration of exercise capacity over a 4 h recovery. These authors reported a 128% increase in the rate of muscle glycogen resynthesis during recovery and a 55% increase in exercise capacity subsequent to recovery when carbohydrate ingestion was increased from $0.15\text{-}0.40 \text{ g}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$ while also adding $0.10 \text{ g}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$ of protein (Williams *et al.*, 2003). However, it cannot be established from these data whether the observed benefits of the carbohydrate-protein mixture were primarily the result of the increased carbohydrate availability or the inclusion of protein. It would therefore be of interest to further consider the potential benefits of ingesting carbohydrate-protein mixtures during recovery from exercise.

The studies described in this thesis attempt to resolve some of the outstanding issues regarding the efficacy of ingesting a mixed carbohydrate-protein supplement during recovery from prolonged exercise. This series of investigations begins with an examination of whether the addition of a small amount of amino acids to a high-carbohydrate recovery solution can improve the restoration of treadmill running capacity at 85% $\dot{V}O_2$ max within 4 h of prior exercise (Chapter 4). This recovery duration and exercise intensity are identical to those applied by Williams *et al.* (2003) in their previous examination of recovery solutions. To gain greater insight into the specific influence of carbohydrate availability on recovery of exercise capacity, a second investigation was conducted to repeat the protocol from Study 1 but involving a more moderate quantity of carbohydrate (Chapter 5). Following Chapters 4 and 5 it was necessary to determine whether any influences of the carbohydrate-protein mixture had been a product of the additional protein *per se* or simply a result of the increased energy content. As such, Study 3 was designed to evaluate a carbohydrate-protein solution in comparison both with a solution matched for carbohydrate content and a solution matched for available energy. Notably, the relative intensity of the exercise capacity test was reduced to 70% $\dot{V}O_2$ max for this study (Chapter 6). In the final investigation of this thesis, the carbohydrate matched solutions that were examined during Study 3 were assessed both in terms of the rate of muscle glycogen resynthesis during recovery and the rate of muscle glycogen degradation during exercise subsequent to recovery (Chapter 7). Finally, the General Discussion reviews how the cumulative information gathered across all the above studies might progress scientific understanding regarding the potential role of carbohydrate-protein ingestion during recovery from exercise.

CHAPTER 2

Review of Literature

2.1 Energy Metabolism During Exercise

2.1.1 Metabolism of Muscle Glycogen

At sub-maximal exercise intensities, a linear relationship exists between exercise intensity and oxygen uptake. Once oxygen demand exceeds approximately 65% of an individual's $\dot{V}O_2$ max, the preferred fuel source to support muscle metabolism will be carbohydrate since this substrate exists in an already partially oxidised form and can therefore be metabolised most efficiently (Wasserman and Cherrington, 1991). The majority of endogenous carbohydrate ($\approx 79\%$) is stored within muscle in the form of glycogen, which provides an essential source of fuel during exercise of moderate to high intensity (Sherman, 1995). Basal concentrations of muscle glycogen have been reported in the region of 300-400 mmol glucosyl units·kg dry mass⁻¹ (Bergstrom *et al.*, 1972) and it has been demonstrated that manipulation of both exercise and diet has the capacity to elevate this concentration closer to 900 mmol glucosyl units·kg dry mass⁻¹ (Sherman *et al.*, 1981). Muscle glycogen is comprised of 2 physiologically distinct sub-glycogen pools: proglycogen and macroglycogen (Jentjens and Jeukendrup, 2003). This heterogeneous structure is known to originate from a self-glucosylating protein (glycogenin) which acts as a primer for muscle glycogen synthesis through the actions of branching enzyme and glycogen synthase (Alonso *et al.*, 1995; Pitcher *et al.*, 1988; Smythe and Cohen, 1991). Following the transcription of glycogenin in muscle (Shearer *et al.*, 2005), the initial rapid addition of glucosyl units to glycogenin forms proglycogen, while a more gradual accumulation over an extended period results in the formation of macroglycogen (Adamo *et al.*, 1998).

The importance of muscle glycogen as a substrate during moderate to high intensity exercise is clearly demonstrated by the large quantity of this fuel that is used during such activity (Ahlborg *et al.*, 1967; Bergstrom *et al.*, 1967; Hermansen *et al.*, 1967) and also by the severe exercise intolerance that is present in patients with McArdle's disease (Nielsen *et al.*, 2002), a condition characterised by the inability to

breakdown glycogen due to phosphorylase deficiency (McArdle, 1951). The rate at which muscle glycogen is degraded during exercise is influenced by a number of factors and, as indicated above, the intensity of exercise is a crucial determinant of substrate selection (Brooks and Mercier, 1994; Romijn *et al.*, 1993). In fact, an exponential increase in the rate of muscle glycogen utilisation can be observed in response to a linear increase in exercise intensity (Hawley *et al.*, 1997b; Sherman, 1995; van Loon *et al.*, 2001). In addition to the relative oxygen efficiency of carbohydrate metabolism, it is likely that the particular increase in muscle glycogen oxidation with increasing exercise intensity is also mediated by the catecholamine stimulated suppression of insulin secretion (Hunt and Ivy, 2002), thus limiting the availability of extracellular carbohydrate sources (Tsintzas and Williams, 1998). Furthermore, Gollnick *et al.* (1974) have demonstrated that an increase in exercise intensity also alters the glycogen depletion pattern across different muscle fibres. In this study it was identified that muscle glycogen metabolism during sub-maximal endurance exercise occurs primarily in those muscle fibres with a high oxidative capacity (type I) and only once these fibres are depleted will those fibres with a high glycolytic capacity (type II) be substantially recruited (Gollnick *et al.*, 1974). Indeed, more recent evidence has confirmed that, following a treadmill run to exhaustion at 70% $\dot{V}O_2$ max, a selective depletion of muscle glycogen in the type I rather than type II fibres is apparent (Tsintzas *et al.*, 1996a).

Another important factor which dictates the rate of muscle glycogen utilisation during exercise is the availability of the substrate itself (Gollnick *et al.*, 1972; Sherman *et al.*, 1981; Weltan *et al.*, 1998a). Specifically, low muscle glycogen concentrations result in an increased contribution of lipid towards energy production and therefore reduce the reliance on muscle glycogen as a substrate (Weltan *et al.*, 1998a, 1998b). This finding is clearly important regarding carbohydrate loading strategies but is also inexorably linked to exercise duration since the progressive decline in muscle glycogen content during exercise at 65-70% $\dot{V}O_2$ max has been associated with a progressive decline in the rate of muscle glycogen utilisation (Coyle *et al.*, 1986; Hargreaves *et al.*, 1995; Romijn *et al.*, 1993).

While relative exercise intensity and substrate availability have been suggested as the major determinants of muscle glycogen utilisation rate (Arkininstall *et al.*, 2004), the specific type of exercise being undertaken along with the presence or absence of exogenous carbohydrate provision also exert an influence (Tsintzas and Williams, 1998). The interaction of these 2 variables appears to be important in that ingestion of exogenous carbohydrate may provide performance benefits through different mechanisms depending on exercise mode. When exogenous carbohydrate is provided during prolonged constant pace running, the rate of blood glucose oxidation increases such that total carbohydrate oxidation can be maintained while the rate of muscle glycogen utilisation is reduced (Tsintzas *et al.*, 1995; Tsintzas *et al.*, 1996b). In contrast, ingestion of carbohydrate during prolonged constant pace cycling produces no such muscle glycogen sparing effect, although an improved maintenance of euglycaemia late in exercise has been reported (Coyle *et al.*, 1986; Hargreaves and Briggs, 1988; Mitchell *et al.*, 1989). The mechanism through which an enhanced rate of blood glucose oxidation might delay fatigue is not immediately apparent but recent evidence suggests that this effect is independent of changes in total carbohydrate oxidation and unrelated to the avoidance of hypoglycaemia (Claassen *et al.*, 2005). A further consideration regarding exercise type is whether that exercise is of constant pace (as discussed above) or is more intermittent in nature. When exercise of moderate to high intensity is interspersed with periods of lower intensity work or complete rest, there may be a greater opportunity for exogenous carbohydrate ingestion to postpone muscle glycogen depletion (Tsintzas and Williams, 1998). One reason for the increased effectiveness of ingested carbohydrate during intermittent exercise is that, unlike during higher intensity activity, plasma insulin is able to elevate transiently during the lower intensity periods (Ivy *et al.*, 2003). In addition, it has been suggested that resynthesis of muscle glycogen may occur in non-exercising muscle fibres during these rest periods (Kuipers *et al.*, 1987; McKenzie *et al.*, 2005). Overall, evidence supports a glycogen sparing effect of carbohydrate ingestion both during intermittent cycling (Yaspelkis *et al.*, 1993) and during an intermittent shuttle running protocol (Nicholas *et al.*, 1999).

There are clearly a myriad of factors which govern the rate at which muscle glycogen will be degraded during physical exercise and the relative influence of each will be mediated by other elements that are specific to each individual. Endurance

training is one such factor that has been suggested to modify the metabolic response across varying exercise intensities and durations (Holloszy and Coyle, 1984). As detailed by Brooks and Mercier (1994), substrate selection will be the product of intensity induced increases in carbohydrate oxidation coupled with a training induced promotion of lipid oxidation. It has been clearly demonstrated that endurance training can enhance lipid oxidation and thus reduce carbohydrate oxidation, but only when the exercise intensity remains relatively low (i.e. $<40\% \dot{V}O_2\text{max}$) and when that activity is performed following an overnight fast (Bergman and Brooks, 1999). The significance of the overnight fast appears to be related to the finding that physical training will also increase both the sensitivity and responsiveness of insulin-mediated glucose uptake (Dela *et al.*, 1992; Mikines *et al.*, 1989), which may account for why exogenous carbohydrate ingestion has been reported to negate the carbohydrate sparing influence of prior training by some (Helge *et al.*, 1996; Jeukendrup *et al.*, 1997) but not all authors (van Loon *et al.*, 1999). One final factor which may contribute to the inter-individual differences in carbohydrate utilisation during exercise is gender (Tarnopolsky *et al.*, 1990). Firstly, it has been reported that females may be less able to supercompensate their muscle glycogen stores through chronic exercise-diet manipulation than males (Tarnopolsky *et al.*, 1995) although the possibility remains that a more acute dietary manipulation in the immediate post-exercise period may reveal the capacity to rapidly synthesise muscle glycogen in females (Tarnopolsky *et al.*, 1997). Furthermore, female athletes have been shown to oxidise comparatively less carbohydrate and more lipid during exercise of moderate to high intensity than do males of matched training status (Tarnopolsky *et al.*, 1995). These authors attribute their observed differences in substrate selection to the lower insulin release in males during exercise (Tarnopolsky *et al.*, 1990) although more recent evidence does not appear to support this hypothesis (Leelayuwat *et al.*, 2005). Alternatively, others have suggested that females may display a blunted sympathetic response to exercise which would restrain their transfer towards a predominance of carbohydrate metabolism at moderate exercise intensities (Brooks and Mercier, 1994).

2.1.2 Metabolism of Other Carbohydrate Sources

As described in the previous section, carbohydrate is the preferred fuel source during exercise of moderate to high intensity but the readily available store of carbohydrate within the working muscle is finite. In addition to the carbohydrate stored within muscle, alternative sources are found in the liver and in the blood. Liver glycogen comprises approximately 14% of total stored carbohydrate while approximately 7% of available carbohydrate circulates as blood glucose (Sherman, 1995). However, these 2 sources are inherently linked since the increase in blood glucose uptake by muscle during exercise is closely matched by an increased rate of hepatic glycogenolysis and gluconeogenesis (Wasserman and Cherrington, 1991). During the early stages of exercise, hepatic glucose production is known to be supported almost entirely by an increased rate of hepatic glycogenolysis (Wahren *et al.*, 1971). This maintenance of euglycaemia through hepatic glycogenolysis results in a progressive decline in liver glycogen concentrations, thus necessitating an increasing reliance on gluconeogenesis such that, after approximately 2 h of moderate exercise, up to 20% of total hepatic glucose output can be derived from other carbon-based compounds (Ahlborg *et al.*, 1974; Decombaz *et al.*, 1979; Wasserman *et al.*, 1988). However, while an increased contribution of gluconeogenesis to hepatic glucose output can undoubtedly delay both muscle and liver glycogen depletion (Turcotte *et al.*, 1990), it cannot entirely compensate for any major decrease in hepatic glycogenesis once liver glycogen depletion eventually occurs (Ahlborg and Felig, 1982). Regulation of gluconeogenic flux during prolonged exercise appears to be principally under hormonal control since a reduction in insulin and/or an increase in glucagon have been shown to be essential for effective maintenance of glucose homeostasis in such situations (Barthel and Schmoll, 2003; Wolfe *et al.*, 1986). Interestingly, this finding may explain why ingestion of exogenous carbohydrate during exercise has been shown to completely suppress endogenous glucose production (Jeukendrup *et al.*, 1999). In addition, recent evidence also suggests that interleukin-6 (IL-6) exert a major influence in mediating endogenous glucose production during exercise, but not at rest (Febbraio *et al.*, 2004). Further information relating to this cytokine is provided in section 2.3.2.2a.

Whether arising from the liver or from an exogenous source, there are many factors which influence the rate at which blood glucose will be oxidised during exercise (Jeukendrup and Jentjens, 2000). Similar to muscle glycogen oxidation, the rate of plasma glucose oxidation can be expected to rise exponentially in response to a linear increase in exercise intensity (Romijn *et al.*, 1993; van Loon *et al.*, 2001). In the transition from low to moderate intensity exercise (i.e. up to 60% $\dot{V}O_2$ max), it is likely that the exponential increase in total carbohydrate oxidation will be met through a concomitant increase both in endogenous and exogenous carbohydrate oxidation rates (Pirnay *et al.*, 1982), although this effect may only be detectable when monitoring the transition upwards of very low intensities when non-oxidative glucose disposal would initially be possible (Jeukendrup and Jentjens, 2000). In contrast, for any increase in exercise intensity beyond approximately 60% $\dot{V}O_2$ max, increases in overall carbohydrate oxidation will predominantly originate from a greater oxidation of muscle glycogen rather than exogenously provided substrate (van Loon *et al.*, 2001).

The rate of blood glucose oxidation at any given exercise intensity does not appear to be determined by muscle glycogen availability (Arkinstall *et al.*, 2004) but instead is controlled primarily by the availability of blood glucose (Weltan *et al.*, 1998a, 1998b). The availability of blood glucose for oxidation during exercise will depend on how quickly ingested carbohydrate sources can be emptied from the stomach and absorbed through the intestine, the degree of hepatic glucose output and the capacity of active muscle to absorb blood glucose (Angus *et al.*, 2002; Jentjens and Jeukendrup, 2003). When ingesting glucose alone, maximal rates of glucose oxidation have been reported in the region of 1.1 g·min⁻¹ (Jeukendrup and Jentjens, 2000; Wagenmakers *et al.*, 1993). The rate of gastric emptying is not believed to dictate the maximal rate of glucose oxidation since the rate of glucose appearance from the stomach exceeds the maximal glucose oxidation rate \approx 3-fold (Moodley *et al.*, 1992; Rehrer *et al.*, 1992). Conversely, evidence does support a limit to the rate of glucose oxidation located at the level of the intestine. When glucose has been infused into the bloodstream, thus bypassing the proposed intestinal limitation, rates of glucose oxidation have been recorded far in excess of 1.1 g·min⁻¹ (Hawley *et al.*, 1994). Furthermore, Jentjens *et al.* (2004) proposed that this limit to intestinal

absorption may occur due to saturation of the sodium-dependent glucose transporters which carry glucose across the brush-border membrane from the intestine into the bloodstream. These authors have subsequently demonstrated rates of exogenous carbohydrate oxidation as high as $1.75 \text{ g}\cdot\text{min}^{-1}$ following ingestion of mixed glucose and fructose (Jentjens and Jeukendrup, 2005; Jentjens *et al.*, 2004) and have attributed this effect to the fact that fructose can be absorbed separately to glucose via sodium-independent facilitative transporters (Ferraris, 2001). Despite this identification of intestinal absorption as a rate limiting factor for blood glucose oxidation, it cannot be ruled out that the liver may also perform a role under certain circumstances to limit the quantity of glucose which is made available for metabolism (Jeukendrup and Jentjens, 2000).

In addition to blood glucose, it has been suggested that blood lactate may provide another particularly mobile source of fuel for aerobic metabolism (Brooks, 2002b). While the proposal that lactate released by an active muscle can either be oxidised by other muscles or used as a gluconeogenic precursor is widely accepted, the extension of this concept to describe intracellular shuttling of lactate has not been as well received (Brooks, 2002a). The key tenets of this theory which have been challenged by other authors are that it would firstly involve transport of lactate into the mitochondria, before subsequently necessitating the presence of lactate dehydrogenase (LDH) to catalyse conversion of lactate into pyruvate for further oxidation to take place (Gladden, 2004). This scepticism stems from evidence that mitochondria may be unable to oxidise lactate due to the absence of intra-mitochondrial LDH (Rasmussen *et al.*, 2002; Sahlin *et al.*, 2002). In contrast, other data have verified the existence of an LDH pool within mitochondria and, more recently, specific lactate transporters have been identified which would facilitate the function of a lactate shuttle (Brooks *et al.*, 1999; Hashimoto *et al.*, 2005). Brooks (2002a) speculates that the discrepancy in these findings may be due to LDH loss during the mitochondrial isolation procedures which were applied by Rasmussen *et al.* (2002) and Sahlin *et al.* (2002). Despite these inconsistent findings, there is now good evidence both for inter- and intra-cellular lactate shuttling mechanisms, thus providing a clear link between the aerobic and anaerobic routes of glycolysis (Gladden, 2004). It is essential, therefore, that any discussion of metabolism during exercise must recognise the contribution of this emerging substrate towards overall

carbohydrate metabolism. Furthermore, evidence also supports that lactate can easily be converted into muscle glycogen during recovery from exercise (Astrand *et al.*, 1986; Bendall and Taylor, 1970; Hermansen and Vaage, 1977), particularly in type II muscle fibres (McLane and Holloszy, 1979).

2.1.3 Metabolism of Lipid

While sections 2.1.1 and 2.1.2 clearly demonstrate the importance of carbohydrate as an energy source during exercise, if it were the sole energy substrate then total depletion of endogenous carbohydrate reserves would occur within ≈ 2 h of physical activity (Sherman, 1995). Fortunately, the body's endogenous triacylglycerol (TAG) stores represent an energy source over 60 times larger than that of glycogen and, once hydrolysed (i.e. lipolysis), these TAG can provide the mitochondria with sufficient free fatty acids (FFA) to sustain physical activity over longer periods of exercise (Horowitz and Klein, 2000). Most of this TAG is stored as sub-cutaneous or deep visceral adipose tissue, although other sites of storage are located within muscle fibres and in the circulation as either FFA or lipoproteins (van Loon, 2004). Initial escalation from low to moderate exercise intensity elevates the absolute rate of lipid oxidation (Romijn *et al.*, 1993) and evidence indicates that this increase is predominantly achieved through an accelerated oxidation of IMTG and circulating lipoprotein, although the greatest contribution is still derived from FFA (van Loon *et al.*, 2001). Further increments in exercise intensity (i.e. $>70\%$ $\dot{V}O_2$ max) result in decreased oxidation of all lipid based substrates (Romijn *et al.*, 1993; van Loon *et al.*, 2001), partly due to a reduced mobilisation of FFA from adipose tissue (Romijn *et al.*, 1995). In addition, however, these relatively low rates of lipid oxidation during intense exercise have also been associated with an increased accumulation of intramuscular fatty acids, which may suggest that the rate of lipid utilisation is limited by transport of lipid sources into the mitochondria rather than their availability in the circulation (Kiens *et al.*, 1999). Importantly, recent evidence has now demonstrated that inhibition of adipose tissue lipolysis can dramatically increase the rate of muscle glycogen degradation during exercise (van Loon *et al.*, 2005), which confirms earlier support for the existence of a glucose-FFA cycle whereby FFA availability exerts a central mediating role in substrate selection (Randle *et al.*, 1963; Randle *et al.*, 1964; Rennie and Holloszy, 1977).

In terms of exercise duration, it has been well documented that metabolism of lipid displays an overall increase as time progresses (Ahlborg *et al.*, 1974; Romijn *et al.*, 1993; van Loon *et al.*, 2003b), largely due to an amplified catecholaminergic stimulation of lipolysis (Horowitz and Klein, 2000). An earlier report from Romijn *et al.* (1993) suggesting that this gradual elevation in lipid oxidation over time is mainly supported by an increased oxidation of FFA has more recently been confirmed (van Loon *et al.*, 2003b). Furthermore, direct measurement of IMTG from muscle biopsy samples has now indicated that utilisation of this lipid source may actually decline with increasing exercise duration (Watt *et al.*, 2002a). Taken together, these findings have been interpreted to suggest that an increased availability of FFA may suppress IMTG oxidation during the latter stages of prolonged exercise (van Loon, 2004). It is also known that the rate of lipid oxidation will remain elevated in the post-exercise period to allow a more rapid replenishment of endogenous carbohydrate stores (Kuo *et al.*, 2005).

Endurance training is known to modify the metabolic response to exercise such that relatively more lipid will be oxidised at low to moderate exercise intensities following training (Bergman and Brooks, 1999). Regarding the effect of training on the different endogenous sources of lipid, one proposed training adaptation is an enhanced myocellular capacity to uptake and oxidise FFA rather than IMTG (Turcotte *et al.*, 1992). However, other studies attribute most of the increased propensity for lipid oxidation when in the trained state to an increased lipolysis of IMTG (Hurley *et al.*, 1986). At least some of the controversy regarding training adaptations in terms of IMTG use during exercise may arise from methodological differences between certain studies and a recent review has identified many of the difficulties in attaining reliable IMTG data using muscle biopsies, especially when untrained individuals have been recruited (Watt *et al.*, 2002b). Specifically, it has been suggested that measuring IMTG in muscle biopsy samples taken before and after exercise may not be a valid method due to the simultaneous utilisation and reesterification of IMTG over the intervening period (Guo, 2004). Overall, data using techniques other than muscle biopsies to quantify IMTG utilisation during exercise (e.g. stable isotope methodology and/or ^1H -MRS) support the contention that IMTG does indeed represent a valuable source of energy for muscular contraction during exercise (van Loon, 2004; Watt *et al.*, 2002b). Interestingly, recent evidence has also clarified that males may in fact

oxidise more intramuscular triacylglycerol (IMTG) than females during prolonged sub-maximal exercise (Zehnder *et al.*, 2005), although it remains to be fully established whether these differences were a direct consequence of gender or an indirect consequence of gender related training habits.

In summary, the oxidation of lipid during prolonged exercise is essential to supplement the body's primary reliance upon relatively limited endogenous carbohydrate stores for energy production. Recent evidence has clarified the specific contributions of the various lipid sources that are used during exercise and further research is warranted regarding the potential need to replace IMTG stores during recovery (Decombaz *et al.*, 2000).

2.1.4 Metabolism of Protein

Protein can support metabolism both through direct oxidation within muscle and also through providing gluconeogenic precursors for hepatic glucose production (Butterfield, 1990). Only the branched chain amino acids (BCAA) along with asparagine, aspartate, and glutamate are available for oxidation within muscle (Rennie and Tipton, 2000). All other amino acids must be transaminated into either glutamate or alanine before subsequent deamination in the liver, at which point the resultant carbon can be oxidised and the amino group disposed of as urea (Felig and Wahren, 1971). Importantly, a dynamic endogenous amino acid pool exists such that the amino acids required for hepatic glucose production during exercise do not necessarily require catabolism of muscle protein (Decombaz *et al.*, 1979).

Early evidence using [U-¹⁴C]alanine and [U-¹⁴C]leucine tracers reported that the metabolism of these 2 amino acids can increase during exercise (White and Brooks, 1981) and this finding has been generalised to the other amino acids for estimations that as much as 5-10% of total energy production may be derived from protein during exercise (Butterfield, 1990). However, data from other authors using [¹⁵N₂]urea as a tracer do not support the contention that exercise elicits an increased rate of amino acid catabolism (Carraro *et al.*, 1993). These apparently discrepant findings may be resolved through the suggestion that the rate of leucine oxidation may not necessarily represent the fate of all other amino acids (Wolfe *et al.*, 1984).

This hypothesis has been confirmed more recently using a multiple-tracer approach (L-[1-¹³C]leucine, L-[²H₅]phenylalanine and [¹⁵N₂]urea) to demonstrate not only the disproportionate oxidation of leucine during exercise, but also the absence of any increase in net protein oxidation above basal levels even during very prolonged activity (Koopman *et al.*, 2004).

More recent estimations regarding the capacity for protein oxidation in humans now assert that amino acids can only ever account for a very minor proportion (i.e. <5%) of overall energy production (Gibala, 2002). Nonetheless, amino acids clearly play an important role in facilitating carbohydrate oxidation through the provision of precursors for gluconeogenesis and possibly also through anaplerotic replenishment of the tricarboxylic acid (TCA) cycle (Ivy *et al.*, 2003; Sahlin *et al.*, 1990). A more comprehensive discussion with respect to this latter possibility is provided in section 2.2.1.

2.2 Mechanisms of Fatigue During Exercise

2.2.1 Muscle Glycogen Depletion

The interaction between exercise intensity and duration will impact profoundly on the precise causes of fatigue during physical exercise. Evidence is available demonstrating that elevated pre-exercise muscle glycogen concentrations can delay fatigue during high intensity exercise lasting less than 10 min (Maughan and Poole, 1981) and it appears that a prior bout of sub-maximal exercise augments this enhancing influence of glycogen availability (Pizza *et al.*, 1995; Tarnopolsky *et al.*, 1995). However, even when preceded by sub-maximal exercise, the effects of glycogen loading regimens on high intensity exercise have typically been small and it is unlikely that absolute muscle glycogen availability was compromised within such short durations (Hawley *et al.*, 1997b). It is more likely, therefore, that the ergogenic benefit of an increased muscle glycogen concentration during maximal or supra-maximal exercise operates through a mechanism other than delaying the point of glycogen depletion, possibly involving some enhancement of fibre excitability (Rockwell *et al.*, 2003; Stephenson *et al.*, 1999).

Similar to the very short duration exercise described above, the availability of muscle glycogen does not appear to be a primary determinant of exercise capacity during a single bout of exercise lasting less than 90 min (Hawley *et al.*, 1997b). Research involving time-trial performance tests have demonstrated that well trained athletes are able to exert themselves at relatively high intensities (i.e. $\approx 85\% \dot{V}O_2 \text{ max}$) over such durations without performance being limited by glycogen availability (Hawley *et al.*, 1997a; Sherman *et al.*, 1981). Correspondingly, when exercise capacity tests following carbohydrate supercompensation are set at an intensity such that run time to fatigue does not exceed 90 min, substantial quantities of muscle glycogen can remain in the active muscles at the point of exhaustion (Madsen *et al.*, 1990). From these data, it seems reasonable to conclude that pre-exercise muscle glycogen availability does not influence exercise capacity during an isolated exercise session lasting less than 90 min because exercise of this duration is not sufficiently prolonged to induce a substantial degree of carbohydrate depletion.

In contrast, when the intensity of exhaustive exercise is reduced to 65-75% $\dot{V}O_2 \text{ max}$, thus permitting sustained physical activity for longer than 90 min, the point of fatigue will often coincide with critically low concentrations of muscle glycogen (Bosch *et al.*, 1993; Sherman, 1995). Compelling evidence in support of this contention has demonstrated a strong positive correlation ($r = 0.92$) between initial muscle glycogen content and subsequent work time to fatigue at 75% $\dot{V}O_2 \text{ max}$ (Bergstrom *et al.*, 1967). Furthermore, at the point of exhaustion during exercise of such intensity and duration it has been a common observation for mixed muscle glycogen concentrations to be depleted below 100 mmol glucosyl units·kg dry mass⁻¹ (Ahlborg *et al.*, 1967; Bergstrom *et al.*, 1967; Hermansen *et al.*, 1967). It has been reported that sub-maximal exercise requires a greater rate of glycogen utilisation in type I as opposed to type II muscle fibres (Costill *et al.*, 1973; Gollnick *et al.*, 1974; Tsintzas *et al.*, 1995) and subsequent work appears to suggest that the onset of fatigue may be associated with the depletion of glycogen specifically in those fibres with a high oxidative capacity (Tsintzas *et al.*, 1996a).

The association described by Bergstrom *et al.* (1967) between pre-exercise muscle glycogen content and exercise capacity permits those studies investigating the effect of carbohydrate supplementation on exercise metabolism to provide indirect evidence for a role of low muscle glycogen availability in the fatigue process. In particular, the study of Tsintzas *et al.* (1996a) investigated the influence of carbohydrate ingestion during exercise on concomitant rates of muscle glycogen utilisation. The increased time to exhaustion in association with carbohydrate ingestion was attributed by these authors to the 24% reduction in muscle glycogen utilisation in comparison with placebo ingestion. Coupled with the finding that mixed muscle glycogen at the point of fatigue was depleted to a similar extent in both the carbohydrate and placebo trials (≈ 55 mmol glucosyl units·kg dry mass⁻¹), this study provides clear support for the contention that fatigue during prolonged exercise can coincide with depletion of muscle glycogen (Tsintzas *et al.*, 1996a). In addition, other evidence has revealed that this ergogenic benefit of an improved muscle glycogen availability is not only manifested during exercise capacity tests. Studies have shown that using high carbohydrate diets to increase pre-exercise muscle glycogen availability can reduce the time taken to complete a 30 km time trial, due primarily to an improved maintenance of running speed over the final 5 km (Karlsson and Saltin, 1971; Williams *et al.*, 1992). However, other findings showing that the provision of exogenous carbohydrate late in exercise can reverse the occurrence of fatigue, without any sparing of muscle glycogen, appear to suggest that fatigue arises as a consequence of compromised overall carbohydrate oxidation rather than limited muscle glycogen availability *per se* (Coggan and Coyle, 1989, 1987; Coyle *et al.*, 1986; Marcil *et al.*, 2005; Widrick *et al.*, 1993). This concept is discussed further in section 2.2.2.

The precise reasons why an adequate supply of muscle glycogen is so essential during moderate to high intensity exercise remain to be fully elucidated. One possibility is that continued muscular contraction may require a steady balance of glycogenolysis and glycogenesis such that a limited availability of intramuscular glucose would necessitate the presence of sufficient muscle glycogen to meet immediate cellular energy demand (Shulman, 2005; Shulman and Rothman, 2001). However, it has also been suggested that glycogen may act as a carbon chain precursor for anaplerosis of certain intermediates in the TCA cycle during exercise (Wagenmakers *et al.*, 1990). Evidence certainly supports that intermediates such as 2-oxoglutarate, succinate, fumarate, malate and oxaloacetate are reduced below normal exercising levels at the point of exhaustion (Gibala *et al.*, 1997) and it has been shown that carbohydrate ingestion might offset this effect (Spencer *et al.*, 1991). Nonetheless, while some authors have affirmed that this consequence of reduced glycogen availability might compromise mitochondrial respiration (Sahlin *et al.*, 1990; Wagenmakers *et al.*, 1991), data from other authors have indicated that manipulation of TCA pool size does not causally effect either aerobic energy production or exercise performance (Dawson *et al.*, 2005; Gibala *et al.*, 1998), possibly indicating that an enriched pool of TCA cycle intermediates is not necessary to maintain cycle flux (Gibala, 2003).

2.2.2 Liver Glycogen Depletion and Hypoglycaemia

While the availability of muscle glycogen at the onset of exercise is undoubtedly an important determinant of exercise capacity and the intensity of exercise must necessarily be reduced once this substrate is depleted (Ahlborg *et al.*, 1967; Bergstrom *et al.*, 1967; Hermansen *et al.*, 1967), there are clearly other mechanisms of fatigue which can be initiated before a muscle glycogen availability reaches critically low levels (Costill *et al.*, 1971b). Evidence for such mechanisms arises from the studies referred to earlier in which provision of exogenous carbohydrate was shown to postpone fatigue without reducing the rate of muscle glycogen utilisation (Coggan and Coyle, 1989, 1987; Coyle *et al.*, 1986; Marcil *et al.*, 2005; Widrick *et al.*, 1993). In the study by Coyle *et al.* (1986), endurance trained participants were required to cycle to the point of exhaustion at approximately 70% $\dot{V}O_2$ max while ingesting either a placebo or a glucose polymer solution. As might be

expected, time to fatigue was significantly enhanced through ingestion of the carbohydrate solution rather than the placebo solution (242 versus 182 min, respectively). Of greater interest was the fact that the additional hour of exercise performed with carbohydrate supplementation was supported almost entirely through blood glucose oxidation, despite approximately 170 mmol glucosyl units·kg dry mass⁻¹ of muscle glycogen still available for metabolism in the active muscles at the point of fatigue. It was concluded from these results that the muscle glycogen concentrations after 3 h of cycling were sufficiently reduced to stimulate an increased rate of muscle glucose uptake and that eventual fatigue therefore coincided with the development of hypoglycaemia (Coyle *et al.*, 1986).

However, as discussed in section 2.1.2, it has subsequently been discovered that the rate of glucose disposal during exercise is regulated by blood glucose availability and not muscle glycogen availability (Arkinstall *et al.*, 2004; Weltan *et al.*, 1998a, 1998b). Furthermore, Claassen *et al.* (2005) recently applied a euglycaemic clamp to demonstrate that the ergogenic influence of glucose administration late in exercise is unrelated to changes in carbohydrate oxidation and that fatigue under such circumstances is not a direct consequence of hypoglycaemia. These authors propose that fatigue during prolonged exercise might instead be the result of a combined depletion of both intramuscular and intrahepatic carbohydrate, which in turn acts as a feed-forward signal to mediate cerebral sensitivity to reductions in blood glucose concentration (Claassen *et al.*, 2005). This suggestion is in agreement with evidence using ¹³C MRS to simultaneously quantify muscle and liver glycogen concentrations during continuous cycling to exhaustion at 70% $\dot{V}O_2$ max following prior depletion (Casey *et al.*, 2000). This evidence demonstrated that, under certain conditions, the relationship between pre-exercise muscle glycogen content and subsequent exercise capacity, first described by Bergstrom *et al.* (1967), may not be as strong as when pre-exercise availability of both muscle and liver glycogen are considered in unison.

The apparently increased influence of liver glycogen on exercise capacity in the study of Casey *et al.* (2000) might be a consequence of the prior bout of prolonged exercise that was performed just 4 h before the capacity test. A study in rats from Terjung *et al.* (1974) verifies that hypoglycaemia secondary to depletion of liver

glycogen will occur before depletion of muscle glycogen when these animals exercise to fatigue in the fasted state. Furthermore, this data also maintains that preferential restoration of liver glycogen is initiated following such exercise because complete recovery of hepatic carbohydrate status takes longer than in muscle and may therefore limit recovery of functional capacity (Terjung *et al.*, 1974). However, due to the relatively small amount of muscle glycogen that is stored in rodent muscle, it may be that this particular animal model might overestimate the importance of blood glucose availability when generalised to humans (Pederson *et al.*, 2005). On the other hand, repeated exercise bouts performed with a limited recovery interval may sensitise the human system to the effects of compromised glucose availability (Casey *et al.*, 2000; Tsintzas *et al.*, 2003), at least in certain individuals (Claassen *et al.*, 2005; Sherman, 1995), thus making the human and rodent models more comparable in this specific situation. The importance of liver glycogen availability during exercise is further highlighted by studies which have demonstrated that the ability to increase hepatic glucose output at the onset of exercise may be essential for maintenance of glucose homeostasis (Jentjens *et al.*, 2003; Moseley *et al.*, 2003).

In summary, the reliance on carbohydrate as an energy substrate during moderate to high intensity exercise dictates that fatigue during such activity will often coincide with reduced carbohydrate availability. However, despite the primary importance of muscle glycogen as a carbohydrate source during exercise, depletion of this particular substrate is not necessarily the central cause of fatigue in all circumstances. Instead, it is apparent that a complex interaction between muscle glycogen utilisation, blood glucose disposal and hepatic glucose output operates to maintain carbohydrate oxidation during exercise. The precise cause of fatigue will therefore depend upon the availability of both muscle and liver glycogen while also taking into account the relative sensitivity of the central nervous system (CNS) to depletion of each respective carbohydrate store.

2.2.3 Dehydration

The clear importance of substrate availability to maintain energy delivery during exercise has been documented in previous sections. However, in some situations increases in body temperature and associated fluid losses can compromise physiological function long before substrate depletion occurs (Nadel *et al.*, 1980). To prevent undue increases in body temperature, the heat which is produced by exercising tissue must be transported to the periphery by the blood and dissipated via the evaporation of sweat from the skin surface (Murray, 1995). This process results losses of body fluid, as evidenced by the strong positive correlation ($r = 0.98$) between increases in oesophageal temperature and body mass losses during exercise when performed at an ambient temperature of 33°C (Montain and Coyle, 1992b). These losses in body fluid have been shown to reduce blood volume such that increases in heart rate are unable to compensate for the resultant decrease in stroke volume, thus reducing cardiac output (Hamilton *et al.*, 1991). Ultimately, this cardiovascular drift is manifested as a reduced peripheral blood flow and, as a result, subsequent thermoregulation is compromised (Montain and Coyle, 1992a, 1992b).

The negative influence of dehydration on fatigue is known to be more profound when exercise is performed in warmer environments. Graded increments in ambient temperature from 11 to 31°C have been reported to exert a progressively detrimental influence on exercise capacity (Galloway and Maughan, 1997). However, it is difficult to establish from the above data whether fatigue at each temperature was in fact a direct consequence of dehydration and resultant hyperthermia. Given that the catecholaminergic response to exercise would be augmented with increasing heat load (Parkin *et al.*, 1999) and that this in turn would be expected to accelerate the rate of muscle glycogen utilisation (Febbraio *et al.*, 1994; Hunt and Ivy, 2002; Jentjens *et al.*, 2002), the data of Galloway and Maughan (1997) cannot refute the possibility that depletion of endogenous carbohydrate may have occurred earlier as environmental temperature approached 31°C. To investigate this hypothesis, the subsequent investigation has now quantified muscle glycogen directly during 135 min of cycling and confirmed that the reduced ability to exercise under heat stress when dehydrated is in fact due to hyperthermia rather than depletion of metabolic substrate (Gonzalez-Alonso *et al.*, 1999). Nonetheless, the majority of evidence implicating fluid

availability as a limiting factor for physical performance has tended to focus on exercise performed under conditions of heat stress. If a euhydrated individual were to perform similar exercise in a thermoneutral environment and with sufficient opportunity for fluid consumption then it is more likely that exercise capacity will depend on carbohydrate rather than fluid availability (Murray, 1995).

2.2.4 Accumulation of Metabolic By-Products

During the performance of dynamic exercise at close to maximal intensity, energy production through anaerobic glycolysis results in the formation of lactic acid which quickly dissociates to yield lactate salts and hydrogen ions, thus contributing to reductions in muscle pH as low as ≈ 6.6 (Sahlin *et al.*, 1976). Reductions in intramuscular pH of similar magnitude have been shown to inhibit further glycolysis *in vitro* (Sutton *et al.*, 1981), with evidence suggesting that this limitation occurs primarily through inhibition of the respective rate-limiting enzymes for glycogenolysis and glycolysis: namely phosphorylase (Chasiotis *et al.*, 1983a) and phosphofructokinase (Trivedi and Danforth, 1966). In addition to a reduced glycolytic rate, an accumulation of hydrogen ions during exercise can also limit muscle contraction more directly through inhibiting both the release of calcium from the sarcoplasmic reticulum (SR) and its subsequent binding to troponin (Metzger and Fitts, 1987; Nakamaru and Schwartz, 1972). As alluded to in section 2.2.1, this inhibition of calcium release may be even more profound in situations where muscle glycogen availability is also compromised (Rockwell *et al.*, 2003; Stephenson *et al.*, 1999).

However, in reviewing this topic, other authors have challenged the notion that metabolic acidosis is the principal cause of fatigue during high intensity exercise (Westerblad *et al.*, 2002). This view stems from findings indicating that the negative impact of elevated intracellular pH on force production is absent when acidosis is induced in intact skeletal muscle at temperatures within the physiological range (Wiseman *et al.*, 1996). Evidence from skeletal muscle *in vivo* certainly supports that elevated muscle acidity does not diminish muscle glycogenolysis or glycolysis and therefore cannot be the sole cause of fatigue during intense exercise (Bangsbo *et al.*, 1996). An alternative cause of fatigue to the more traditional metabolic acidosis

explanation has been suggested following reports of deteriorated muscle fibre contractility following exposure to high physiological concentrations of lactate (Andrews *et al.*, 1996). However, subsequent investigations attempting to verify this potential influence of lactate on muscular fatigue have only identified a modest detrimental effect of lactate on excitation-contraction coupling (Posterino and Fryer, 2000). Some evidence even supports a protective effect of lactic acidosis against fatigue, supposedly related to a reversal of the negative influence exerted by elevated potassium concentrations (Nielsen *et al.*, 2001).

Inorganic phosphate is another by-product of anaerobic metabolism for which evidence in support of a potential role in depressed contractile function is more convincing (Allen and Westerblad, 2001; Westerblad *et al.*, 2002). The concentration of inorganic phosphate in skeletal muscle increases rapidly with the onset of exercise as creatine kinase (CK) catalyses the breakdown of phosphocreatine to maintain an adequate supply of adenosine triphosphate (Sahlin *et al.*, 1997). Support for the role of inorganic phosphate in the fatigue process comes from research showing that repeated tetanic stimulation of isolated muscle fibres in CK-deficient mice neither increases inorganic phosphate concentration nor produces a decline in maximal force production over time (Dahlstedt *et al.*, 2000). One mechanism through which inorganic phosphate accumulation is believed to inhibit contractile function involves an inhibition of calcium uptake by the SR, although this effect is not straightforward since inorganic phosphate would simultaneously facilitate opening of SR calcium release channels (Balog *et al.*, 2000; Duke and Steele, 2000).

While inorganic phosphate accumulation clearly provides a credible alternative to the current paradigm implicating metabolic acidosis as the primary cause of fatigue during dynamic exercise of high intensity, certain issues remain to be resolved concerning the specific relationship between increases in inorganic phosphate concentration and the time-course of fatigue across various muscle groups (Gladden, 2004). Even once the precise role of inorganic phosphate has been clarified, the more traditional perspective regarding reduced intramuscular pH should not be entirely discounted since evidence strongly supports a beneficial role of induced alkalosis on physical performance (Bird *et al.*, 1995; Wilkes *et al.*, 1983).

2.2.5 Reduced Central Drive

The causes of fatigue described so far have tended to involve peripheral factors which might impede or at least result in dysfunction of contraction in the working muscles during exercise. Intuitively, however, the CNS can be expected to play a major role in terms of deciphering the many peripheral signals during exercise and ultimately initiating fatigue (Blomstrand, 2001). This process is likely to be highly complex and potential mediating factors may involve either efferent stimulation from the corticospinal tract or afferent signals from active tissue (Davis and Bailey, 1997). Regarding this latter possibility, it has been suggested that the synthesis of certain neurotransmitters from amino acid precursors may dictate the central drive for exercise and, although perhaps overly reductionist, efforts have been made to identify the specific neurotransmitters which might induce fatigue (Davis, 1995). One such neurotransmitter, 5-hydroxytryptamine (5-HT), has been implicated in the development of fatigue since it has been found to be released from the hippocampus of rats following exercise (Meeusen *et al.*, 1996). Causal evidence, again in the rat model, supporting a role of 5-HT in the fatigue process arises from the finding that exercise capacity can either be deteriorated or enhanced by approximately 30% through administration of 5-HT agonists or antagonists, respectively (Bailey *et al.*, 1993).

The study referenced above from Meeusen *et al.* (1996) also demonstrated that the release of 5-HT in the brain is stimulated by treatment with its amino acid precursor tryptophan. However, for 5-HT to be synthesised without tryptophan administration, circulating tryptophan must first be displaced by FFA from its binding site on albumin (Yamamoto *et al.*, 1997) before subsequently competing with the other large neutral amino acids (LNAA) for transport across the blood-brain barrier (Blomstrand *et al.*, 2005; Pardridge, 1983). Therefore, the increased mobilisation of FFA during exercise (see section 2.1.3) and preferential oxidation of certain LNAA (namely leucine, see section 2.1.4) might be expected to favour the synthesis of 5-HT and thus reduce the central drive to exercise (Blomstrand, 2001; Davis and Bailey, 1997).

While manipulations of 5-HT or its amino acid precursor have been consistently shown to influence fatigue in rodents, evidence relating this and other isolated neurotransmitters to fatigue in humans has been less conclusive (Chinevere *et al.*, 2002). Research in this area using humans has typically involved BCAA supplementation during exercise, under the rationale that increased plasma concentrations of these particular amino acids might inhibit the transport of free tryptophan into the brain and therefore alleviate sensations of fatigue. Blomstrand and colleagues have demonstrated a reduction in the free tryptophan:BCAA ratio using such supplements, which has been associated both with a reduced rating of perceived exertion at a given work rate (Blomstrand *et al.*, 1997) and with an improved performance during a competitive marathon in non-elite individuals (Blomstrand *et al.*, 1991). Attempts to replicate this latter finding under laboratory conditions have tended to involve exercise in warm environments to increase the central component of fatigue and, while some authors have corroborated the enhancing effect of BCAA on performance (Mittleman *et al.*, 1998), others have not (Watson *et al.*, 2004a). However, in the study of Watson *et al.* (2004), a large inter-individual response to BCAA supplementation was noted and therefore the possibility remains that BCAA supplementation might improve the central drive for exercise in certain individuals. The question has also been raised as to whether coingestion of carbohydrate along with BCAA during exercise may further ameliorate the central component of fatigue through simultaneously reducing the circulating concentrations of both free tryptophan and FFA (Davis *et al.*, 1992). The available evidence does not appear to support this hypothesis during continuous exercise (Madsen *et al.*, 1996) and it is not clear whether the improved performance of intermittent exercise with carbohydrate-protein ingestion is the result of central or peripheral mechanisms (Ivy *et al.*, 2003).

As stated earlier, it is likely that overall CNS control of exercise motivation is a highly complex process and attempts to determine the cause of fatigue in terms of a single neurotransmitter may be an overly reductionist approach (Noakes *et al.*, 2005). In this sense, many peripheral factors contributing to fatigue are likely to exert a direct influence on the brain in addition to any indirect neurotransmitter mediated signalling pathways. For instance, brain carbohydrate metabolism is known to increase in the transition from rest to exercise (Ide *et al.*, 2000) and it may be that

hypoglycaemia as a consequence of increases in peripheral glucose uptake during exercise might reduce CNS activation (Bequet *et al.*, 2002; Nybo, 2003), possibly in conjunction with a depletion of glycogen within the type 2 astrocytes of the brain (Kong *et al.*, 2002). In summary, it must be accepted that fatigue in any circumstance in which an individual exercises to the point of *voluntary* exhaustion will necessarily be dictated to some extent by the CNS (Noakes and St. Clair Gibson, 2004).

2.3 Short-Term Recovery from Prolonged Exercise

As described in the previous sections, prolonged exercise can deplete the body's limited stores of endogenous carbohydrate while also eliciting some degree of dehydration. An effective recovery therefore requires the replacement of these essential resources as rapidly as possible in preparation for subsequent performance. If the interval between exercise sessions is 24 h or longer, then simply ingesting a carbohydrate-rich diet along with sufficient fluid can often completely restore both carbohydrate and fluid status (Casey *et al.*, 1995; Costill *et al.*, 1981; Piehl, 1974) such that exercise capacity can also be entirely recovered within this time (Nicholas *et al.*, 1997). Furthermore, provided that adequate quantities of carbohydrate and fluid are consumed over a 24 h recovery, it appears that no further considerations are necessary regarding the specific type of diet that is consumed (Burke *et al.*, 1995; Kiens *et al.*, 1990; Reed *et al.*, 1989) or indeed the time-course over which it is provided (Ivy *et al.*, 1988a; Parkin *et al.*, 1997). In contrast, many athletes are required to train or compete on multiple occasions within a single 24 h period, in which case considerations regarding the timing, type and amount of carbohydrate ingested during recovery become quantitatively more important (Ivy, 2001, 1998; Jentjens and Jeukendrup, 2003; Sherman, 1995). The following sections will address the physiological mechanisms that are involved during recovery, with specific reference to those nutritional strategies which might be implemented to accelerate these processes when recovery time is limited (i.e. <8 h).

2.3.1 Rehydration

The degree to which an individual will be rehydrated following exercise is dependent upon how much fluid they consume along with the specific composition of that fluid. These 2 factors are inextricably linked since factors relating to the composition of a solution can also directly influence both the palatability of that solution and the subsequent effect of ingestion on the thirst mechanism, thus dictating the volume which will be voluntarily consumed (Shirreffs *et al.*, 2004). The inclusion of certain electrolytes can improve the palatability of rehydration solutions following exercise induced dehydration. One such electrolyte is sodium, which has been demonstrated to encourage fluid consumption while also improving the absorption and retention of fluid (Shirreffs and Maughan, 1998b). Evidence also exists supporting the inclusion of potassium in rehydration solutions, although it is thought to be less effective than sodium in terms of recovering extracellular fluid volume following exercise (Yawata, 1990).

It is known that the amount of fluid evacuated from the stomach in a given unit of time will correlate positively with the volume of gastric contents (Murray, 1995), which suggests that fluid should be consumed at regular intervals during recovery to maintain a high gastric volume and therefore maximise fluid delivery. In addition to gastric volume, other factors such as the pH, osmolality, energy content and macronutrient composition of gastric contents are also known to influence gastric emptying rate with varying effect (Costill and Saltin, 1974; Piehl Aulin *et al.*, 2000). For example, when the carbohydrate content of a solution is elevated, the rate of gastric emptying will be delayed more as a consequence of the increase in energy density than as a result of increased osmolality (Vist and Maughan, 1995, 1994). Regarding macronutrient content, it has long been known that carbohydrate empties from the stomach much more rapidly than fat and somewhat faster than intact protein (Thomas, 1957). However, a more recent investigation has reported no difference in the rate of gastric emptying and intestinal fluid flux following ingestion of iso-energetic glucose and soy protein hydrolysate solutions (Maughan *et al.*, 2004). In addition, certain amino acids are known to have physiologically distinct mechanisms for transport across the intestinal brush-border (Taylor *et al.*, 1989) and it has been

suggested that the inclusion of such amino acids in a recovery solution might therefore actually facilitate net fluid absorption (Bowtell *et al.*, 1999).

2.3.2 Repletion of Endogenous Carbohydrate

2.3.2.1 Autoregulation of Glycogen Concentration

The rate at which muscle glycogen will be resynthesised following prolonged exercise is determined both by the rate of glucose transport into muscle and by the conversion of this glucose into glycogen (Fisher *et al.*, 2002b). The literature supports that the degree of carbohydrate depletion may play an important role in mediating both these processes (Jentjens and Jeukendrup, 2003). Specifically, Zachwieja *et al.* (1991) have demonstrated that the rate of muscle glycogen resynthesis following exercise will become more rapid with an increasing magnitude of glycogen depletion and this finding has been attributed both to a stimulation of glucose uptake (Fell *et al.*, 1982; Ivy and Holloszy, 1981; Ploug *et al.*, 1984) and an increase in glycogen synthase activity (Mikines *et al.*, 1988; Zachwieja *et al.*, 1991). Notably, this stimulus for rapid carbohydrate storage in the immediate post-exercise period operates primarily to increase the concentrations of muscle glycogen rather than hepatic carbohydrate stores (Fell *et al.*, 1980), possibly linked to the ability of skeletal muscle to increase IMTG utilisation during recovery to allow maximal utilisation of glucose for glycogenesis (Kiens and Richter, 1998; van Loon *et al.*, 2003b). In an attempt to clarify whether the observed increase in muscle glycogen storage following exercise was related to the high glycogen utilisation rate during that exercise or due to the amount of muscle glycogen remaining afterwards, Price *et al.* (2000) compared the glycogen resynthesis rate between normal and glycogen loaded muscles following a similarly demanding exercise regimen. It was concluded from this study that it is low muscle glycogen content *per se* which stimulates post-exercise increases in carbohydrate storage, rather than the amount by which initial glycogen levels were previously reduced (Price *et al.*, 2000).

Interestingly, it has been demonstrated that this stimulatory influence of low muscle glycogen availability on glycogen resynthesis is not dependent on insulin (Price *et al.*, 1994) and that the factors which are responsible appear to become less influential over time following exercise, even if muscle glycogen concentrations

remain low (Ploug *et al.*, 1987). Indeed, the biphasic nature of glycogen resynthesis has previously been identified to involve an initial rapid insulin-independent phase followed by a more gradual accumulation of glucosyl units which is dependent on insulin (Piehl, 1974). This effect may be related to the 2 distinct pools of muscle glycogen that were described in section 2.1.1 since proglycogen has been shown to be resynthesised more rapidly than macroglycogen (Adamo *et al.*, 1998). More importantly, a large body of literature now provides compelling evidence in support of an additive effect of exercise and insulin on the rate of muscle glycogen synthesis, which indicates that glycogen resynthesis is stimulated by different mechanisms at various stages in recovery (Gao *et al.*, 1994; Lund *et al.*, 1995; Ploug *et al.*, 1984; Ploug *et al.*, 1987; Ploug *et al.*, 1993; Price *et al.*, 1996; Thorell *et al.*, 1999; Wallberg-Henriksson *et al.*, 1988; Wallberg-Henriksson and Holloszy, 1984; Zorzano *et al.*, 1986). The processes of glucose uptake and subsequent conversion of glucose into glycogen are described in the following sections 2.3.2.2 and 2.3.2.3, respectively, with particular reference to the independent influences of exercise and insulin on each process (sub-sections a and b, respectively).

2.3.2.2 Glucose Uptake

2.3.2.2a Exercise Induced Glucose Uptake

In addition to the references cited in the previous section regarding the additive effect of exercise and insulin on glucose uptake, further support for the existence of specific exercise induced mechanisms comes from the finding that translocation of glucose transporter 4 (GLUT4) becomes normal in type 2 diabetic subjects following exercise (Kennedy *et al.*, 1999). GLUT4 is thought to be the primary glucose carrier in skeletal muscle (Bell *et al.*, 1993; Fushiki *et al.*, 1989) and pharmacological inhibition of this transporter has been shown to reduce overall glucose uptake by 70-80% (Rudich *et al.*, 2003). Both exercise and insulin are believed to increase glucose uptake through translocation of GLUT4 from an intracellular storage vesicle onto the plasma membrane (Hirshman *et al.*, 1990; Lund *et al.*, 1995; Rodnick *et al.*, 1992; Thorell *et al.*, 1999; Wardzala and Jeanrenaud, 1981) and data demonstrating the additive effects of these stimuli have led to the suggestion that 2 separate pools of GLUT4 transporters may exist (Douen *et al.*, 1990; Goodyear and Kahn, 1998; Hayashi *et al.*, 1997; Holman *et al.*, 1994; Ploug *et al.*,

1998) which are likely to be stimulated via distinct signaling pathways (Brozinick *et al.*, 1994; Sternlicht *et al.*, 1989). Further suggestions that the intrinsic activity of this glucose transporter might also be increased are less convincing since increases in glucose uptake can often be completely accounted for by the increased number of GLUT4 carrier proteins at the cell surface (Hansen *et al.*, 1998; King *et al.*, 1989; Lund *et al.*, 1995; Nesher *et al.*, 1985).

The availability of glucose is clearly an essential requirement for muscle glycogen resynthesis to take place and a direct relationship ($r = 0.63$) has been identified between the concentration of GLUT4 carrier proteins on the plasma membrane and the rate of post-exercise glycogen storage (McCoy *et al.*, 1996). Furthermore, it has been suggested that it is glucose transport rather than subsequent conversion of glucose into glycogen which may ultimately limit the rate of muscle glycogen synthesis (Holloszy, 2003). This suggestion is based on findings from transgenic mice showing that increased glucose transport can produce large increments in the rate of glycogen storage despite a 50% reduction in the active form (I form) of glycogen synthase (Ren *et al.*, 1993). In fact, the activity of glycogen synthase is only thought to impose a limit on the rate of muscle glycogen storage when the percent of glycogen synthase in the I form is below fasted values (Fisher *et al.*, 2002b).

There are a number of mechanisms through which exercise might operate to stimulate the increments in the rate of glucose uptake, primarily involving a direct influence of increased availability of interstitial glucose as a result of exercise induced increases in blood flow (MacLean *et al.*, 1999). An increased cytoplasmic concentration of calcium (Ca^{2+}) has also been reported to contribute to the increased rates of glucose transport during exercise (Holloszy and Narahara, 1967). However, it has been difficult to determine whether Ca^{2+} acts directly to translocate GLUT4 or if it is Ca^{2+} induced contraction which provides the principle stimulus for glucose uptake (Holloszy, 2003; Zorzano *et al.*, 2005). The direct signalling pathways downstream of Ca^{2+} are thought to involve protein kinase C (PKC) and calmodulin-dependent protein kinase (CAMK) since inhibition of these protein kinases has been shown to inhibit exercise induced glucose uptake (Ihlemann *et al.*, 1999a). Evidence supporting this direct influence of calcium *per se* originates from the finding that

glucose uptake can be stimulated by levels of calcium release that are insufficient to induce muscular contraction (Youn *et al.*, 1991). In support for an effect of Ca^{2+} stimulated contraction on glucose uptake, Holloszy and Narahara (1965) have described a good correlation between the permeability of isolated frog muscle to glucose and the frequency to which that muscle is stimulated to contract, although more recent evidence has clarified that stimulation frequency is not the sole determinant of contraction induced glucose uptake since force development is also a mediating factor (Holloszy and Narahara, 1965; Ihlemann *et al.*, 1999b). A subsequent investigation to determine the relative impact of stimulation frequency and force development on glucose uptake found that the influence of both these factors was in fact dependent on other metabolic consequences of exercise (Ihlemann *et al.*, 2000).

In summarising the available evidence regarding exercise induced glucose uptake, Richter *et al.* (2001) have stated that “If glucose transport were only activated by a feed-forward Ca^{2+} -sensitive mechanism, the regulatory and adaptive capacity of the system would be limited” (Richter *et al.*, 2001, p. 314). This statement arises primarily from the findings of Ihleman *et al.* (2000) and concisely describes the necessity for some feed-back mechanism through which fuel depletion might maintain the stimulus for glucose uptake even once muscle contraction ceases (i.e. the persistent increase in glucose uptake during recovery). The reductions in muscle glycogen content that occur during exercise are known to increase glucose uptake and a negative correlation ($r = -0.53$) has been observed between glycogen content and the expression of GLUT4 on the cell membrane (Derave *et al.*, 1999). This finding has led some authors to speculate that glycogen may be structurally attached to GLUT4 (Coderre *et al.*, 1995; Richter *et al.*, 2001), although direct biochemical evidence to support such an association has not been published to date.

All the factors which are discussed above in relation to glucose uptake are illustrated in Figure 2.1. However, the lower portion of this figure also depicts the interaction of these factors with the activation of adenosine monophosphate-activated protein kinase (AMPK). This particular enzyme has received great interest in recent years and has been proposed to be *the* contraction-activated kinase responsible for increasing glucose transport (Richter *et al.*, 2003). As presented in Figure 2.1, even if

muscle glycogen is structurally attached to GLUT4, it is likely that a secondary function of reduced muscle glycogen availability is to activate AMPK during the insulin independent phase of glycogen resynthesis. Evidence clearly shows that low muscle glycogen concentrations are associated with an increased AMPK activity both at rest and during exercise (Wojtaszewski *et al.*, 2002; Wojtaszewski *et al.*, 2003b). However, patients suffering from McArdle's disease appear to have hyper-activated AMPK despite high muscle glycogen concentrations (Nielsen *et al.*, 2002), which leads to the conclusion that it may be a low capability to oxidise glycogen rather than a low glycogen content *per se* which causes AMPK activation (Hardie, 2004). In addition, the dephosphorylation of ATP and PCr during exercise is known to result in allosteric activation of AMPK (Kemp *et al.*, 1999) and, when exercise is of sufficient intensity, hypoxia has been found to be entirely dependent on the presence of AMPK to stimulate increased glucose transport (Mu *et al.*, 2001).

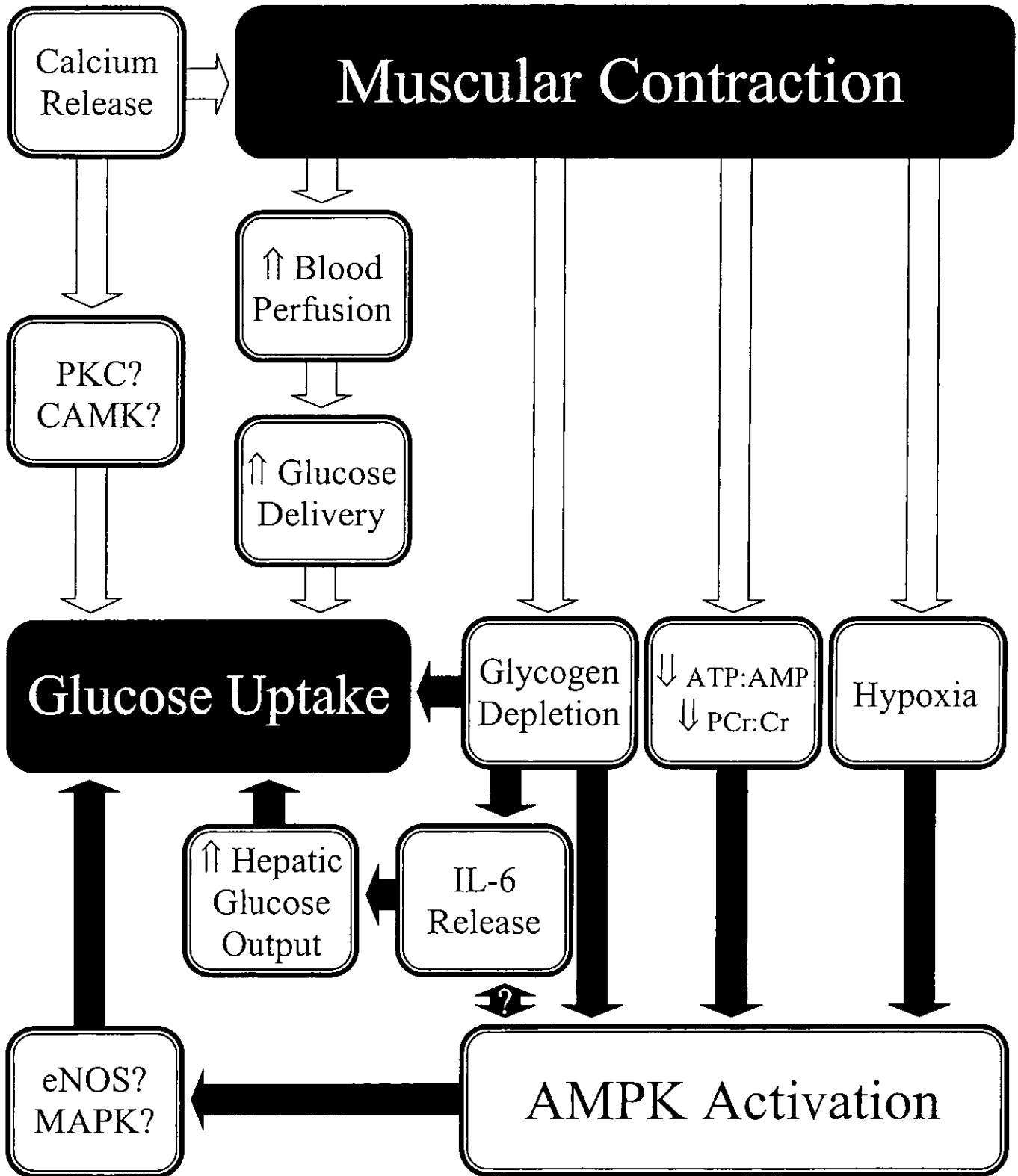


Figure 2.1: Regulation of glucose uptake during/after exercise. Open arrows represent feed-forward mechanisms and closed arrows represent feedback mechanisms.

Once activated, the specific process through which AMPK ultimately stimulates the translocation of GLUT4 onto the plasma membrane remains unclear (Musi and Goodyear, 2003). It has been suggested that nitric oxide synthase (NOS) may perform an intermediary role (Balon and Nadler, 1997; Fryer *et al.*, 2000) since evidence has reported the phosphorylation of endothelial NOS (eNOS) by AMPK (Chen *et al.*, 1999). Alternatively, AMPK might target the p38 mitogen-activated protein kinase (MAPK). In support of this, the increase in glucose uptake that is usually observed following activation of AMPK using 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR) can be substantially blunted without simultaneous activation of MAPK (Xi *et al.*, 2001). Another potential AMPK related pathway has been identified through the positive correlation ($r = 0.86$) between the activity of AMPK and the release of IL-6 from skeletal muscle during exercise (MacDonald *et al.*, 2003), although the possibility that IL-6 release might also be directly stimulated by low muscle glycogen availability precludes the establishment of a causal relationship (Pedersen and Febbraio, 2005). Indeed, it is unlikely that AMPK is the only contraction induced stimulus for glucose uptake following exercise (Jessen and Goodyear, 2005). Nonetheless, given that IL-6 has been implicated in both the regulation of hepatic glucose production (Febbraio *et al.*, 2004) and signalling peripheral fuel status to the CNS (Gleeson, 2000), any potential interaction with AMPK is likely to be a central component of metabolic regulation during exercise. Establishing the precise signalling pathways through which AMPK and IL-6 stimulate glucose uptake during and after exercise will therefore be crucial for further progression of understanding in this area.

Regardless of the mechanism of action, it is clear that AMPK plays an essential role in the regulation of glucose transport during exercise, acting as an intracellular fuel gauge to stimulate glucose uptake in the early stage of recovery. However, it is also evident that AMPK can only account for a portion of the increase in glucose transport following muscular contraction (Mu *et al.*, 2001). This suggests that overall control of glucose uptake during and after exercise is the net product of an integrated intracellular system whereby Ca^{2+} release and fuel depletion can both stimulate glucose uptake either directly or indirectly via AMPK (Holloszy, 2003; Richter *et al.*, 2001). Recent evidence seems to suggest that the relative influence of Ca^{2+} and AMPK dependent mechanisms on glucose transport may be determined

primarily by muscle fibre type. Specifically, it has been suggested that exercise stimulated glucose uptake into type II muscle fibres may involve both Ca^{2+} and AMPK mediated mechanisms (Wright *et al.*, 2004), while glucose uptake into type I fibres may be entirely dependent on the former (Wright *et al.*, 2005). Importantly, the effect of exercise on glucose uptake is sustained during recovery and manifested as the insulin independent rapid phase of glycogen resynthesis (Goodyear *et al.*, 1990b). Despite being independent of insulin, this early stage of recovery is also associated with a period of insulin sensitivity (Cartee *et al.*, 1989; Holloszy, 2005; Wojtaszewski *et al.*, 2003a) which has emerged as yet another factor that may be mediated by the activation of AMPK (Fisher *et al.*, 2002a; Musi and Goodyear, 2003). Insulin sensitivity during the early hours of recovery allows for elevations in insulin and glucose arising from carbohydrate supplementation to further increase the translocation of GLUT4 over this period (Kuo *et al.*, 1999). This separate influence of insulin on the rate of glucose uptake in the post-exercise period is addressed in subsection b.

2.3.2.2b Insulin Induced Glucose Uptake

Similar to contraction induced glucose uptake, insulin also stimulates glucose transport through evoking translocation of GLUT4 from its intracellular storage site onto the plasma membrane (Rodnick *et al.*, 1992; Thorell *et al.*, 1999; Wardzala and Jeanrenaud, 1981). Also similar to the glucose uptake initiated by muscular contraction is the belief that GLUT4 translocation can entirely account for increases in glucose uptake, therefore negating any role for an increased intrinsic activity of these transporters (Hansen *et al.*, 1998; Lund *et al.*, 1995; Nesher *et al.*, 1985). However, it has recently been proposed that intrinsic activation of GLUT4 subsequent to translocation may be necessary for glucose transport to be stimulated maximally, at least in relation to insulin induced glucose uptake (Furtado *et al.*, 2003). This proposal arises from a series of studies in which a myc tagged GLUT4 carrier protein was used to detect the effects of insulin on GLUT 4 translocation and glucose uptake independently; this novel methodology made it possible to identify a temporal divergence between the translocation of GLUT4 and increases in glucose uptake (Somwar *et al.*, 2001).

The above mentioned study by Somwar *et al.* (2001) also highlighted some integration between the glucose uptake pathways that are initiated by insulin and exercise respectively, since insulin was shown to stimulate GLUT4 activity via the same MAPK (p38) that is responsible for exercise induced increases in glucose transport (Somwar *et al.*, 2001). In their review, Rudich and Klip (2003) neatly describe the combined operation of 2 other factors which are known to play a major role in the stimulation of glucose transport by insulin, these are phosphatidylinositol 3-kinase (PI3K) and protein kinase B (PKB). At rest without insulin stimulation, PI3K and PKB (along with the PI3K sub-units p85 α and p110) are distributed throughout the cytosol and 90% of GLUT4 remains within its intracellular storage vesicle (Li *et al.*, 2001), as can be seen in Figure 2.2a. Upon stimulation with insulin, certain insulin receptor substrates (IRS) will be phosphorylated which, through the p85 α and p110 sub-units of PI3K, will generate phosphatidylinositol-3,4,5-triphosphate (PIP₃) on the cell surface (Zorzano *et al.*, 2005). The essential role of the IRS for insulin mediated glucose transport is evidenced by the finding of impaired glucose disposal in mice that are deficient in IRS (Withers *et al.*, 1998). The initial role of PIP₃ is to encourage the remodelling of filamentous actin, thus drawing the plasma membrane closer to GLUT4 containing vesicles (Rudich and Klip, 2003). This ‘ruffling’ of the plasma membrane is illustrated in Figure 2.2b. In addition to this effect, Figure 2.2c shows how PIP₃ simultaneously functions to facilitate PKB in accelerating the transport of GLUT4 through its intracellular recycling endosome compartment (Foster *et al.*, 2001; Watson *et al.*, 2004b). Finally, GLUT4 must be transported from its intracellular recycling endosome to the cell surface (Figure 2.2d) and vesicle-associated membrane-2 (VAMP2) has been suggested as the ‘specialised GLUT4 vesicle compartment’ responsible for this transportation across the actin mesh (Rudich and Klip, 2003). This suggestion is supported by evidence showing that inhibition of VAMP2 results in a markedly reduced insulin stimulated incorporation of GLUT4 into the cell surface (Randhawa *et al.*, 2000).

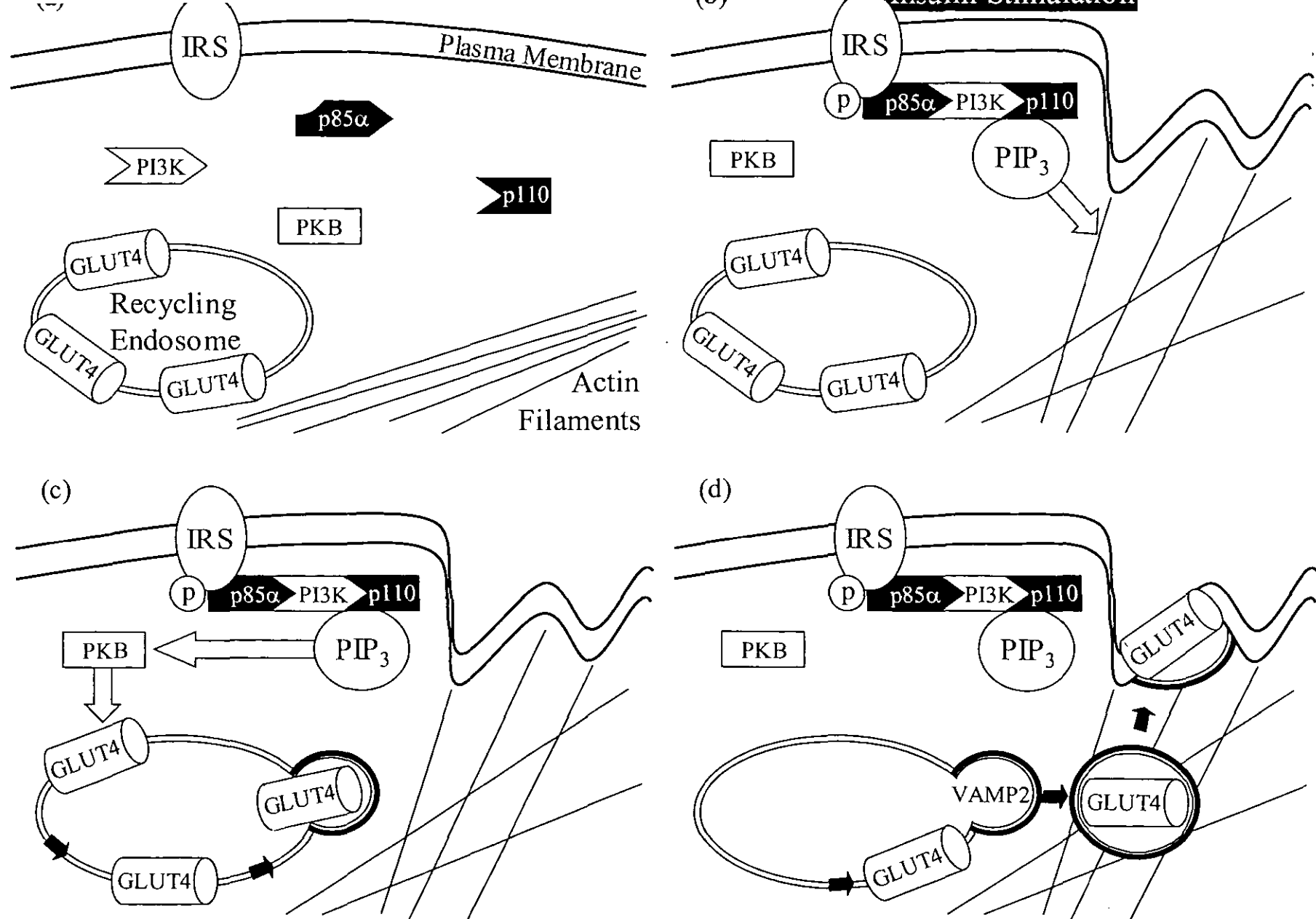


Figure 2.2: Push and pull mechanisms of insulin induced GLUT4 translocation. (a) In the absence of insulin, 90% of GLUT4 remains within its intracellular storage vesicle. (b) Insulin stimulates the remodelling of actin through PIP₃, thus drawing the plasma membrane closer to the recycling endosome. (c) PIP₃ then provides a platform for PKB to accelerate intracellular recycling of GLUT4. (d) GLUT4 molecules 'bud out' of the recycling endosome and are transported across the actin mesh by VAMP2. Figure adapted from Rudich and Klip (2003).

While the process through which insulin stimulates glucose uptake is undoubtedly complex, what is clear is that increasing glucose and insulin concentrations in the immediate post-exercise period can result in large increases in glucose transport (Kuo *et al.*, 1999). As stated earlier, the stimulatory effect of insulin is even more potent following prolonged exercise due to the relative insulin sensitivity which occurs in the post-exercise period (Holloszy, 2005; Wojtaszewski *et al.*, 2003a). Notably, in the absence of carbohydrate ingestion, this elevated insulin sensitivity has been observed to persist in rats for at least 48 h following exercise (Cartee *et al.*, 1989). The finding that glucose uptake in the exercised leg following single-legged cycling is far greater than in the unexercised leg suggests that this enhanced influence of insulin on glucose uptake following exercise is primarily due to local factors (Richter *et al.*, 1984; Richter *et al.*, 1989). One such factor is muscle glycogen depletion and evidence has demonstrated that low muscle glycogen concentrations can stimulate an enhanced sensitivity of insulin induced glucose transport through activation of the PKB signalling pathway (Derave *et al.*, 2000). In addition, the possibility that GLUT4 might be structurally attached to glycogen could also explain the progressive increase in the rate of glucose uptake as glycogen concentrations fall (Coderre *et al.*, 1995; Richter *et al.*, 2001). Aside from these direct influences of muscle glycogen on insulin induced glucose uptake, the reports that post-exercise insulin sensitivity may be mediated by AMPK activation suggest that muscle glycogen depletion (among other exercise associated factors, see Figure 2.1) may also exert an indirect effect on insulin action (Fisher *et al.*, 2002a; Holloszy, 2005; Musi and Goodyear, 2003; Olsen and Hansen, 2002; Wojtaszewski *et al.*, 2003a).

The magnitude of insulin sensitivity following exercise is also mediated by other more applied factors relating to the specific nature of the exercise session which preceded recovery. In particular, evidence suggests that a period of relative insulin resistance can occur following eccentric or unaccustomed exercise (Asp and Richter, 1996; Asp *et al.*, 1997b; King *et al.*, 1993; Kirwan *et al.*, 1992). Such physical activity can potentially induce a substantial degree of muscle damage and it seems reasonable to suggest that this tissue injury might inhibit GLUT4 translocation due to disruption of the plasma membrane, with evidence also supporting a potential role for reduced insulin signalling via MAPK under conditions of oxidative stress (Blair *et al.*, 1999). This reduced capacity to develop insulin sensitivity following damaging

exercise can ultimately result in a reduced rate of muscle glycogen resynthesis during recovery (Costill *et al.*, 1990; Doyle *et al.*, 1993; O'Reilly *et al.*, 1987) or even a net degradation of muscle glycogen in the first 2 h following exercise (Zehnder *et al.*, 2004), although it has been suggested that impaired carbohydrate storage following muscle damage probably involves more than reduced GLUT4 translocation alone (Asp *et al.*, 1997a). Further to the negative impact during recovery of prior eccentric contractions, it would also be expected that muscle glycogen utilisation during subsequent exercise would be higher in damaged muscle groups (Asp *et al.*, 1998). A final consideration is whether the prior exercise bout was an isolated episode or was part of a continued program of training. Similar to the effects of eccentric or unaccustomed exercise, repeated depletion of muscle glycogen over a series of days may also compromise the stimulatory influence of prior exercise on the rate of glycogen resynthesis (McInerney *et al.*, 2005), although such repeated depletion would also be expected to reduce the rate of muscle glycogen degradation during each sequential exercise session (Costill *et al.*, 1971a).

Replenishment of muscle glycogen following prolonged exercise is a critical component of recovery and it appears that the rate at which this substrate will be replaced is limited to a large extent by the rate at which glucose can be transported into muscle (Fisher *et al.*, 2002b; Holloszy, 2003; Ren *et al.*, 1993), which in turn will vary according to fibre type (Henriksen *et al.*, 1990; James *et al.*, 1985). The respective mechanisms through which exercise and insulin independently promote glucose uptake have been discussed. However, to be stored within muscle as an available fuel source, the absorbed glucose must first be synthesised into muscle glycogen. The rate limiting enzyme for this process is glycogen synthase (GS) and the relative influences of exercise and insulin on GS activity will be reviewed in the following sub-sections.

2.3.2.3 Conversion of Glucose to Glycogen

2.3.2.3a Exercise Induced Activation of Glycogen Synthase

While glucose transport is believed to be the rate limiting factor for glycogen resynthesis under most circumstances (Ren *et al.*, 1993), it has been shown that GS activity also has the potential to limit the rate of muscle glycogen resynthesis when insufficiently activated (Conlee *et al.*, 1978; Fisher *et al.*, 2002b), even when the rate of glucose uptake is also sub-maximal (Ryder *et al.*, 1999). The potential for this occurrence is further supported by findings that transgenic over-expression of GS can result in at least a 2-fold increase in glycogen synthetic rate across all types of skeletal muscle (Azpiazu *et al.*, 2000). However, it should be acknowledged that the processes of glucose uptake and glycogen synthesis *in vivo* are not mutually exclusive since the glucose that is transported into muscle is immediately converted by hexokinase into glucose-6-phosphate (G-6-P) which is known to be a powerful allosteric effector of all forms of GS (Shulman *et al.*, 1995). Following this conversion, G-6-P is subsequently converted into G-1-P by phosphoglucomutase before merging with uridine triphosphate via the action of G-1-P uridtransferase to yield uridine diphosphate (U-D-P)-glucose (Ivy, 1991). The rate limiting reaction in the formation of muscle glycogen occurs next as U-D-P is released and the glucose residue is attached to a pre-existing branch of glycogen, it is this final incorporation of glucose into glycogen which is catalysed by GS (Jentjens and Jeukendrup, 2003).

GS is known to exist in 2 enzymatically interconvertible forms, of which the I form is thought to be most active because, unlike the D form, it can operate even in the absence of G-6-P (Danforth, 1965). Exercise is known to stimulate increases in GS-I through dephosphorylation of GS-D, which would explain why increments in G-6-P are not essential for exercise induced GS activation (Montell *et al.*, 1999). In fact, consistent with so many factors relating to muscle glycogen resynthesis, it appears that the most potent regulator of GS activity is a reduced availability of muscle glycogen (Laurent *et al.*, 2000; Montell *et al.*, 1999; Nielsen and Richter, 2003; Zachwieja *et al.*, 1991). Muscle glycogen content remains high following exercise in patients with McArdle's disease and it has been noted that these individuals are also unable to activate GS in the post-exercise period (Nielsen *et al.*, 2002). In such situations where muscle glycogen is readily available, GS would be located on the

outer branches of macroglycogen and it has been suggested that a high level of GS activation can only occur when GS is positioned within the inner core of glycogen, bound to glycogenin (Pitcher *et al.*, 1988; Smythe and Cohen, 1991). A further explanation for the inverse relationship between muscle glycogen content and GS activity is that glycogen might cause allosteric inhibition of both GS phosphatase (GSP) and indeed GS itself (Fischer *et al.*, 1971). In this scenario, the depletion of muscle glycogen during exercise would therefore release both enzymes and GS would then be converted into its active form by GSP (Ivy and Kuo, 1998).

Contrary to the above findings, it is also likely that compromised muscle glycogen availability, and indeed exercise *per se*, will also stimulate a number of inhibitory regulators of GS activity (Nielsen and Richter, 2003). As discussed in section 2.3.2.2a, AMPK is emerging as an important exercise induced factor regulating metabolic responses both during and following exercise (Hardie, 2004) and it appears that this particular kinase operates not only to increase glucose uptake (see Figure 2.1) but also to inhibit GS activity (Halse *et al.*, 2003; Nielsen *et al.*, 2002; Wojtaszewski *et al.*, 2002). Furthermore, sympathetic stimulation from the CNS during exercise results in increased circulating levels of adrenaline which have also been associated with a decrease in the ratio of GS-I:GS-D (Chasiotis *et al.*, 1983b). This effect of adrenaline is thought to operate through protein kinase A (PKA) and evidence supports that this pathway is distinct to that of contraction mediated GS inhibition (Sakamoto *et al.*, 2002). Notably, while not essential to stimulate GS activation, increases in G-6-P concentration following exercise are known to attenuate the inhibitory effects of kinases such as AMPK and PKA while at the same time increasing the susceptibility of GS-D to dephosphorylation by GSP (Nielsen and Richter, 2003). In addition, it has been shown in rodents that exercise increases GS activity through deactivating other inhibitory kinases such as glycogen synthase kinase (GSK)-3 (Markuns *et al.*, 1999; Sakamoto *et al.*, 2002). However, this negative influence of exercise on GSK-3 activity has not been consistently demonstrated in human muscle (Sakamoto *et al.*, 2004; Wojtaszewski *et al.*, 2001), possibly because the stimulation of AMPK activity during exercise can potentiate the ability of GSK-3 to phosphorylate GS (Zhang *et al.*, 1993). Figure 2.3 summarises the regulation of GS activity following exercise and clearly illustrates that the activation status of GS at any given time will be determined by the relative intensity of a number

of opposing stimuli. It can be seen that the balance of these stimuli will favour the activation of GS during recovery from prolonged exercise since both muscle glycogen depletion and the increase in G-6-P availability will exert a positive influence, while the latter would also act to inhibit those kinases which would otherwise deactivate GS.

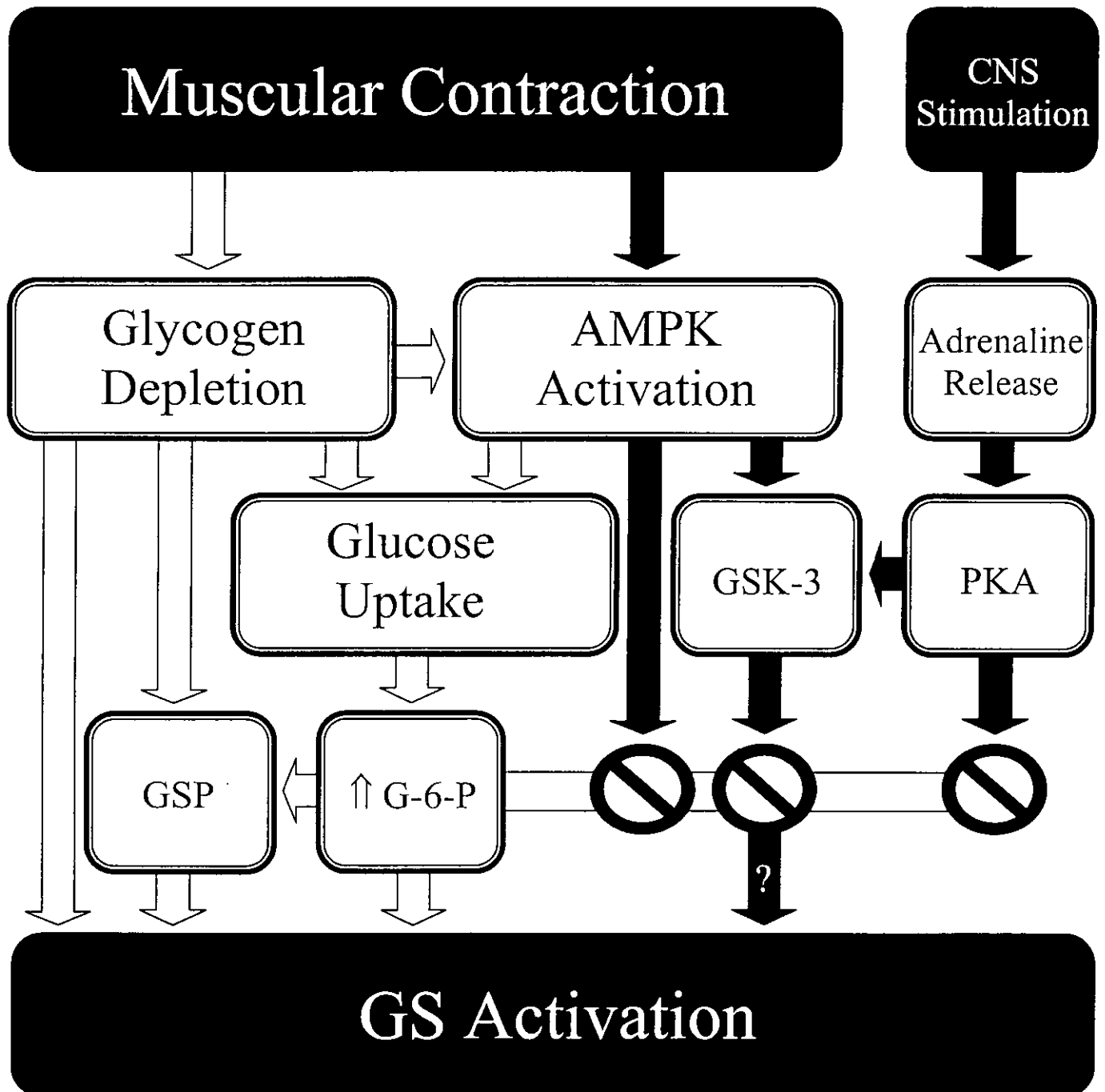


Figure 2.3: Regulation of glycogen synthase (GS) activity following exercise. Open arrows represent signalling pathways culminating in GS activation and closed arrows represent signalling pathways culminating in GS deactivation. GS is often activated following prolonged exercise since many positive influences will be initiated, while increments in G-6-P also serve to inhibit the negative influences of AMPK and PKA (N.B: it remains unclear whether a similar inhibition of GSK-3 occurs following exercise in human skeletal muscle).

2.3.2.3b Insulin Induced Activation of Glycogen Synthase

In contrast to exercise induced GS activation, the precise mechanisms through which insulin stimulates conversion of glucose into glycogen are far less well understood (Wojtaszewski *et al.*, 2003a). The fact that combined exercise and insulin stimulation do not always produce an additive effect on GS activation suggests that the signalling pathways of these 2 stimuli converge at some point (Nielsen and Richter, 2003; Wojtaszewski *et al.*, 1999). This convergence may occur quite late in the respective signalling pathways of exercise and insulin since both processes must ultimately dephosphorylate GS at the same C-terminal residues (Lawrence and Roach, 1997). However, it is thought that the more distal factors leading up to this eventual activation of GS are likely to differ between the exercise and insulin stimulated pathways. For instance, insulin stimulated GS activation appears to involve IRS, PI3K, 3-phosphoinositide dependent protein kinase (PDK)-1 and PKB (Cross *et al.*, 1997; Mora *et al.*, 2005), yet muscular contraction can maintain GS activity even when this signalling network is inhibited (Sakamoto *et al.*, 2002; Wojtaszewski *et al.*, 1999). In parallel to this finding that certain pathways may be exclusive to insulin signalling, it has also been identified that the dephosphorylation of GS by GSP that occurs in response to exercise is not necessary when GS is activated by insulin (Suzuki *et al.*, 2001).

A signalling mechanism which may potentially be common to both insulin and exercise stimulated GS activation is that of GSK-3 (Nielsen and Richter, 2003). This kinase operates to phosphorylate and therefore deactivate GS and it is thought that this process may be inhibited by insulin (Lawrence and Roach, 1997). However, as illustrated in Figure 2.3, it has not yet been fully established whether a similar deactivation of GSK-3 also occurs following exercise in humans (Sakamoto *et al.*, 2004; Wojtaszewski *et al.*, 2001). Even if exercise is assumed to down-regulate GSK-3 activity, evidence suggests that the mechanism through which this is achieved does not involve the PKB pathway that is initiated by insulin (Markuns *et al.*, 1999). Nonetheless, the possibility that GSK-3 might be involved in GS activation both by exercise and insulin stimulation has led some authors to implicate this particular signalling pathway in the enhanced action of insulin on GS in the post-exercise period (Christ-Roberts *et al.*, 2003). Indeed, more recent evidence has now established that

exercise induced IL-6 release can increase insulin sensitivity by facilitating the phosphorylation of GSK-3 by insulin (Weigert *et al.*, 2005). However, this effect is likely to operate in combination with an increased insulin delivery to muscle during exercise due to elevated blood flow (Clark *et al.*, 2003).

This section (2.3.2) has described the specific signalling mechanisms through which exercise and insulin promote the uptake and storage of glucose during recovery. The fact that insulin promotes muscle glycogen resynthesis through a mechanism that is distinct from that of exercise is important when considering various nutritional strategies which might facilitate carbohydrate storage following exercise, especially since factors initiated by prior exercise have been shown to increase insulin sensitivity during the early hours of recovery (Wojtaszewski *et al.*, 2003a). Specifically, any nutritional strategy which rapidly increases both glucose and insulin concentrations during recovery will augment the rate of muscle glycogen storage since the exercise and insulin mediated signalling pathways will operate synergistically during the initial rapid phase of resynthesis (Narahara and Ozand, 1963). In addition, increasing the availability of blood glucose in the immediate post-exercise period may also be important in terms of decreasing the influence of factors such as adrenaline release that would otherwise oppose the rapid resynthesis of muscle glycogen (Galbo *et al.*, 1977). The benefit of hyperglycaemia and hyperinsulinaemia becomes even more important later in recovery when the residual influence of exercise related factors has diminished and muscle glycogen resynthesis progresses into the insulin dependent phase (Young *et al.*, 1987). The ingestion of carbohydrate following prolonged exercise has the potential to promote muscle glycogen storage through the mechanism described above and what follows is an account of the various studies that have demonstrated such an effect.

2.4 Carbohydrate Ingestion

The belief that muscle glycogen should be resynthesised as rapidly as possible during recovery stems from the initial findings of Bergstrom *et al.* (1967) which demonstrated a direct association between pre-exercise muscle glycogen content and exercise capacity. Many studies certainly support that physical performance can be improved over a range of exercise intensities through glycogen loading prior to exercise (Brewer *et al.*, 1988; Karlsson and Saltin, 1971; Maughan and Poole, 1981; Pizza *et al.*, 1995; Simonsen *et al.*, 1991; Starling *et al.*, 1997; Tarnopolsky *et al.*, 1995; Williams *et al.*, 1992), although it appears that acute carbohydrate loading regimens may be more effective in terms of performance enhancement than more chronic dietary manipulation (Jacobs and Sherman, 1999). However, when the primary focus of nutritional supplementation is to restore exercise capacity within a matter of hours following prolonged exercise, these carbohydrate loading regimens will need to be condensed to maximise the rate of muscle glycogen storage during recovery while also minimising any potential metabolic disturbance during subsequent exercise. In this sense, nutritional interventions during a short-term recovery necessitate a combined appreciation of pre- and post-exercise nutrition since the most effective feeding strategy will be required to maximise muscle glycogen availability following recovery while simultaneously reducing the rate of muscle glycogen utilisation during a repeated performance (Chryssanthopoulos *et al.*, 1994b; Wright *et al.*, 1991).

It has been consistently demonstrated that carbohydrate ingestion over a short-term recovery period can increase the rate of carbohydrate storage above that of a placebo (Casey *et al.*, 2000; Tarnopolsky *et al.*, 1997; Van Hall *et al.*, 2000b). However, attempts to identify the most effective carbohydrate feeding strategy have shown that the rate of muscle glycogen resynthesis can vary greatly depending on the relative timing, type and amount of carbohydrate that is ingested during recovery (Ivy, 2001, 1998; Jentjens and Jeukendrup, 2003; Sherman, 1995). In terms of exercise capacity, while some authors have reported an improved exercise time to exhaustion at 70% $\dot{V}O_2$ max when carbohydrate rather than flavoured water was ingested during a preceding 4 h recovery (Fallowfield *et al.*, 1995), other authors have observed no such effect (Casey *et al.*, 2000). The following sections will address considerations

relating to the timing, type and amount of carbohydrate that should be ingested during a short-term recovery with particular reference to the impact of these varying feeding regimens on muscle glycogen resynthesis and the restoration of exercise capacity.

2.4.1 Considerations Regarding Timing of Carbohydrate Ingested

There are 3 considerations in terms of supplement timing during recovery: how soon after the initial exercise session should supplementation begin; how frequently during the recovery period should supplements be ingested; and how soon before subsequent exercise should supplementation be terminated? In answer to the first of these considerations, it is thought that the rate of muscle glycogen resynthesis will be most rapid during a short-term recovery if carbohydrate is consumed immediately following prior exercise rather than delayed by several hours (Ivy, 2001). This might be expected since the exercise induced increase in insulin sensitivity is greatest in the early hours of recovery and the capacity to translocate and activate GLUT4 through carbohydrate ingestion will therefore gradually diminish over time (Cartee *et al.*, 1989; Goodyear *et al.*, 1990a; Young *et al.*, 1987). It has been demonstrated that delaying the intake of a mixed macronutrient supplement by 3 h rather than feeding immediately post-exercise can reduce net leg glucose uptake during recovery by approximately 65% (Levenhagen *et al.*, 2001). Furthermore, a study by Ivy *et al.* (1988a) demonstrated a rate of muscle glycogen resynthesis of ≈ 25 mmol glucosyl units \cdot kg dry mass $^{-1}\cdot$ h $^{-1}$ over a 4 h recovery when carbohydrate was provided immediately following exercise. Importantly, this rate of resynthesis was reduced by almost half to ≈ 14 mmol glucosyl units \cdot kg dry mass $^{-1}\cdot$ h $^{-1}$ when carbohydrate provision was delayed by just 2 h (Ivy *et al.*, 1988a). Contrary to this finding, Parkin *et al.* (1997) monitored the effects of delayed carbohydrate feeding, again by 2 h, on the resynthesis of glycogen over an 8 h recovery and reported that the rate of muscle glycogen resynthesis was similar between treatments (Parkin *et al.*, 1997). While at first glance it appears that these studies are in direct opposition, it may be that the rate of muscle glycogen resynthesis is greater with immediate feeding over the first 4 h of recovery but slows thereafter due to a greater feedback inhibition of GS activity (Laurent *et al.*, 2000). This pattern of resynthesis is shown in Figure 2.4 and would suggest that prompt ingestion of carbohydrate following exercise becomes of increasing importance when recovery time is more restricted. Of course,

aside from issues relating to the development of insulin resistance later in recovery, it would be prudent to begin carbohydrate feeding as early as possible following exercise to maximise the time available for the resynthesis of muscle glycogen to occur (Ivy, 2001).

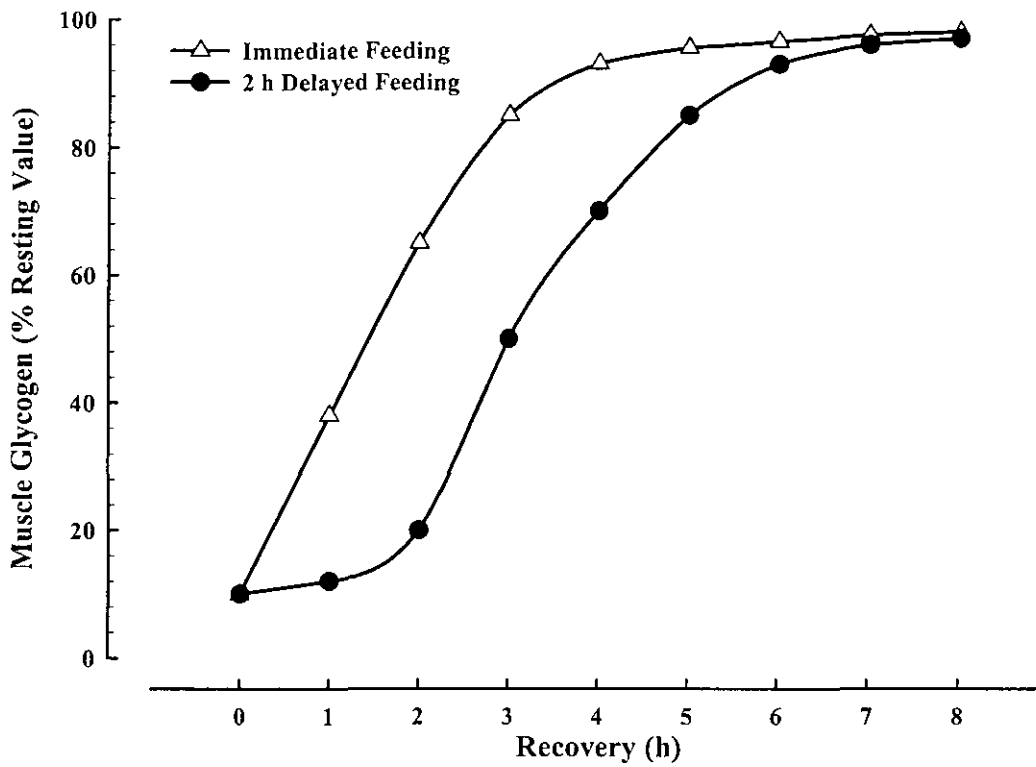


Figure 2.4: Expected time-course of muscle glycogen resynthesis when carbohydrate is provided either immediately following exercise or after a 2 h delay. Based on the combined findings of Ivy *et al.* (1988a) and Parkin *et al.* (1997), this figure illustrates that shorter recovery periods increase the necessity for carbohydrate feeding immediately following exercise.

In relation to the issue regarding how frequently carbohydrate should be consumed following exercise, studies from Blom *et al.* (1987) and Ivy *et al.* (1988b) have both provided carbohydrate at 2 h intervals during recovery and subsequently reported muscle glycogen resynthesis rates in the region of 22-25 mmol glucosyl units·kg dry mass⁻¹·h⁻¹. Notably, these rates of muscle glycogen resynthesis were achieved using relatively moderate carbohydrate intakes (i.e. 0.35-0.75 g CHO·kg⁻¹·h⁻¹) but doubling the amount of carbohydrate did not increase the rate of muscle glycogen storage any further (Blom *et al.*, 1987; Ivy *et al.*, 1988b). It has been suggested that providing carbohydrate at 2 h intervals during recovery may not sustain elevations in circulating insulin sufficiently between feedings, resulting in sub-optimal activation of GLUT4 and GS (Ivy, 1998). This suggestion has subsequently been upheld by evidence showing that, when carbohydrate is ingested at 15-30 min intervals during a short-term recovery, the rate of muscle glycogen resynthesis can be elevated to 30-45 mmol glucosyl units·kg dry mass⁻¹·h⁻¹ (Doyle *et al.*, 1993; Jentjens *et al.*, 2001; Piehl Aulin *et al.*, 2000; van Loon *et al.*, 2000a). However, more applied evidence has shown that more frequent feeding during a short-term recovery can also increase the reliance on carbohydrate oxidation during subsequent exercise, although this effect was not found to be detrimental in terms of exercise capacity (Siu *et al.*, 2004).

The final consideration regarding the timing of carbohydrate ingestion during a short-term recovery is whether feeding should cease at least 1 h prior to subsequent performance to prevent metabolic disturbances during that activity. Specifically, ingesting 75 g of carbohydrate 45 min prior to a 30 min treadmill run can potentially increase the rate of muscle glycogen utilisation during that exercise by 17% as a result of hyperinsulinaemia and hypoglycaemia (Costill *et al.*, 1977). On the basis of this finding, these authors hypothesised that exercise capacity might be compromised if carbohydrate is ingested within 1 h of performance and they consequently repeated their protocol with a cycling capacity test following feeding. This follow-up study confirmed the hypothesis in that time to exhaustion at 80% $\dot{V}O_2$ max was reduced by 19% when carbohydrate rather than plain water was ingested prior to exercise (Foster *et al.*, 1979). While these hyperinsulinaemic and hypoglycaemic influences of pre-exercise carbohydrate feeding have now been demonstrated at a range of exercise intensities (Achten and Jeukendrup, 2003), the negative effect on performance shown

during cycling to has yet to be replicated using a treadmill running protocol (Chryssanthopoulos *et al.*, 1994a), possibly because maintenance of euglycaemia during exercise is less important during running than during cycling (see section 2.1.1). More recent evidence has now established that, although hypoglycaemia can occur during exercise if large quantities of carbohydrate are consumed within 1 h of performance, this effect is often a transient response (i.e. 'rebound' hypoglycaemia) and would not therefore be expected to contribute to eventual fatigue (Jentjens *et al.*, 2003; Moseley *et al.*, 2003). However, all the above studies relate specifically to pre-exercise feeding, which may not necessarily reflect the metabolic response to carbohydrate ingestion when exercise is performed following recovery from an earlier exercise session. The specific question as to whether continuous ingestion of carbohydrate during *recovery* would influence subsequent glycogen metabolism was addressed in the study of Tsintzas *et al.* (2003). This study demonstrated that ingesting moderate (≈ 0.5 g CHO \cdot kg $^{-1}\cdot$ h $^{-1}$) rather than low (≈ 0.2 g CHO \cdot kg $^{-1}\cdot$ h $^{-1}$) amounts of carbohydrate at frequent intervals during recovery can significantly increase glycogen storage prior to subsequent exercise without affecting glycogen utilisation during the ensuing activity (Tsintzas *et al.*, 2003). Nonetheless, even if carbohydrate ingestion late in recovery does not influence subsequent exercise metabolism, from a practical perspective it seems reasonable to suggest that feeding should cease at least 15-30 min prior to exercise in order to minimise the risk of gastrointestinal discomfort.

2.4.2 Considerations Regarding Type of Carbohydrate Ingested

There are a number of considerations regarding the type or form of carbohydrate that should be ingested during a short-term recovery (Williams, 2004). One issue which has received much attention in recent years is the issue of glycaemic index (GI). The GI is a measure of the glucose response to a given carbohydrate source (expressed as incremental area under curve; see Appendix F) in relation to the maximal response observed following ingestion of a matched quantity of pure glucose (Wolever, 2004). Given the influential role of insulin in the promotion of carbohydrate storage following exercise, it has been suggested that the increased insulin response following ingestion of a mixed high GI meal might result in a greater rate of muscle glycogen resynthesis than following ingestion of a mixed low GI meal

(Burke *et al.*, 1998). A study from Kiens *et al.* (1990) investigated the influence of high versus low GI carbohydrate feeding during recovery from prolonged cycling and confirmed that the almost doubled insulin response with high GI as opposed to low GI was associated with a significantly higher muscle glycogen concentration 6 h later. Notably, as with the delayed carbohydrate feeding discussed above (Ivy *et al.*, 1988a; Parkin *et al.*, 1997), the issue of glycaemic index seems to become of greater importance in the early stages of recovery since the differences in muscle glycogen content that have been reported at 6 h have been found to be normalised 14 h later (Kiens *et al.*, 1990). Conversely, however, other authors have found differences in muscle glycogen resynthesis in favour of high GI to persist for up to 24 h following exercise (Burke *et al.*, 1993).

Despite the positive effect of high GI meals on glycogen storage during recovery, it is important to consider whether ingesting these highly insulinotropic foods prior to exercise may exacerbate the proposed metabolic disturbances which can occur during subsequent exercise (Costill *et al.*, 1977; Foster *et al.*, 1979). It has therefore been suggested that ingestion of low GI carbohydrates during recovery may result in muscle glycogen sparing during subsequent exercise and a study from Thomas *et al.* (1991) has demonstrated that this reduction in carbohydrate oxidation with low GI can prolong exercise capacity (Thomas *et al.*, 1991). However, while subsequent investigations have verified the proposed metabolic benefits of low GI ingestion, the earlier reports of performance enhancement from Thomas *et al.* (1991) have not yet been confirmed (Febbraio *et al.*, 2000; Sparks *et al.*, 1998; Wee *et al.*, 1999; Wee *et al.*, 2005).

Studies that have investigated the influence of ingesting various forms of sugar during recovery provide some insight into the specific fate of high and low GI carbohydrates following mixed meals. In particular, fructose has a lower GI than glucose and consequently also results in a lower rate of muscle glycogen resynthesis during recovery (Blom *et al.*, 1987). It is thought that the rate of muscle glycogen synthesis when fructose is ingested may be limited by the conversion of fructose into glucose by the liver and it is therefore possible that liver glycogen resynthesis may be greater in this situation (Nilsson and Hultman, 1974). This possibility was assessed in the study of Casey *et al.* (2000) which examined the effect of ingesting either glucose

or sucrose (i.e. equimolar fractions of glucose and fructose) during recovery on the combined resynthesis of both muscle and liver glycogen. A trend was apparent in favour of greater liver glycogen resynthesis following ingestion of sucrose and, similar to the findings of others (Blom *et al.*, 1987), there were no differences in muscle glycogen resynthesis between the glucose and sucrose treatments (Casey *et al.*, 2000). It has been hypothesised that the fructose component of sucrose may decrease liver glucose uptake and therefore render a greater fraction of the glucose component available for muscle glycogen synthesis (Blom *et al.*, 1987), although subsequent investigations have demonstrated that fructose can actually increase hepatic glucose uptake (Shiota *et al.*, 2005). Contrary to evidence promoting the use of sucrose over glucose, another study has reported reduced whole body and skeletal muscle glycogen storage with sucrose ingestion as opposed to glucose ingestion (Bowtell *et al.*, 2000). However, the data gathered during this latter study are questionable because the acid extraction method used to assess muscle glycogen would have disregarded the acid soluble fraction of glycogen (Jansson, 1981). Therefore, it cannot be discounted that this soluble fraction of glycogen (i.e. proglycogen) may have responded differently to the respective glucose and sucrose treatments since it is known to be highly sensitive to dietary intervention and would be expected to account for the majority of muscle glycogen resynthesis during a short-term recovery (Adamo *et al.*, 1998). On balance, especially given the suggestion that restoration of exercise capacity may require the rapid resynthesis of both muscle and liver glycogen stores (Casey *et al.*, 2000; Claassen *et al.*, 2005; Terjung *et al.*, 1974), it appears reasonable to conclude that sucrose ingestion represents the most effective method of carbohydrate supplementation during recovery.

Whether carbohydrate is ingested in solid or liquid form is another important consideration when attempting to enhance the rate of post-exercise carbohydrate storage. Early evidence suggested that ingestion of a mixed macronutrient meal or a carbohydrate-rich liquid can achieve a similar rate of muscle glycogen resynthesis over a 5 h recovery period, although the carbohydrate content of the solid meal in this particular study was greater than that of the carbohydrate solution (Keizer *et al.*, 1987). A subsequent investigation by Reed *et al.* (1989) compared the effects of solid versus liquid feedings during a 4 h recovery when the carbohydrate content of the 2 supplements was matched ($0.75 \text{ g CHO} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$). This investigation confirmed earlier

findings in that rates of muscle glycogen resynthesis were not different between treatments and a further comparison of these oral feedings against intravenous glucose infusion suggested that the rate of gastric emptying did not limit the rate of muscle glycogen resynthesis following either feeding method (Reed *et al.*, 1989). Successive investigations have now supported the contention that the gastric emptying rate of ingested carbohydrate is not likely to limit the rate of muscle glycogen resynthesis following exercise (Moodley *et al.*, 1992; Rehrer *et al.*, 1992), although the possibility remains that gastric emptying may begin to limit carbohydrate storage when the rate of carbohydrate intake is in excess of $0.75 \text{ g CHO} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$ (Jentjens and Jeukendrup, 2003). From a practical perspective, even if solid and liquid carbohydrate sources can be assumed to stimulate muscle glycogen resynthesis with equal effect, fluid supplements will achieve this aim while also encouraging rehydration and are therefore recommended (Coleman, 1994). Of course, plain water can be ingested alongside solid meals to satisfy both objectives but care should be taken because many solid foods do not contain carbohydrate in isolation and the presence of other macronutrients can compromise the gastric emptying rate of the liquid component (Berry *et al.*, 2002). One final consideration which warrants attention is whether the osmolality of a given carbohydrate solution will influence the rate of glycogenesis during recovery. Indeed, recent evidence from Piehl Aulin *et al.* (2000) has demonstrated a notably higher rate of muscle glycogen resynthesis over the initial 2 h of recovery when a glucose polymer solution (low osmolality) was ingested as opposed to a glucose monomer solution (high osmolality), with this effect attributed to a more rapid rate of gastric emptying together with an increase in exercise induced glucose uptake (Piehl Aulin *et al.*, 2000). However, a subsequent study has reported that carbohydrate oxidation rates during exercise may be unaffected by the osmolality of a given carbohydrate solution (Rowlands *et al.*, 2005).

2.4.3 Considerations Regarding Amount of Carbohydrate Ingested

While the above considerations regarding the timing and type of carbohydrate ingested during recovery are undoubtedly of importance, it is perhaps of most practical value to establish the optimal amount of carbohydrate to ingest during recovery. The finding that carbohydrate supplementation of any substantial magnitude during recovery can stimulate far greater rates of muscle glycogen

resynthesis than when no carbohydrate at all is ingested has been well established (Casey *et al.*, 2000; Ivy *et al.*, 1988b; Tarnopolsky *et al.*, 1997; Van Hall *et al.*, 2000b). What is less clear is the precise effect of increasing carbohydrate intake on subsequent glycogen storage rates, particularly with reference to the factors which may limit the rate of muscle glycogen resynthesis when large quantities of carbohydrate are ingested. However, it is likely that the difficulty in ascertaining the smallest quantity of carbohydrate necessary to maximise muscle glycogen resynthesis is at least partially due to the confounding influence of the many factors discussed above regarding the timing and type of carbohydrate ingested and, most importantly, the degree of prior depletion.

It was initially suggested by Blom *et al.* (1987) that ingesting $0.35 \text{ g CHO}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$ might be sufficient to maximise the rate of muscle glycogen resynthesis following exhaustive cycling. This opinion was based on these authors' finding that doubling the rate of carbohydrate ingestion from $\approx 0.18\text{-}0.35 \text{ g CHO}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$ resulted in a linear increase in muscle glycogen resynthesis during recovery ($r = 0.99$) but with no further elevation in glycogen storage when carbohydrate intake was increased to $0.7 \text{ g CHO}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$ (Blom *et al.*, 1987). Even when the carbohydrate ingestion rate has been further raised from $0.75\text{-}1.5 \text{ g CHO}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$, glycogen synthetic rate has been unaffected (Ivy *et al.*, 1988b). In retrospect, however, the rate of glycogen resynthesis reported by Blom *et al.* (1987) in response to $0.35 \text{ g CHO}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$ was rather high ($\approx 25 \text{ mmol glucosyl units}\cdot\text{kg dry mass}^{-1}\cdot\text{h}^{-1}$), possibly as a result of lower absolute glycogen concentrations at the beginning of recovery in this trial. As discussed in section 2.3.2.1, the degree of glycogen depletion is thought to be the most powerful determinant of glycogen resynthesis (Price *et al.*, 2000). Furthermore, it is likely that the plateau in muscle glycogen resynthesis with increasing carbohydrate intake in the studies of Blom *et al.* (1987) and Ivy *et al.* (1988) was also due to the relative infrequency of feedings in these studies (i.e. 2 h intervals) rather than attainment of the optimal carbohydrate ingestion rate (see section 2.4.1).

In support of this contention, subsequent research has demonstrated that ingesting $\geq 1 \text{ g CHO}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$ at more frequent intervals (i.e. 15-30 min) during a short-term recovery can elevate the rate of muscle glycogen resynthesis above that

previously proposed to be maximal following lower carbohydrate ingestion rates (Doyle *et al.*, 1993; Jentjens *et al.*, 2001; Piehl Aulin *et al.*, 2000; van Loon *et al.*, 2000a). A prime example of such evidence is the investigation of van Loon *et al.* (2000a), which showed that an increase in carbohydrate ingestion rate from 0.8-1.2 g CHO·kg⁻¹·h⁻¹ can in fact increase the rate of muscle glycogen resynthesis from ≈17 mmol glucosyl units·kg dry mass⁻¹·h⁻¹ up to ≈45 mmol glucosyl units·kg dry mass⁻¹·h⁻¹ when supplements are provided every 30 min during recovery. On the basis of this finding, it has been suggested that the carbohydrate ingestion rate implemented by these authors (i.e. 1.2 g CHO·kg⁻¹·h⁻¹) may have achieved the maximal possible rate of muscle glycogen synthesis in response to oral carbohydrate intake (Jentjens and Jeukendrup, 2003).

The above suggestion from Jentjens and Jeukendrup (2003) is supported when the majority of available literature regarding carbohydrate ingestion and muscle glycogen resynthesis during short-term recovery is examined collectively (Figure 2.5). All the studies presented in Figure 2.5 involve supplementation of carbohydrate in isolation over recovery durations ranging from 2-6 h and a positive correlation is clearly apparent between the 2 variables ($r = 0.65$; $P < 0.01$). The protocols adopted across this range of studies varied greatly with respect to a number of factors that are known to impact upon muscle glycogen resynthesis, in particular, the muscle glycogen concentrations at the start of recovery ranged from ≈16-260 mmol glucosyl units·kg dry mass⁻¹ (Price *et al.*, 2000; Tsintzas *et al.*, 2003). Given the effective autoregulation of muscle glycogen that was described in section 2.3.2.1, it is highly likely that the large variation in terms of glycogen resynthesis at any given rate of carbohydrate ingestion is mainly the result of these differences at the beginning of recovery. Interestingly, when the studies displayed in Figure 2.5 are streamed according to the absolute degree of depletion prior to recovery (see trendlines), it is apparent that the capacity of glycogen availability to mediate the glycogen storage may be eradicated at higher rates of carbohydrate intake (i.e. >1.4 g CHO·kg⁻¹·h⁻¹). Of primary importance, however, is that the general dose-response relationship between carbohydrate intake and glycogen resynthesis appears to be limited at a maximal rate of ≈45 mmol glucosyl units·kg dry mass⁻¹·h⁻¹, a rate which can be accomplished by ingesting 1.2 g CHO·kg⁻¹·h⁻¹ (van Loon *et al.*, 2000a).

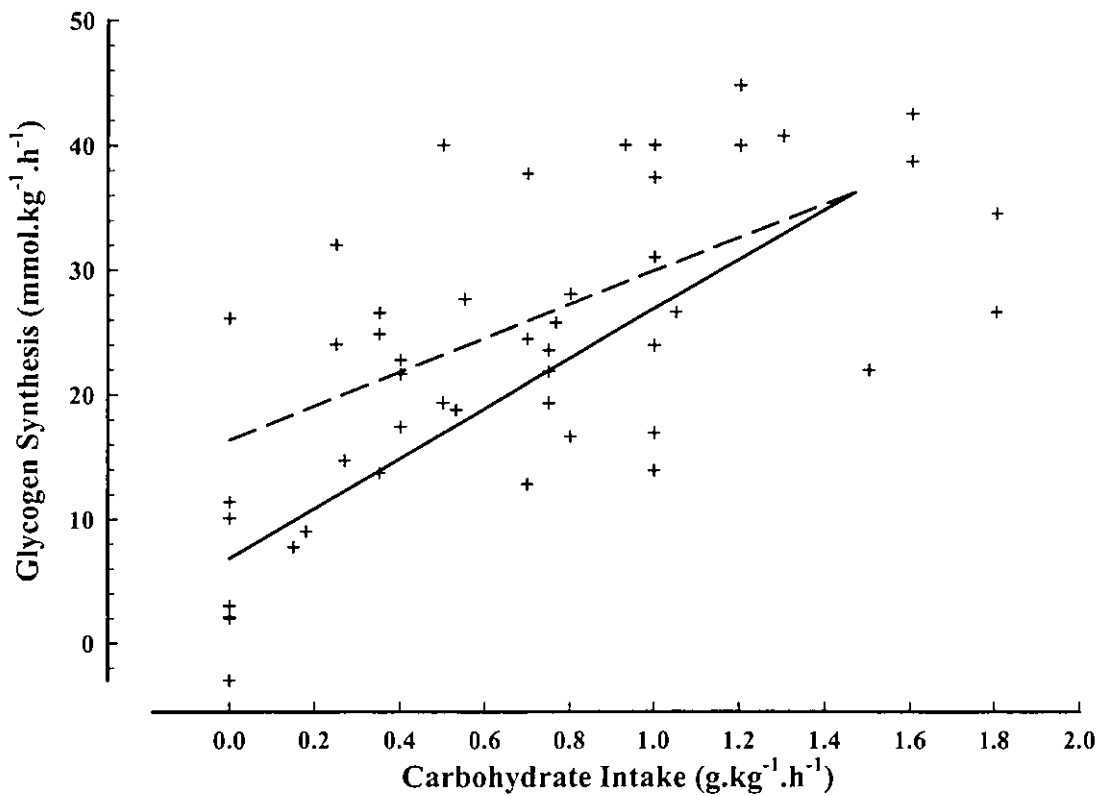


Figure 2.5: Reported rates of muscle glycogen resynthesis during 2-6 h recoveries across 25 different studies involving varying rates of carbohydrate intake. The solid trendline denotes the correlation coefficient ($r = 0.76$; $P < 0.01$) for the 22 data points which represent the upper 50% of muscle glycogen concentrations prior to recovery (Blom *et al.*, 1987; Carrithers *et al.*, 2000; Doyle *et al.*, 1993; Ivy *et al.*, 1988a; Ivy *et al.*, 1988b; McCoy *et al.*, 1996; Reed *et al.*, 1989; Roy and Tarnopolsky, 1998; Tarnopolsky *et al.*, 1997; Tsintzas *et al.*, 2003; Van Hall *et al.*, 2000a; van Loon *et al.*, 2000a; Yaspelkis and Ivy, 1999; Zawadzki *et al.*, 1992), while the broken trendline denotes the correlation coefficient ($r = 0.69$; $P < 0.01$) for the 22 data points which represent the lower 50% of muscle glycogen concentrations prior to recovery (Blom, 1989; Blom *et al.*, 1987; Bowtell *et al.*, 2000, 1999; Casey *et al.*, 2000; Casey *et al.*, 1995; Jentjens *et al.*, 2001; Maehlum *et al.*, 1978; Piehl Aulin *et al.*, 2000; Price *et al.*, 2000; Reed *et al.*, 1989; Van Hall *et al.*, 2000b). The study of Zachwieja *et al.* (1991) is represented as data points but does not contribute to either trendline since no absolute muscle glycogen concentrations were reported in this study (Zachwieja *et al.*, 1991).

The relationship shown in Figure 2.5 together with the proposed association between pre-exercise muscle glycogen availability and exercise capacity (Bergstrom *et al.*, 1967) may account for the enhanced recovery of endurance running capacity when $0.5 \text{ g CHO}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$ is ingested during a 4 h recovery in comparison with ingestion of a placebo (Fallowfield *et al.*, 1995). Given these same correlations, however, it is puzzling why increasing the carbohydrate dose from $0.5\text{-}1.5 \text{ g CHO}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$ did not result in a further enhancement of exercise capacity (Fallowfield and Williams, 1997). It is possible that the rate of muscle glycogen resynthesis may have been limited by the frequency of ingestion as in the earlier studies of Blom *et al.* (1987) and Ivy *et al.* (1988b) since the carbohydrate solutions in the studies of Fallowfield *et al.* were only provided at 2 h intervals during recovery. However, ensuing investigations have fed carbohydrate at 30 min intervals during recovery and supported the findings of Fallowfield *et al.* (1997) in that subsequent endurance capacity at $70\% \dot{V}O_2 \text{ max}$ was not different between solutions providing either 0.2 or $0.6 \text{ g CHO}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$ (Wong and Williams, 2000). These findings remain somewhat surprising since muscle glycogen resynthesis has been shown to be different between treatments when this experiment has been repeated using muscle biopsies to quantify glycogen content directly (Tsintzas *et al.*, 2003). The fact that restoration of exercise capacity has not been found to reflect muscle glycogen availability following recovery suggests either that factors other than muscle glycogen depletion contributed to fatigue or that the exercise capacity tests employed in these studies were not sufficiently sensitive to detect less substantial differences in muscle glycogen concentration (Fallowfield and Williams, 1997; Fallowfield *et al.*, 1995; Wong and Williams, 2000). Therefore, it remains to be established whether a more complete recovery of exercise capacity can be accomplished through increasing the amount of carbohydrate that is ingested during recovery.

The rate of muscle glycogen resynthesis can be limited to the proposed maximal rate of $\approx 45 \text{ mmol glucosyl units}\cdot\text{kg dry mass}^{-1}\cdot\text{h}^{-1}$ by a number of factors between the moment a large volume of exogenous carbohydrate is ingested to the eventual conversion of the glucose into glycogen within muscle. As was discussed in relation to solid versus liquid carbohydrate feedings in section 2.4.2, while consumption of large quantities of carbohydrate can delay carbohydrate storage when

ingested in conjunction with solid non-carbohydrate sources (Berry *et al.*, 2002; Jentjens and Jeukendrup, 2003), it is not thought that gastric emptying is likely to limit the rate of muscle glycogen resynthesis under most circumstances (Moodley *et al.*, 1992; Reed *et al.*, 1989; Rehrer *et al.*, 1992). In contrast, the rate of intestinal absorption may well impose a limitation to the maximal rate of muscle glycogen storage since infusion of a glucose solution directly into the duodenum has revealed intestinal absorption of glucose to be almost maximal at a rate of just $\approx 1.3 \text{ g}\cdot\text{min}^{-1}$ (Duchman *et al.*, 1997). However, more recent findings showing that rate of exogenous carbohydrate oxidation can approach $1.75 \text{ g}\cdot\text{min}^{-1}$ during exercise argues that intestinal absorption of glucose can in fact exceed $1.3 \text{ g}\cdot\text{min}^{-1}$ (Jentjens and Jeukendrup, 2005), but possibly only when mixed glucose and fructose are ingested (Ferraris, 2001; Jentjens *et al.*, 2004) or when supplements are provided immediately following exercise (Rose *et al.*, 2001). Overall, it is likely that muscle glycogen resynthesis is at least partly limited at some stage of gastrointestinal transit since arterial infusion of glucose and insulin over an 8 h recovery from exercise has been found to elicit extremely high muscle glycogen storage rates ($\approx 98 \text{ mmol glucosyl units}\cdot\text{kg dry mass}^{-1}\cdot\text{h}^{-1}$) in comparison with carbohydrate ingestion (Hansen *et al.*, 1999).

Interestingly, the above study from Hansen *et al.* (1999) also indicated that, in addition to the restriction imposed by intestinal transport, the rate of muscle glycogen accumulation may be further limited by the transport capacity of exercised muscle. This is because only $\approx 30\%$ of the glucose infused during recovery was actually taken up by muscle, despite glucose and insulin concentrations being elevated to $\approx 22 \text{ mmol}\cdot\text{l}^{-1}$ and $\approx 11.4 \text{ nmol}\cdot\text{l}^{-1}$, respectively (Hansen *et al.*, 1999). In agreement, a study from Bowtell *et al.* (2000) assessed the proportion of ingested carbohydrate that is directed towards muscle glycogen synthesis and concluded that storage of glucose within previously exercised muscles accounted for less than one-third of total carbohydrate storage (Bowtell *et al.*, 2000). Given that the maximal rate of muscle glycogen storage from oral glucose intake during recovery is thought to be only $\approx 0.3 \text{ g}\cdot\text{min}^{-1}$, the remaining glucose that appears from the gastrointestinal tract would be taken up by other tissues and either oxidised or stored (Jentjens and Jeukendrup, 2003). This latter possibility is certainly supported by the studies cited earlier from

Casey *et al.* (2000) and Terjung *et al.* (1974) in which the predominant route for carbohydrate disposal during recovery was storage in the form of liver glycogen (Casey *et al.*, 2000).

The finding that transport capacity of previously exercised muscle can restrict the rate of muscle glycogen resynthesis during recovery raises a number of interesting possibilities in terms of nutritional intervention following exercise. Specifically, in the study of Reed *et al.* (1989) there were no differences in the rate of muscle glycogen storage when glucose was infused intravenously rather than being ingested during recovery. This was surprising since the peak blood glucose concentrations during recovery were almost twice as high following infusion as opposed to ingestion, although perhaps most relevant is the fact that insulin concentrations were not different between treatments (Reed *et al.*, 1989). In contrast, the remarkably high rates of glycogen resynthesis observed by Hansen *et al.* (1999) were achieved through the application of both hyperglycaemic *and* hyperinsulinaemic clamps (Hansen *et al.*, 1999). Taken together, these observations have led to the suggestion that insulin concentration may limit the rate of carbohydrate storage in situations where glucose availability is adequate (Ivy, 2001). As discussed at length in sub-sections 2.3.2.2b and 2.3.2.3b, insulin functions to facilitate both the uptake of glucose into muscle and its subsequent conversion into glycogen. Moreover, evidence has demonstrated that the latter of these processes may require insulin concentrations $>340 \text{ pmol}\cdot\text{l}^{-1}$ before the glucose that is transported into muscle is stored rather than oxidised (Young *et al.*, 1988). Given these findings it becomes understandable why a number of investigators have explored the possibility that increasing the insulinaemic response to a carbohydrate solution may promote a greater rate of muscle glycogen resynthesis during recovery.

2.5 Influence of Carbohydrate-Protein Mixtures on Recovery

2.5.1 Insulinaemic Response to Amino Acid Ingestion

It has been known for a relatively long time that pancreatic insulin secretion can be induced both through intravenous infusion or ingestion of certain amino acids (Floyd *et al.*, 1966; Rabinowitz *et al.*, 1966). More importantly, it has been shown that when amino acids or proteins are provided in conjunction with carbohydrate, a synergistic influence on insulin release can occur (Floyd *et al.*, 1970a, 1970b; Rabinowitz *et al.*, 1966). More recent research by van Loon *et al.* (2000b) has examined the specific magnitude of insulinaemic responses following ingestion of 10 different amino acid/protein mixtures when ingested alongside carbohydrate. The results obtained from this investigation suggested that the insulinaemic response to a carbohydrate-protein (CHO-PRO) mixture will be strongly dependent on the amounts of leucine, phenylalanine and tyrosine that are included in the medium (van Loon *et al.*, 2000b). Interestingly, despite the fact that arginine is known to be highly insulinotropic when infused intravenously (Floyd *et al.*, 1966), evidence contests that arginine may not be an effective means of elevating circulating insulin when provided orally (Gannon *et al.*, 2002; Robinson *et al.*, 2003), especially in light of the gastrointestinal discomfort that is associated with ingestion of this amino acid (van Loon *et al.*, 2000b). Finally, the study of van Loon *et al.* (2000b) has also indicated that ingestion of protein hydrolysates may increase circulating amino acid concentrations more effectively than ingestion of intact casein. Indeed, subsequent research by these authors has now confirmed that coingestion of carbohydrate, protein hydrolysate, leucine and phenylalanine provides an effective means of increasing plasma insulin concentrations during a 3 h recovery (van Loon *et al.*, 2000c).

In addition to findings regarding the most effective mixture of amino acids to ingest during recovery, the latter study cited above from van Loon *et al.* also found that a greater insulinaemic response can be achieved through increasing the amount of protein in a given CHO-PRO mixture from 0.2-0.4 g·kg⁻¹·h⁻¹ (van Loon *et al.*, 2000c). While no significant dose-response relationship between protein intake and insulin release was reported in an earlier study which assessed a range of protein intakes alongside carbohydrate, an inverse relationship between elevations in plasma glucose

and protein intake was apparent (Spiller *et al.*, 1987). However, it cannot be established from this data whether the lower blood glucose concentrations at higher rates of protein intake were the result of increased glucose uptake or to a reduced appearance of glucose due to a delayed rate of gastric emptying (Thomas, 1957). Nonetheless, the likelihood is that CHO-PRO ingestion will be most effective in elevating circulating insulin when the protein component is ingested at rates in excess of $\approx 0.3 \text{ g}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$. When the available evidence reporting insulinaemic responses to CHO-PRO ingestion is compiled, it is apparent that those studies reporting insulin concentrations to be enhanced following the addition of protein to a carbohydrate solution have all included between $0.3\text{-}0.5 \text{ g PRO}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$ (Jentjens *et al.*, 2001; Van Hall *et al.*, 2000a; Van Hall *et al.*, 2000b; van Loon *et al.*, 2000a; van Loon *et al.*, 2000c; Zawadzki *et al.*, 1992). Conversely, those studies that have not reported a significant enhancement of insulinaemic response following ingestion of CHO-PRO rather than carbohydrate alone have tended to provide protein in quantities closer to $0.1 \text{ g}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$ (Carrithers *et al.*, 2000; Ivy *et al.*, 2002; Ivy *et al.*, 2003; Tamopolsky *et al.*, 1997). Furthermore, similar to the findings of Spiller *et al.* (1987), 7 out of the 10 investigations cited above have reported significantly lower blood glucose concentrations following ingestion of CHO-PRO rather than carbohydrate alone (Carrithers *et al.*, 2000; Ivy *et al.*, 2002; Ivy *et al.*, 2003; Van Hall *et al.*, 2000a; Van Hall *et al.*, 2000b; van Loon *et al.*, 2000a; Zawadzki *et al.*, 1992). It should also be noted that 2 of the 3 remaining studies also observed a reduced glycaemic response when protein was added but that this difference did not reach statistical significance (Tamopolsky *et al.*, 1997; van Loon *et al.*, 2000c), probably due to the diminished statistical power associated with the multi-factorial designs that were applied in these experiments (Atkinson, 2002). Again, however, it is difficult to determine whether these attenuated elevations in blood glucose concentration are the consequence of decreased glucose appearance or increased glucose uptake, although the study of Van Hall *et al.* (2000b) appears to support the former explanation (Van Hall *et al.*, 2000b). This evidence notwithstanding, it cannot be entirely discounted that the synergistic influence of carbohydrate and protein on insulin secretion might increase glucose uptake and thus facilitate muscle glycogen storage during recovery, a possibility that is addressed in section 2.5.3.

2.5.2 Carbohydrate-Protein Mixtures and Protein Synthesis

Before moving on to discuss the potential influence of CHO-PRO mixtures on muscle glycogen resynthesis, it is appropriate at this juncture to consider the influence of such solutions on protein balance in the post-exercise period. Evidence gathered using rats has demonstrated that prolonged treadmill running can reduce post-exercise muscle protein synthesis by 18% (Anthony *et al.*, 1999) and increase non-myofibrillar protein degradation by 37% (Kasperek and Snider, 1989) in comparison with resting levels. As a result, it has been suggested that a transient period of negative protein balance occurs during recovery from such exercise (Rennie and Tipton, 2000). Importantly, it has now been well established that ingestion of amino acids following exercise can encourage a transition from net protein breakdown to net protein synthesis (Biolo *et al.*, 1997; Borsheim *et al.*, 2002; Tipton *et al.*, 2004; Tipton *et al.*, 1999). It is of further relevance that this effect has also been observed when carbohydrate and protein are ingested together either during or following various forms of exercise (Koopman *et al.*, 2004; Koopman *et al.*, 2005; Rasmussen *et al.*, 2000). Amino acid ingestion will promote anabolism both via direct stimulation of net protein synthesis by amino acids such as leucine (Anthony *et al.*, 1999; Anthony *et al.*, 2002; Rennie, 2005) and through stimulating the release of anabolic hormones such as insulin, insulin-like growth factor (IGF)-I and growth hormone (Balon *et al.*, 1990; Chromiak and Antonio, 2002; Fryburg *et al.*, 1995). The insulin independent pathways involving leucine, IGF-I and growth hormone are thought to be responsible for stimulation of protein synthesis, while the insulin dependent pathway improves protein balance solely by inhibition of proteolysis (Fryburg *et al.*, 1995; Vary *et al.*, 2005). This synergism between insulin and amino acids advocates the combined ingestion of carbohydrate plus protein during recovery to induce a more favourable influence on muscle protein synthesis than when either nutrient is consumed in isolation (Roy *et al.*, 1997; Tipton and Wolfe, 2004).

The considerations regarding how much protein is necessary to achieve this benefit are relatively uncomplicated in comparison with the earlier discussion regarding carbohydrate ingestion during recovery. No dose dependent effect was apparent regarding the extent of protein synthesis when the rate of essential amino acid ingestion has been doubled from $\approx 0.07-0.14 \text{ g}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$ during a 4.5 h recovery

(Tipton *et al.*, 1999) and considerable increments in muscle protein synthesis have been achieved with ingestion of far smaller quantities of essential amino acids ($\approx 0.04 \text{ g}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$) over a similar duration of recovery (Borsheim *et al.*, 2002). It is known that this sensitivity is at least partially due to the fact that the insulin mediated suppression of proteolysis is maximal even with relatively low doses of amino acids (Louard *et al.*, 1992). The finding that protein synthesis is highly sensitive to insulin may explain why, similar to muscle glycogen storage, a more rapid accretion of muscle protein occurs if CHO-PRO supplements are provided immediately after exercise rather than being delayed by 3 h when insulin sensitivity would have diminished (Levenhagen *et al.*, 2001). However, this effect was not evident in an earlier study in which CHO-PRO solutions were ingested at either 1 h or 3 h post-exercise and no difference in net muscle protein balance was identified (Rasmussen *et al.*, 2000). Perhaps more relevant in terms of supplement timing in relation to amino acid ingestion is the actual sequence of exercise and ingestion. To be precise, it has been established that a superior rate of net muscle protein synthesis occurs when a CHO-PRO mixture is consumed before as opposed to after resistance exercise, with this effect being attributed to an increased delivery of amino acids to exercising tissue during muscular contraction (Tipton *et al.*, 2001). It is therefore reasonable to suggest that an additional benefit of ingesting CHO-PRO during recovery could be to prime the musculo-skeletal system with an increased availability of amino acids in preparation for the succeeding recovery from a repeated performance or even just to maintain a higher habitual intake of protein to facilitate training adaptation (Bolster *et al.*, 2005).

Aside from the obvious positive influence of protein accretion in terms of muscular hypertrophy, the anabolic stimulus provided by ingestion of amino acids also has the potential to impart a number of associated benefits. For example, if the exercise bout that was performed prior to recovery involved a large component of eccentric muscle contractions, then it is likely that a substantial amount of damage to muscle fibres will have occurred as a consequence (Armstrong *et al.*, 1983; Cleak and Eston, 1992; Eston *et al.*, 1995). This mechanical tissue injury is often associated with the appearance of intracellular proteins and enzymes in the circulation due to membrane disruption (Armstrong, 1984; Friden and Lieber, 2001) which subsequently leads to secondary oxidative stress, as evidenced by increased

concentrations of thiobarbituric acid reactive substrates (T-BARS), malondialdehyde and lipid hydroperoxide (Alessio, 1993; Dekkers *et al.*, 1996; Pyne, 1994). In relation to CHO-PRO ingestion, it has been suggested by some authors that the ingestion of carbohydrate coincident with protein may maintain cell membrane integrity more effectively than ingestion of either a placebo (Seifert *et al.*, 2005) or just carbohydrate in isolation (Ready *et al.*, 1999). This suggestion is supported by studies in which the post-exercise concentration of plasma creatine kinase (an ordinarily intracellular enzyme) has been attenuated when protein was included in a standard carbohydrate solution (Ready *et al.*, 1999; Saunders *et al.*, 2004). Further support comes from a study in which the ingestion of CHO-PRO resulted in a decreased appearance of certain biomarkers that are associated with oxidative stress (Williams *et al.*, 1999). On the other hand, another study from Wojcik *et al.* (2001) reported that markers of muscle damage and inflammation following exercise were not alleviated any more effectively when CHO-PRO was ingested following exercise than when carbohydrate alone was ingested. It is possible, however, that the prior exercise bout in this latter study did not induce a sufficient degree of muscle damage to identify the beneficial effects of the additional protein (Wojcik *et al.*, 2001). From a practical perspective, it has been demonstrated that this potential impact of post-exercise CHO-PRO ingestion in terms of muscle damage may be translated into reduced sensations of muscular soreness when consumed daily over ≈ 8 weeks of basic training in a large cohort of marine recruits (Flakoll *et al.*, 2004).

It was also observed in the above study involving marine recruits that those individuals who consumed the CHO-PRO mixture required 33% fewer visits to the medical clinic than those individuals who consumed either placebo or carbohydrate alone (Flakoll *et al.*, 2004). Prolonged exercise is known to depress immune cell function (Gleeson *et al.*, 2004) and proteolysis of the gastrointestinal tract following exercise may compound this effect through an increased translocation of gut bacteria (Flakoll *et al.*, 2004; Halseth *et al.*, 1997). It is therefore possible that the reduced incidence of bacterial/viral infections reported by Flakoll *et al.* (2004) was a direct result of decreased gut catabolism arising from the ingestion of amino acids (Hamada *et al.*, 1999).

The studies presented throughout this section provide clear evidence that including amino acids in a post-exercise carbohydrate supplement may provide a number of benefits associated with the stimulation of protein synthesis. However, it is unlikely that any of these benefits would be of value when the primary focus of nutritional supplementation is to restore functional capacity as rapidly as possible following prolonged exercise. In such circumstances, a more desirable outcome would be to accelerate the rate at which carbohydrate is stored during recovery and the following section will discuss how effectively CHO-PRO supplements might achieve this aim.

2.5.3 Carbohydrate-Protein Mixtures and Carbohydrate Storage

As was addressed in section 2.5.1, it is thought that the greatest insulinaemic response will be achieved when the protein fraction of a CHO-PRO mixture is composed of protein hydrolysate along with certain essential amino acids (van Loon *et al.*, 2000b). The addition of individual amino acids such as leucine, glutamine or arginine to a carbohydrate supplement has not been found to substantially increase circulating insulin concentrations and, while adding leucine to carbohydrate has been shown to enhance muscle glycogen storage in rats (Anthony *et al.*, 1999), ingestion of either glutamine or arginine has failed to accelerate muscle glycogen accumulation during recovery in humans (Van Hall *et al.*, 2000a; Yaspelkis and Ivy, 1999). It cannot be ruled out, however, that amino acids such as glutamine might be deaminated and converted into muscle glycogen directly rather than promoting glycogenesis from glucose (Varnier *et al.*, 1995). In contrast, a number of studies have reported that the augmented insulin concentrations when mixed amino acids have been added to carbohydrate recovery solution can in fact increase the rate of muscle glycogen resynthesis following exercise (Fogt and Ivy, 2000; van Loon *et al.*, 2000a; Williams *et al.*, 2003; Zawadzki *et al.*, 1992). However, in 2 of these studies the CHO-PRO mixture was assessed in relation to a carbohydrate solution which was not matched for carbohydrate content, which makes it difficult to determine if the glycogenic effect of the CHO-PRO solution was due to the amino acids or to the additional carbohydrate (Fogt and Ivy, 2000; Williams *et al.*, 2003). Of the other studies cited above, the study from Zawadzki *et al.* (1992) examined whether ingesting $\approx 0.8 \text{ g CHO}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$ plus an additional $\approx 0.3 \text{ g}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$ of protein at 2 h

intervals during a 4 h recovery from prolonged exercise would result in a faster rate of muscle glycogen storage than when ingesting the carbohydrate fraction alone. Although the absolute concentrations of muscle glycogen were not different between trials by the end of recovery in this study, the rate at which glycogen was stored was 38% greater when protein was included in the recovery solution (Zawadzki *et al.*, 1992). Again, however, it cannot be established whether the enhanced rate of glycogenesis reported by Zawadzki *et al.* (1992) was purely a result of the increased insulin response to the CHO-PRO solution or a consequence of the 43% increase in energy provision when protein was added. Other authors have subsequently demonstrated that the energy content of a supplement can enhance the rate of muscle glycogen storage irrespective of macronutrient composition (Roy and Tarnopolsky, 1998). Therefore, the only remaining study to show an increase in the rate of muscle glycogen resynthesis following amino acid induced hyperinsulinaemia is that of van Loon *et al.* (2000a). In this study a CHO-PRO solution providing $0.8 \text{ g}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$ of carbohydrate and $0.4 \text{ g}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$ of protein was ingested at 30 min intervals during a 4 h recovery from exhaustive cycling. Fortunately, the effectiveness of the CHO-PRO solution was evaluated in comparison with 2 separate carbohydrate solutions: 1 matched for carbohydrate content and the other for total energy content. In this way it was possible to determine that the rate of muscle glycogen accumulation can be increased equally effectively when either amino acids or additional carbohydrate are added to an existing recovery solution that provides carbohydrate in sub-optimal quantities (van Loon *et al.*, 2000a).

There are also a similar number of investigations that have observed the increase in insulinaemic response when mixed amino acids have been added to a standard carbohydrate solution but have reported no concomitant increase in the rate of muscle glycogen storage during recovery (Jentjens *et al.*, 2001; Van Hall *et al.*, 2000a; Van Hall *et al.*, 2000b). The studies of Jentjens *et al.* (2001) and Van Hall *et al.* (2000b) assessed whether the proposed 'maximal' rate of muscle glycogen resynthesis in response to ingesting $\approx 1.2 \text{ g}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$ of carbohydrate could be exceeded if additional protein, rather than additional carbohydrate, was ingested during a 3-4 h recovery. It was concluded by both authors that the additional protein did not further increase the rate of carbohydrate storage during recovery despite a significant enhancement of insulin release (Jentjens *et al.*, 2001; Van Hall *et al.*, 2000b). It

appears that the important distinction between these studies and those cited previously, in which muscle glycogen resynthesis was accelerated, is the precise quantity of carbohydrate to which the protein was added. When plotted graphically (Figure 2.6), it becomes apparent that those studies which have provided ≥ 1 g CHO \cdot kg $^{-1}\cdot$ h $^{-1}$ have not observed any increase in muscle glycogen resynthesis when amino acids were added (Jentjens *et al.*, 2001; Van Hall *et al.*, 2000b). Conversely, muscle glycogen resynthesis has more commonly been accelerated when amino acids have been added to matched quantities of carbohydrate providing ≤ 0.8 g CHO \cdot kg $^{-1}\cdot$ h $^{-1}$ (Ivy *et al.*, 2002; van Loon *et al.*, 2000a; Zawadzki *et al.*, 1992). The only study which does not conform with this explanation is that of Van Hall *et al.* (2000a), who concluded that adding protein to 0.8 g CHO \cdot kg $^{-1}\cdot$ h $^{-1}$ did not significantly increase the rate of muscle glycogen resynthesis over the course of a 3 h recovery. However, it is clearly illustrated in Figure 2.6 that the differences in muscle glycogen resynthesis reported by these authors (represented as circular data points) are meaningful even if not statistically significant (Van Hall *et al.*, 2000a).

In combination with the fact that the rate of muscle glycogen resynthesis appears to be maximal following ingestion of 1.2 g CHO \cdot kg $^{-1}\cdot$ h $^{-1}$ alone (see Figure 2.5), the above findings have led some authors to suggest that regular ingestion of relatively high doses of carbohydrate during recovery will maximally stimulate glucose uptake such that further increases in insulin concentration as a result of added amino acids are obsolete (Carrithers *et al.*, 2000; Jentjens *et al.*, 2001; van Loon *et al.*, 2000a). Nonetheless, the addition of protein to a carbohydrate solution can potentially increase the rate of muscle glycogen storage in circumstances where insulin is likely to limit the rate of muscle glycogen resynthesis, i.e. when insufficient carbohydrate is consumed to maximise glucose transport.

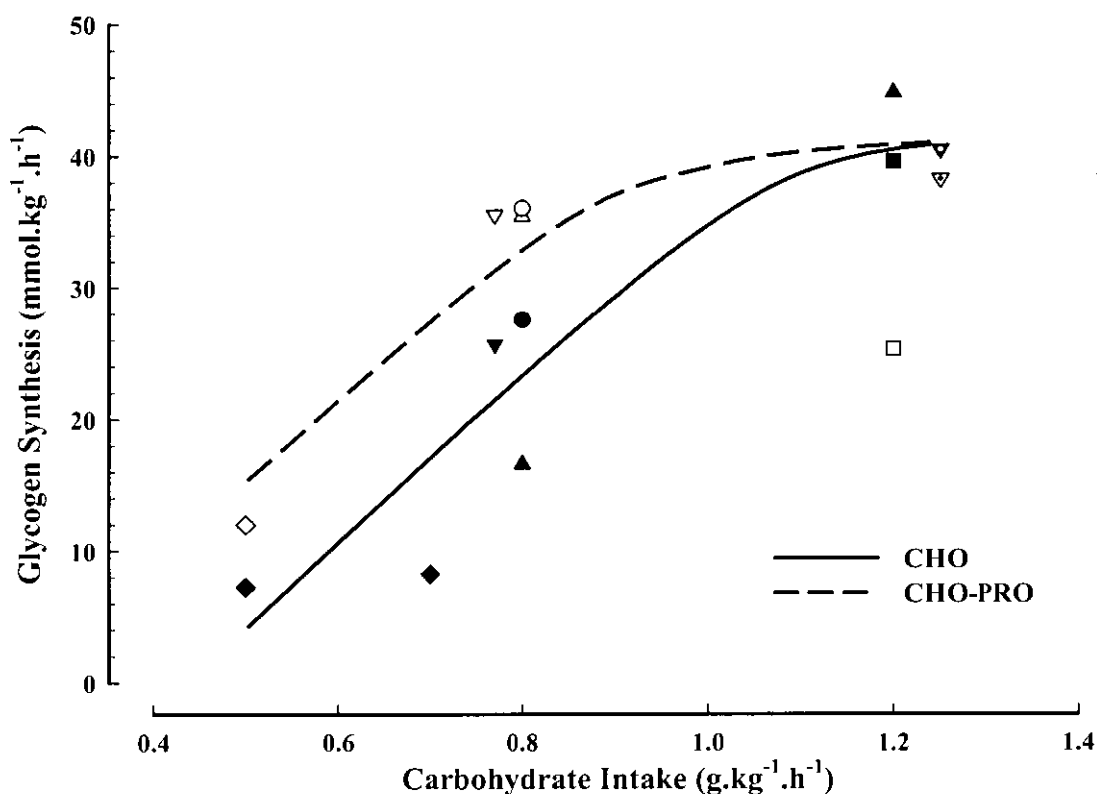


Figure 2.6: Reported rates of muscle glycogen resynthesis during 3-5 h recoveries across 6 different studies involving varying rates of carbohydrate intake both with and without mixed amino acids. Each study is represented by a different symbol with filled data points representing carbohydrate alone and open data points representing carbohydrate plus protein (Ivy *et al.*, 2002; Jentjens *et al.*, 2001; Van Hall *et al.*, 2000a; Van Hall *et al.*, 2000b; van Loon *et al.*, 2000a; Zawadzki *et al.*, 1992). The trendlines denote the suggested patterns of muscle glycogen resynthesis with each treatment based on all 6 studies (N.B: the apparent treatment effect in the study of Jentjens *et al.* (2001), represented as squares, is a product of large inter-individual variation during the exercise induced component of glycogen resynthesis and is not statistically significant).

As discussed in section 2.5.1, it is likely that ingestion of $\geq 0.3 \text{ g}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$ of protein is necessary to achieve the synergistic effect of combined CHO-PRO ingestion on insulin secretion (van Loon *et al.*, 2000c). This finding would explain why some other investigators have failed to increase glycogen storage when adding less than this critical amount of protein to carbohydrate recovery solutions, since insulin stimulated glucose transport would not be expected to differ between trials (Carrithers *et al.*, 2000; Tarnopolsky *et al.*, 1997). However, a study by Ivy *et al.* (2002) has demonstrated that ingestion of just $\approx 0.2 \text{ g PRO}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$ in combination with $\approx 0.5 \text{ g CHO}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$ can increase the rate of muscle glycogen storage above that of either $\approx 0.5 \text{ g CHO}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$ alone or even an isoenergetic carbohydrate feeding of $\approx 0.7 \text{ g CHO}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$. Perhaps of most interest is the fact that this effect was not associated with any significant increase in insulin concentration (Ivy *et al.*, 2002), thus presenting the interesting possibility that enhanced insulin mediated glucose uptake may not be the only mechanism through which CHO-PRO ingestion can operate to increase carbohydrate storage. Indeed, it is highly possible that the protein fraction of a CHO-PRO solution might also be used as gluconeogenic substrate during recovery to provide additional glucose both for liver and muscle glycogen resynthesis (Butterfield, 1990).

In summary, it appears that the rate of muscle glycogen resynthesis during short-term recovery can be maximised either through ingesting $\geq 1 \text{ g CHO}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$ or through the ingestion of smaller quantities of carbohydrate in combination with mixed amino acids. The primary mechanism through which additional amino acids increase muscle glycogen storage is likely to be related to the synergistic influence of carbohydrate and protein on insulin secretion, especially when $\geq 0.3 \text{ g}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$ of amino acids are ingested. However, the possibility remains that the ingested amino acids may also provide an indirect substrate for synthesis of muscle glycogen through conversion into glucose by the liver. Regardless of the mechanism, the potential for amino acids to accelerate glycogen resynthesis when ingested alongside carbohydrate during recovery introduces the possibility that subsequent physical performance might also be enhanced. The final section of this chapter will present those studies which have examined the effectiveness of CHO-PRO ingestion in terms of restoring the capacity for physical exercise within 8 h of prior activity.

2.5.4 Carbohydrate-Protein Mixtures and Physical Performance

If the addition of amino acids to a carbohydrate recovery solution does in fact increase the rate of muscle glycogen resynthesis above that achieved through ingestion of carbohydrate alone, then it would seem reasonable to suggest that subsequent exercise capacity might also be improved given the proposed association between pre-exercise muscle glycogen availability and exercise capacity (Bergstrom *et al.*, 1967). Furthermore, the potential interaction of ingested amino acids with the liver might also be relevant in terms of short-term recovery since it has been suggested that resynthesis of liver glycogen might be another crucial element dictating subsequent performance (Casey *et al.*, 2000; Terjung *et al.*, 1974). Despite the above possibilities, however, there is a relative dearth of information relating to the influence of CHO-PRO mixtures on recovery of exercise capacity.

Tentative evidence supporting the efficacy of CHO-PRO ingestion during recovery can be derived from the recent study of Saunders *et al.* (2004). This study involved the ingestion of carbohydrate either with or without additional whey protein both during and after a prolonged bout of cycling to exhaustion at 75% $\dot{V}O_2$ max, followed 12-15 h later by another exercise capacity test at 85% $\dot{V}O_2$ max. Including protein in the solution was reported to increase cycle time to exhaustion by 29% during the first exercise test and by 40% during the second exercise test. However, while the 2 solutions provided in this study were matched for carbohydrate content, the inclusion of protein resulted in a 20% increment in total energy provision (Saunders *et al.*, 2004). Therefore, similar to much of the research on muscle glycogen resynthesis, it cannot be established whether the ergogenic benefit of the CHO-PRO solution was due to the increase in available energy or the additional protein *per se*. Regardless of this limitation, the above study clearly demonstrates that recovery over a more extended period can benefit from CHO-PRO ingestion and Saunders *et al.* (2004) suggest that this performance enhancing effect may be the result of improved protein synthesis and consequent repair of damaged tissue during recovery. Therefore, since muscular contraction during cycling is exclusively concentric, it is possible that even greater performance benefits might arise from CHO-PRO ingestion when other more damaging forms of exercise are performed. Nonetheless, it is less likely that any substantial repair of muscle tissue will occur

during a more short-term recovery, at least not sufficiently to impact upon physical performance within 8 h.

The study of Saunders *et al.* (2004) also warrants further discussion in relation to the observation that exercise capacity during the first exercise session was increased from 82.3 min up to 106.3 min when CHO-PRO rather than carbohydrate alone was consumed. The fact that physical performance was enhanced without any prior manipulation of muscle glycogen availability leads to the possibility that CHO-PRO might positively affect metabolism through other mechanisms. Further support for this contention comes from a study by Ivy *et al.* (2003) in which either CHO-PRO or a supplement matched for carbohydrate content was ingested during 3 h of variable intensity cycling (45-75% $\dot{V}O_2$ max), following which the exercise intensity was increased to 85% $\dot{V}O_2$ max until the point of fatigue. In agreement with Saunders *et al.* (2004), it was identified in the study of Ivy *et al.* (2003) that the CHO-PRO mixture improved time to fatigue above that of the carbohydrate matched solution by 7.2 min (36%). Importantly, there were no significant differences in insulin concentrations during the exercise performed in this study, despite the intermittent periods of low intensity activity, which would tend to argue against the possibility that muscle glycogen was spared in the CHO-PRO trial (Ivy *et al.*, 2003). Alternative explanations for the differences in exercise capacity between trials may therefore involve specific protein mediated mechanisms such as an increased central drive for exercise (Davis, 1995) or anaplerotic replenishment of TCA cycle intermediates (Ivy *et al.*, 2003; Wagenmakers *et al.*, 1990). The former of these hypotheses was addressed earlier in section 2.2.5 where it was concluded that the coingestion of glucose along with branched-chain amino acids was unlikely to improve performance through attenuated sensations of fatigue (Madsen *et al.*, 1996). Likewise, recent evidence (Dawson *et al.*, 2005; Gibala, 2003) has also challenged the latter hypothesis that amino acids might maintain TCA cycle flux during prolonged exercise (see section 2.2.1). However, even if these mechanisms can be discounted, the findings of Saunders *et al.* (2004) and Ivy *et al.* (2003) remain in their contention that an enhanced rate of muscle glycogen resynthesis during recovery might not be the only factor contributing to improved performance with CHO-PRO ingestion.

There is actually very little evidence to support the proposal that an enhanced rate of muscle glycogen resynthesis during recovery will result in an improved recovery of exercise capacity. It is known that the rate of muscle glycogen resynthesis will be very low if no carbohydrate is consumed during recovery (Ivy *et al.*, 1988b; Tarnopolsky *et al.*, 1997; Van Hall *et al.*, 2000b), yet increasing the rate of muscle glycogen storage through the provision of carbohydrate has not been consistently found to enhance subsequent exercise capacity (Casey *et al.*, 2000; Fallowfield *et al.*, 1995). However, it is possible that one of the above studies may not have provided sufficient carbohydrate to reveal an effect of carbohydrate ingestion on physical performance (Casey *et al.*, 2000). In summary, the apparent dose-response relationship between carbohydrate intake and muscle glycogen resynthesis has not yet been reflected in a similar relationship between carbohydrate intake and exercise capacity (Fallowfield and Williams, 1997; Wong and Williams, 2000).

However, there is an investigation which has demonstrated that an increased rate of muscle glycogen resynthesis during a short-term recovery will also improve exercise capacity during subsequent exercise (Williams *et al.*, 2003). The investigation of Williams *et al.* (2003) is also of particular relevance given that it is presently the only study to date which has investigated the effect of CHO-PRO ingestion during recovery on the capacity to exercise within 8 h of prior exertion. Specifically, in this investigation participants were required to cycle at 65-75% $\dot{V}O_2$ max for >105 min in order to deplete muscle glycogen stores and reduce blood glucose concentrations below 4.0 mmol·l⁻¹. Once glucose homeostasis had been sufficiently challenged, participants began a 4 h recovery during which they consumed either carbohydrate alone (0.15 g·kg⁻¹·h⁻¹) or a mixture of carbohydrate (0.40 g·kg⁻¹·h⁻¹) plus additional protein (0.10 g·kg⁻¹·h⁻¹). Perhaps unsurprisingly given the large differences in carbohydrate content, the CHO-PRO mixture resulted in a 92% greater insulinaemic response and 128% greater rate of muscle glycogen resynthesis than the solution which provided less carbohydrate with no amino acids. Of primary importance, however, was that participants were able to exercise for 55% longer (i.e. 20.0 versus 31.1 min) during a subsequent exercise capacity test at 85% $\dot{V}O_2$ max when CHO-PRO rather than carbohydrate alone was ingested during

recovery (Williams *et al.*, 2003). Despite the obvious bias towards the CHO-PRO solution both in terms of carbohydrate and total energy provision, this study provides convincing evidence that elevations in muscle glycogen resynthesis during recovery above that induced by a standard carbohydrate solution can actually be translated into an enhancement of exercise capacity.

What remains to be established is whether this enhancement of exercise capacity subsequent to recovery would also occur if a CHO-PRO solution was evaluated in comparison with a solution that was matched for either total carbohydrate or total energy content. Such a comparison would not be expected to induce differences in muscle glycogen resynthesis of the magnitude reported by Williams *et al.* (2003) but, in combination with the other potential mechanisms which might also facilitate performance, it remains a possibility that ingestion of carbohydrate along with amino acids during short-term recovery might restore exercise capacity more effectively than ingestion of carbohydrate alone. What follows is an account of a series of studies in which this possibility is explored and the potential mechanisms underlying any differences in physical performance are considered.

CHAPTER 3

General Methods

3.1 Introduction

This chapter provides an account of the generic methodologies that were implemented during the various studies which compose this thesis. Each of these investigations was conducted in the Exercise Physiology Laboratories of Loughborough University and received approval from the University Ethics Committee prior to participant recruitment. The target population for recruitment were male, non-smokers who included running as part of their habitual training. These participants were recruited via advertisements located around the university campus and tended to be undergraduate students aged between 18 & 25 years. After volunteering for any study, participants were fully briefed both verbally and in writing regarding what would be required of them for that particular study. Any volunteers who felt they could adequately fulfil these requirements were asked to give written informed consent and were made aware that they could withdraw from the procedures at any time without giving a reason. Participants completed a compulsory health screen questionnaire (Appendix Ai) and were excluded from the study if they reported any medical condition that either posed an undue personal risk or introduced bias into the investigation. Further health questionnaires were completed by each participant upon every visit to the laboratory to ensure that no deterioration in either well-being or commitment to the study had occurred over the course of testing (Appendix Aii).

3.2 Anthropometry

Post-void nude body mass was recorded as part of each participant's initial visit to the laboratory so that carbohydrate provision during subsequent main trials could be calculated relative to body mass. Body mass was also recorded before and after any exercise session to assess hydration status through percentage change in mass, this measurement was again made nude and only after participants had used a towel to remove excess moisture from the skin surface. All body mass measurements were made using a balance scale (Avery Ltd., UK) that had a capacity of 120 kg and was accurate to ± 0.05 kg.

Height was measured using a wall mounted stadiometer (Holtain Ltd., UK) that had a maximum range of 200 cm and was accurate to ± 0.01 cm. Each participant's height was recorded barefoot, with heels together and resting against the stadiometer. The moveable indicator was lowered until in contact with the superior point of the head while each participant inspired deeply. Both height and mass were then used to calculate body mass index ($BMI = \text{kg/m}^2$).

3.3 Preliminary Measurements

Prior to main trials, all participants were required to make 2 visits to the laboratory for preliminary testing. The first of these visits was used to assess each participant's sub-maximal and maximal oxygen uptakes (Taylor *et al.*, 1955) while the second was designed to familiarise participants with the experimental procedures. Exercise during all preliminary tests, along with main trials, was performed on a motorised treadmill (Technogym HC1200, Italy) that had been calibrated prior to each testing period to confirm the validity of the analogue speedometer and gradient display.

3.3.1 Sub-maximal and Maximal Oxygen Uptake

Participants' preliminary visit to the laboratory comprised 2 exercise tests. The first of these tests determined each participant's oxygen uptake ($\dot{V}O_2$) at a variety of sub-maximal running speeds. Typically this test was completed within 16 min, with the treadmill speed increasing at 4 min intervals such that a range of at least 4 intensities could be monitored. For most participants, treadmill speeds of 11, 12, 13 and 14 $\text{km}\cdot\text{h}^{-1}$ were sufficient to encompass all the relative exercise intensities employed during subsequent main trials. During the final minute of each 4 min stage, expired gas samples along with ratings of perceived exertion (Borg, 1973) and heart rates were recorded. Heart rates were recorded throughout all these investigations via short range telemetry (Polar 8810, Finland).

Approximately 30 min following the sub-maximal oxygen uptake test participants completed a second test to assess their maximal oxygen uptake ($\dot{V}O_{2\text{max}}$). This test involved incremental continuous treadmill running until the point of volitional exhaustion. The speed at which this test was conducted was dictated by the data recorded during the sub-maximal test and a speed eliciting approximately 85% of maximum heart rate was selected. Participants ran at this speed against a 3.5% gradient which was increased by 2.5% after every 3 min stage. One minute expired air samples, ratings of perceived exertion (Borg, 1973) and heart rates were measured in the final minute of each stage and also at the point of volitional exhaustion, defined as when the participant indicated that only 1 minute remained until fatigue. A number of criteria were applied to determine whether this endpoint was reflective of a valid $\dot{V}O_{2\text{max}}$ value, these were: attainment of age predicted maximal heart rate ($\pm 10 \text{ beats}\cdot\text{min}^{-1}$); a respiratory exchange ratio (RER) in excess of 1.15; and an increase in $\dot{V}O_2 \leq 5 \text{ ml}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ in response to an increased gradient. While progressive incline protocols may be less likely to fulfill all 3 of these criteria in comparison with level running protocols, the resultant $\dot{V}O_{2\text{max}}$ value is not thought to differ significantly between the 2 methods (St. Clair Gibson *et al.*, 1999).

3.3.2 Familiarisation

The sub-maximal and maximal oxygen uptake data obtained from the first preliminary tests were subjected to a simple linear regression against running speed to determine what speed would be required to elicit given relative exercise intensities. Participants' second visit to the laboratory was scheduled within 2 weeks of the first main trial and involved running at speeds eliciting 70% $\dot{V}O_2$ max for at least 60 min (an additional 15 min at 85% $\dot{V}O_2$ max was included in Chapters 4 and 5 due to the intensity of exercise performed during these investigations). This test provided confirmation that the calculated speeds corresponded to the proper relative exercise intensities (\pm 2-3%). An additional purpose of this visit was to familiarise all participants with the experimental procedures and apparatus.

3.4 Experimental Design

All studies reported in this thesis involved a cross over design that was fully randomised and counterbalanced. All main trials were separated by at least 1 week to avoid any carry-over effects and the interventions were applied in a double blind manner. Common to all these investigations was an overall main trial format involving a 90 min treadmill run at 70% $\dot{V}O_2$ max (R_1) followed by a 4 h recovery period, during which 8 experimental solutions were consumed at 30 min intervals beginning immediately after the initial run. The precise intensity and nature of the exercise session (R_2) which proceeded this recovery period was manipulated specifically according to the aims of each study and details of these protocols are included in the methods sections of each respective experimental chapter.

3.5 Physiological Measurements

3.5.1 Expired Gas Analysis

Participants were provided with a nose clip and a respiratory valve 45 s prior to any expired gas collection to clear all atmospheric air from the apparatus prior to sampling. The respiratory valve was attached via falconia tubing (Baxter, Woodhouse and Taylor, UK) to a 200 l Douglas bag (Harvard Apparatus, UK) for collection of expired gases (Williams and Nute, 1983). The relative gas fractions of oxygen and carbon dioxide were measured using paramagnetic and infra-red analysers, respectively (Servomex 1440, UK). These analysers were calibrated on the morning of each trial with a two point calibration using gas cylinders of known composition within the physiological range (O_2 0-16%; CO_2 0-4%) which had been previously checked for accuracy via gravimetric analysis (British Oxygen Company, UK). Each analyser was then validated against atmospheric air and was recalibrated at 4 h intervals during main trials. Expired gas volumes (\dot{V}_E) were determined while each Douglas bag was evacuated using a dry gas meter (Harvard Apparatus, UK) which was routinely checked against a precision 3 l calibration syringe (5530 Hans Rudolph Inc., USA). The temperature of expired gases was recorded during evacuation using a thermistor probe (Edale type 2984, Model C, UK) and all values were converted to the standard temperature and pressure for a dry gas (i.e. 0°C and 760 mmHg). The assumptions of the Haldane transformation (Wilmore and Costill, 1973) were applied to establish inspired gas volumes (\dot{V}_I) in order that rates of oxygen utilisation ($\dot{V}O_2$) and carbon dioxide production ($\dot{V}CO_2$) could be calculated (Appendix Di). Using these data, it was then possible to estimate both the RER ($\dot{V}CO_2/\dot{V}O_2$) and the relative contributions of carbohydrate and lipid towards overall energy expenditure via indirect calorimetry (Appendix Dii). In addition, during the study described in Chapter 7, the predicted $\dot{V}O_2$ and $\dot{V}CO_2$ for protein was incorporated into subsequent equations (Appendix Diii) such that non-protein respiratory exchange ratios (NPRER) and corrected rates of substrate utilisation could be determined for the recovery period (Frayn, 1983; Jequier *et al.*, 1987; Weir, 1949).

3.5.2 Urine Collection and Analysis

Urine samples were collected before all main trials and analysed for osmolality by freezing point depression using a cryoscopic osmometer (Gonometer 030, Gonotec, Germany) to assess whether participants were euhydrated upon arrival in the laboratory. Adequate hydration was assumed for urine osmolality values below 900 mosmol·kg⁻¹ (Shirreffs and Maughan, 1998a). During the study described in Chapter 7, each participant's urine was collected throughout the 4 h recovery period and was stored in a vessel containing 5 ml of 10% thymol-isopropanol as a preservative. In this study, the total volume of urine produced during recovery was recorded and a mixed 5 ml sample of this total volume was stored at -80 °C. As an estimate of total urine nitrogen excretion, the urea concentration of this sample was determined spectrophotometrically using procedures identical to those applied during analysis for plasma urea concentrations (see section 3.5.4.3). Plasma urea concentrations were then used to correct urinary urea excretion for changes in the total body urea pool (Jequier *et al.*, 1987) and NPRER was calculated according to the estimated protein oxidation rate (Frayn, 1983; Weir, 1949). The precise methods and equations used in this process are detailed in Appendix Diii.

3.5.3 Blood Sampling

Blood samples were drawn during main trials through an indwelling cannula (Venflon, 18G, BOC Ohmeda, Sweden). This cannula was inserted into an antecubital vein under local anaesthetic (1% lignocaine, Antigen Pharmaceuticals Ltd., Ireland) and was kept patent throughout each trial by frequent flushing with non-heparinised isotonic saline (0.9% Sodium Chloride, Steripak Ltd., UK). Participants were required to stand for 15 min prior to all resting blood samples to reduce the effect of body position on localised haemoconcentration and those blood samples taken during recovery were drawn prior to each feeding. Of the 10 ml whole blood drawn at each sampling point, 5 ml was dispensed into a non-anticoagulant blood collection tube (Sarstedt Ltd., UK) where it was left to clot for ≈45 min at room temperature and centrifuged at 2000 xg for 10 min at 4 °C (Beckman-Coulter Allegra X-22R, Germany) before the serum fraction was removed and stored at -80 °C. The other 5 ml of whole blood was transferred into another blood collection tube (Sarstedt

Ltd., UK) containing the anti-coagulant ethylenediaminetetraacetic acid (EDTA) from which 4 x 20 µl along with 3 x 50 µl aliquots of blood were removed using micropipette tubes and non-heparinised microhaematocrit tubes, respectively (Blaubrand, Germany). Two of the 20 µl samples of whole blood were deproteinised in 2.5% perchloric acid (200 µl) and centrifuged at 7000 xg for 3 min (Eppendorf Centrifuge 5415c, Germany) prior to storage at -80 °C, the other 5 aliquotes were analysed immediately to determine plasma volume (see section 3.5.4.1). The remaining EDTA treated blood was centrifuged at 2000 xg for 10 min at 4 °C (Beckman-Coulter Allegra X-22R, Germany) before plasma was abstracted and stored at -80 °C.

3.5.4 Blood Analysis

3.5.4.1 Plasma Volume

Duplicate 20 µl aliquots of EDTA treated whole blood were mixed with 5 ml of Drabkin's Reagent (GmbH Diagnostica, Boehringer Mannheim, Germany) and haemoglobin concentration was calculated using a standard cyanomethaemoglobin method and a spectrophotometer (Shimadzu 1240, Japan) to measure light absorbency. Haematocrit was then determined from 3 further 50 µl aliquots of EDTA treated whole blood using a micro-haematocrit centrifuge and reader (Hawksley Ltd., UK) to assess packed cell volume. Equations were subsequently applied to these haemoglobin and haematocrit values to monitor changes in plasma volume during main trials (Dill and Costill, 1974).

3.5.4.2 Blood Lactate

Blood lactate concentrations were determined via fluorimetric analysis (Locarte 8.9, UK) using 20 μl of perchloric acid extract derived from the previously deproteinised whole blood samples (Maughan, 1982). The fluorescence of each sample was then converted into a blood lactate concentration using a standard linear regression programme. The lactate standards used to construct this regression line ranged from 0.65 – 13.2 $\text{mmol}\cdot\text{l}^{-1}$ (ABX diagnostics, France), a range which was sufficient to encompass all values recorded in this thesis.

3.5.4.3 Plasma Glucose, Free Fatty Acids, Glycerol, Myoglobin and Urea

An automated spectrophotometer (COBAS Mira Plus, Roche Diagnostic Systems, Switzerland) was used to analyse the absorbency of all plasma samples at 37 $^{\circ}\text{C}$ using commercially available techniques: glucose (Randox, Ireland); free fatty acids (Wako NEFA C, Germany); glycerol (Randox, Ireland); myoglobin (Randox, Ireland); and urea (Randox Ireland). The principle of this method is that the amount of light absorbed by any sample will be proportional to the concentration of analyte in that sample. Prior to each assay, this automated analyser was calibrated against a number of commercially available standards (Randox, Ireland; Wako, Germany) and checked against quality controls (Preciset, Boehringer Mannheim, Germany) in both the physiological and pathological range.

3.5.4.4 Serum Insulin, Cortisol and Growth Hormone

Commercially available radioimmunoassays (MP Biomedicals Ltd., USA) were used to analyse sera for insulin (Coat-Count Insulin), cortisol (Corti-Cote) and growth hormone (hGH) using an automated gamma counter (Cobra 5000, Packard Instruments, USA). All these assays operate on the principle that the sample antigen will compete for a limited number of specific antibody binding sites with the radioactive form of that antigen (Iodine-125). The result is an inverse relationship between radioactivity and hormone concentration that, when plotted graphically using standards (MP Biomedicals Ltd., USA), allows the precise quantification of analyte concentration in each sample. These radioimmunoassays were conducted either in the Radiochemistry Laboratories at Loughborough University or in the School of Biomedical Sciences, Queen's Medical Centre, Nottingham, UK.

3.5.5 Muscle Biopsy Sampling and Storage

During the study described in Chapter 7, biopsy samples were taken from the vastus lateralis muscle on 3 occasions during each trial. This specific site was selected for muscle biopsy sampling because the quadriceps can be assumed to contribute substantially during treadmill running. Early evidence suggested that the lateral portion of the quadriceps femoris, the gastrocnemius and the lateral gluteals contribute equally to running activity (Karlsson and Saltin, 1971). However, while total depletion of glycogen in the vastus lateralis has commonly been reported following prolonged exhaustive cycling (Hermansen *et al.*, 1967), it has been questioned whether long-distance running focuses a comparable emphasis on the quadriceps (Costill *et al.*, 1974). Indeed, a more recent study has confirmed that treadmill running actually elicits a greater rate of muscle glycogen utilisation in the triceps surae than in the vastus lateralis (Krssak *et al.*, 2000). Nonetheless, previous findings do support the contention that prolonged treadmill running exerts a substantial, even if not principal, demand on the quadriceps (Tsintzas *et al.*, 1996a). In concordance with these latter findings, when calculating the total contribution of muscle glycogen towards overall metabolism it was assumed that the dry muscle mass of both legs was 5% of body mass and that the average glycogen utilisation for these limbs was reflected by that observed in the vastus lateralis (Tsintzas *et al.*, 1996a).

Upon completion of R_1 in the relevant investigation, participants rested in a supine position while a 3-5 mm skin incision was made under local anaesthetic (2-3 ml of 1% lignocaine, Antigen Pharmaceuticals Ltd., Ireland) using a surgical blade. The percutaneous needle biopsy technique (Bergstrom, 1962) was then used to obtain between 30 and 100 mg of wet muscle tissue from this site. In a similar manner, a further 2 skin incisions were made to the same leg approximately 3 h 45 min later such that subsequent biopsies could be acquired after precisely 4 h of recovery and then again immediately following R_2 . During participants first trials, the times elapsed between: completion of R_1 and the first muscle biopsy; the second muscle biopsy and the onset of R_2 ; and completion of R_2 and the final biopsy, were recorded and then matched in each participant's second trial (904 ± 314 s; 548 ± 94 s; and 339 ± 48 s, respectively; mean \pm SD). In addition, muscle biopsies were taken from each participant's opposite leg during trial 2 and the use of dominant/non-dominant limbs was counterbalanced.

Once removed from the leg, muscle samples were immediately placed in a ventilated eppendorf tube and snap-frozen in liquid nitrogen where they were stored for ≈ 8 weeks. Upon removal from storage but while still immersed in liquid nitrogen, each sample was dissected to remove a 15-20 mg fragment of muscle which was placed in a freeze-dryer (Pirani 10, Edwards, UK) for 24 h at -50°C and between -10^{-1} and -10^{-2} mmHg. Following freeze-drying, the muscle fragments were placed in plastic eppendorf tubes and washed with 1 ml of petroleum ether to remove any excess fat before being left to dry in a fume cupboard. All visible blood and connective tissue was removed from each fragment using a surgical blade and an agate pestle and mortar was used to reduce each sample into a fine powder. The resultant powder obtained from each fragment was divided equally between 2 new eppendorf tubes and the precise quantity in each was assessed using an electrical balance (Mettler AE240, Switzerland). These powdered samples were then stored at -80°C along with silica gel pending analysis for mixed muscle metabolites.

3.5.6 Muscle Biopsy Analysis

On the first day of analysis, mixed muscle metabolites were extracted by adding to each powder sample $100 \mu\text{l}\cdot\text{mg}^{-1}$ of $0.5 \text{ mmol}\cdot\text{l}^{-1}$ perchloric acid (HClO_4) which also contained $1 \text{ mmol}\cdot\text{l}^{-1}$ ethylenediaminetetraacetic sodium salt ($\text{EDTA}\cdot\text{Na}_2$). All samples were then kept on ice for 10 minutes while being agitated regularly using a vortex (Stuart Scientific, UK), before centrifugation at $20000 \times g$ for 10 min at $0-4^\circ\text{C}$ (Hawk 15/05, Sanyo, USA). A known quantity ($>150 \mu\text{l}$) of supernatant (extract) was transferred to a new eppendorf tube and the remaining precipitate stored at -80°C pending later analysis for insoluble glycogen (i.e. proglycogen). The extracted fraction was then quickly neutralised by adding $25 \mu\text{l}$ of $2.2 \text{ mmol}\cdot\text{l}^{-1}$ potassium hydrogen carbonate (KHCO_3) per $100 \mu\text{l}$ of extract. Each sample was then vortexed (Stuart Scientific, UK) and left on ice for 5 minutes with lids fitted loosely to permit the liberated CO_2 to escape. Litmus paper was used to verify that the reaction had completed before samples were centrifuged once again at $20000 \times g$ for 10 min at $0-4^\circ\text{C}$ (Hawk 15/05, Sanyo, USA). Finally, the clear supernatant (neutralised extract) was transferred into a new eppendorf tube and stored at -80°C pending analysis for mixed muscle metabolites, based on the calculation that 1 ml of this neutralised extract was equivalent to 8 mg of powdered muscle tissue (Harris *et al.*, 1974).

All assays were performed within 96 h of extraction. A fraction of the neutralised extract was diluted for glucose-6-phosphate (G-6-P), adenosine triphosphate (ATP) and phosphocreatine (PCr) analysis in the first 24 h following extraction, with analysis for remaining creatine (Cr) being performed a further 24 h later. On the third day of analysis, muscle lactate concentrations were measured using the undiluted extracts and samples to be used for glycogen analyses were hydrolysed. Acid hydrolysis of the soluble fraction of glycogen (i.e. macroglycogen) required the addition of $100 \mu\text{l}$ of $1 \text{ mmol}\cdot\text{l}^{-1}$ hydrochloric acid (HCl) to a $20 \mu\text{l}$ volume of each neutralised extract. Similarly, the insoluble fraction of glycogen (i.e. proglycogen) involved adding $100 \mu\text{l}$ of $1 \text{ mmol}\cdot\text{l}^{-1}$ HCl for every 1 mg of powder originally weighed. These mixtures were then incubated at 100°C in a dry bath (Grant BTS, UK) for 2 h, before centrifugation (Eppendorf minispin plus, Germany) for 1 min at $11000 \times g$. Following incubation, the soluble fraction was then neutralised with $15 \mu\text{l}$

of sodium hydroxide (NaOH). On the day following acid hydrolysis, the hydrolysed extracts and powder pellets were diluted as necessary and total glycogen content was determined as the combined glucose concentrations recorded in each (Jansson, 1981). Furthermore, the glucose concentration in the condensed neutralised extracts was also assessed in order that the soluble glycogen concentrations could be corrected for any free glucose within muscle.

The relative concentrations of all the metabolites cited above were determined according to the methods described by Harris *et al.* (1974) using a spectrophotometric plate reader (SpectraMax 190, Molecular Devices, USA). These methods are based on enzyme catalysed reactions with the coenzymes NAD^+ and NADP^+ being simultaneously reduced to NADH and NADPH, respectively (or *vice versa* in the case of the creatine assay). Using the coefficient of extinction of NADH and NADPH, metabolite concentrations can therefore be calculated from the change in sample absorbency in relation to double-distilled water blanks (Rnase free H_2O , Sigma-Aldrich Ltd., UK) using the equations presented in Appendix E. All buffers, co-factors, standards and enzymes were prepared immediately prior to each assay using Grade-I chemicals and all samples were analysed at least in duplicate (both glycogen assays in triplicate). Standards were prepared to a specified molarity and, where necessary, metabolite concentrations were adjusted according to the calculated result. Finally, to account for any contamination of the muscle samples with blood, fat or connective tissue, all metabolites other than lactate were normalised in relation to each participant's mean total creatine ($\text{PCr} + \text{Cr}$) across both trials. All muscle metabolite data are reported as $\text{units}\cdot\text{kg dry mass}^{-1}$ to avoid changes in concentration due to fluid shift during exercise. A more detailed account of all muscle analysis procedures including relevant stoichiometry, calculations and corrections is provided in Appendix E.

3.5.7 Subjective Data

Participants' sensations of fatigue were monitored during all exercise sessions using a rating of perceived exertion (RPE) scale as described by Borg (1973). The RPE scale is graded from 6-20, with these numbers being anchored by expressions ranging from 7 being 'Very, very light' to 20 which infers 'Maximum' effort (Appendix Bi). Both heart rate and relative exercise intensity have been found to correlate positively with the RPE (Borg, 1973). Furthermore, to assess each participant's perceptions of gut fullness and thirst, 2 adapted versions of the conventional RPE scale were constructed such that the anchor terms ranged from 'not full' and 'not thirsty' to 'very very full' and 'very very thirsty', respectively (Appendices Bii and Biii).

3.6 Intra-Assay Variation

Coefficients of variation ($CV = SD/mean \times 100$) were calculated to evaluate the error variation present in each assay. The CV for each analyte that was measured during the current series of studies is presented in Table 3.1, with these calculations arising from the results of at least 10 separate analyses of a given sample. However, due to the limited availability of muscle tissue, the CV for muscle glycogen represents the variance across triplicate absorbencies when averaged for all participants.

Table 3.1: Coefficient of variation for each assay.

Analyte	Method	CV (%)
Lactate	Fluorometry	2.0
Haemaglobin	Spectrophotometry	0.6
Glucose	Spectrophotometry	1.2
Free Fatty Acids	Spectrophotometry	0.5
Glycerol	Spectrophotometry	1.0
Urea	Spectrophotometry	1.8
Myoglobin	Spectrophotometry	1.3
Insulin	Radioimmunoassay	7.4
Cortisol	Radioimmunoassay	4.8
Growth Hormone	Radioimmunoassay	4.4
Urine Osmolality	Osmometry	0.4
Proglycogen	Spectrophotometry	1.5
Macroglycogen	Spectrophotometry	1.4

3.7 Standardisation Procedures

3.7.1 Ambient Conditions

The ambient temperature and humidity in the laboratory were controlled where possible such that all main trials were performed under similar conditions between treatments. A whirling hygrometer (Zeal, UK) was operated in close proximity to the treadmill every 30 min to measure the wet and dry bulb temperatures in this area of the laboratory. Relative humidity was then calculated from the difference between these 2 readings. A wall mounted barometer (Griffin and George BHL-340-X, UK) was used to monitor atmospheric pressure at regular intervals during trials so that expired gases could be corrected to standard volumes at 760 mmHg.

3.7.2 Dietary Control

To ensure that each participant's nutritional status was standardised between experimental conditions, each participant was provided with a dietary record book (Appendix C) and weighing scales (Salter 3007, UK) with which to record their dietary intake over the 48 h prior to their first main trial. This diet was then strictly adhered to over the 48 h prior to subsequent main trials. Each dietary record was assessed using a nutritional software package (COMP-EAT 4.0, Nutritional Systems, UK) to calculate both total energy intake and a profile of various macronutrients, vitamins and minerals. In addition, participants were required to abstain from consuming both alcoholic and caffeinated drinks over the 48 h prior to main trials. All food intake ceased at 10 pm on the night before main trials and participants fasted overnight, consuming only water in order to remain adequately hydrated.

3.7.3 Habitual Training

While participants were permitted to continue the majority of their habitual training throughout the testing period, strenuous exercise was avoided in the week leading up to each main trial. Participants were asked to record any low-intensity training that was performed during this period prior to trial 1 and then repeat this activity pattern in preparation for ensuing trials. However, due to the demanding nature of the investigations described in this thesis, all physical exertion was entirely prohibited during the 48 h prior to any visit to the laboratory.

3.8 Statistical Analyses

Any data in this thesis which required a single comparison of 2 level measurements was tested for normality of distribution using a Shapiro-Wilk test to examine the respective mean and median of each respective trial. A paired t-test with a 1 sided significance level was then applied to those data sets deemed to be normally distributed, while any data requiring a non-parametric statistical method was assessed using a Wilcoxon test. Where multiple comparisons were necessary, a 2 way general linear model for repeated measures (Drink \times Time) was used to identify differences between experimental conditions, regardless of normality, since the type 1 error rate of this model is usually close to the nominal value even when data are non-normally distributed (Maxwell and Delaney, 1990, p. 109). The degrees of freedom for all significant F values were Greenhouse-Geisser corrected for epsilon < 0.75 , while the Huynh-Feldt correction was adopted for less severe asphericity (Atkinson, 2001); the F values quoted in the text reflect interaction effects unless main/time effects are specifically stated. Where a significant interaction effect was identified, comparisons between the level means of interest were made using multiple paired t-tests to determine the location of variance. The Holm-Bonferroni step-wise method was then used to prevent these multiple comparisons inflating the type 1 error rate (Atkinson, 2002; Ludbrook, 1998).

A Priori statistical power tests were conducted to determine the number of participants required in each study to provide at least an 80% power of correctly rejecting the null hypothesis and every effort was made to attain the necessary sample

size. No order effects were detected between trials for any variable. All statistical analyses were performed using commercially available software (SPSS 11.0, USA) and significance was accepted at the $P \leq 0.05$ level. Unless otherwise stated, all data are expressed in text as mean \pm SEM. Specifically, descriptive data are expressed as mean \pm SD because no inference to the target population is intended, while non-normally distributed data are expressed as median and range.

The error bars displayed on figures are confidence intervals (CI) that have been corrected for the repeated measures design to remove between subject variance (Masson and Loftus, 2003). These normalised confidence intervals are computed using an equation (Appendix G) that incorporates the same error term as was involved during statistical testing. The magnitude of these confidence intervals therefore directly infers the difference between means in the target population (i.e. statistical significance) and not the variance of individual values around the mean. Due to the 3 trial study design described in Chapter 6, the confidence intervals included in this chapter were corrected for violations of sphericity. This correction was made as described above using either Greenhouse-Geisser or Huynh-Feldt epsilons to reduce the relevant degrees of freedom for the omnibus error term, thus maintaining a valid reflection of the overall pattern across all 3 means. However, regarding the run times observed in Chapter 6, it was of particular theoretical interest to illustrate the comparisons between the CHO-PRO trial and each other trial independently. Thus, on this figure (6.3, p. 158) the specific error terms from each contrast were used to calculate separate pairs of confidence intervals for each comparison (Loftus and Masson, 1994).

CHAPTER 4

The Influence of Carbohydrate-Protein Mixtures on Recovery of Running Capacity.

4.1 Introduction

It has been established that exercise capacity during exercise of moderate to high intensity is closely related to the magnitude of pre-exercise muscle glycogen stores and that fatigue during such exercise often coincides with muscle glycogen depletion (Ahlborg *et al.*, 1967; Bergstrom *et al.*, 1967). These findings have placed great focus on the determination of nutritional strategies to maximise pre-exercise muscle glycogen concentrations (Ivy, 1991; Tsintzas and Williams, 1998).

While exhaustive exercise has been shown to stimulate muscle glycogen resynthesis independent of nutritional interventions (Bergstrom and Hultman, 1966; Zachwieja *et al.*, 1991), ingesting carbohydrate during recovery is known to enhance the rate of post-exercise muscle glycogen resynthesis (Bergstrom *et al.*, 1967). More recent experimental data has now revealed the optimum amount (Ivy *et al.*, 1988b; Wong and Williams, 2000), timing (Ivy *et al.*, 1988a; Parkin *et al.*, 1997) and type (Blom *et al.*, 1987; Burke *et al.*, 1993; Reed *et al.*, 1989) of carbohydrate intake required to effectively promote recovery. The most rapid rates of muscle glycogen resynthesis for a 4-6 h recovery have now been reported slightly in excess of 40 mmol·kg dry mass⁻¹·h⁻¹ following ingestion of 1.2 g CHO·kg⁻¹·h⁻¹ at 30 min intervals (van Loon *et al.*, 2000a). However, it has been suggested that a 'glycogen synthesis threshold' may occur at this optimal level of carbohydrate intake (Jentjens and Jeukendrup, 2003) and attention has subsequently focused on the possibility that ingesting a mixed macronutrient supplement may accelerate the rate of muscle glycogen resynthesis beyond this threshold (Zawadzki *et al.*, 1992).

Early evidence revealed that the ingestion of carbohydrate plus protein can stimulate pancreatic insulin secretion to a greater extent than ingestion of either substrate alone (Rabinowitz *et al.*, 1966). These initial findings have now been extended and the precise mixtures of various amino acids and proteins with the greatest insulinotropic properties have been identified (van Loon *et al.*, 2000b; van

Loon *et al.*, 2000c). There are 2 mechanisms through which these elevated insulin concentrations may increase the rate of muscle glycogen synthesis. Firstly, insulin can stimulate an increased rate of glucose uptake into the muscle cell both through translocating glucose transporters (GLUT4) to the cell membrane (McCoy *et al.*, 1996; Rudich and Klip, 2003) and potentially also through increasing the intrinsic activity of these glucose transporters (Furtado *et al.*, 2003; Somwar *et al.*, 2001). Secondly, insulin is known to convert the rate limiting enzyme for glycogenesis (glycogen synthase) into its active form, thus facilitating the conversion of glucose-6-phosphate into glycogen (Danforth, 1965; Markuns *et al.*, 1999).

The study of Zawadzki *et al.* (1992) demonstrated both an elevated insulin concentration and a 38% greater rate of muscle glycogen resynthesis when a carbohydrate-protein (CHO-PRO) mixture was ingested during a 4 h recovery rather than carbohydrate (CHO) alone (Zawadzki *et al.*, 1992). However, the CHO-PRO supplement provided in this study contained 43% more energy than the CHO supplement; the energy content of a supplement has since been found to enhance muscle glycogen storage irrespective of macronutrient composition (Roy and Tarnopolsky, 1998). Nonetheless, these early findings suggesting a beneficial effect of adding protein to carbohydrate recovery solutions have been corroborated by some more recent studies (Fogt and Ivy, 2000; Ivy *et al.*, 2002; van Loon *et al.*, 2000a), although other studies have found similar rates of muscle glycogen resynthesis following either CHO or CHO-PRO ingestion (Jentjens *et al.*, 2001; Van Hall *et al.*, 2000a; Van Hall *et al.*, 2000b). Furthermore, other authors have reported that CHO-PRO mixtures fail even to elevate insulin release to a greater extent than solutions containing CHO alone (Carrithers *et al.*, 2000; Ivy *et al.*, 2002; Tarnopolsky *et al.*, 1997; Wojcik *et al.*, 2001).

Given the previously described correlation between exercise capacity and pre-exercise muscle glycogen content (Ahlborg *et al.*, 1967; Bergstrom *et al.*, 1967), it is reasonable to assume that any supplement increasing the rate of glycogen resynthesis during recovery might also restore the capacity for aerobic exercise more rapidly. While information on this topic is certainly limited, evidence from Williams *et al.* (2003) has supported that the addition of protein to a carbohydrate solution can enhance recovery of exercise capacity during continuous cycling. However, the 55%

greater time to exhaustion at an intensity of 85% $\dot{V}O_2$ max reported in this study may be attributable to the quantity of carbohydrate included in each supplement. Specifically, the CHO-PRO solution provided in this study contained more carbohydrate than the solution containing CHO alone (Williams *et al.*, 2003).

Therefore, the main aim of this first study was to determine whether the ingestion of a CHO-PRO solution results in a more rapid recovery of running capacity at 85% $\dot{V}O_2$ max than a solution containing a matched quantity of CHO (1.2 g CHO·kg⁻¹·h⁻¹) without any additional protein.

4.2 Methods

4.2.1 Participants

Nine healthy male participants volunteered to take part in this study. These individuals participated in a wide range of sporting activities but all reported running to represent a substantial component of their habitual training. The physiological characteristics of these participants are presented in Table 4.1.

Table 4.1: Participant details.

Characteristic	Mean \pm SD
Age (yr)	21 \pm 1
Height (m)	1.81 \pm 0.1
Mass (kg)	79.6 \pm 11.2
$\dot{V}O_2$ max (ml·kg ⁻¹ ·min ⁻¹)	60 \pm 4

4.2.2 Experimental Design

Participants performed 2 main trials in a randomised, counterbalanced design that were separated by at least 1 week and applied in a double blind manner. A 2 day dietary record was completed over the 48 h prior to trial 1 and then the same diet was consumed prior to trial 2 (2458 \pm 670 kcal·day⁻¹, 53 \pm 9% CHO, 25 \pm 6% fat, 19 \pm 5% protein: mean \pm SD). Each main trial involved a 90 min run at 70% $\dot{V}O_2$ max (R_1) followed by a 4 h recovery. During the recovery period, participants rested while consuming either CHO alone or a CHO-PRO mixture before a treadmill run to exhaustion was performed at an intensity of 85% $\dot{V}O_2$ max (R_2).

4.2.3 Experimental Protocol

A schematic representation of the experimental protocol is included in Figure 4.1. All participants arrived in the laboratory between 8-8.30 am following a 10 h overnight fast and completed a pre-trial consent form (Appendix Aii). After providing a urine sample, each participant's post-void nude body mass was recorded (Avery Ltd., UK) before a cannula was inserted into an antecubital vein and a 10 ml

resting venous blood sample obtained. The cannula was kept patent throughout each trial by frequent flushing with isotonic saline. Prior to exercise, the Douglas bag technique was used to collect a 5 min resting expired gas sample (Williams and Nute, 1983). Participants were required to stand for 15 min prior to the collection of all resting gas and blood samples. A 5 min run at 60% $\dot{V}O_2$ max was used as a standardised warm-up prior to running at a speed equivalent to 70% $\dot{V}O_2$ max for 90 min (R_1). One minute expired gas samples, heart rates (Polar 8810, Finland) and RPE (Borg, 1973) followed by 10 ml venous blood samples were taken at 30 min intervals throughout R_1 . Water intake was permitted *ad libitum* during trial 1 and matched in trial 2 (0.46 ± 0.15 l; mean \pm SD). Nude body mass was recorded immediately following R_1 to assess hydration status through percentage change in mass.

The first volume of prescribed solution was provided as soon as post R_1 nude body mass had been recorded. The remaining 7 volumes were provided at 30 min intervals during the 4 h recovery such that the last solution was ingested 30 min prior to R_2 , participants were permitted 15 min to consume each volume. Expired gas samples and venous blood samples, along with ratings of gut fullness and thirst (6-20 scale; Appendices Bii and Biii), were taken during the recovery period every hour prior to feedings. Nude body mass was again recorded and, after the standard warm-up, participants began the run to exhaustion at 85% $\dot{V}O_2$ max (R_2) with any background music and verbal encouragement being standardised between trials (Atkinson *et al.*, 2004). As in R_1 , water intake was *ad libitum* during trial 1 and matched in trial 2 (0.12 ± 0.05 l; mean \pm SD). Physiological measurements were obtained at regular intervals (10 min) and at the point of volitional fatigue. Nude body mass was recorded immediately after R_2 , again to assess hydration status through changes in body mass. Ambient temperature and humidity were recorded at 30 min intervals throughout all testing periods using a hygrometer (Zeal, UK) and were not different between trials. Average values recorded across both trials were 22.6 ± 1.9 °C and $42.3 \pm 6.7\%$ (mean \pm SD). Full details of all sampling and analysis are included in the General Methods section 3.5.

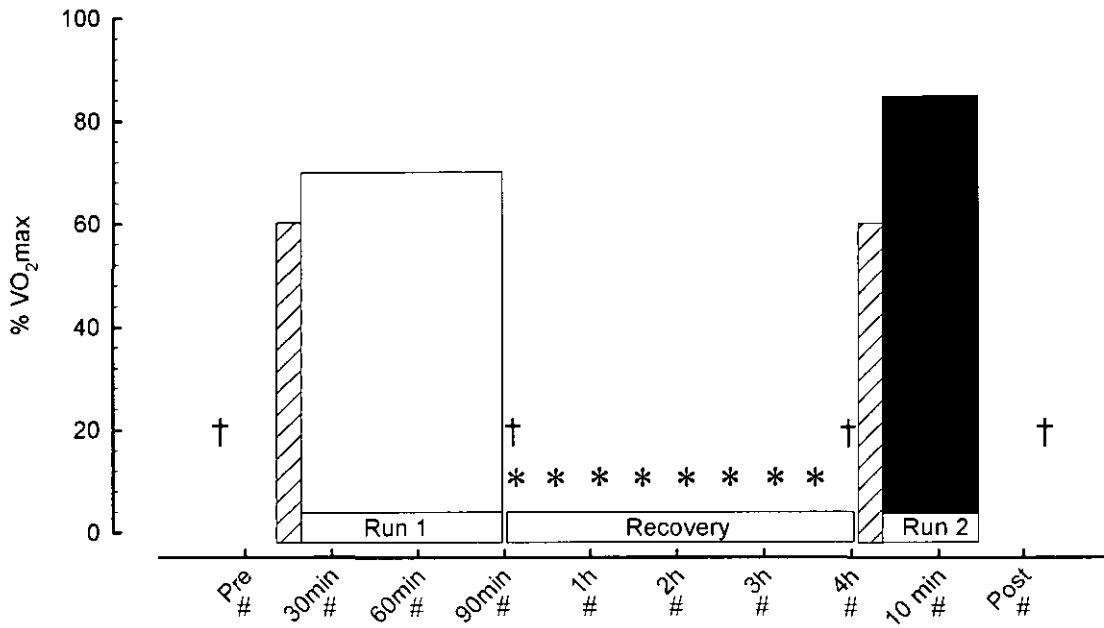


Figure 4.1: A schematic representation of the experimental protocol. Key: * = solution provision, # = expired gas sample/blood sample & RPE, † = body mass, hatched area = warm-up (5 min at 60% $\dot{V}O_{2\max}$), open area = R₁ (90 min at 70% $\dot{V}O_{2\max}$) and closed area = R₂ (85% $\dot{V}O_{2\max}$ until volitional exhaustion).

4.2.4 Solution Composition

The CHO and CHO-PRO supplements were both 9.3% carbohydrate solutions composed of 6.2% glucose and 3.1% fructose (GlaxoSmithKline, UK). These solutions were provided in matched volumes between trials ($1031 \pm 144 \text{ ml}\cdot\text{h}^{-1}$; mean \pm SD) relative to each participant's body mass such that both solutions provided a matched quantity of carbohydrate ($1.2 \text{ g CHO}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$). Participants in this study therefore ingested a total of $382 \pm 54 \text{ g CHO}$ during recovery (mean \pm SD). The CHO-PRO solution included an additional 1.5% ($0.2 \text{ g PRO}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$) of wheat protein hydrolysate (Hyprol 4107, Quest International, The Netherlands), rich in peptide bound glutamine (i.e. $\approx 6:1$ carbohydrate-protein ratio). Thus, $64 \pm 9 \text{ g PRO}$ (mean \pm SD) was ingested during recovery, which made approximately 16.7% more energy available for metabolism in the CHO-PRO trial (4.8 versus $5.6 \text{ kcal}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$). The specific amino acid profile of the wheat protein hydrolysate is included in Figure 4.2. These solutions were taste matched (orange and passion fruit flavour).

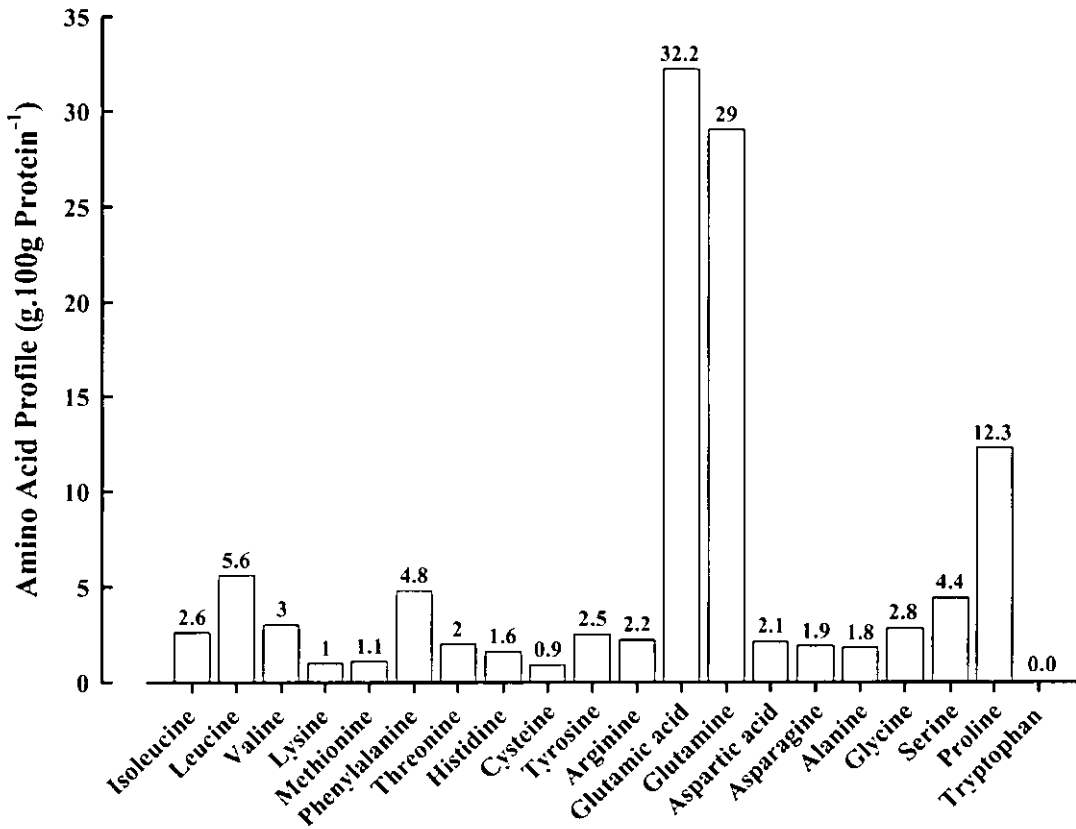


Figure 4.2: Amino acid profile of the wheat protein hydrolysate added to CHO solutions during CHO-PRO trials. (N.B: approximately 90% of the glutamic acid and aspartic acid fraction comprise glutamine and asparagine).

4.2.5 Statistical Analyses

The exercise capacity data of Williams *et al.* (2003) were utilised to estimate that a sample size of 9 has a 94% power to detect a difference in means of 11.1 min, assuming a SD of differences of approximately 9.4, using a paired t-test with a 1 sided significance level. The exercise capacity data recorded during R₂ were found to be non-normally distributed and a Wilcoxon test was therefore applied to compare the median run times between trials, along with any other ordinal/discrete variables (i.e. RPE, gut fullness and thirst) requiring non-parametric statistical analysis. Paired t-tests were used to analyse the incremental area under curve data that was calculated to assess the glycaemic and insulinaemic responses to each solution during recovery (Wolever, 2004). A 2 way general linear model for repeated measures (Drink x Time) was used to identify differences between experimental conditions. The Greenhouse-Geisser correction was utilised for epsilon <0.75, while the Huynh-Feldt correction was adopted for less severe asphericity. Where significant F values were found, the Holm-Bonferroni step-wise correction was applied to determine the location of variance where necessary (Atkinson, 2002; Ludbrook, 1998). Unless otherwise stated, all data are expressed in text as mean \pm SEM and the error bars included on figures are confidence intervals (CI) that have been corrected to remove between subject variance (Masson and Loftus, 2003). Specific details of, and justification for, these statistical methods are included in the General Methods section 3.8.

4.3 Results

4.3.1 Exercise Capacity

The recovery of exercise capacity was not different following ingestion of the CHO-PRO solution than following ingestion of CHO alone (Effect size = 0.3) and was highly variable between participants. Median run times to exhaustion at 85% $\dot{V}O_2$ max (R_2) were 14.5 (7-57) min during the CHO trial and 18 (7-72) min during the CHO-PRO trial (Figure 4.3).

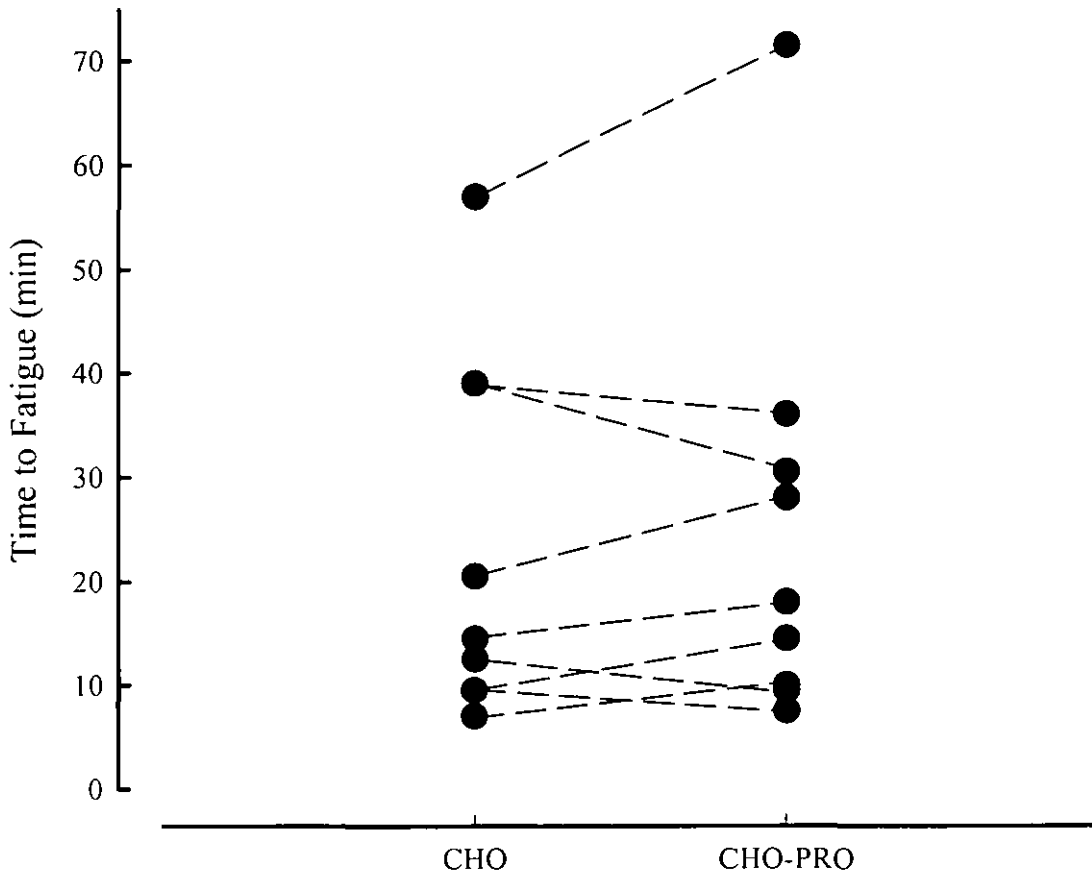


Figure 4.3: Run times to exhaustion for each individual during R_2 following ingestion of either CHO or CHO-PRO during recovery ($n = 9$).

4.3.2 Serum Insulin

Serum insulin concentrations were higher during the CHO-PRO trial than during the CHO trial (main effect: $F = 6.8$, $P = 0.03$; Figure 4.4). The values observed during recovery were then converted into an insulinaemic response (Appendix F), expressed as incremental area under the curve. In concordance with the observed main effect, this insulinaemic response was also higher in the CHO-PRO trial than in the CHO trial ($P = 0.02$; Figure 4.5).

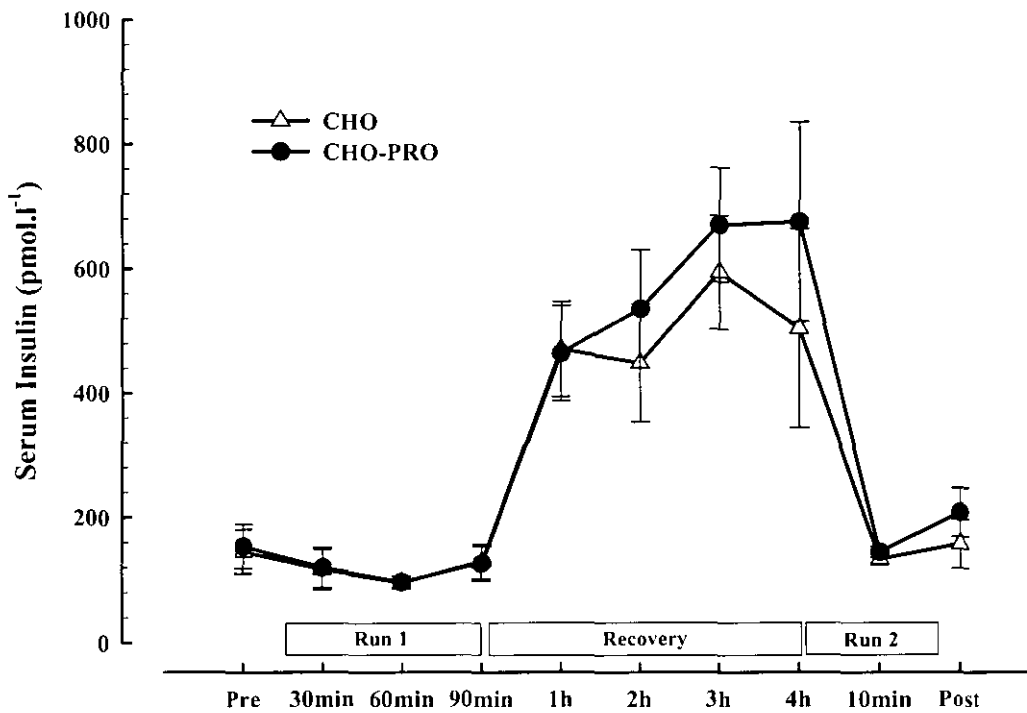


Figure 4.4: Serum insulin concentrations during R₁, recovery and R₂. Participants ($n = 9$) received either CHO or CHO-PRO supplements during recovery. (N.B: at 10 min R₂ $n = 4$). Values are means \pm CI and are different between trials ($P = 0.03$). To convert from SI units into $\mu\text{IU}\cdot\text{ml}^{-1}$ divide by 6.95.

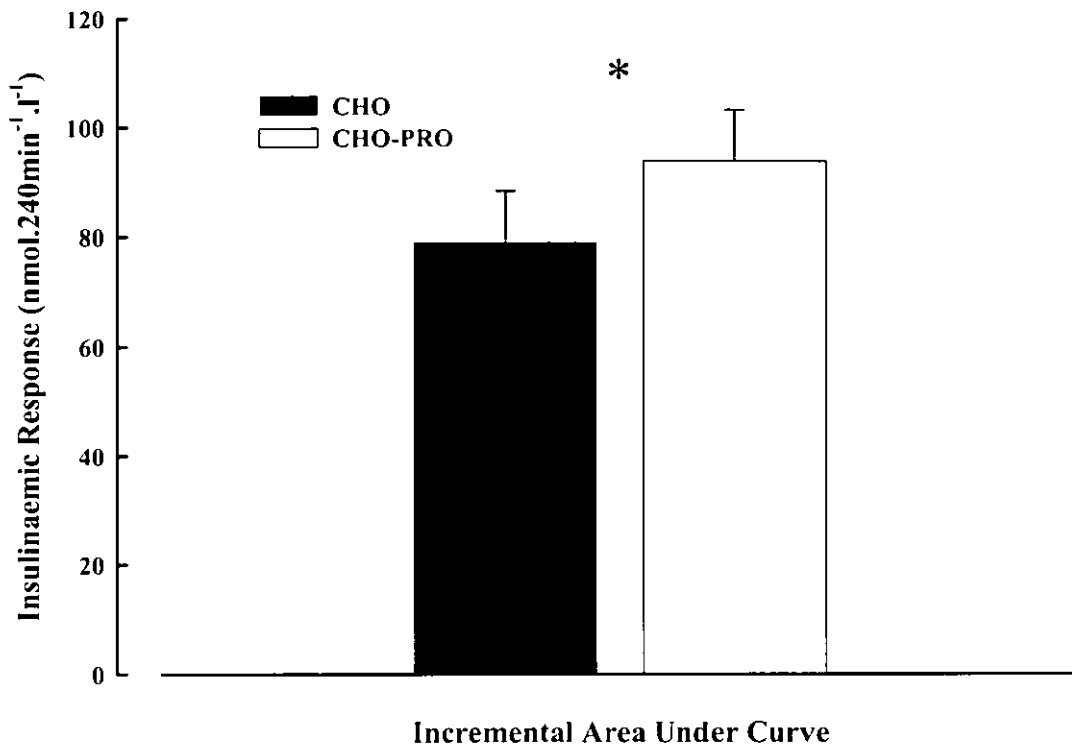


Figure 4.5: Insulinaemic responses during 4 h recovery (n = 9). Values are means \pm CI and * denotes values different between trials ($P = 0.02$). To convert from SI units into mIU·ml⁻¹ divide by 6.95.

4.3.3 Plasma Glucose

Plasma glucose concentrations were not statistically different between the CHO and CHO-PRO treatments at any time point (Figure 4.6) although a slightly lower peak concentration after 1 h of recovery was apparent when ingesting the CHO-PRO mixture. In both trials, the blood samples taken 10 min into R₂ revealed glucose concentrations to be below 3.5 mmol·l⁻¹ for most participants and there was an insignificant trend for this hypoglycaemia to be more severe during the CHO trial than during the CHO-PRO trial. When the incremental area under the curve during recovery was calculated for this glucose data, the resultant glycaemic response did not identify any differences between the experimental treatments (Figure 4.7).

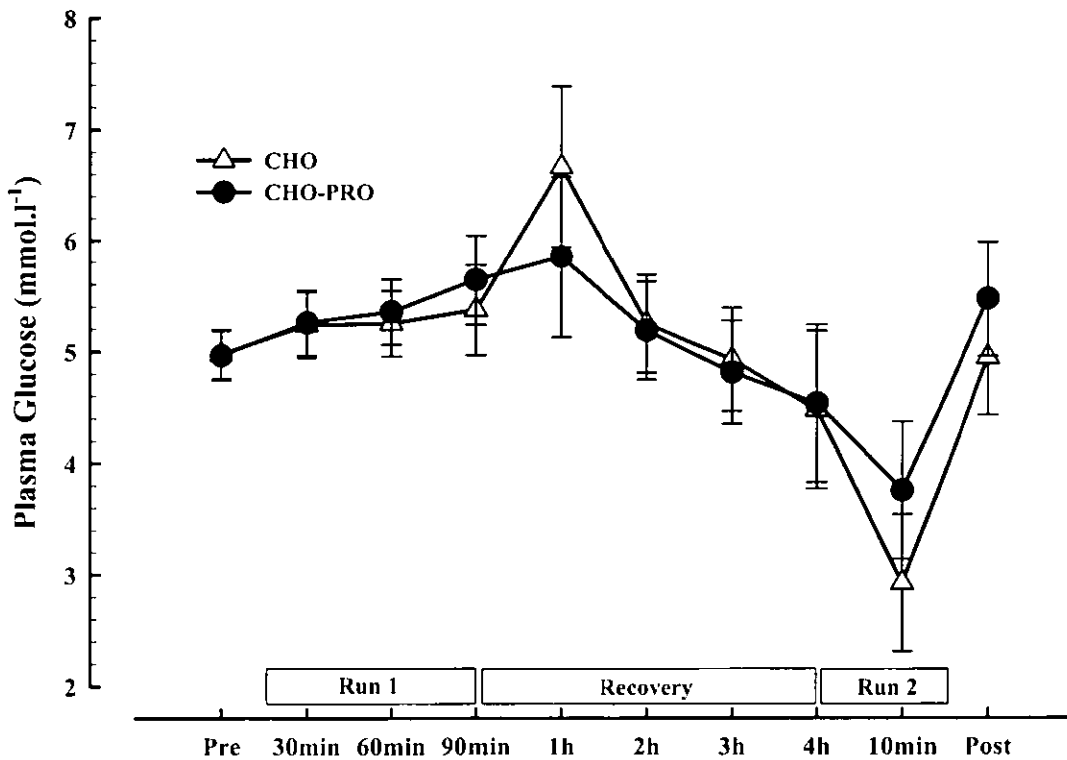


Figure 4.6: Plasma glucose concentrations during R₁, recovery and R₂. Participants (n = 9) received either CHO or CHO-PRO supplements during recovery. (N.B: at 10 min R₂ n = 4). Values are means ± CI.

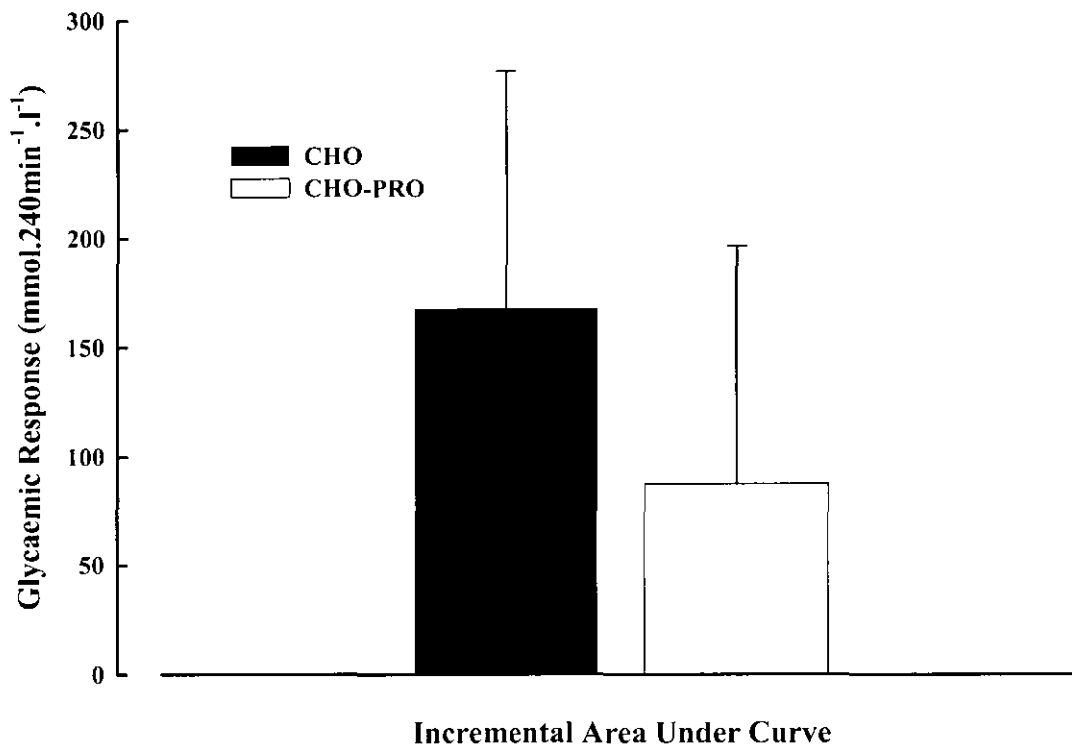


Figure 4.7: Glycaemic responses during 4 h recovery (n = 9). Values are means \pm CI.

4.3.4 Plasma Urea

Plasma urea concentrations began to dissociate between trials after approximately 1 h of recovery and were significantly greater ($F = 6.7$, $P = 0.01$) during R_2 following ingestion of CHO-PRO rather than CHO (Figure 4.8).

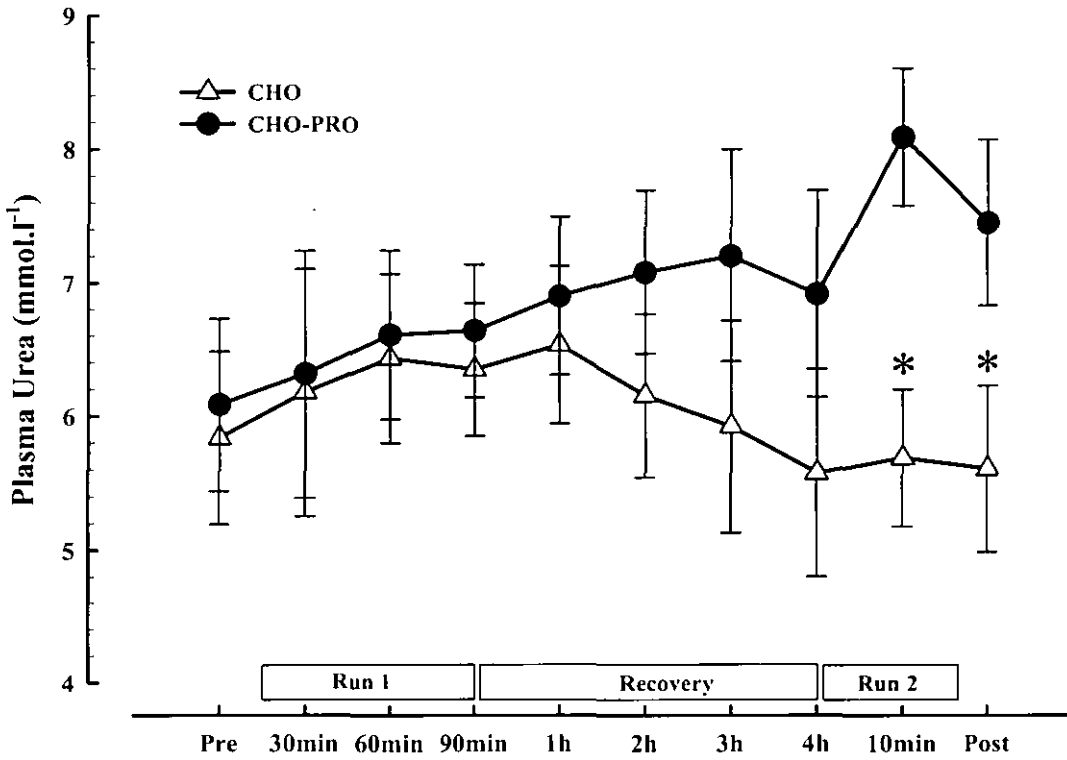


Figure 4.8: Plasma urea concentrations during R_1 , recovery and R_2 . Participants ($n = 9$) received either CHO or CHO-PRO supplements during recovery. (N.B: at 10 min R_2 $n = 3$). Values are means \pm CI and * denotes values different between trials ($P \leq 0.02$).

4.3.5 Plasma Free Fatty Acids and Glycerol

Ingestion of either the CHO or the CHO-PRO solutions did not effect free fatty acid or glycerol concentrations differently between trials. Concentrations of both metabolites increased gradually during R₁ before decreasing sharply upon ingestion of the first carbohydrate mixture, thereafter remaining close to zero throughout the recovery period (Figures 4.9 and 4.10).

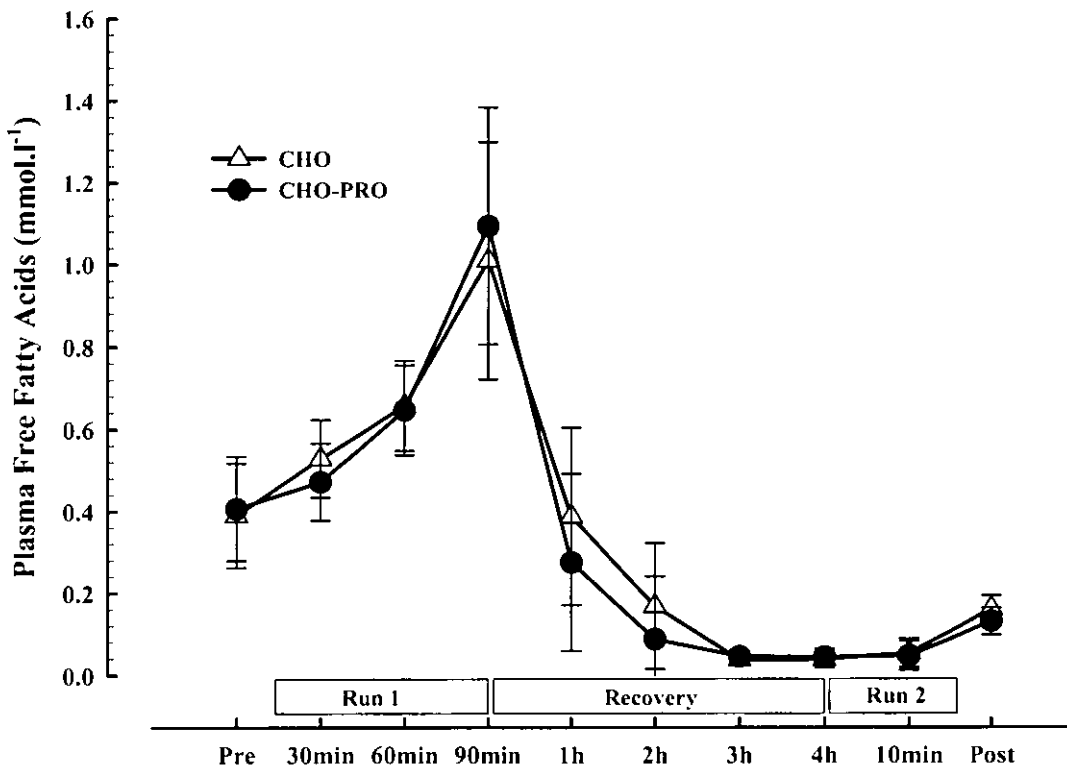


Figure 4.9: Plasma free fatty acid concentrations during R₁, recovery and R₂. Participants (n = 9) received either CHO or CHO-PRO supplements during recovery. (N.B: at 10 min R₂ n = 4). Values are means \pm CI.

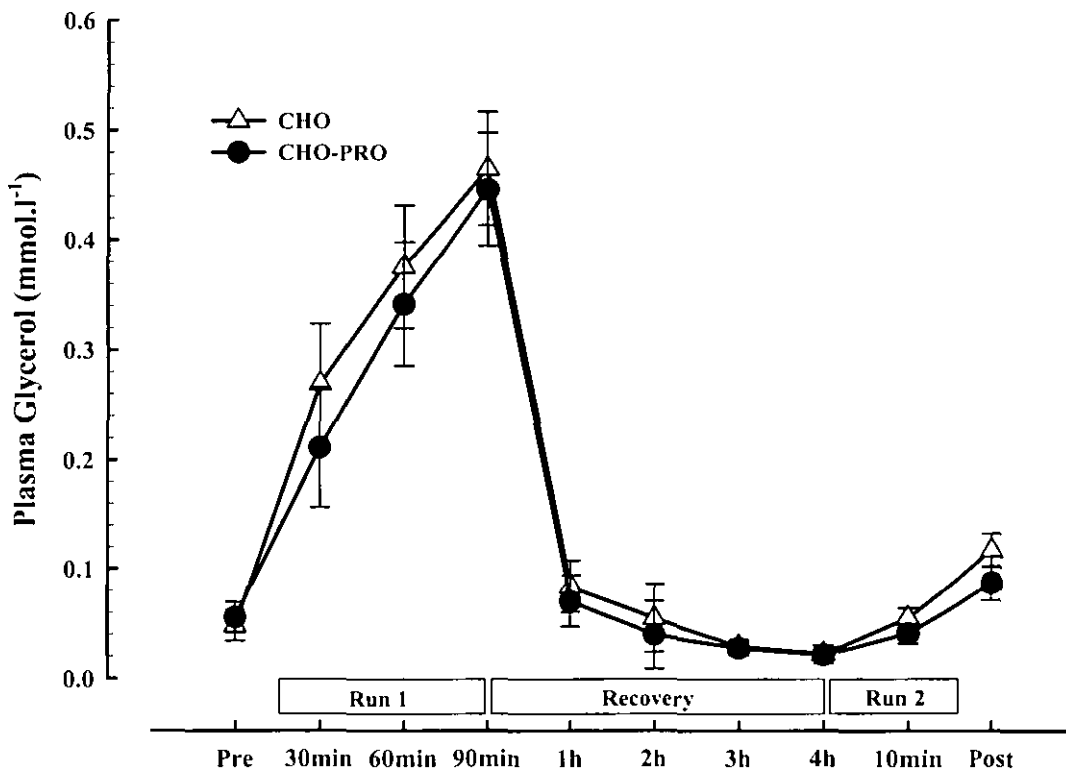


Figure 4.10: Plasma glycerol concentrations during R₁, recovery and R₂. Participants (n = 9) received either CHO or CHO-PRO supplements during recovery. (N.B: at 10 min R₂ n = 4). Values are means \pm CI.

4.3.6 Blood Lactate

Blood lactate concentrations increased slightly upon commencement of R_1 and were maintained at approximately $1.5 \text{ mmol}\cdot\text{l}^{-1}$ throughout this exercise session and the subsequent recovery period. These values increased during R_2 such that samples taken immediately post-exercise had reached concentrations of $5.0 \pm 0.3 \text{ mmol}\cdot\text{l}^{-1}$ during the CHO trial and $4.4 \pm 0.4 \text{ mmol}\cdot\text{l}^{-1}$ during the CHO-PRO trial. However, there were no statistical differences between the CHO and CHO-PRO treatments (Figure 4.11).

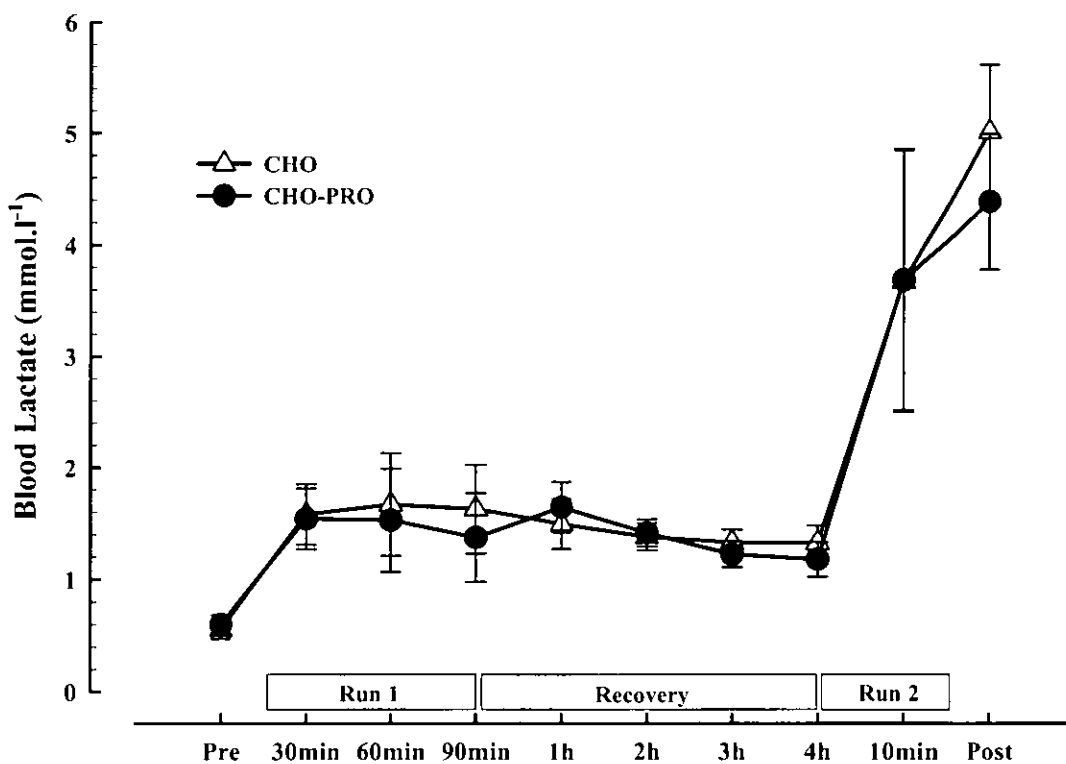


Figure 4.11: Blood lactate concentrations during R_1 , recovery and R_2 . Participants ($n = 9$) received either CHO or CHO-PRO supplements during recovery. (N.B: at 10 min R_2 $n = 4$). Values are means \pm CI.

4.3.7 Substrate Metabolism

The estimated contributions of lipid and carbohydrate oxidation towards overall energy expenditure were not different between the CHO and CHO-PRO trials (Table 4.2). However, the respiratory exchange ratio (RER) tended to be lower during the CHO-PRO trial than during the CHO trial, although this difference could not be detected statistically (main effect: $F = 4.7$, $P = 0.06$).

Table 4.2: Substrate metabolism and respiratory exchange ratios during R₁, recovery and R₂. Participants (n = 9) received either CHO or CHO-PRO supplements during recovery (N.B: at 10 min R₂ n = 4). Values are means ± SEM.

	Pre	30 min	Run 1		Recovery				Run 2	
			60 min	90 min	1 hr	2 hr	3 hr	4 hr	10 min	Last min
Carbohydrate oxidation (g.min⁻¹)										
CHO	0.3±0.1	3.0±0.4	2.7±0.4	2.8±0.3	0.4±0.1	0.4±0.1	0.5±0.04	0.5±0.04	4.3±0.3	4.8±0.4
CHO-PRO	0.3±0.1	2.9±0.3	2.6±0.3	2.5±0.3	0.4±0.1	0.4±0.1	0.5±0.1	0.5±0.1	4.2±0.6	4.6±0.4
Lipid oxidation (g.min⁻¹)										
CHO	0.1±0.02	0.4±0.1	0.6±0.1	0.6±0.1	0.1±0.02	0.1±0.01	0.04±0.01	0.03±0.01	0.01±0.01	0.1±0.03
CHO-PRO	0.1±0.02	0.5±0.1	0.6±0.1	0.7±0.1	0.1±0.02	0.1±0.01	0.1±0.02	0.1±0.01	0.1±0.04	0.2±0.1
RER										
CHO	0.88±0.04	0.90±0.01	0.89±0.02	0.89±0.01	0.94±0.03	0.95±0.03	0.97±0.02	0.98±0.02	1.02±0.02	1.01±0.01
CHO-PRO	0.87±0.03	0.90±0.01	0.89±0.01	0.88±0.01	0.90±0.02	0.90±0.02	0.93±0.02	0.95±0.02	0.99±0.02	0.98±0.01

4.3.8 Serum Cortisol

Serum cortisol concentrations were not different between the CHO and CHO-PRO treatments. Levels of this hormone increased during R₁ before decreasing in the latter stages of recovery and then, primarily in the CHO trial, elevating once again at the end of R₂ (Figure 4.12).

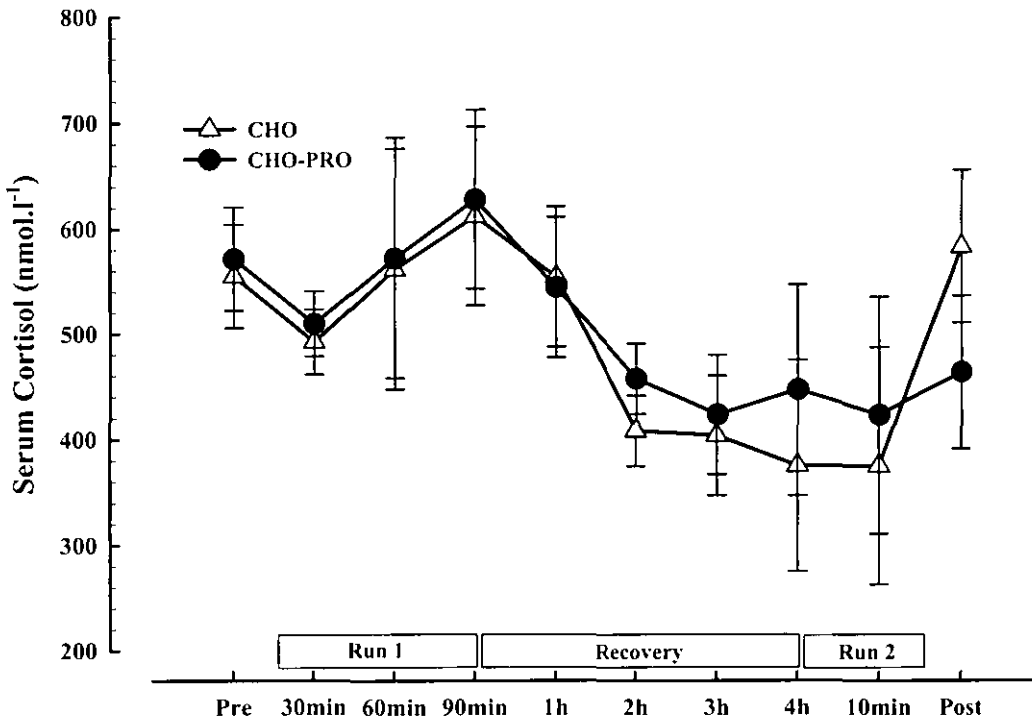


Figure 4.12: Serum cortisol concentrations during R₁, recovery and R₂. Participants (n = 9) received either CHO or CHO-PRO supplements during recovery. (N.B: at 10 min R₂ n = 4). Values are means ± CI.

4.3.9 Plasma Myoglobin

Concentrations of myoglobin increased with both exercise sessions (R_1 and R_2) and decreased slightly during recovery. This profile was uniform between the CHO and CHO-PRO treatments (Figure 4.13).

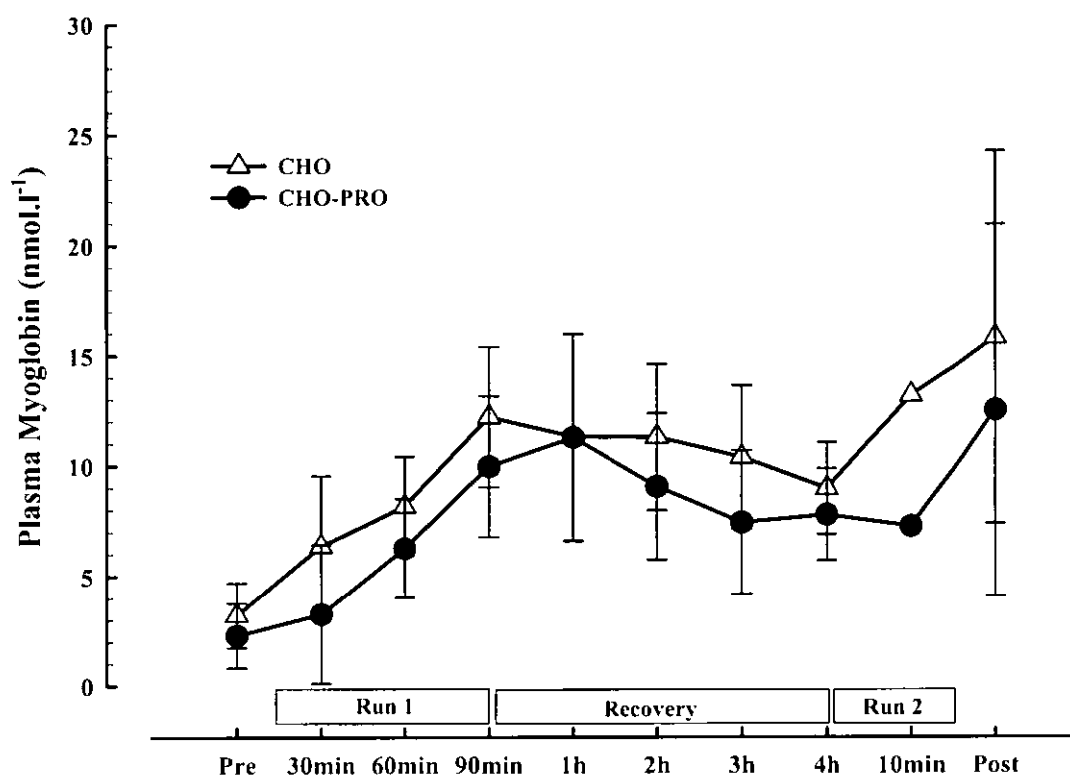


Figure 4.13: Plasma myoglobin concentrations during R_1 , recovery and R_2 . Participants ($n = 8$) received either CHO or CHO-PRO supplements during recovery. (N.B: insufficient degrees of freedom to construct CI at 10 min R_2 , $n = 4$). Values are means \pm CI.

4.3.10 Indices of Hydration Status

Urine osmolality was 747 ± 344 mosmol·kg⁻¹ prior to CHO trials and 797 ± 319 mosmol·kg⁻¹ prior to CHO-PRO trials (mean \pm SD), this difference was not statistically significant. Changes in each participant's body mass (BM) revealed losses in the CHO trial of $-1.7 \pm 0.2\%$ BM in R₁ and $-0.8 \pm 0.2\%$ BM in R₂. Similar shifts in hydration status were recorded during the CHO-PRO trials: $-1.7 \pm 0.2\%$ BM (R₁) and $-1.0 \pm 0.3\%$ BM (R₂). Fluctuations in plasma volume during main trials were not different between the CHO and CHO-PRO treatments (Figure 4.14) but an effect of time was established ($F = 5.4, P = 0.004$).

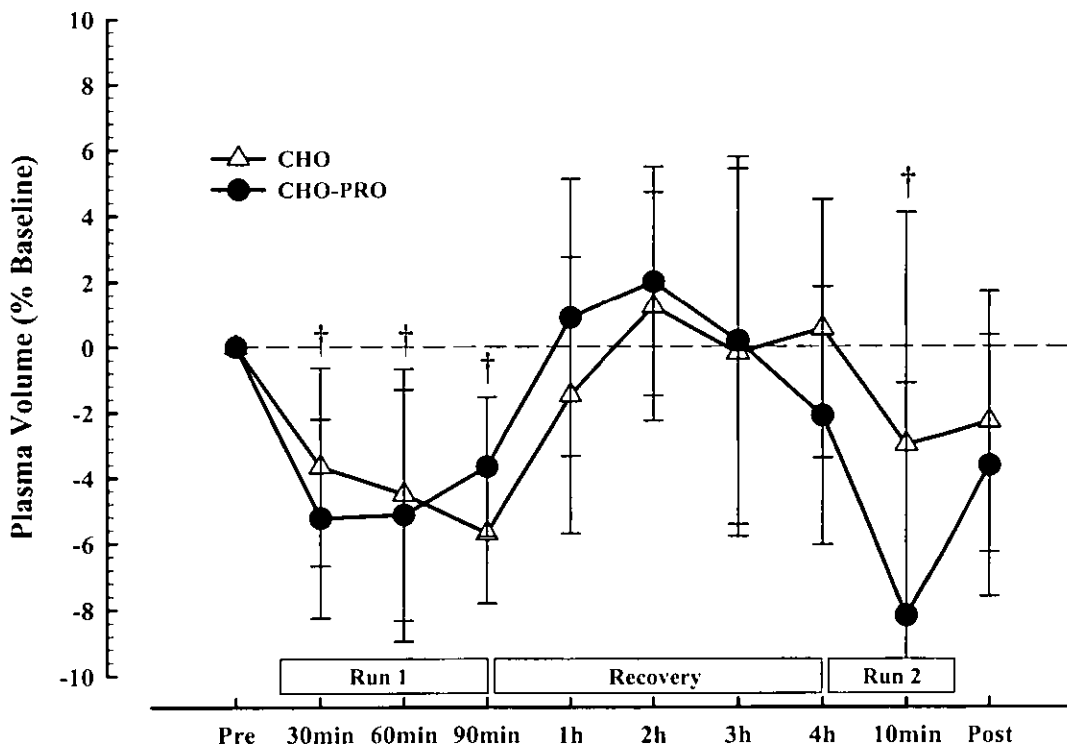


Figure 4.14: Changes in plasma volume during R₁, recovery and R₂. Participants ($n = 9$) received either CHO or CHO-PRO supplements during recovery. (N.B: at 10 min R₂ $n = 4$). Values are means \pm CI and † denotes values different from baseline ($P \leq 0.02$).

4.3.11 Subjective Data

The RPE indicated by participants (scale 6-20) during both R₁ and R₂ were not different between the CHO and CHO-PRO trials. Likewise, thirst ratings during the recovery and R₂ were unaffected by the independent variable. However, the cumulative rating for gut fullness throughout each trial was greater following ingestion of CHO-PRO as opposed to ingesting CHO alone ($P = 0.02$; Table 4.3).

Table 4.3: Subjective ratings of gut fullness during recovery and R₂. Participants ($n = 9$) received either CHO or CHO-PRO supplements during recovery. (N.B: at 10 min R₂ $n = 4$). Values are means \pm SEM and are different between trials ($P = 0.02$).

	Recovery				Run 2	
	1 h	2 h	3 h	4 h	10 min	Last min
Gut Fullness (scale 6-20)						
CHO	9 \pm 1	11 \pm 1	11 \pm 1	12 \pm 1	12 \pm 1	12 \pm 1
CHO-PRO	9 \pm 1	13 \pm 1	14 \pm 1	15 \pm 1	12 \pm 2	13 \pm 1

4.3.12 Control Data

The intensity of exercise performed in the CHO and CHO-PRO trials was controlled and kept consistent between each participant's first and second trial. During R₁ the average relative exercise intensity was $71.0 \pm 4\% \dot{V}O_2 \text{ max}$ in the CHO trial and $71.3 \pm 3\% \dot{V}O_2 \text{ max}$ in the CHO-PRO trial (mean \pm SD). This intensity was increased for R₂ but remained similar between trials: CHO = $84.0 \pm 5.7\% \dot{V}O_2 \text{ max}$ and CHO-PRO = $85.6 \pm 2.8\% \dot{V}O_2 \text{ max}$ (mean \pm SD). Accordingly, mean heart rates were very similar between trials and were $169 \pm 10 \text{ beats}\cdot\text{min}^{-1}$ overall during R₁ and $182 \pm 6 \text{ beats}\cdot\text{min}^{-1}$ overall during R₂ (mean \pm SD).

4.4 Discussion

The main finding of this study was that exercise capacity following 4 h of recovery from prolonged exercise was similar following ingestion of either a CHO solution or an identical solution with added protein. However, the addition of protein did result in a higher insulinaemic response during recovery, although no associated effect on plasma glucose concentrations was apparent.

A higher rate of glycogen resynthesis during recovery will result in a larger pre-exercise glycogen availability, therefore it is reasonable to assume that this may be reflected in an enhanced capacity to perform subsequent exercise (Ahlborg *et al.*, 1967; Bergstrom *et al.*, 1967). The improved cycling capacity following CHO-PRO rather than CHO ingestion observed by Williams *et al.* (2003) was attributed to the increased rate of muscle glycogen storage during recovery. Given the similarity of run times in the present study it might be concluded that glycogen accumulation occurred at similar rates in the CHO and CHO-PRO trials. However, since glycogen concentrations were not directly measured in this investigation, it cannot be ruled out that factors other than muscle glycogen depletion may have contributed to eventual fatigue. Indeed, recent evidence using ^{13}C magnetic resonance spectroscopy to quantify both muscle and liver glycogen has indicated that, when individuals perform repeated exercise sessions⁰ with a limited (i.e. 4 h) recovery, the depletion of liver glycogen may be the principle factor initiating fatigue (Casey *et al.*, 2000).

While the data presented in section 4.3.10 negates the suggestion that dehydration directly influenced time to exhaustion during R_2 , it remains a possibility that an accumulation of metabolic by-products may have contributed to fatigue. Blood lactate values immediately following R_2 were $5.0 \pm 0.3 \text{ mmol}\cdot\text{l}^{-1}$ (CHO) and $4.4 \pm 0.4 \text{ mmol}\cdot\text{l}^{-1}$ (CHO-PRO), which may suggest significant intramuscular accumulation of metabolic by-products that are associated with high-intensity activity (e.g. hydrogen ions, lactate, inorganic phosphate and adenosine diphosphate). These metabolites can potentially induce fatigue both through an inhibition of key glycolytic enzymes (Chasiotis *et al.*, 1983a; Sutton *et al.*, 1981; Trivedi and Danforth, 1966) and through interference with excitation-contraction coupling (Allen and Westerblad, 2001; Nakamaru and Schwartz, 1972). The present results may therefore suggest that

factors other than carbohydrate availability contributed to fatigue during the treadmill run to exhaustion (R_2). Clearly, if fatigue during this second run was primarily the consequence of increased anaerobic metabolism rather than compromised substrate availability, then this may question the validity of a capacity test at 85% $\dot{V}O_2$ max as a proxy measure for muscle glycogen availability. However, there is evidence that increased pre-exercise muscle glycogen availability can improve exercise capacity even at maximal and supra-maximal workloads (Maughan and Poole, 1981; Pizza *et al.*, 1995), possibly due to an enhancing effect of muscle glycogen either on H^+ buffering capacity or fibre excitability (Stephenson *et al.*, 1999).

If the run times recorded during R_2 were in fact dictated by pre-exercise muscle glycogen content, then it is important to elucidate why the rate of muscle glycogen resynthesis would be similar between the CHO and CHO-PRO trials when the insulinaemic responses were different. It would be expected that the higher insulin concentrations following CHO-PRO ingestion should increase glucose uptake into muscle and also the conversion of this glucose into glycogen. However, plasma glucose concentrations were not different between trials which may suggest that glucose uptake occurred at a similar rate under both experimental conditions. One possibility is that the rate of glucose uptake was already maximal following ingestion of 1.2 g CHO·kg⁻¹·h⁻¹ (Carrithers *et al.*, 2000; Jentjens *et al.*, 2001; van Loon *et al.*, 2000a). If this were the case then it becomes understandable why the additional protein might fail to increase both glucose uptake and glycogen resynthesis, since insulin would no longer be a rate limiting factor for these processes (Jentjens *et al.*, 2001).

The sharp fall in plasma glucose concentrations upon commencement of R_2 was interpreted as 'rebound' hypoglycaemia in response to the combination of high carbohydrate intake and the commencement of exercise. In agreement with the literature (Jentjens *et al.*, 2003; Moseley *et al.*, 2003), this challenge to glucose homeostasis was quickly compensated for and would therefore not be expected to contribute to eventual fatigue. However, the observed trend for higher glucose values at 10 min R_2 following ingestion of CHO-PRO rather than ingestion of CHO (3.8 ± 0.5 versus 2.9 ± 0.4 mmol·l⁻¹, respectively) may be of physiological relevance in terms of skilled performance since many participants were noticeably less alert at this

stage of the CHO trial. The mechanism underlying this improved maintenance of plasma glucose availability in the CHO-PRO trial may be an increased hepatic glucose output. In support of this, the elevated plasma urea concentrations following CHO-PRO ingestion could indicate an increased deamination of the exogenously provided amino acids in the liver, thus supplying carbon for synthesis of glucose through gluconeogenesis (Wahren *et al.*, 1973).

The observed increase in urea concentrations upon ingestion of the CHO-PRO solutions may also help explain the observed trend towards a lower respiratory exchange ratio (RER) during the CHO-PRO trial than during the CHO trial (main effect: $P = 0.06$). The indirect calorimetric method used to estimate both rates of substrate metabolism and the RER assumes protein metabolism to be negligible. However, it is likely that ingestion of the CHO-PRO solution increased amino acid catabolism in the liver, thus simultaneously overestimating lipid metabolism (i.e. reducing the RER) and increasing urea production.

Participants' reported perceptions of gut fullness were more intense when the CHO-PRO solution was ingested during recovery than when the CHO solution was ingested, possibly as a consequence of a delayed rate of gastric emptying. In general, intact protein is thought to empty from the stomach slower than carbohydrate (Thomas, 1957) and certain amino acids are known to inhibit intestinal absorption (van Loon *et al.*, 2000b). In a more recent study, however, isoenergetic glucose or soy protein hydrolysate solutions were instilled into the stomach and similar rates both of gastric emptying and intestinal absorption were observed via gastric aspiration and deuterium oxide tracer methods, respectively (Maughan *et al.*, 2004). Conversely, increased energy density has consistently been shown to delay gastric emptying (Vist and Maughan, 1995, 1994) and it is therefore likely that any impairment of gastric emptying rate following CHO-PRO ingestion in the present study would arise from the increased energy content of this solution rather than the specific macronutrient composition. It is therefore also conceivable that the slightly reduced peak in plasma glucose following CHO-PRO ingestion may reflect a more gradual appearance of exogenous glucose from the gastrointestinal tract.

In conclusion, consumption of the CHO-PRO solutions elicited greater serum insulin concentrations during recovery than consumption of CHO alone. The glycaemic response was not different between the CHO and CHO-PRO trials, possibly because the provision of $1.2 \text{ g CHO}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$ alone was sufficient to stimulate maximal rates of glucose uptake. Ultimately, the conclusion of this study is that, converse to the findings of Williams *et al.* (2003) involving continuous cycling, treadmill running capacity was not improved by CHO-PRO ingestion any more effectively than following ingestion of CHO alone.

CHAPTER 5

Carbohydrate-Protein Ingestion During Recovery: Effect of Carbohydrate Amount.

5.1 Introduction

A previous study by Williams *et al.* (2003) has indicated that ingestion of a carbohydrate-protein supplement (CHO-PRO) during short-term recovery (i.e. <8 h) may promote a more rapid restoration of exercise capacity than a supplement containing carbohydrate (CHO) with no additional protein. Since exercise capacity is believed to correlate positively with the magnitude of pre-exercise muscle glycogen stores (Ahlborg *et al.*, 1967; Bergstrom *et al.*, 1967), the improved time to exhaustion following CHO-PRO was attributed to the increased rate of glycogen resynthesis observed during recovery (Williams *et al.*, 2003). However, it remains unclear whether the increased rate of glycogen resynthesis and concomitant ergogenic benefit reported by Williams *et al.* (2003) was the result of the additional protein or a product of the higher carbohydrate content of the CHO-PRO supplement. Therefore, the previous study of this thesis compared the effects of a CHO-PRO solution and a CHO solution on recovery of exercise capacity but when the solutions were matched for carbohydrate content (i.e. 1.2 g CHO·kg⁻¹·h⁻¹).

The data recorded during this initial study revealed serum insulin concentrations to be higher while ingesting the CHO-PRO solution during recovery rather than while ingesting CHO alone. In contrast, plasma glucose responses did not appear to be influenced differently between trials and exercise capacity subsequent to recovery was also similar between the CHO-PRO and CHO treatments. There is currently inconsistency within the literature regarding the effect of ingesting CHO-PRO during recovery on muscle glycogen resynthesis (Ivy *et al.*, 2002; Jentjens *et al.*, 2001) and some authors have suggested that these inconsistencies may be resolved through an identification of the precise quantities of carbohydrate included in CHO-PRO supplements (Carrithers *et al.*, 2000; Jentjens *et al.*, 2001; van Loon *et al.*, 2000a). Specifically, it appears that the rate of muscle glycogen resynthesis has most commonly been accelerated following the addition of protein to ≤ 0.8 g CHO·kg⁻¹·h⁻¹

(Ivy *et al.*, 2002; van Loon *et al.*, 2000a; Zawadzki *et al.*, 1992) while adding protein to ≥ 1.2 g CHO \cdot kg $^{-1}\cdot$ h $^{-1}$ has produced no such effect (Jentjens *et al.*, 2001; Van Hall *et al.*, 2000b).

This suggestion leaves open the possibility that reducing the rate of carbohydrate ingestion below that applied during Study 1 might be less likely to maximise the rate of muscle glycogen resynthesis during recovery. Reducing the rate of muscle glycogen resynthesis in response to carbohydrate ingestion alone might therefore provide some scope for the additional protein to exert a positive influence on recovery. In concordance with this possibility, the study described in this chapter replicated the procedures of Study 1 exactly except that the CHO and CHO-PRO solutions that were ingested during recovery provided carbohydrate in amounts likely to be insufficient to maximise the rate of muscle glycogen resynthesis (i.e. 0.8 g CHO \cdot kg $^{-1}\cdot$ h $^{-1}$). It was therefore hypothesised that the additional protein would enhance the sub-optimal rate of muscle glycogen resynthesis expected in response to CHO ingestion alone and that this enhancement would be manifested as an increased capacity for physical exercise subsequent to recovery.

5.2 Methods

5.2.1 Participants

All 7 participants who volunteered to take part in this study were healthy males and included running as part of their habitual training. The physiological characteristics of these participants are presented in Table 5.1.

Table 5.1: Participant details

Characteristic	Mean \pm SD
Age (yr)	22 \pm 0.5
Height (m)	1.83 \pm 0.1
Mass (kg)	83.5 \pm 11.8
$\dot{V}O_2$ max (ml·kg ⁻¹ ·min ⁻¹)	55 \pm 4

5.2.2 Experimental Design

Each participant performed 2 main trials separated by at least 1 week in a randomised and counterbalanced design whereby interventions were applied in a double blind manner. Over the 48 h prior to participants' first trial a dietary record was completed and then the same diet was consumed prior to trial 2 (3623 \pm 1630 kcal·day⁻¹, 49 \pm 8% CHO, 30 \pm 8% fat, 21 \pm 3% protein: mean \pm SD). Each of these main trials involved a 90 min run at 70% $\dot{V}O_2$ max (R_1) followed by a 4 h recovery, during which participants rested in the laboratory while consuming either CHO alone or a CHO-PRO mixture. Exercise capacity was then assessed as run-time to exhaustion at an intensity of 85% $\dot{V}O_2$ max (R_2).

5.2.3 Experimental protocol

The experimental procedures applied during this study were identical to those described in Chapter 4 and a schematic representation of this protocol is included on page 100. All participants arrived in the laboratory between 8-8.30 am following a 10 h overnight fast and were asked to complete a pre-trial consent form (Appendix Aii). After providing a urine sample, each participant's post-void nude body mass was

recorded (Avery Ltd., UK) before a cannula was inserted into an antecubital vein and a 10 ml resting venous blood sample obtained. The cannula was kept patent throughout each trial by frequent flushing with isotonic saline. Prior to exercise, the Douglas bag technique was used to collect a 5 min resting expired gas sample (Williams and Nute, 1983). Participants were required to stand for 15 min prior to the collection of all resting gas and blood samples. A 5 min run at 60% $\dot{V}O_2$ max was used as a standardised warm-up prior to running at a speed equivalent to 70% $\dot{V}O_2$ max for 90 min (R_1). One minute expired gas samples, heart rates (Polar 8810, Finland) and RPE (Borg, 1973) followed by 10 ml venous blood samples were taken at 30 min intervals throughout R_1 . Water intake was permitted *ad libitum* during trial 1 and matched in trial 2 (1.24 ± 0.42 l; mean \pm SD). Nude body mass was recorded immediately following R_1 to assess hydration status through percentage change in mass.

The first volume of prescribed solution was provided as soon as post R_1 nude body mass had been recorded. The remaining 7 volumes were provided at 30 min intervals during the 4 h recovery such that the last solution was ingested 30 min prior to R_2 , participants were permitted 15 min to consume each volume. Expired gas samples and venous blood samples, along with ratings of gut fullness and thirst (6-20 scale; Appendices Bii and Biii), were taken during the recovery period every hour prior to feedings. Nude body mass was again recorded and, after the standard warm-up, participants began the run to exhaustion at 85% $\dot{V}O_2$ max (R_2) with any background music and verbal encouragement being standardised between trials (Atkinson *et al.*, 2004). As in R_1 , water intake was *ad libitum* during trial 1 and matched in trial 2 (0.50 ± 0.33 l; mean \pm SD). Physiological measurements were obtained at regular intervals (10 min) and at the point of volitional fatigue. Nude body mass was recorded immediately after R_2 , again to assess hydration status through changes in body mass. Ambient temperature and humidity were recorded at 30 min intervals throughout all testing periods using a hygrometer (Zeal, UK) and were not different between trials. Average values recorded were 23.2 ± 1.3 °C and $43.1 \pm 5.7\%$ (mean \pm SD). Full details of all sampling and analysis are included in the General Methods section 3.5.

5.2.4 Solution Composition

The CHO and CHO-PRO solutions ingested during this study were identical to those provided during Study 1 (i.e. 9.3% carbohydrate solutions: 6.2% glucose and 3.1% fructose). However, the total volume of solution provided relative to each participant's body mass was reduced in the current investigation ($722 \pm 102 \text{ ml}\cdot\text{h}^{-1}$) such that a lower quantity of carbohydrate was ingested ($0.8 \text{ g CHO}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$). Therefore, the total amount of carbohydrate provided during recovery was $267 \pm 38 \text{ g}$ (mean \pm SD). The protein fraction was also the same as in Study 1 (1.5%) resulting in a lower relative quantity of protein being ingested ($0.13 \text{ g PRO}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$) but maintaining the carbohydrate-protein ratio at $\approx 6:1$. Again, including this additional quantity of protein ($43 \pm 6 \text{ g}$ in total; mean \pm SD) made approximately 16.7% more energy available in the CHO-PRO mixture ($3.7 \text{ kcal}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$) than in the solution containing CHO alone ($3.2 \text{ kcal}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$). As before, the CHO and CHO-PRO solutions were taste matched (orange and passion fruit flavour) and the amino acid profile of the CHO-PRO solution is included in Chapter 4 (Figure 4.2, p. 102).

5.2.5 Statistical Analyses

The exercise capacity data of Williams *et al.* (2003) were utilised to estimate that a sample size of 7 has an 87% power to detect a difference in means of 11.1 min, assuming a SD of differences of approximately 9.4, using a paired t-test with a 1 sided significance level. The exercise capacity data recorded during R₂ were found to be non-normally distributed and a Wilcoxon test was therefore applied to compare the median run times between trials, along with any other ordinal/discrete variables (i.e. RPE, gut fullness and thirst) requiring non-parametric statistical analysis. Paired t-tests were used to analyse the incremental area under curve data that was calculated to assess the glycaemic and insulinaemic responses to each solution during recovery (Wolever, 2004). A 2 way general linear model for repeated measures (Drink x Time) was used to identify differences between experimental conditions. The Greenhouse-Geisser correction was utilised for epsilon <0.75, while the Huynh-Feldt correction was adopted for less severe asphericity. Where significant F values were found, the Holm-Bonferroni step-wise correction was applied to determine the location of variance where necessary (Atkinson, 2002; Ludbrook, 1998). Unless otherwise stated, all data are expressed in text as mean \pm SEM and the error bars included on figures are confidence intervals (CI) that have been corrected to remove between subject variance (Masson and Loftus, 2003). Specific details of, and justification for, these statistical methods are included in the General Methods section 3.8.

5.3 Results

5.3.1 Exercise Capacity

There was no difference in exercise capacity following ingestion of the CHO or CHO-PRO solutions (Effect size = 0.1) and a large degree of inter-individual variability was apparent. Median run times to exhaustion at 85% $\dot{V}O_2$ max (R_2) were 18 (12-52) min during the CHO trial and 19.5 (15-35) min during the CHO-PRO trial (Figure 5.1).

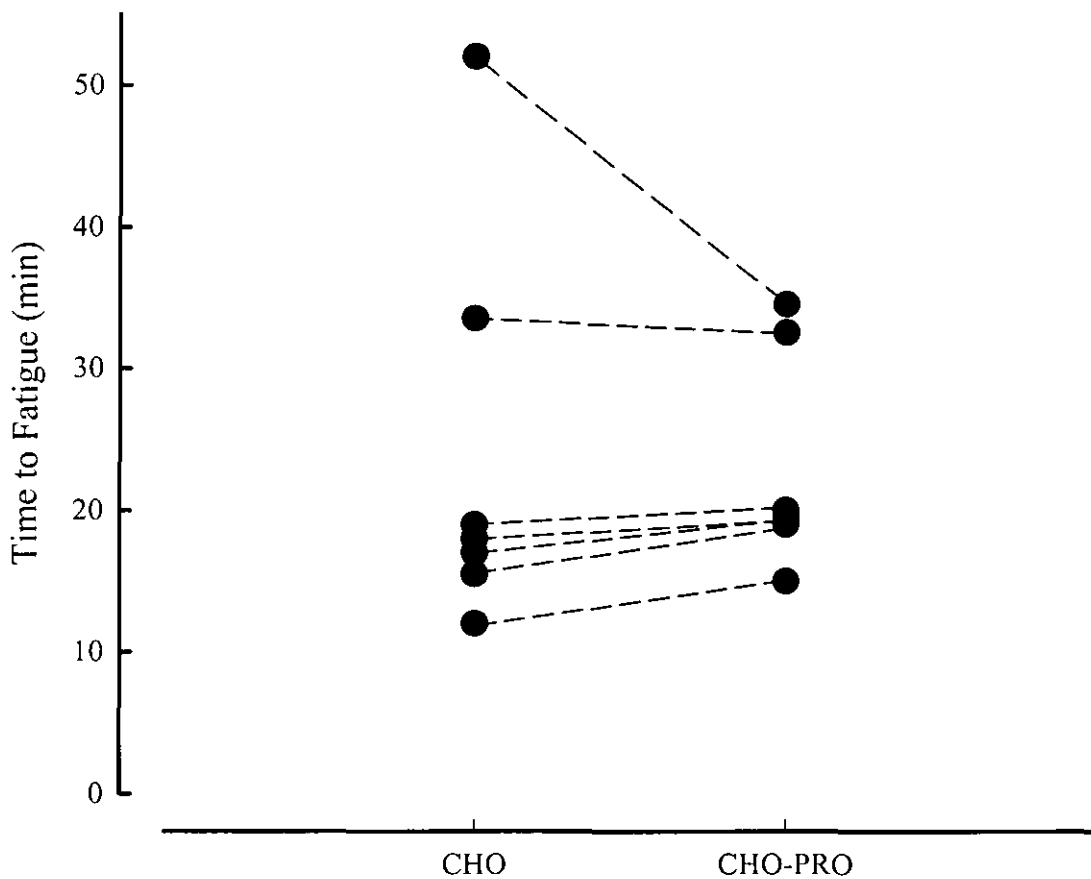


Figure 5.1: Run times to exhaustion for each individual during R_2 following ingestion of either CHO or CHO-PRO during recovery ($n = 7$).

5.3.2 Serum Insulin

Ingestion of CHO-PRO produced greater serum insulin concentrations than ingestion of CHO alone ($F = 4.1$, $P = 0.02$) and *post hoc* statistical analysis of this data revealed that differences reached statistical significance after 3 h of recovery ($P = 0.03$; Figure 5.2). However, this finding of higher insulin concentrations with CHO-PRO ingestion rather than CHO ingestion was consistent for the entire duration of recovery and the insulinaemic response for the 4 h period reflected this pattern ($P = 0.01$; Figure 5.3).

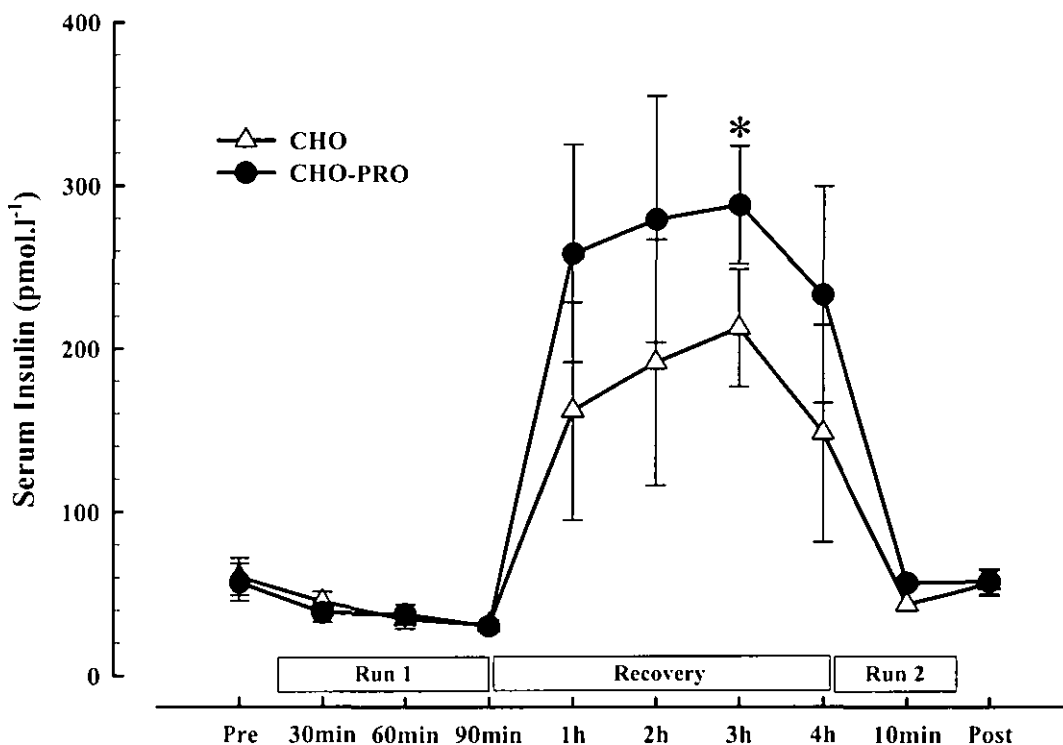


Figure 5.2: Serum insulin concentrations during R₁, recovery and R₂. Participants ($n = 7$) received either CHO or CHO-PRO supplements during recovery. (N.B: insufficient degrees of freedom to construct CI at 10 min R₂, $n = 4$). Values are means \pm CI and * denotes values different between trials ($P = 0.03$). To convert from SI units into $\mu\text{IU}\cdot\text{ml}^{-1}$ divide by 6.95.

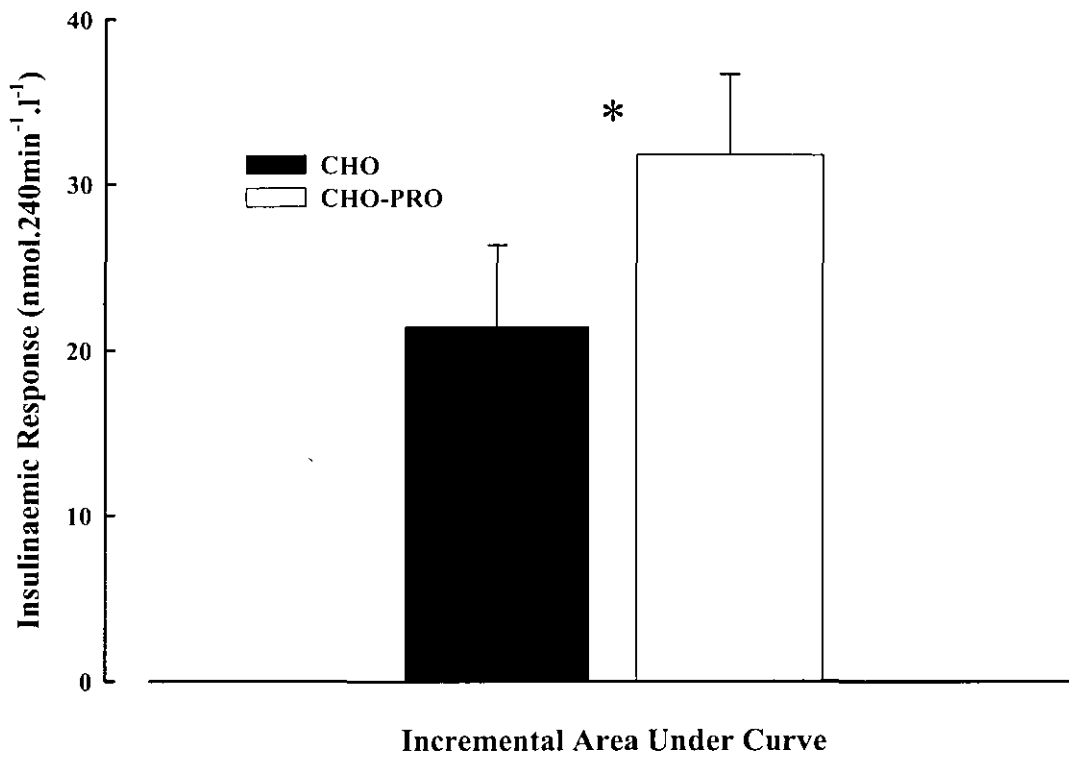


Figure 5.3: Insulinaemic responses during 4 h recovery ($n = 7$). Values are means \pm SE and * denotes values different between trials ($P = 0.01$). To convert from SI units into $\text{mIU}\cdot\text{ml}^{-1}$ divide by 6.95.

5.3.3 Plasma Glucose

Glucose concentrations peaked in both trials after 1 h of recovery and gradually reduced over the remaining 3 h (Figure 5.4). While no significant differences between trials were identified at any specific sampling point, there was a trend for more a more rapid reduction in plasma glucose concentrations while ingesting the CHO-PRO rather than the CHO solutions. This trend resulted in a significantly lower glycaemic response with the CHO-PRO trial when expressed as incremental area under the glucose concentration curve for the entire recovery ($P = 0.02$; Figure 5.5). After 10 min of subsequent exercise (R_2), plasma glucose concentrations had fallen to $3.5 \pm 0.3 \text{ mmol}\cdot\text{l}^{-1}$ in the CHO trial and $3.9 \pm 0.2 \text{ mmol}\cdot\text{l}^{-1}$ in the CHO-PRO trial, although these values were not significantly different from the concentrations recorded at baseline.

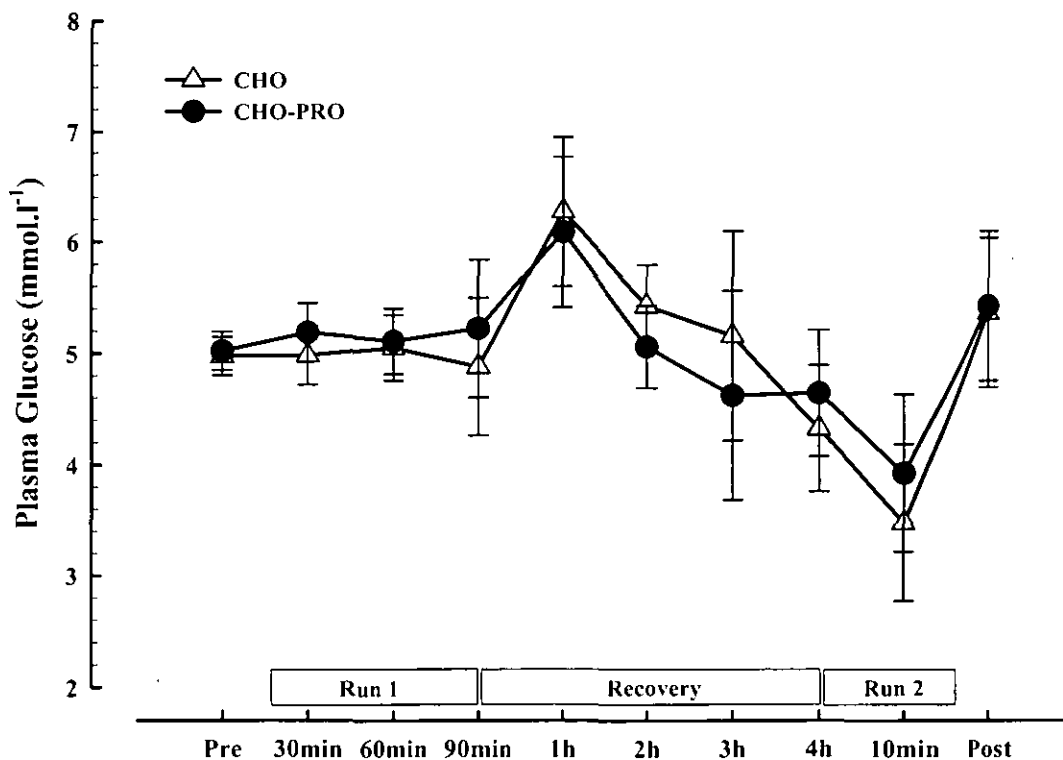


Figure 5.4: Plasma glucose concentrations during R_1 , recovery and R_2 . Participants ($n = 7$) received either CHO or CHO-PRO supplements during recovery. (N.B: at 10 min R_2 $n = 5$). Values are means \pm CI.

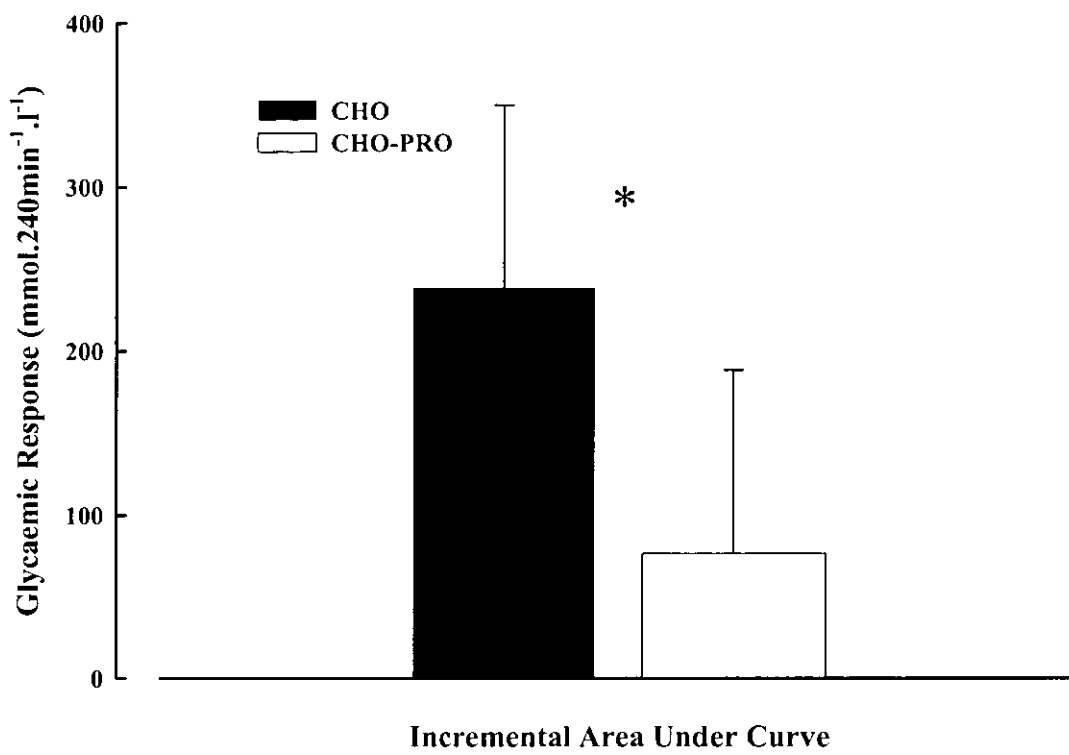


Figure 5.5: Glycaemic responses during 4 h recovery ($n = 7$). Values are means \pm CI and * denotes values different between trials ($P = 0.02$).

5.3.4 Plasma Urea

During the CHO-PRO trial, plasma urea concentrations began to increase immediately upon ingestion of the first solution and had reached a concentration of $6.7 \pm 0.4 \text{ mmol}\cdot\text{l}^{-1}$ by the end of R₂. Conversely, after 1 h of ingesting the CHO solution, plasma urea concentrations decreased such that values were $5.6 \pm 0.3 \text{ mmol}\cdot\text{l}^{-1}$ after R₂ (Figure 5.6). Although a strongly significant interaction effect was identified ($F = 11.6, P < 0.001$), the mean effects at any given time point were not large enough to attain statistical significance. Notably, however, significant effects could be revealed at 3 h, 4 h and Post R₂ ($P \leq 0.02$) if the data was normalised to remove baseline differences between treatments.

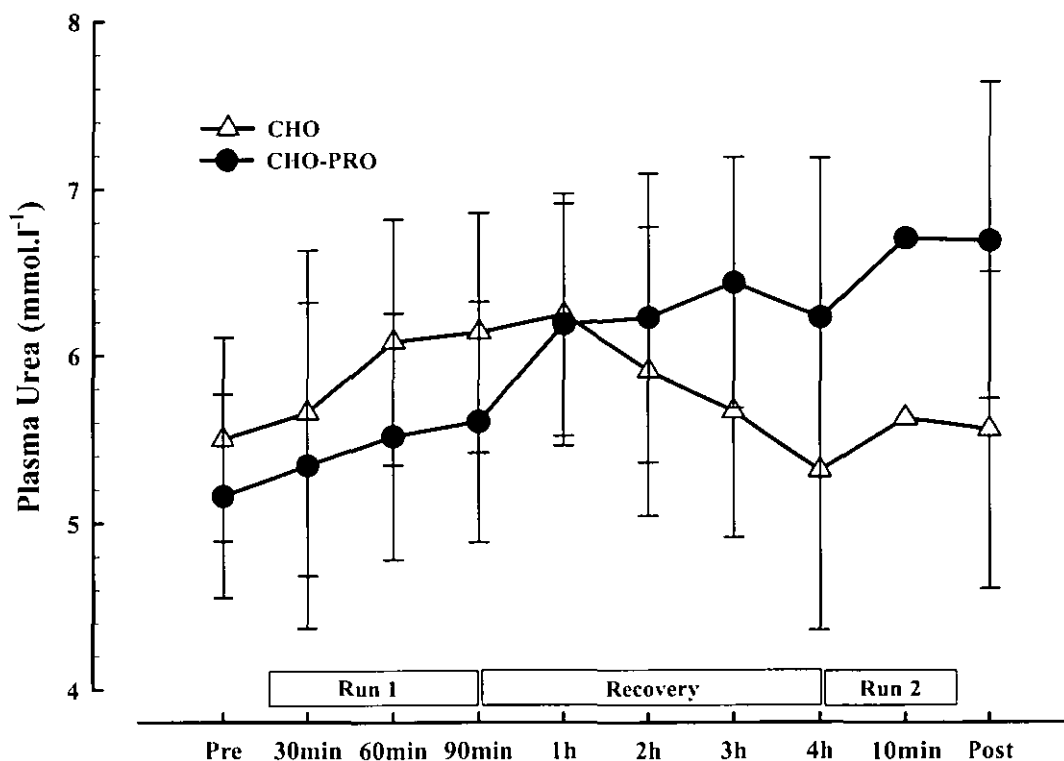


Figure 5.6: Plasma urea concentrations during R₁, recovery and R₂. Participants ($n = 7$) received either CHO or CHO-PRO supplements during recovery. (N.B: insufficient degrees of freedom to construct CI at 10 min R₂, $n = 5$). Values are means \pm CI.

5.3.5 Plasma Free Fatty Acids and Glycerol

Free fatty acid and glycerol concentrations increased with each exercise session and were reduced substantially during feeding. There were no differences in the concentrations of either metabolite between the CHO and CHO-PRO treatments (Figures 5.7 and 5.8).

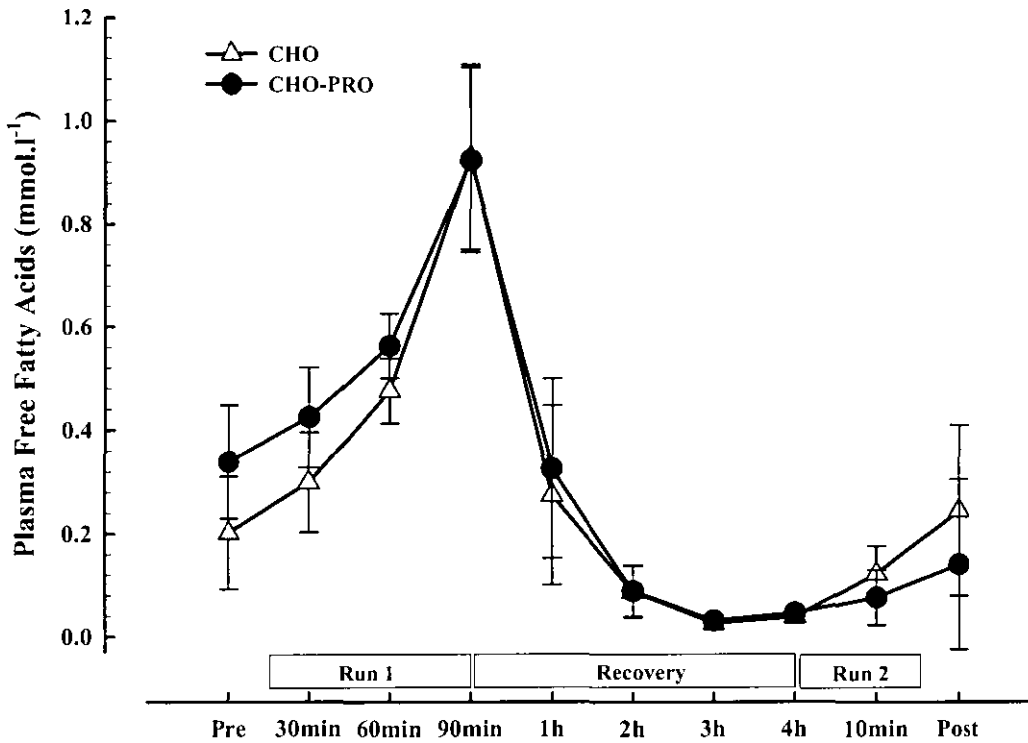


Figure 5.7: Plasma free fatty acid concentrations during R₁, recovery and R₂. Participants (n = 7) received either CHO or CHO-PRO supplements during recovery. (N.B: at 10 min R₂ n = 5). Values are means ± CI.

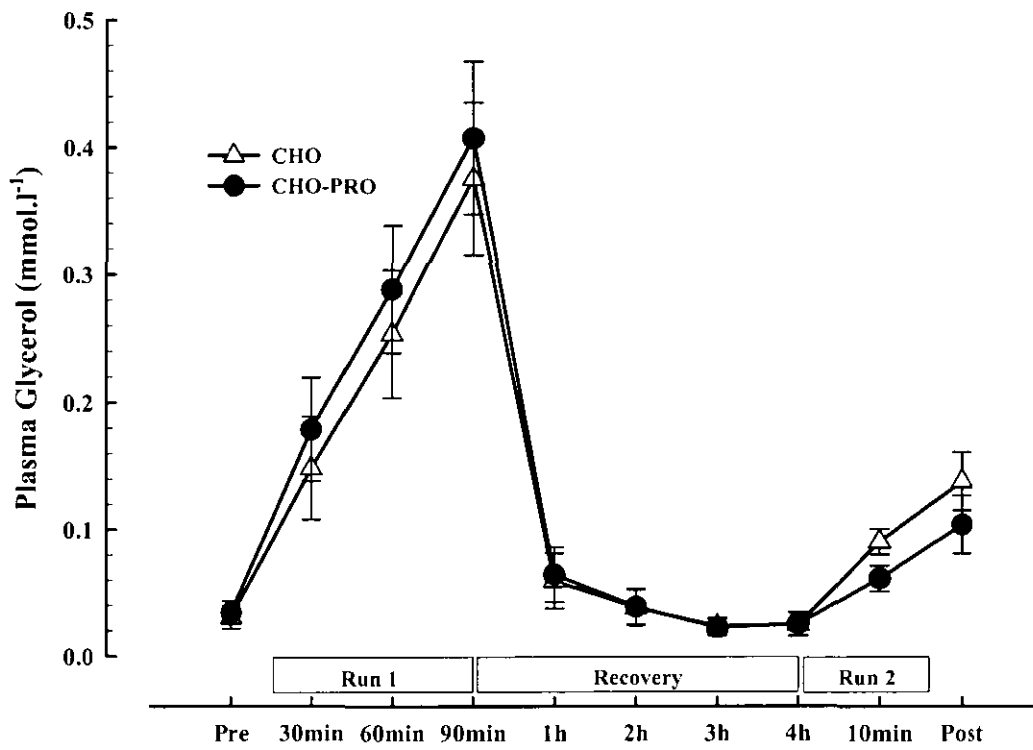


Figure 5.8: Plasma glycerol concentrations during R₁, recovery and R₂. Participants (n = 7) received either CHO or CHO-PRO supplements during recovery. (N.B: at 10 min R₂ n = 5). Values are means ± CI.

5.3.6 Blood Lactate

No differences were found between the blood lactate concentrations recorded during the CHO and CHO-PRO trials. Concentrations increased slightly above baseline during R_1 and recovery before further increasing during R_2 (Figure 5.9).

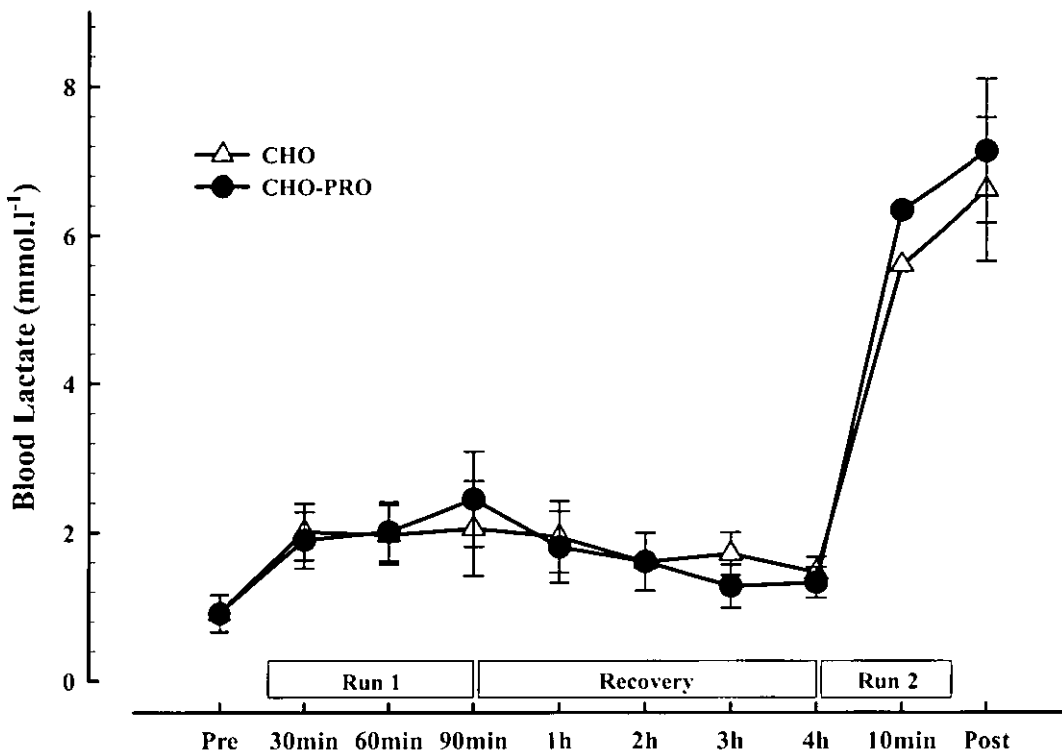


Figure 5.9: Blood lactate concentrations during R_1 , recovery and R_2 . Participants ($n = 7$) received either CHO or CHO-PRO supplements during recovery. (N.B: insufficient degrees of freedom to construct CI at 10 min R_2 , $n = 5$). Values are means \pm CI.

5.3.7 Substrate Metabolism

The respiratory exchange ratio (RER) along with estimations of carbohydrate and lipid metabolism were not different between the CHO and CHO-PRO trials. Total metabolism of both substrates increased during R_1 and then decreased during feeding. Metabolism of lipid remained minimal during the second exercise session (R_2) while carbohydrate metabolism again increased during exercise (Table 5.2).

Table 5.2: Substrate metabolism and respiratory exchange ratios during R₁, recovery and R₂. Participants (n = 7) received either CHO or CHO-PRO supplements during recovery (N.B: at 10 min R₂ n = 6). Values are means ± SEM.

	Pre	30 min	Run 1		Recovery				Run 2	
			60 min	90 min	1 hr	2 hr	3 hr	4 hr	10 min	Last min
Carbohydrate oxidation (g.min⁻¹)										
CHO	0.3±0.1	3.2±0.2	2.8±0.2	2.5±0.2	0.3±0.04	0.3±0.04	0.4±0.1	0.5±0.04	4.4±0.3	4.5±0.3
CHO-PRO	0.3±0.03	3.0±0.2	2.8±0.1	2.8±0.2	0.3±0.1	0.4±0.1	0.4±0.04	0.5±0.1	4.7±0.3	4.7±0.3
Lipid oxidation (g.min⁻¹)										
CHO	0.1±0.02	0.3±0.1	0.5±0.1	0.6±0.1	0.1±0.02	0.1±0.01	0.1±0.01	0.04±0.01	0.1±0.07	0.1±0.1
CHO-PRO	0.1±0.02	0.3±0.04	0.5±0.1	0.5±0.04	0.1±0.03	0.1±0.02	0.1±0.02	0.04±0.02	0.04±0.03	0.03±0.03
RER										
CHO	0.86±0.03	0.95±0.01	0.92±0.01	0.90±0.01	0.86±0.02	0.88±0.01	0.91±0.02	0.96±0.01	1.02±0.02	1.01±0.02
CHO-PRO	0.91±0.04	0.94±0.01	0.92±0.01	0.91±0.01	0.86±0.03	0.89±0.02	0.90±0.02	0.97±0.02	1.03±0.02	1.03±0.02

5.3.8 Serum Growth Hormone

Serum concentrations of human growth hormone were very similar during CHO and CHO-PRO trials. The increases observed during R_1 were reduced to baseline levels upon ingestion of the first solution. Concentrations then elevated again when the second exercise bout (R_2) was initiated (Figure 5.10).

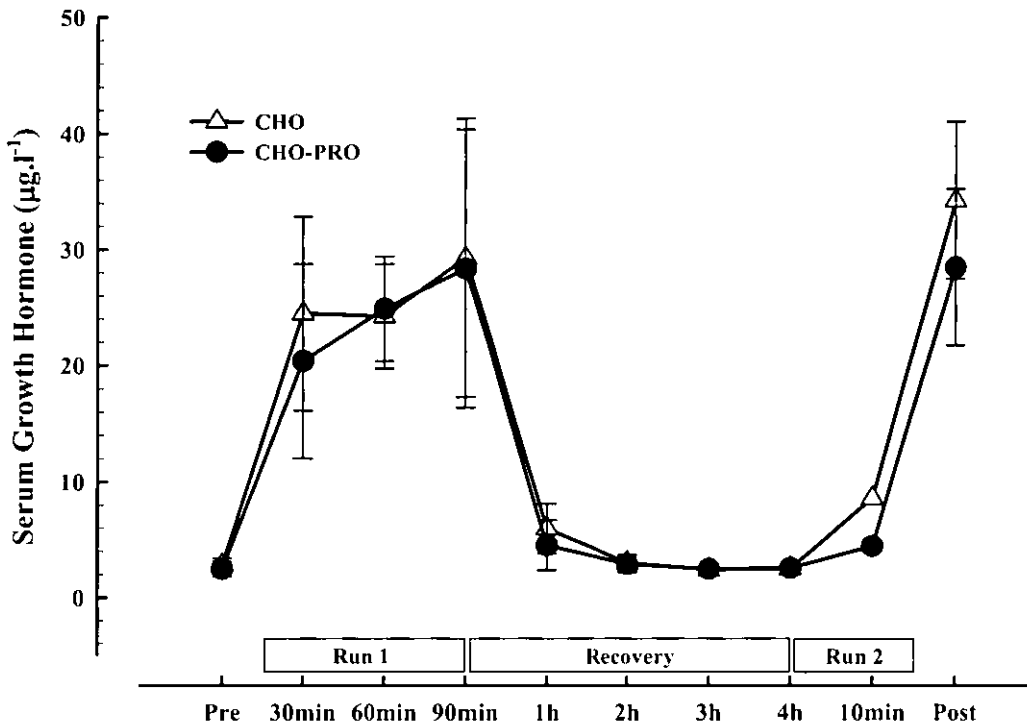


Figure 5.10: Serum growth hormone concentrations during R_1 , recovery and R_2 . Participants ($n = 7$) received either CHO or CHO-PRO supplements during recovery. (N.B: insufficient degrees of freedom to construct CI at 10 min R_2 , $n = 4$). Values are means \pm CI.

5.3.9 Serum Cortisol

Serum cortisol concentrations were not different between main trials at any time point, concentrations elevated with increasing exercise duration in both R₁ and R₂ but declined over time during recovery (Figure 5.11).

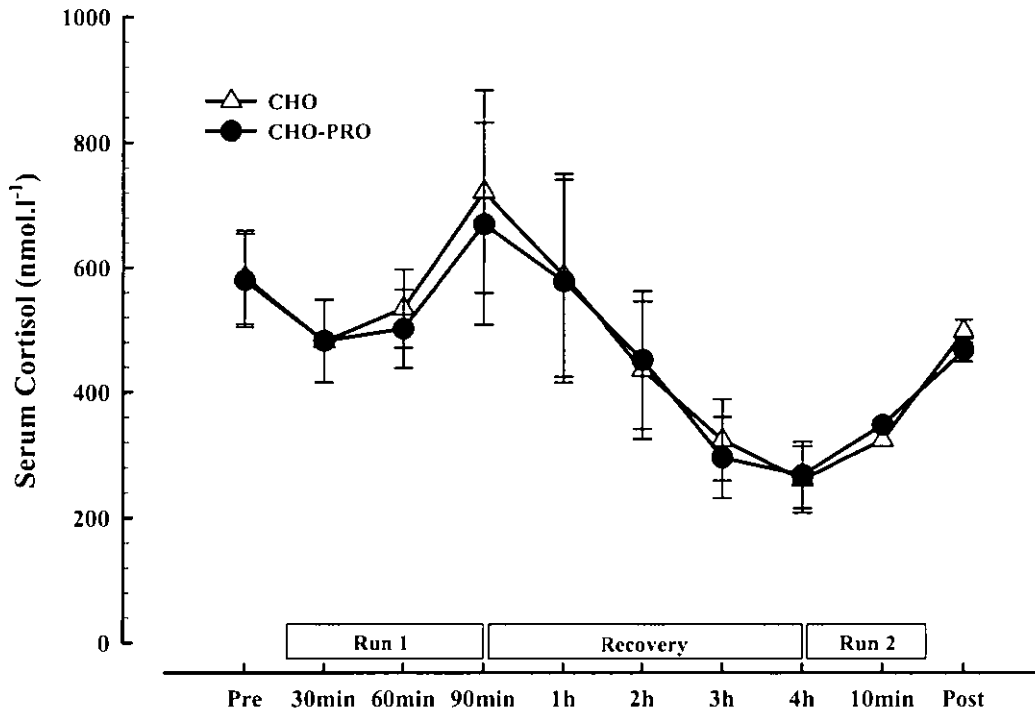


Figure 5.11: Serum cortisol concentrations during R₁, recovery and R₂. Participants (n = 7) received either CHO or CHO-PRO supplements during recovery. (N.B: insufficient degrees of freedom to construct CI at 10 min R₂, n = 5). Values are means ± CI.

5.3.10 Plasma Myoglobin

There were no differences in plasma myoglobin concentrations following either CHO or CHO-PRO ingestion (Figure 5.12). Concentrations reached 14.3 ± 2.8 $\text{nmol}\cdot\text{l}^{-1}$ (CHO) and 18.2 ± 4.2 $\text{nmol}\cdot\text{l}^{-1}$ (CHO-PRO) 1 h following R_1 before reducing gradually during the remainder of recovery and finally increasing to concentrations of 20.4 ± 5.0 $\text{nmol}\cdot\text{l}^{-1}$ (CHO) and 21.3 ± 6.4 $\text{nmol}\cdot\text{l}^{-1}$ (CHO-PRO) immediately following R_2 .

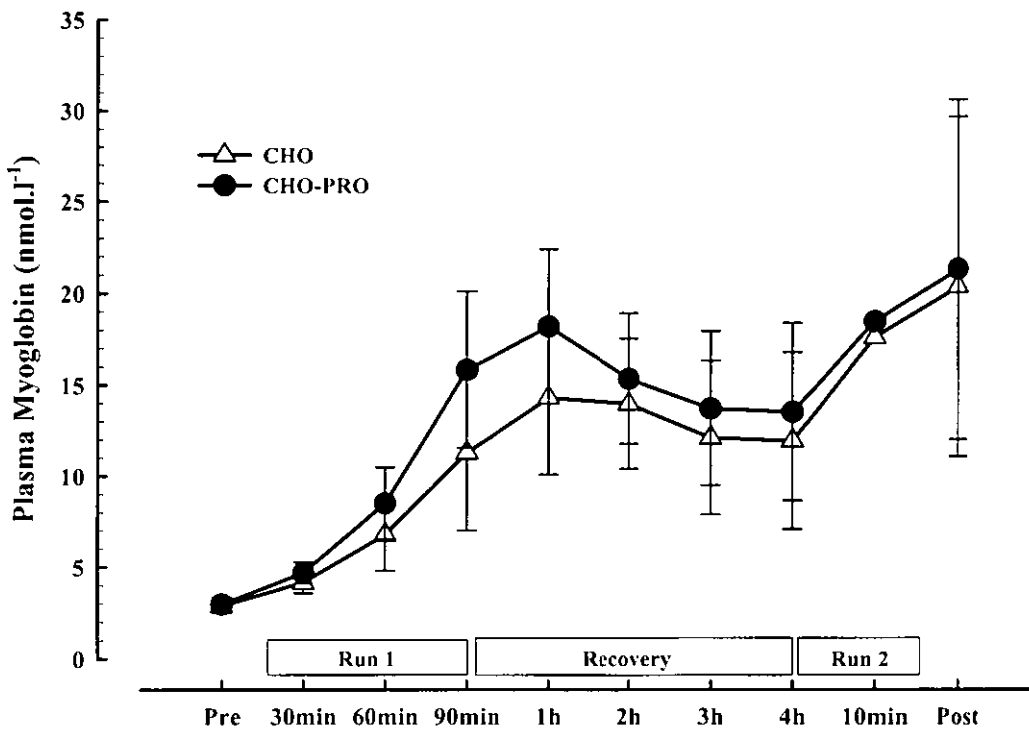


Figure 5.12: Plasma myoglobin concentrations during R_1 , recovery and R_2 . Participants ($n = 7$) received either CHO or CHO-PRO supplements during recovery. (N.B: insufficient degrees of freedom to construct CI at 10 min R_2 , $n = 5$). Values are means \pm CI.

5.3.11 Indices of Hydration Status

The urine osmolality measurements made prior to main trials did not reveal any differences between the CHO and CHO-PRO trials: 724.9 ± 331.5 mosmol·kg⁻¹ and 639.3 ± 83.3 mosmol·kg⁻¹ (mean \pm SD), respectively. Changes in body mass (BM) during R₁ were $-1.4 \pm 0.2\%$ BM in the CHO trial and $-1.6 \pm 0.2\%$ BM in the CHO-PRO trial. During R₂, a decrease of $-0.8 \pm 0.2\%$ BM was recorded both in the CHO and CHO-PRO trials. Figure 5.13 illustrates the changes in plasma volume throughout each trial, there were no differences between the CHO and CHO-PRO treatments but an effect of time was established ($F = 9.0$, $P = 0.001$).

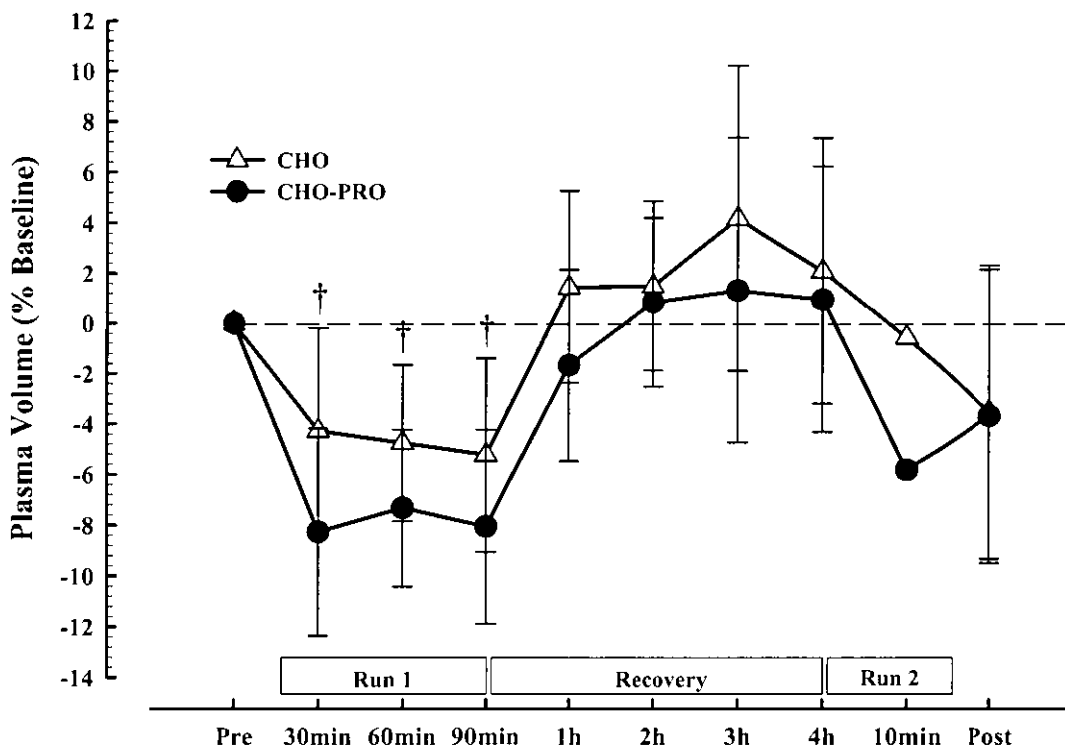


Figure 5.13: Changes in plasma volume during R₁, recovery and R₂. Participants ($n = 7$) received either CHO or CHO-PRO supplements during recovery. (N.B: insufficient degrees of freedom to construct CI at 10 min R₂, $n = 5$). Values are means \pm CI and † denotes values different from baseline ($P \leq 0.003$).

5.3.12 Subjective Data

Participants' subjective ratings of perceived exertion (scale 6-20) increased steadily during R₁ to eventual values of 14.7 ± 0.6 in the CHO trial and 14.7 ± 0.5 in the CHO-PRO trial. The higher intensity of R₂ was reflected by increased sensations of perceived effort, with ratings noted in the final minute of this run being 17.9 ± 0.3 (CHO) and 17.7 ± 0.4 (CHO-PRO). There were no statistical differences between these scores. Neither were differences apparent between trials for any other subjective variables. Perceptions of thirst were found to decrease during recovery and increase during R₂, while gut fullness increased during recovery and decreased slightly during subsequent exercise (Table 5.3).

Table 5.3: Subjective ratings of gut fullness during recovery and R₂. Participants (n = 7) received either CHO or CHO-PRO supplements during recovery. (N.B: at 10 min R₂ n = 6). Values are means \pm SEM.

	Recovery				Run 2	
	1 h	2 h	3 h	4 h	10 min	Last min
Gut Fullness (scale 6-20)						
CHO	10 ± 1	11 ± 1	12 ± 1	11 ± 1	11 ± 1	10 ± 1
CHO-PRO	9 ± 1	9 ± 1	11 ± 1	10 ± 1	10 ± 2	10 ± 1

5.3.13 Control Data

The average exercise intensities of R₁ (mean \pm SD) were $71.1 \pm 2.1\%$ $\dot{V}O_2$ max and $70.5 \pm 3.1\%$ $\dot{V}O_2$ max in the CHO and CHO-PRO trials, respectively. The exercise intensities elicited for R₂ were $84.8 \pm 2.7\%$ $\dot{V}O_2$ max in the CHO trial and $86.6 \pm 4.4\%$ $\dot{V}O_2$ max in the CHO-PRO trial (mean \pm SD). Heart rates recorded during these periods were almost identical between the CHO and CHO-PRO trials, overall averaging 164 ± 11 beats·min⁻¹ during R₁ and 178 ± 8 beats·min⁻¹ during R₂ (mean \pm SD).

5.4 Discussion

The ingestion of the CHO-PRO mixture during 4 h of recovery from prolonged exercise did not enhance subsequent exercise capacity any more effectively than ingesting a solution containing a matched quantity of carbohydrate alone. Insulin concentrations were higher during recovery while ingesting the solution with added protein and glycaemic responses during this period were lower following ingestion of CHO-PRO as opposed to ingestion of CHO.

The amount of carbohydrate provided was reduced from 1.2 g CHO·kg⁻¹·h⁻¹ (Study 1) to the amount provided in the current investigation (i.e. 0.8 g CHO·kg⁻¹·h⁻¹) in concordance with the suggestion that ingesting 1.2 g CHO·kg⁻¹·h⁻¹ may be sufficient to maximise glucose uptake even without protein (Carrithers *et al.*, 2000; Jentjens *et al.*, 2001; van Loon *et al.*, 2000a). In contrast to the results of the earlier study, the present results revealed glycaemic responses to be lower while ingesting the CHO-PRO solution rather than the CHO solution. This finding is in agreement with the findings of other authors (Ivy *et al.*, 2002; Van Hall *et al.*, 2000a; Van Hall *et al.*, 2000b; van Loon *et al.*, 2000a; Zawadzki *et al.*, 1992) and may indicate an increased rate of glucose uptake, potentially stimulated by the elevated insulin concentrations during the CHO-PRO trial. However, this suggestion can only be made tentatively since blood glucose concentration actually provides a reflection of whole body glucose turnover and not glucose uptake *per se*. Indeed, Van Hall *et al.* (2000b) report that the lower glucose concentrations they observed following CHO-PRO rather than CHO ingestion were not the product of an increased rate of glucose uptake, although it should be noted that the amount of carbohydrate provided in the above study (1.25 g CHO·kg⁻¹·h⁻¹) was probably sufficient to maximise glucose transport irrespective of the inclusion of protein (Van Hall *et al.*, 2000b).

Those studies which have reported higher insulin and lower glucose concentrations following the addition of protein to ≤ 0.8 g CHO·kg⁻¹·h⁻¹ have all found the rate of muscle glycogen resynthesis to be enhanced during recovery in comparison with ingestion of the carbohydrate fraction alone (Ivy *et al.*, 2002; Van Hall *et al.*, 2000a; van Loon *et al.*, 2000a; Zawadzki *et al.*, 1992). In light of these findings, it is possible that insulin may only represent a rate limiting factor for glycogen resynthesis

when carbohydrate provision is insufficient to elicit maximal GLUT4 migration to the muscle cell membrane (Jentjens *et al.*, 2001; Van Hall *et al.*, 2000b). Since the amount of carbohydrate provided in the present study would not be expected to maximise glucose uptake, the higher insulinaemic response to the additional protein might have accelerated the rate of glycogen resynthesis during recovery. It is therefore interesting that exercise capacity was similar between trials despite the observed differences in insulin concentrations.

While it remains a possibility that the rate of muscle glycogen resynthesis was in fact similar between treatments, another plausible interpretation would be that fatigue during the capacity test did not coincide with muscle glycogen depletion. Assuming the latter interpretation to be true, any differences between trials in the rate of muscle glycogen resynthesis would not necessarily be manifested as an improved exercise capacity during R₂. Similar to Study 1, the possibility cannot be refuted that fatigue during R₂ was actually the product of either inhibited glycolysis (Chasiotis *et al.*, 1983a; Sutton *et al.*, 1981; Trivedi and Danforth, 1966) or impaired excitation-contraction coupling (Allen and Westerblad, 2001; Nakamaru and Schwartz, 1972). Blood lactate concentrations immediately following the run to exhaustion at 85% $\dot{V}O_2$ max were 6.6 ± 1 mmol·l⁻¹ in the CHO trial and 7.1 ± 0.9 mmol·l⁻¹ in the CHO-PRO trial which may reflect an increased accumulation of metabolic by-products within active muscle fibres (i.e. hydrogen ions, lactate, inorganic phosphate and adenosine diphosphate). Although some evidence does support an ergogenic benefit of improved muscle glycogen availability even when the causes of fatigue originate primarily from anaerobic glycolysis (Maughan and Poole, 1981; Pizza *et al.*, 1995; Stephenson *et al.*, 1999), it has been suggested that prolonged exercise capacity tests (i.e. ≥ 90 min) at lower relative exercise intensities might provide a more valid reflection of pre-exercise muscle glycogen concentrations since fatigue would be more likely to coincide with depletion of these stores (Hawley *et al.*, 1997b).

A common feature to both the current results and those presented in Chapter 4 is the relatively low plasma glucose concentrations recorded during R₂. After 10 min of this second exercise session, plasma glucose had fallen to concentrations of 3.5 ± 0.3 mmol·l⁻¹ and 3.9 ± 0.2 mmol·l⁻¹ in the CHO and CHO-PRO trials, respectively. However, this 'rebound' hypoglycaemia was a transient response to the onset of

exercise following feeding and would therefore not be expected to contribute to eventual fatigue (Jentjens *et al.*, 2003; Moseley *et al.*, 2003). As discussed in Chapter 4, the rise in plasma urea concentrations following CHO-PRO ingestion may reflect an increased deamination of amino acids in the liver. This would liberate α -keto acids for hepatic glucose production through gluconeogenesis, potentially supplementing plasma glucose concentrations during R₂.

Overall, the results of this study demonstrate that the addition of protein to a carbohydrate solution can enhance the insulinaemic effect of this solution during recovery from prolonged exercise. Ingestion of the CHO-PRO solution was also associated with lower plasma glucose concentrations during recovery than ingestion of CHO alone, possibly indicating an increased rate of glucose uptake into muscle. Exercise capacity subsequent to recovery was similar following ingestion of the CHO and CHO-PRO solutions, which may suggest that factors other than carbohydrate availability contributed to fatigue during R₂. This conclusion is in agreement with the view that pre-exercise muscle glycogen availability may not dictate exercise capacity in tests lasting only ≈ 20 min (Hawley *et al.*, 1997b). Furthermore, there is evidence that exercise capacity tests conducted at intensities in excess of $\approx 80\%$ $\dot{V}O_2$ max may not be reliable (Krebs and Powers, 1989). This might explain the relatively large within subject variance in run times both in the current study and in Study 1, which would support the view that a full familiarisation with such high intensity capacity tests may be necessary to reveal a treatment effect (Hopkins *et al.*, 2001). In addition, the large between subject variation in both studies might also indicate that not all of the participants in these investigations were exercising at the same relative exercise intensity during R₂, possibly as a result of using a progressive incline $\dot{V}O_2$ max protocol to determine relative exercise intensities during level running (St. Clair Gibson *et al.*, 1999). Clearly, given that an intensity of 85% $\dot{V}O_2$ max is likely to have been close to most participants' anaerobic threshold, even a very minor overestimation of $\dot{V}O_2$ max could impact considerably upon metabolism during that exercise. It is therefore proposed that an exercise capacity test at a lower relative exercise intensity (e.g. 70% $\dot{V}O_2$ max) might identify any ergogenic effect of the CHO-PRO solution more reliably whilst also providing a more valid reflection of muscle glycogen resynthesis rates during recovery.

CHAPTER 6

Recovery of Prolonged Running Capacity Following Ingestion of Carbohydrate Plus Protein.

6.1 Introduction

Exercise capacity during exercise of moderate to high intensity is positively correlated with the availability of muscle glycogen and fatigue during such exercise often coincides with depletion of this energy source (Ahlborg *et al.*, 1967; Bergstrom *et al.*, 1967). Given this relationship, it is therefore reasonable to assume that any supplement which increases the rate of muscle glycogen resynthesis during recovery will also restore the capacity for aerobic exercise more rapidly. Carbohydrate ingestion has been demonstrated to accelerate the rate of muscle glycogen resynthesis following prolonged exercise (Bergstrom *et al.*, 1967; Ivy, 1998) and to promote a more rapid recovery of endurance capacity (Fallowfield *et al.*, 1995). Furthermore, there is some evidence that adding protein to more moderate carbohydrate loads ($\leq 0.8 \text{ g CHO} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$) may further enhance the glycogenic effect of such solutions (Fogt and Ivy, 2000; van Loon *et al.*, 2000a; Zawadzki *et al.*, 1992).

The effect of additional protein on muscle glycogen storage probably originates from the synergistic influence of carbohydrate and amino acids on insulin secretion (Floyd *et al.*, 1970a, 1970b; Rabinowitz *et al.*, 1966) and it appears that the precise quantity of protein provided will determine the extent of insulin release (van Loon *et al.*, 2000c). Adding $\geq 0.3 \text{ g} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$ of mixed amino acids has been consistently found to substantially enhance the insulinotropic effect of carbohydrate solutions (Jentjens *et al.*, 2001; Van Hall *et al.*, 2000a; Van Hall *et al.*, 2000b; van Loon *et al.*, 2000a; Zawadzki *et al.*, 1992), while studies that have added less than this amount of protein have reported a considerably smaller influence on insulin response (Carrithers *et al.*, 2000; Ivy *et al.*, 2002; Ivy *et al.*, 2003). In addition, it is likely that the magnitude of insulinaemic response to any given quantity of protein will be strongly dependent on the specific amino acid profile of that protein (van Loon *et al.*, 2000b).

Williams *et al.* (2003) have recently reported a 128% greater rate of muscle glycogen storage when their participants ingested CHO-PRO as opposed to CHO alone throughout a 4 h recovery. Participants in this study were then able to cycle 55% longer during a subsequent exercise capacity test following CHO-PRO ingestion, although this ergogenic effect may largely be attributed to the increased amount of carbohydrate provided by the CHO-PRO supplement (Williams *et al.*, 2003). Similarly, Saunders *et al.* (2004) have reported an increased cycle time to exhaustion following ingestion of CHO-PRO rather than CHO during a more prolonged recovery period (12-15 h). However, while the solutions in this study were matched for carbohydrate content, the CHO-PRO solution contained 25% more energy and it therefore remains to be established whether the improved performance was the consequence of this additional energy or the presence of protein *per se* (Saunders *et al.*, 2004).

The total energy content of a supplement is known to influence muscle glycogen synthesis regardless of the inclusion of protein (Roy and Tarnopolsky, 1998). For example, van Loon *et al.* (2000a) have demonstrated increased rates of muscle glycogen resynthesis when protein is *added* to a carbohydrate solution, while *replacing* a fraction of the carbohydrate with protein (i.e. isoenergetic supplements) produced no such effect (van Loon *et al.*, 2000a). In a similar study, Ivy *et al.* (2002) found CHO-PRO ingestion to produce greater rates of muscle glycogen resynthesis not only in comparison with a carbohydrate matched solution but also a solution matched for total energy content (Ivy *et al.*, 2002).

The aim of the current study was to examine the effect of increasing the energy content of a standard CHO solution, either through adding protein or including further carbohydrate. This experimental design was therefore able to assess the CHO-PRO mixture in comparison with a carbohydrate matched solution while simultaneously evaluating whether an isoenergetic solution would produce similar effects. Thus, it can be established whether the proposed effect of such supplements on muscle glycogen resynthesis (Ivy *et al.*, 2002; van Loon *et al.*, 2000a) can be translated into a comparable effect on exercise capacity.

6.2 Methods

6.2.1 Participants

Six healthy men volunteered for this study, all of whom included 6 ± 2 h·week⁻¹ of endurance exercise as part of their habitual training. The physiological characteristics of these participants are presented in Table 6.1.

Table 6.1: Participant details

Characteristic	Mean \pm SD
Age (yr)	21 \pm 2.6
Height (m)	1.77 \pm 0.1
Mass (kg)	72.6 \pm 8.4
$\dot{V}O_2$ max (ml·kg ⁻¹ ·min ⁻¹)	61 \pm 7

6.2.2 Experimental Design

Each participant performed 3 main trials in a randomised, counterbalanced design that were separated by at least 1 week and applied in a double blind manner. A dietary record was completed over the 48 h prior to trial 1 and then participants were asked to adhere to this diet prior to subsequent trials (2497 ± 535 kcal·day⁻¹, $53 \pm 9\%$ CHO, $31 \pm 9\%$ fat, $16 \pm 3\%$ protein: mean \pm SD). These main trials involved a 90 min run at 70% $\dot{V}O_2$ max (R_1) followed by a 4 h recovery. During recovery, participants rested in the laboratory while ingesting either: a carbohydrate-protein mixture; a solution containing a moderate amount of carbohydrate (0.8 g CHO·kg⁻¹·h⁻¹); or a solution containing a high amount of carbohydrate (1.1 g CHO·kg⁻¹·h⁻¹). The latter 2 solutions matched the former for carbohydrate and for total energy, respectively. Exercise capacity was subsequently assessed as run-time to exhaustion at an intensity of 70% $\dot{V}O_2$ max (R_2).

6.2.3 Experimental protocol

The experimental protocol adopted for this study is illustrated graphically in Figure 6.1. All participants arrived in the laboratory between 8-8.30 am following a 10 h overnight fast and were asked to complete a pre-trial consent form (Appendix Aii). After providing a urine sample, each participant's post-void nude body mass was recorded (Avery Ltd., UK) before a cannula was inserted into an antecubital vein and a 10 ml resting venous blood sample obtained. The cannula was kept patent throughout each trial by frequent flushing with isotonic saline. Prior to exercise, the Douglas bag technique was used to collect a 5 min resting expired gas sample (Williams and Nute, 1983). Participants were required to stand for 15 min prior to the collection of all resting gas and blood samples. A 5 min run at 60% $\dot{V}O_2$ max was used as a standardised warm-up prior to running at a speed equivalent to 70% $\dot{V}O_2$ max for 90 min (R_1). One minute expired gas samples, heart rates (Polar 8810, Finland) and RPE (Borg, 1973) followed by 10 ml venous blood samples were taken at 30 min intervals throughout R_1 . Water intake was permitted *ad libitum* during trial 1 and matched during ensuing trials (0.50 ± 0.38 l; mean \pm SD). Nude body mass was recorded immediately following R_1 to assess hydration status through percentage change in mass.

The first volume of prescribed solution was provided as soon as post R_1 nude body mass had been recorded. The remaining 7 volumes were provided at 30 min intervals during the 4 h recovery such that the last solution was ingested 30 min prior to R_2 , participants were permitted 15 min to consume each volume. Expired gas samples and venous blood samples, along with ratings of gut fullness and thirst (6-20 scale; Appendices Bii and Biii), were taken during the recovery period every hour prior to feedings. Nude body mass was again recorded and, after the standard warm-up, participants began the run to exhaustion at 70% $\dot{V}O_2$ max (R_2) with any background music and verbal encouragement being standardised between trials (Atkinson *et al.*, 2004). As in R_1 , water intake was *ad libitum* during trial 1 and matched in subsequent trials (0.76 ± 0.52 l; mean \pm SD). Physiological measurements were obtained at 3 time points during the capacity test (10 min, 30 min and 60 min) and at the point of volitional fatigue. When participants first indicated that they could

no longer continue to run at the required intensity, the treadmill speed was reduced to $4.4 \text{ km}\cdot\text{h}^{-1}$ for a period of 2 min. Thereafter, the speed was increased to the previously applied intensity (i.e. $70\% \dot{V}\text{O}_2 \text{ max}$) and the exercise test resumed. This process was repeated on the second occasion that participants could not maintain the required exercise intensity and only on the third occasion was a final expired air collection made, volitional exhaustion accepted, and run time recorded. The post R_2 blood sample was drawn immediately following the point of volitional fatigue (i.e. within 30 s) and nude body mass was recorded within 5 min, again to assess hydration status through changes in body mass. Ambient temperature and humidity were recorded at 30 min intervals throughout all testing periods using a hygrometer (Zeal, UK) and were not different between trials. Average values recorded were $20.5 \pm 1.2 \text{ }^\circ\text{C}$ and $41.5 \pm 9.8\%$ (mean \pm SD). Full details of all sampling and analysis are included in the General Methods section 3.5.

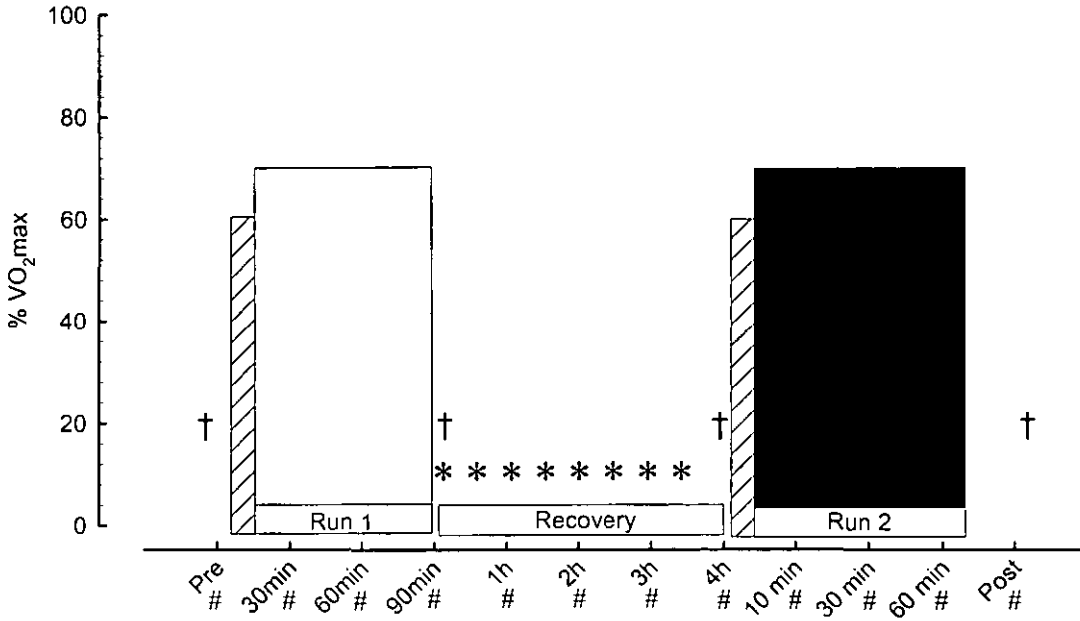


Figure 6.1: A schematic representation of the experimental protocol. Key: * = solution provision, # = expired gas sample/blood sample & RPE, † = body mass, hatched area = warm-up (5 min at 60% $\dot{V}O_2$ max), open area = R₁ (90 min at 70% $\dot{V}O_2$ max) and closed area = R₂ (70% $\dot{V}O_2$ max until volitional exhaustion).

6.2.4 Solution Composition

All solutions were provided in equal volumes between trials ($581 \pm 67 \text{ ml}\cdot\text{h}^{-1}$; mean \pm SD) relative to each participant's body mass such that the amount of carbohydrate (sucrose; GlaxoSmithKline, UK) included in the CHO and CHO-PRO solutions was $0.8 \text{ g}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$, a total of $232 \pm 27 \text{ g}$ CHO during recovery (mean \pm SD). These 2 solutions were identical except for the inclusion of 3.3% ($0.3 \text{ g PRO}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$) whey protein isolate (Protarmor 907 LSI, Armor Proteines, France) in the CHO-PRO solution (i.e. $\approx 3:1$ carbohydrate-protein ratio), thus providing $87 \pm 10 \text{ g}$ PRO during recovery (mean \pm SD) and increasing the metabolisable energy content by 37.5%. To control for the influence of this additional energy, a third trial was introduced (CHO-CHO) whereby the carbohydrate concentration of the CHO solution was increased from 10% to 13.3%. Accordingly, this third trial provided $1.1 \text{ g CHO}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$, equivalent to $320 \pm 37 \text{ g}$ CHO during recovery (mean \pm SD). Therefore, the estimated amount of energy that each solution made available for metabolism was $3.2 \text{ kcal}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$ in the CHO trial and $4.3 \text{ kcal}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$ in both the CHO-PRO and CHO-CHO trials. The specific amino acid profile of the whey protein isolate is illustrated in Figure 6.2. All solutions were matched for flavour (orange and passion fruit), consistency and odour.

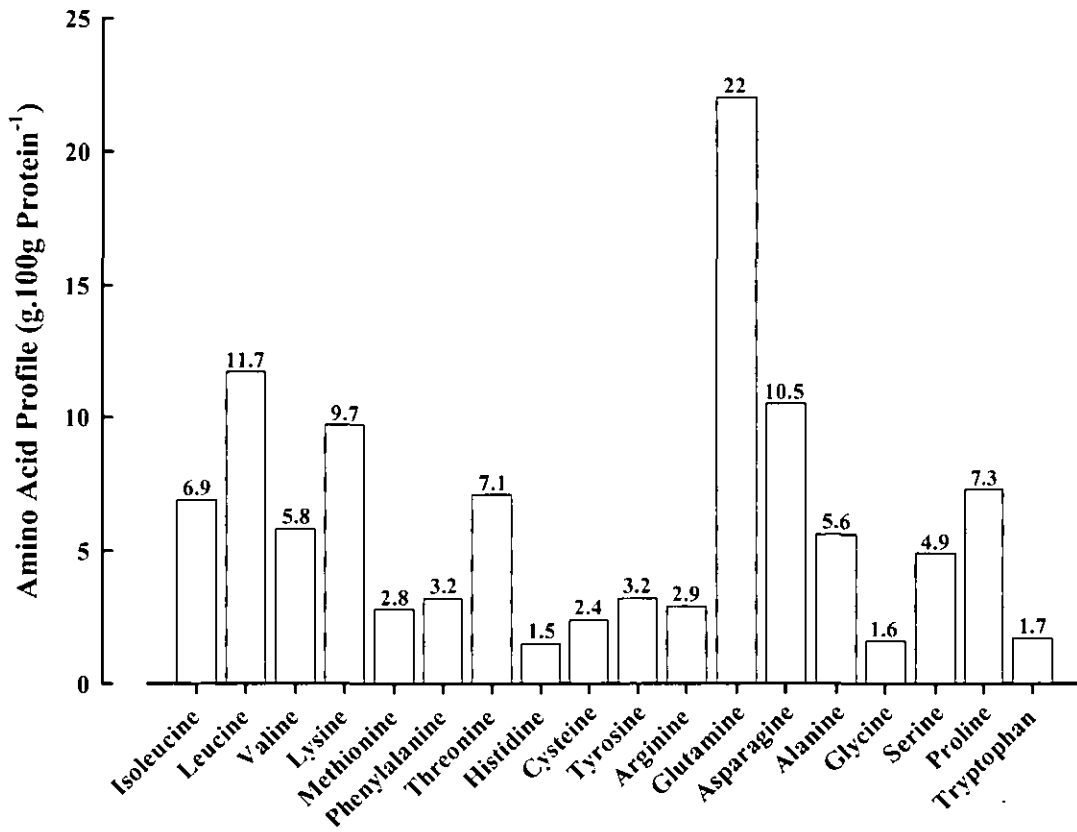


Figure 6.2: Amino acid profile of the whey protein isolate added to CHO solutions during CHO-PRO trials.

6.2.5 Statistical Analyses

The endurance capacity data of Fallowfield *et al.* (1995) were utilised to estimate that a sample size of 6 has a 99.9% power to detect a difference in means of 22.2 min, assuming a SD of differences of approximately 9.9, using a paired t-test with a 1 sided significance level. A 2 way general linear model for repeated measures (Drink x Time) was used to identify differences between experimental conditions. The Greenhouse-Geisser correction was utilised for epsilon <0.75, while the Huynh-Feldt correction was adopted for less severe asphericity. Where significant F values were found, the Holm-Bonferroni step-wise correction was applied to determine the location of variance where necessary (Atkinson, 2002; Ludbrook, 1998). Unless otherwise stated, all data are expressed in text as mean \pm SEM and the error bars included on figures are confidence intervals (CI) that have been corrected to remove between subject variance (Masson and Loftus, 2003). Where appropriate, these confidence intervals have been corrected for violations to sphericity through adjustment of the relevant degrees of freedom. With respect to the exercise capacity data however, it was of particular theoretical interest to illustrate the specific comparisons between the CHO-PRO solution and each other solution independently rather than to portray the general pattern between the 3 means. As such, 2 separate pairs of confidence intervals surround the CHO-PRO bar on Figure 6.3 (see over) based on the specific error terms from each contrast. Specific details of, and justification for, these statistical methods are included in the General Methods section 3.8.

6.3 Results

6.3.1 Exercise Capacity

In all 6 participants, ingestion of the CHO-PRO solution resulted in a significantly improved recovery of exercise capacity in comparison with CHO alone ($P = 0.02$; Effect Size = 1.5). The CHO-CHO solution also restored exercise capacity more effectively than the CHO solution in all but 1 of the participants in this study ($P = 0.05$; Effect Size = 1.1), although no statistical difference could be detected between the CHO-PRO and CHO-CHO solutions ($P = 0.07$; Effect Size 0.7). Specifically, the run times to exhaustion at 70% $\dot{V}O_2$ max (R_2) were: 83.7 ± 6.9 min in the CHO trial; 91.2 ± 6.5 min in the CHO-PRO trial (9% improvement versus CHO); and 99.9 ± 8.1 min in the CHO-CHO trial (19% improvement versus CHO; Figure 6.3).

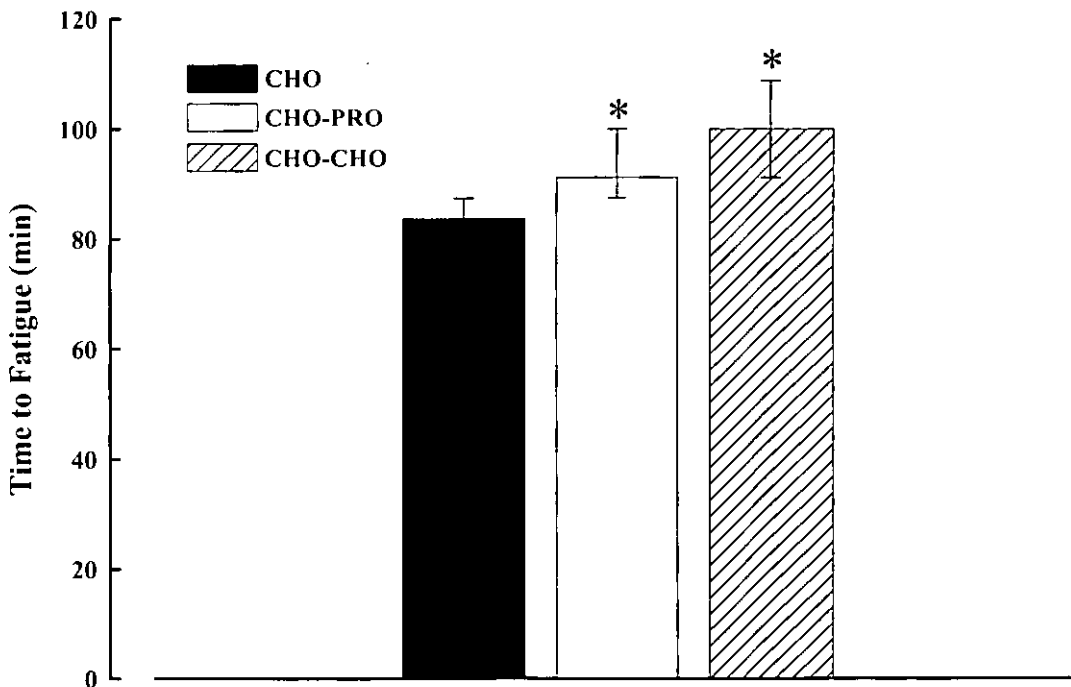


Figure 6.3: Run times to exhaustion recorded during R_2 ($n = 6$). Values are means \pm CI and * denotes values greater than CHO trial ($P \leq 0.05$).

6.3.2 Serum Insulin

Serum insulin concentrations significantly increased in all 3 trials during recovery. However, this increase was most pronounced during the CHO-PRO and CHO-CHO trials such that statistical analysis detected an effect of treatment (main effect: $F = 9.8$, $P = 0.05$; Figure 6.4). Conversion of this insulin concentration data into an insulinaemic response for the 4 h recovery period, expressed as incremental area under the curve, revealed differences between the CHO-PRO and CHO trials ($P = 0.03$; Figure 6.5).

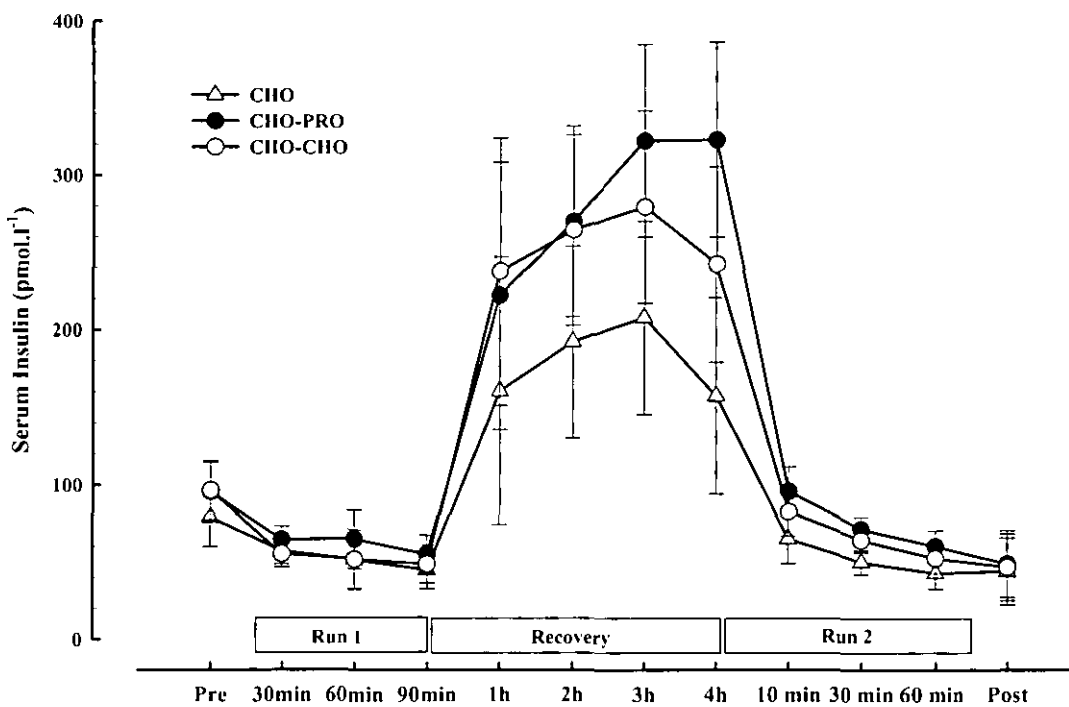


Figure 6.4: Serum insulin concentrations during R₁, recovery and R₂. Participants ($n = 6$) received either CHO, CHO-PRO or CHO-CHO supplements during recovery. (N.B: at 60 min R₂ and Post R₂ $n = 5$). Values are means \pm CI and are different between trials ($P = 0.05$). To convert SI units into $\mu\text{IU}\cdot\text{ml}^{-1}$ divide by 6.95.

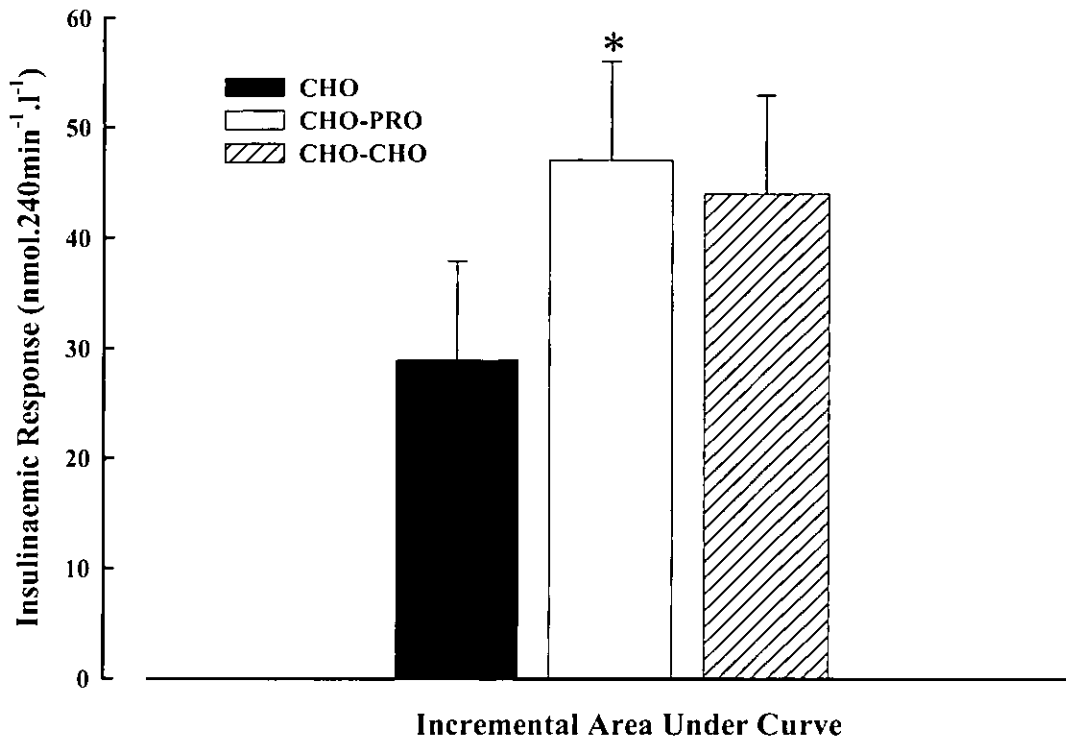


Figure 6.5: Insulinaemic responses during 4 h recovery ($n = 6$). Values are means \pm CI and * denotes values greater than CHO trial ($P = 0.03$). To convert SI units into $\text{mIU} \cdot \text{ml}^{-1}$ divide by 6.95.

6.3.3 Plasma Glucose

Plasma glucose concentrations peaked after 1 h of recovery in all trials and were lower at this point during the CHO-PRO trial than during the CHO-CHO trial ($P = 0.01$). Concentrations then decreased in both the CHO and CHO-CHO trials, while the CHO-PRO trials displayed stable glucose concentrations throughout recovery ($F = 3.7$, $P = 0.03$; Figure 6.6). Upon commencement of the second exercise bout (R_2), blood glucose concentrations in all 3 trials decreased markedly. This hypoglycaemia at 10 min R_2 was more severe in the CHO trial than in the CHO-PRO trial ($P = 0.03$) and the difference between these 2 treatments was maintained until 30 min of this exercise session ($P = 0.004$). Conversion of these data into a glycaemic response for the 4 h recovery period did not expose any treatment differences (Figure 6.7).

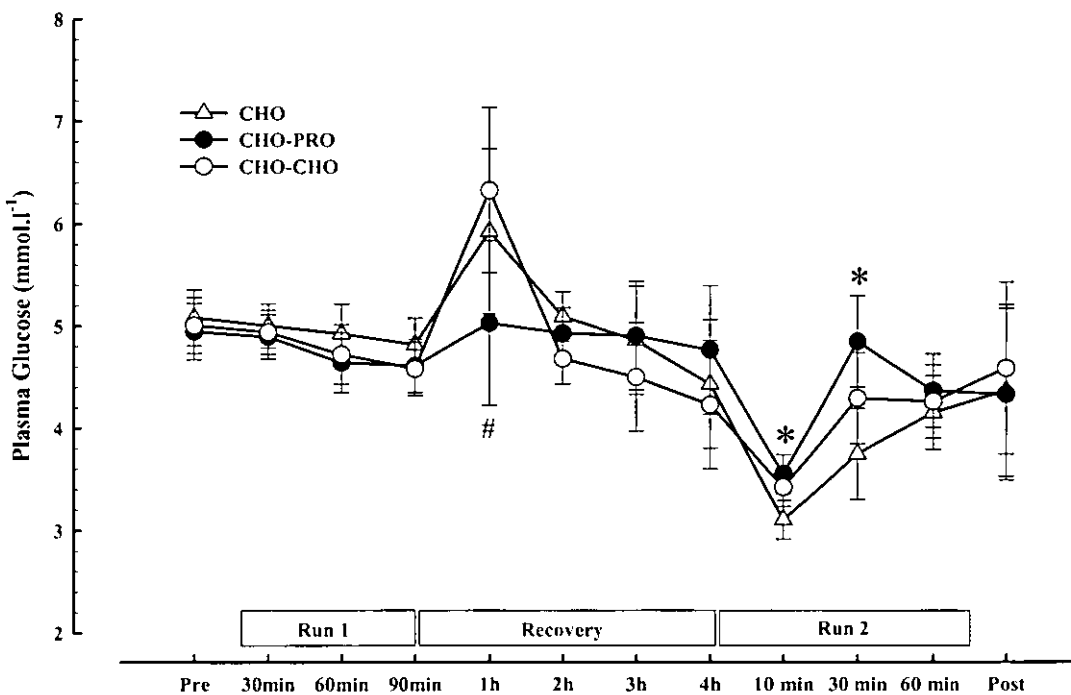


Figure 6.6: Plasma glucose concentrations during R_1 , recovery and R_2 . Participants ($n = 6$) received either CHO, CHO-PRO or CHO-CHO supplements during recovery. (N.B: at 60 min R_2 and Post R_2 $n = 5$). Values are means \pm CI. # denotes values different CHO-PRO versus CHO-CHO ($P = 0.01$); * denotes values different CHO-PRO versus CHO ($P \leq 0.03$).

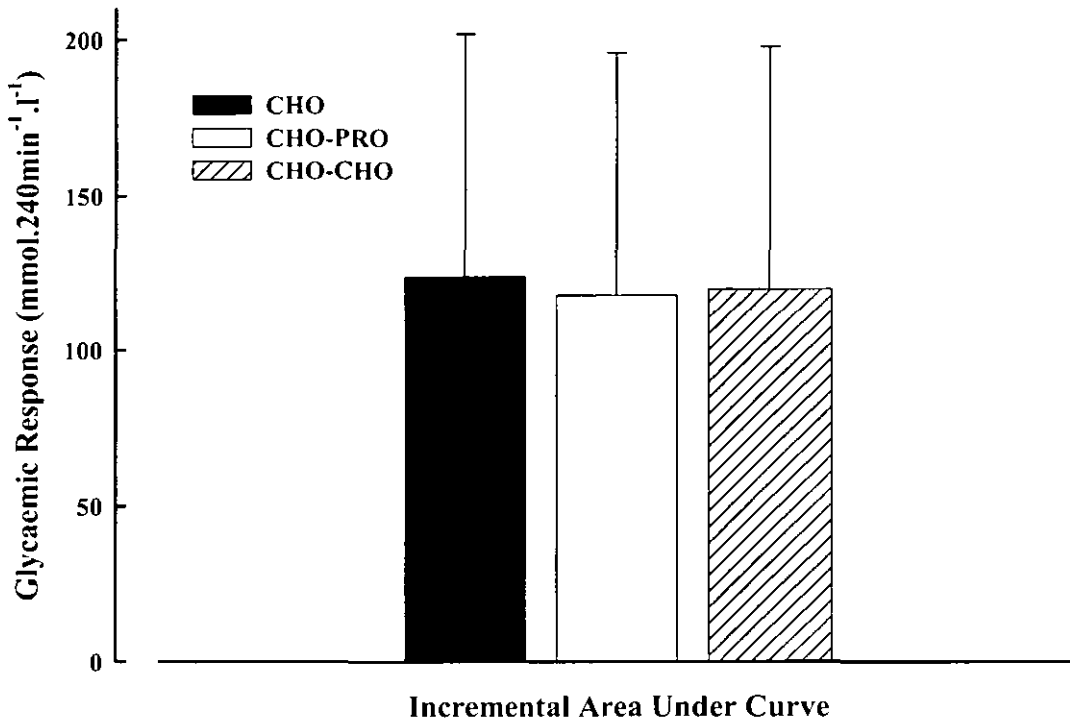


Figure 6.7: Glycaemic responses during 4 h recovery (n = 6). Values are means \pm CI.

6.3.4 Plasma Urea

Concentrations of plasma urea were relatively stable during R_1 in under all 3 treatments. However, circulating urea levels began to rise immediately upon ingestion of the first CHO-PRO solution ($F = 24.6$, $P = 0.001$) and became significantly higher than the CHO-CHO trial after 2 h of recovery and higher than the CHO trial after 4 h of recovery (Figure 6.8).

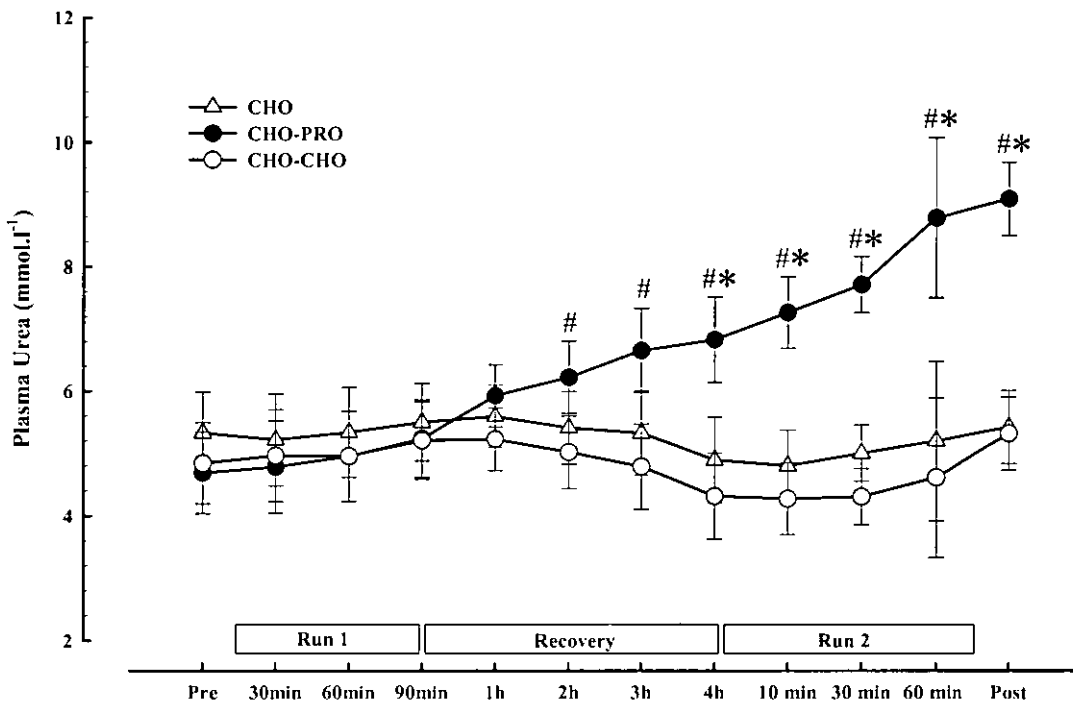


Figure 6.8: Plasma urea concentrations during R_1 , recovery and R_2 . Participants ($n = 6$) received either CHO, CHO-PRO or CHO-CHO supplements during recovery. (N.B: at 60 min R_2 and Post R_2 $n = 5$). Values are means \pm CI. # denotes values different CHO-PRO versus CHO-CHO ($P \leq 0.04$); * denotes values different CHO-PRO versus CHO ($P \leq 0.01$).

6.3.5 Plasma Free Fatty Acids and Glycerol

Free fatty acid and glycerol concentrations responded similarly to the treatments during all 3 trials (Figures 6.9 and 6.10). Average values across trials increased gradually during R₁ (peak at 90 min: FFA = 1.03 ± 0.57 mmol·l⁻¹; glycerol = 0.45 ± 0.18 mmol·l⁻¹), decreased to basal levels throughout the duration of recovery and increased once more during R₂ (peak post R₂: FFA = 1.08 ± 0.49 mmol·l⁻¹; glycerol = 0.39 ± 0.15 mmol·l⁻¹).

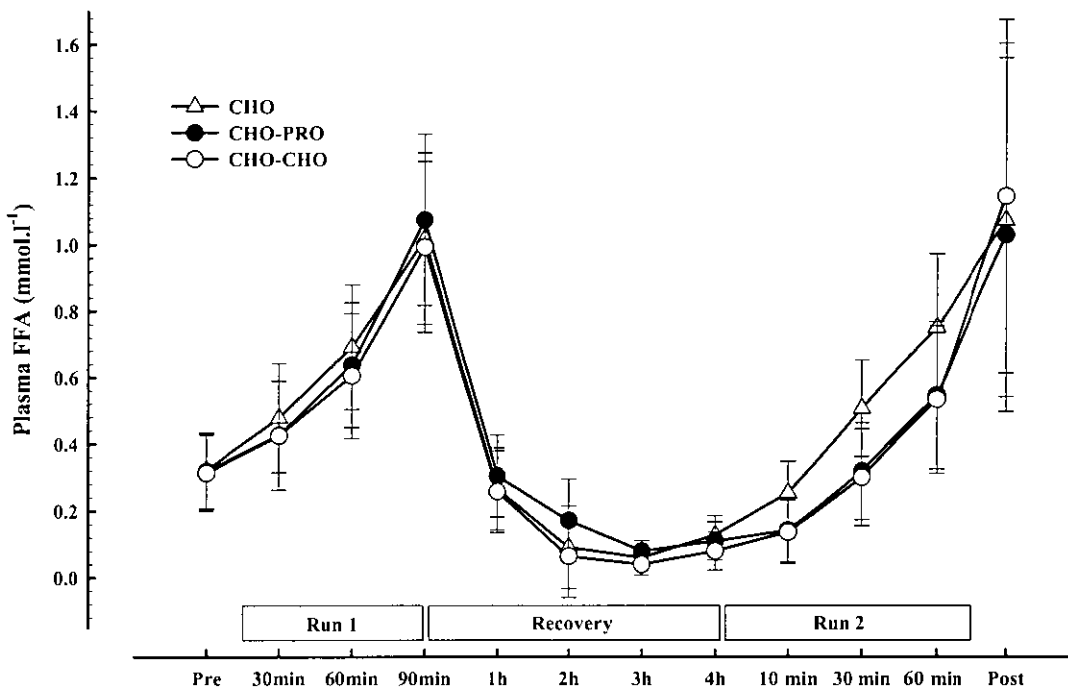


Figure 6.9: Plasma free fatty acid concentrations during R₁, recovery and R₂. Participants (n = 6) received either CHO, CHO-PRO or CHO-CHO supplements during recovery. (N.B: at 60 min R₂ and Post R₂ n = 5). Values are means \pm CI.

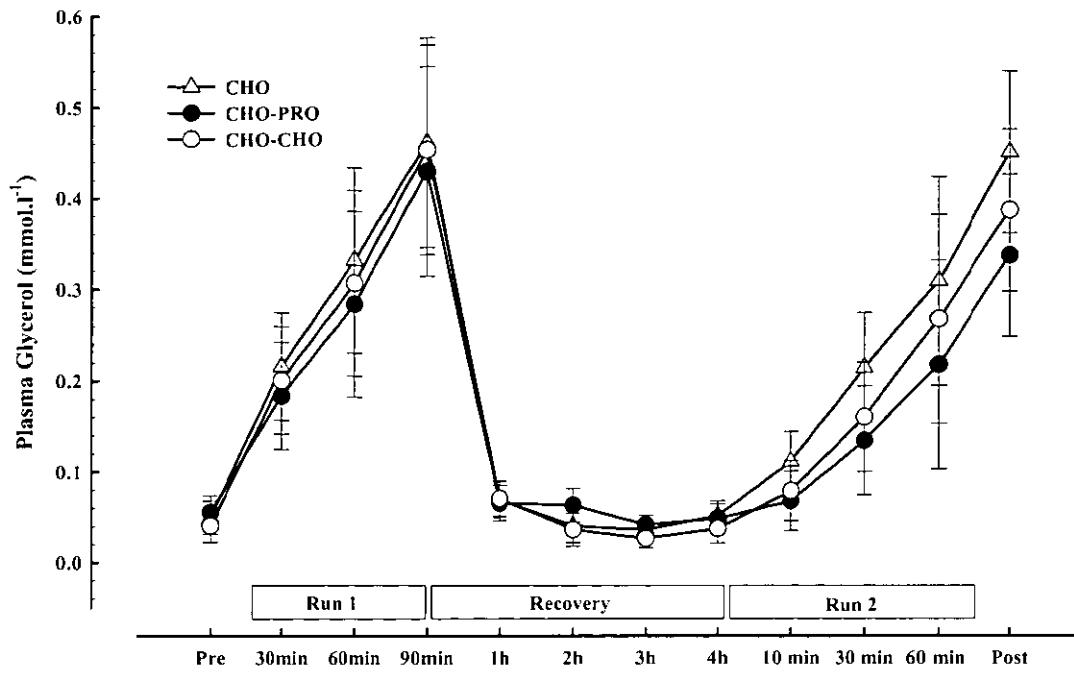


Figure 6.10: Plasma glycerol concentrations during R₁, recovery and R₂. Participants (n = 6) received either CHO, CHO-PRO or CHO-CHO supplements during recovery. (N.B: at 60 min R₂ and Post R₂ n = 5). Values are means ± CI.

6.3.6 Blood Lactate

Upon commencement of R₁, blood lactate concentrations increased above resting concentrations in all 3 trials and mean values were maintained between 1.35 and 1.67 mmol·l⁻¹ for the duration of this exercise session (Figure 6.11). Thereafter, an insignificant trend was apparent for higher lactate concentrations during recovery with CHO-CHO ingestion and lower lactate concentrations with CHO-PRO ingestion. The onset of exercise subsequent to recovery (R₂) initiated a more noticeable accumulation of blood lactate in the CHO trial than in the other 2 trials, although this difference did not attain statistical significance.

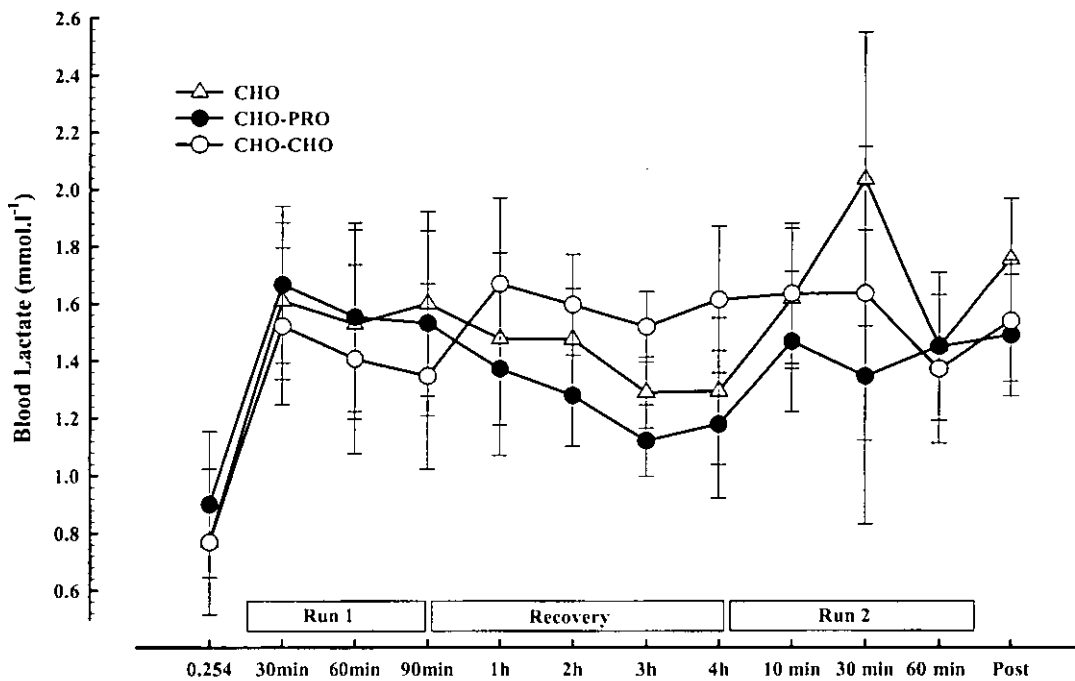


Figure 6.11: Blood lactate concentrations during R₁, recovery and R₂. Participants (n = 6) received either CHO, CHO-PRO or CHO-CHO supplements during recovery. (N.B: at 60 min R₂ and Post R₂ n = 5). Values are means ± CI.

6.3.7 Substrate Metabolism

Estimations of lipid and carbohydrate oxidation rates via indirect calorimetry were not statistically different between trials (Table 6.2). Nonetheless, a trend for higher carbohydrate oxidation and lower lipid oxidation was evident following CHO-CHO ingestion in comparison with ingestion of the other 2 solutions. This pattern was reflected in the respiratory exchange ratios (RER) recorded during recovery and R₂ ($F = 3.8$, $P = 0.04$), with significantly lower values recorded with CHO-PRO ingestion than with CHO-CHO ingestion at 2 h, 3 h and 4 h of recovery ($P \leq 0.05$).

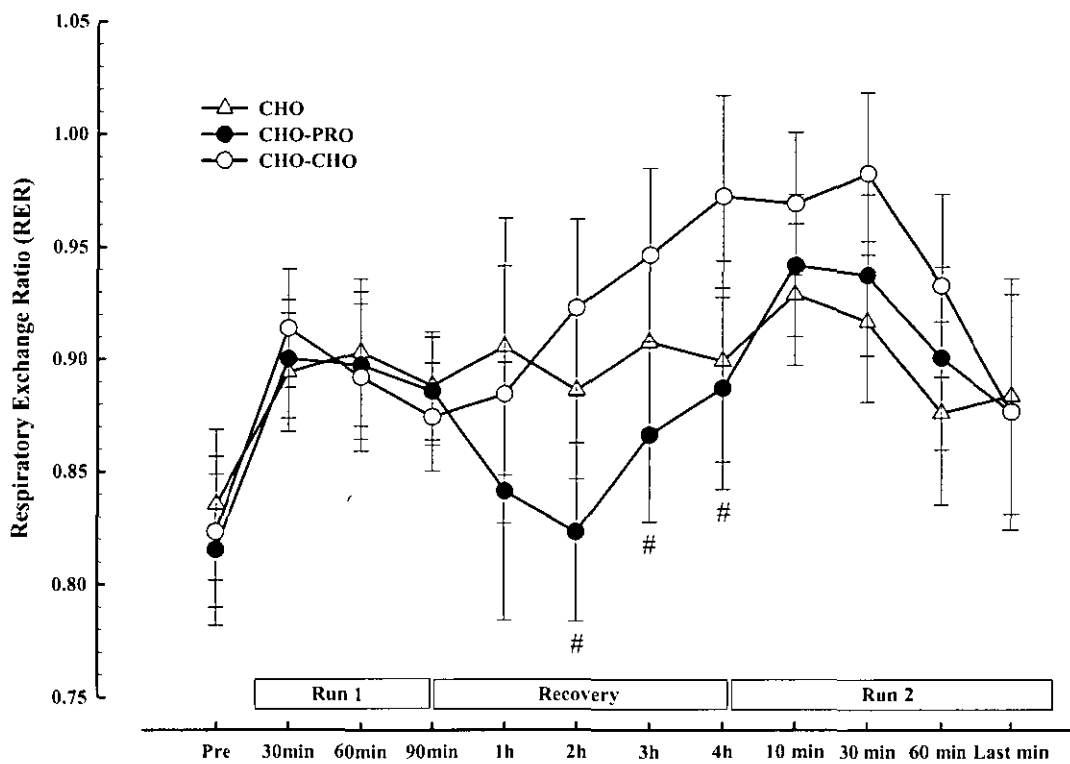


Figure 6.12: Respiratory exchange ratios during R₁, recovery and R₂. Participants ($n = 6$) received either CHO, CHO-PRO or CHO-CHO supplements during recovery. (N.B: at 60 min R₂ $n = 5$). Values are means \pm CI and # denotes values different CHO-PRO versus CHO-CHO ($P \leq 0.05$).

Table 6.2: Substrate metabolism during R₁, recovery and R₂. Participants (n = 6) received either CHO or CHO-PRO supplements during recovery (N.B: at 60 min R₂ n = 5). Values are means ± SEM.

		Run 1				Recovery				Run 2		
	Pre	30 min	60 min	90 min	1 hr	2 hr	3 hr	4 hr	10 min	30 min	60 min	Last min
Carbohydrate												
oxidation (g.min⁻¹)												
CHO	0.2±0.1	2.4±0.2	2.5±0.2	2.3±0.2	0.3±0.04	0.3±0.04	0.3±0.03	0.4±0.1	2.8±0.2	2.7±0.2	2.3±0.3	2.4±0.4
CHO-PRO	0.2±0.1	2.5±0.4	2.5±0.4	2.4±0.4	0.3±0.1	0.2±0.03	0.3±0.03	0.4±0.1	3.1±0.3	3.1±0.3	2.6±0.3	2.3±0.3
CHO-CHO	0.2±0.04	2.7±0.3	2.4±0.3	2.2±0.3	0.3±0.1	0.4±0.04	0.4±0.03	0.5±0.04	3.3±0.3	3.4±0.3	2.9±0.3	2.1±0.3
Lipid												
oxidation (g.min⁻¹)												
CHO	0.1±0.02	0.6±0.1	0.5±0.1	0.6±0.1	0.1±0.01	0.1±0.01	0.1±0.01	0.1±0.02	0.4±0.1	0.5±0.1	0.7±0.1	0.7±0.2
CHO-PRO	0.1±0.02	0.5±0.1	0.5±0.1	0.6±0.1	0.1±0.03	0.1±0.01	0.1±0.02	0.1±0.01	0.3±0.1	0.3±0.1	0.6±0.1	0.7±0.1
CHO-CHO	0.1±0.01	0.5±0.1	0.6±0.1	0.7±0.1	0.1±0.02	0.1±0.02	0.04±0.01	0.02±0.01	0.2±0.1	0.1±0.1	0.4±0.1	0.7±0.1

6.3.8 Serum Growth Hormone

The initial exercise bout of each trial (R_1) increased serum concentrations of human growth hormone to a similar extent in all 3 trials (Figure 6.13). As participants began ingesting the experimental solutions during recovery, growth hormone concentrations returned to basal levels and remained as such until at least 10 min into the second exercise session (R_2). Growth hormone measurements taken immediately following R_2 were higher when CHO-PRO had been ingested during recovery as opposed to either of the carbohydrate solutions with no additional protein ($P \leq 0.02$).

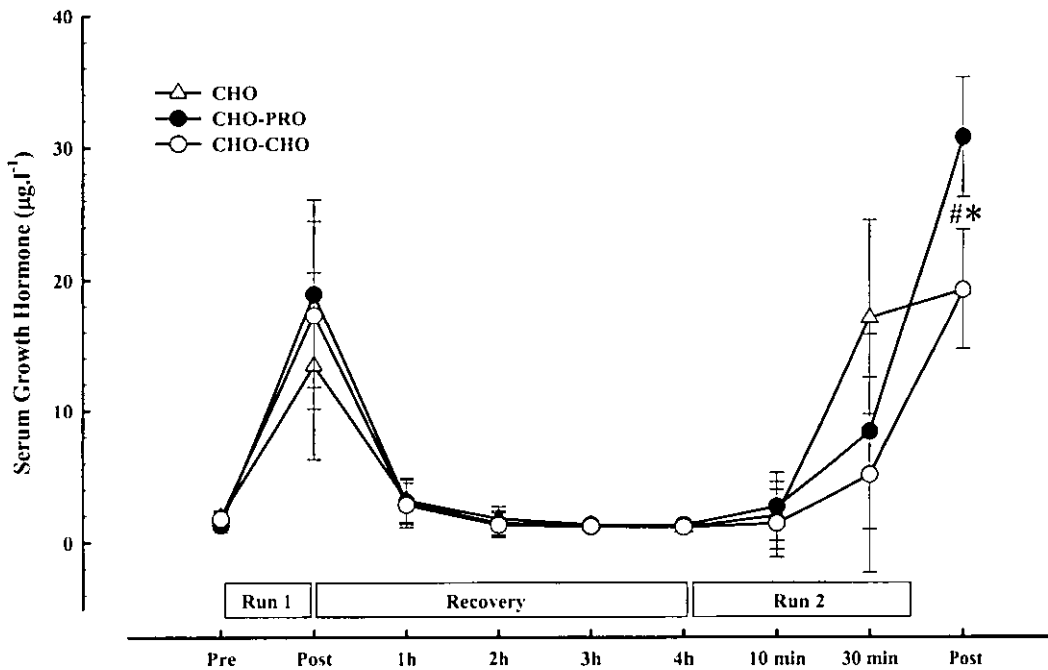


Figure 6.13: Serum growth hormone concentrations during R_1 , recovery and R_2 . Participants ($n = 6$) received either CHO, CHO-PRO or CHO-CHO supplements during recovery. (N.B: at Post R_2 $n = 5$). Values are means \pm CI. # denotes values different CHO-PRO versus CHO-CHO ($P = 0.02$); * denotes values different CHO-PRO versus CHO ($P = 0.01$).

6.3.9 Serum Cortisol

Concentrations of serum cortisol increased over time during R_1 , reduced below resting levels during recovery and were elevated a second time towards the end of R_2 (Figure 6.14). While no differences could be detected between trials at any specific time-point ($F = 2.0$, $P = 0.2$), an overall effect of treatment was identified (main effect: $F = 5.2$, $P = 0.05$). In contrast to the growth hormone data, there was a trend for serum cortisol concentrations to be lower immediately following R_2 following CHO-PRO ingestion in comparison with the CHO and CHO-CHO treatments.

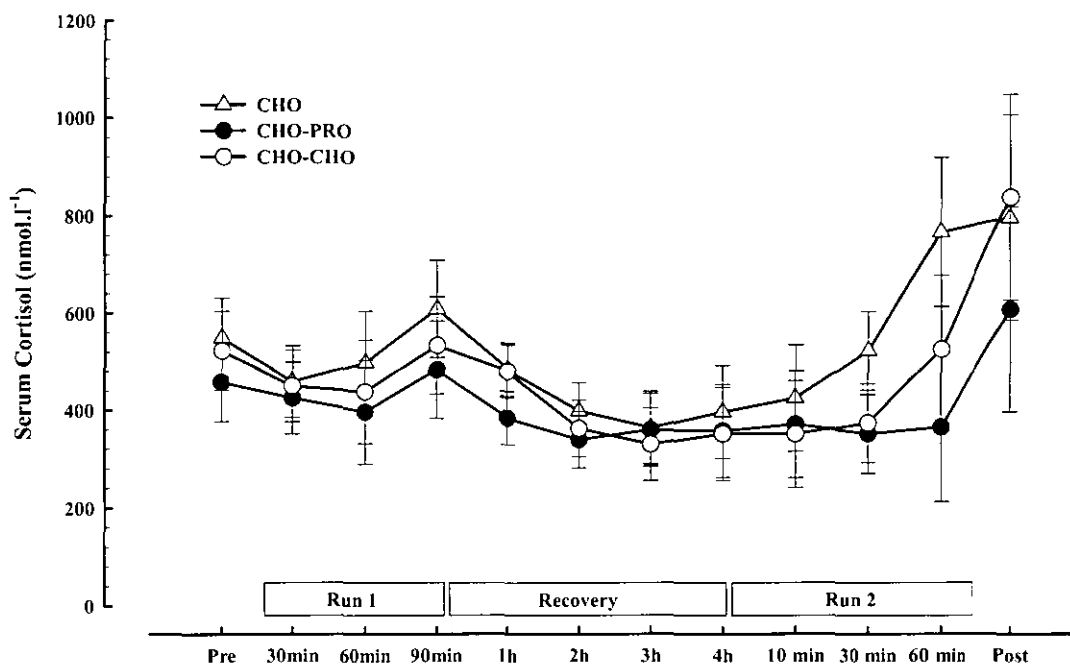


Figure 6.14: Serum cortisol concentrations during R_1 , recovery and R_2 . Participants ($n = 6$) received either CHO or CHO-PRO supplements during recovery. (N.B: at 60 min R_2 and Post R_2 $n = 5$). Values are means \pm CI and are different between trials ($P = 0.05$).

6.3.10 Plasma Myoglobin

Plasma concentrations of this ordinarily intracellular haemprotein increased during R_1 and peaked initially 1 h following this exercise bout (Figure 6.15). A gradual decline in myoglobin concentration was evident during recovery before a second more pronounced increase occurred upon commencement of R_2 . This pattern of response was unaffected by treatment.

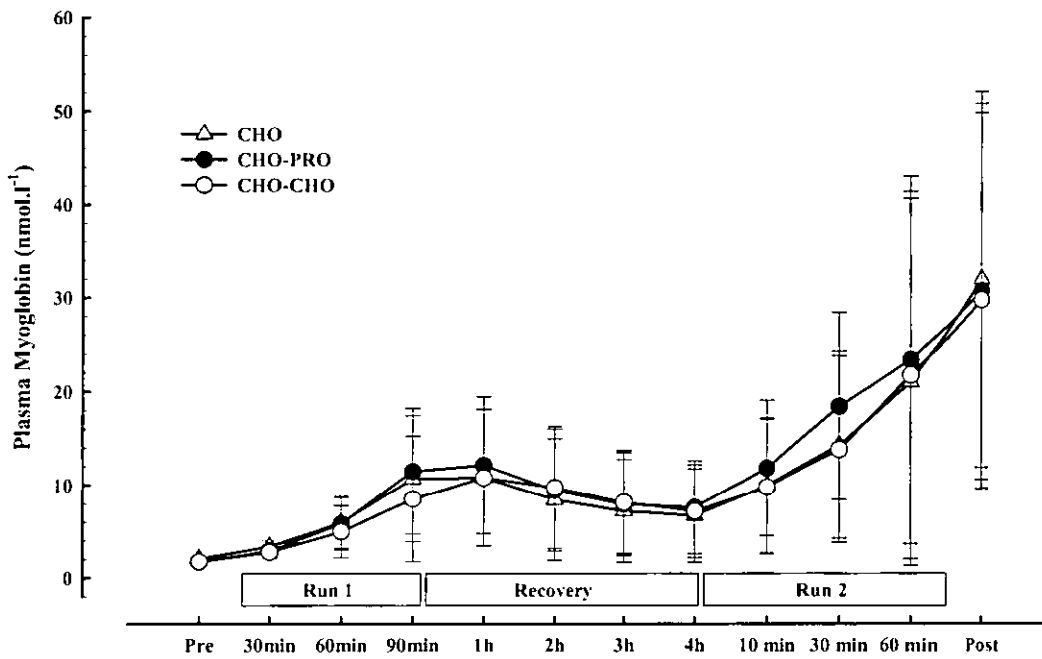


Figure 6.15: Plasma myoglobin concentrations during R_1 , recovery and R_2 . Participants ($n = 6$) received either CHO, CHO-PRO or CHO-CHO supplements during recovery. (N.B: at 60 min R_2 and Post R_2 $n = 5$). Values are means \pm CI.

6.3.11 Indices of Hydration Status

No participant's pre-trial urine sample was found to have an osmolality in excess of $900 \text{ mosmol}\cdot\text{kg}^{-1}$, with a mean value across all 3 trials of $530.3 \pm 183.6 \text{ mosmol}\cdot\text{kg}^{-1}$ (mean \pm SD). Hydration status, determined through changes in body mass (BM), was found to be reduced during R_1 by $-2.1 \pm 0.5\%$ BM, $-1.5 \pm 0.3\%$ BM and $-1.6 \pm 0.3\%$ BM in the CHO, CHO-PRO and CHO-CHO trials, respectively. The second exercise session (R_2) elicited similar reductions in body mass: $-1.9 \pm 0.7\%$ BM (CHO); $-2.1 \pm 0.6\%$ BM (CHO-PRO); and $-1.6 \pm 0.3\%$ BM (CHO-CHO). Plasma volume responses were not different between treatments but deviated substantially from baseline measurements over the course of each trial (time effect: $F = 18.4$, $P = 0.001$). The reduced plasma volume recorded during R_1 was returned to baseline levels after 1 h of recovery before declining once more and remaining significantly reduced from this point onwards (Figure 6.16).

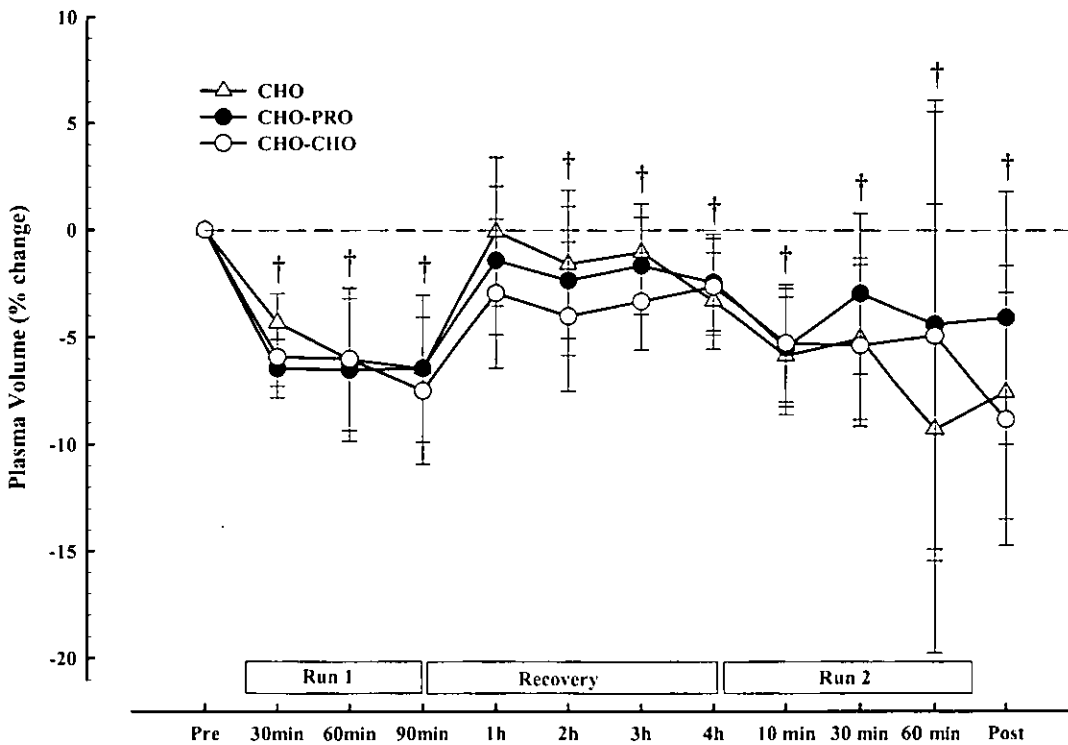


Figure 6.16: Changes in plasma volume during R_1 , recovery and R_2 . Participants ($n = 6$) received either CHO, CHO-PRO or CH-CHO supplements during recovery. (N.B: at 60 min R_2 and Post R_2 $n = 5$). Values are means \pm CI and † denotes values different from baseline ($P \leq 0.02$).

6.3.12 Subjective Data

The ratings of perceived exertion indicated by participants during each exercise bout was not influenced by experimental treatment. However, at the point of exhaustion during R₂, there was a slight tendency for participants to report lower RPE scores (scale 6–20) following ingestion of the CHO-PRO mixture (16.8 ± 0.5) rather than the CHO or CHO-CHO solutions (18.3 ± 0.6 and 18.2 ± 1.1 , respectively). Participants' reported sensations of thirst were not found to be different between trials, generally decreasing during recovery and increasing with subsequent exercise. The degree of gut fullness reported by participants in response to each solution was highly variable and, as a result, no clear pattern across the mean values was apparent (Table 6.3).

Table 6.3: Subjective ratings of gut fullness during recovery and R₂. Participants (n = 6) received either CHO, CHO-PRO or CHO-CHO supplements during recovery.

(N.B: at 60 min R₂ n = 5). Values are means \pm SEM.

	Recovery				Run 2			
	1 h	2 h	3 h	4 h	10 min	30 min	60 min	Last min
Gut Fullness (scale 6-20)								
CHO	9 ± 0.4	10 ± 1	11 ± 1	11 ± 2	11 ± 1	10 ± 1	9 ± 1	9 ± 1
CHO-PRO	8 ± 1	11 ± 1	12 ± 1	11 ± 1	12 ± 1	11 ± 1	10 ± 1	10 ± 1
CHO-CHO	8 ± 1	9 ± 1	11 ± 1	12 ± 2	10 ± 1	10 ± 1	8 ± 0.4	9 ± 1

Despite the lack of treatment differences in terms of gut fullness, it was noted that ingestion of the CHO-CHO solution was associated with reports of severe gastrointestinal discomfort in 3 of the 6 participants who completed this study. In addition, the data gathered from a further 2 participants who were initially recruited for this study was disregarded because these participants experienced such severe gastrointestinal distress in the CHO-CHO trial that the exercise capacity test was terminated before a metabolic endpoint could be established. Notably, no such adverse effects were reported by any participant in response to either the CHO or CHO-PRO solutions.

6.3.13 Control Data

The relative exercise intensity of each exercise session was successfully standardised between experimental conditions and the heart rate data recorded during these periods reflected this. Table 6.4 provides an overview of this information.

Table 6.4: Overall relative exercise intensities and heart rates recorded during R₁ and R₂. Participants (n = 6) received either CHO, CHO-PRO or CHO-CHO supplements during recovery. Values are means \pm SD.

	Run 1	Run 2
Exercise Intensity (% $\dot{V}O_2$ max)		
CHO	70.0 \pm 2.2	71.8 \pm 2.9
CHO-PRO	70.2 \pm 3.0	72.4 \pm 3.9
CHO-CHO	69.2 \pm 3.1	69.3 \pm 3.9
Heart Rate (beats·min⁻¹)		
CHO	170 \pm 6	173 \pm 8
CHO-PRO	169 \pm 9	172 \pm 9
CHO-CHO	166 \pm 9	171 \pm 9

6.4 Discussion

The primary finding of this study was that the recovery of endurance running capacity following ingestion of a solution providing $0.8 \text{ g CHO} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$ (CHO) can be enhanced either through the addition of $0.3 \text{ g} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$ of protein (CHO-PRO) or through adding further carbohydrate (CHO-CHO). The CHO-PRO and CHO-CHO solutions were equally effective in achieving this benefit.

This finding of an increased capacity to perform subsequent exercise following CHO-PRO ingestion rather than ingestion of CHO alone is in agreement with the findings of Williams *et al.* (2003). These authors reported a 55% increased cycle time to fatigue through the addition of protein to carbohydrate recovery solutions and attributed this improvement to the 128% greater rate of glycogen storage during recovery (Williams *et al.*, 2003). It is therefore possible that the observed differences in exercise capacity in the present study were also the consequence of an increased rate of glycogen synthesis. While no direct measurement of muscle glycogen was made to assess this possibility, the higher insulin concentrations stimulated by the additional protein would be expected to increase both the uptake of blood glucose into skeletal muscle and the conversion of this glucose into muscle glycogen. Notably, the enhanced recovery observed in the present study was not as profound as that previously reported by Williams *et al.* (2003). A likely explanation for this difference is that, in the present study, the CHO and CHO-PRO solutions were matched for carbohydrate content whereas in the study of Williams *et al.* (2003) the solution with added protein included more than twice as much CHO ($0.4 \text{ g} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$) as the solution containing CHO alone ($0.15 \text{ g} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$).

In addition, the observation in the current study of more stable plasma glucose concentrations during recovery and higher plasma glucose concentrations during R_2 following CHO-PRO ingestion may be indicative of an increased hepatic appearance of glucose. In support of this, the lower respiratory exchange ratios recorded during recovery in the CHO-PRO trial might reflect increased catabolism of amino acids in the liver rather than increased lipid metabolism, while the elevated concentrations of plasma urea during this trial might therefore indicate an increased availability of α -keto acids for gluconeogenesis. Furthermore, it is likely that a substantial quantity of

this glucose would be stored as glycogen within the liver during recovery and recent evidence has demonstrated a positive correlation between liver glycogen resynthesis and subsequent exercise capacity (Casey *et al.*, 2000). It is therefore possible that the differences in exercise capacity observed in the present study may be at least partly due to an increased availability of blood glucose during R₂.

Another potential benefit of adding protein to carbohydrate recovery solutions may be an increased rate of muscle protein synthesis. Prolonged dynamic exercise, as was performed in the present study, is known to produce an acute phase of negative protein balance in the immediate post-exercise period (Rennie and Tipton, 2000). It has been well established that post-exercise ingestion of amino acids encourages a transition from net protein degradation to net protein synthesis following resistance exercise (Borsheim *et al.*, 2002; Tipton *et al.*, 1999) and more recently a similar anabolic effect of amino acid ingestion has been demonstrated following endurance exercise (Koopman *et al.*, 2004). The present results illustrate that the CHO-PRO solution elicited greater growth hormone concentrations and lower cortisol concentrations following R₂ than either of the solutions containing carbohydrate with no additional protein. The wealth of available evidence certainly supports that amino acid ingestion can induce growth hormone release to some extent (Chromiak and Antonio, 2002; Jacobson, 1990), although the precise magnitude of growth hormone secretion is likely to be mediated by individual factors such as training status and gender (Mejri *et al.*, 2005; Wideman *et al.*, 2000b). Furthermore, it is possible that relatively high concentrations of certain amino acids may be necessary to impart this effect given that chronic oral supplementation with leucine appears to be a less effective stimuli than acute arginine infusion (Merimee *et al.*, 1969; Mero *et al.*, 1997).

It is also interesting to note in the present study that the treatment effects in terms of growth hormone concentration were apparent following R₂ rather than during the feeding period. This is in contrast to evidence from Suminski *et al.* (1997) who have reported the stimulatory influence of amino acid ingestion on growth hormone release to be more potent at rest than during exercise. Similar to the present study, however, these authors also observed higher absolute concentrations of growth hormone during exercise than at rest (Suminski *et al.*, 1997). This effect is consistent

with the now well recognised exercise-induced growth hormone response (Godfrey *et al.*, 2003), which is thought to be regulated through the inhibitory control of somatostatin balanced against the stimulatory influence of growth hormone releasing-hormone (de Vries *et al.*, 2003). Importantly, the finding in the present study that growth hormone concentrations were only different between trials during exercise may be related to evidence that arginine stimulated growth hormone release operates through an inhibition of somatostatin release combined with an increased responsiveness of somatotropes to growth hormone releasing-hormone (Ghigo *et al.*, 1991; Wideman *et al.*, 2000a), although the elevated insulin concentrations during recovery could equally account for the lack of treatment differences in growth hormone release while feeding (Lanzi *et al.*, 1997). Overall, the results of the present study support the contention that including protein in a carbohydrate recovery solution may attenuate the catabolic cellular environment that occurs following prolonged exhaustive exercise.

Finally, it cannot be ruled out that fatigue during R₂ may have coincided not only with compromised substrate availability but also with a reduced motor neuron stimulation from the CNS. A number of neurotransmitters have been identified which may induce fatigue through decreased CNS activation and the synthesis of one such neurotransmitter (5-hydroxytryptamine) is stimulated by increases in the ratio of free tryptophan to other branched chain amino acids (Blomstrand, 2001). While some evidence does support the hypothesis that the amino acids provided in the present study may have offset fatigue through delaying the increase in blood tryptophan: BCAA ratios (Mittleman *et al.*, 1998), more recent evidence appears to oppose this contention (Watson *et al.*, 2004a). However, it should be noted that a large inter-individual variation was reported in this latter study and therefore the possibility remains that BCAA supplementation might improve the central drive for exercise in certain individuals.

In conclusion, the addition of 0.3 g·kg⁻¹·h⁻¹ of protein to a solution providing 0.8 g CHO·kg⁻¹·h⁻¹ resulted in an improved recovery of exercise capacity 4 h following prolonged exercise. The insulin concentrations observed during this period along with muscle biopsy data reported in other studies (Ivy *et al.*, 2002; van Loon *et al.*, 2000a; Zawadzki *et al.*, 1992) suggest that this improved performance might be

the result of an increased rate of muscle glycogen resynthesis during recovery. However, there are other potential mechanisms which might also account for this benefit and it is unlikely that the increase in exercise capacity can be entirely attributed to any single mechanism. Finally, the comparison of the CHO-PRO solution with an isoenergetic carbohydrate solution (CHO-CHO) revealed that the addition of protein to a carbohydrate solution is no more effective in restoring exercise capacity than simply adding an equal amount of carbohydrate, although an increased incidence of gastrointestinal discomfort was associated with the concentrated CHO solution.

CHAPTER 7

Effect of Carbohydrate-Protein Ingestion on Muscle Glycogen Resynthesis During Recovery.

7.1 Introduction

The resynthesis of muscle glycogen following prolonged exercise of moderate to high intensity is an essential component of recovery, especially when the interval before a repeated performance is of short duration (i.e. <8 h). It has been demonstrated that carbohydrate ingestion is an effective means of enhancing the rate of muscle glycogen resynthesis following exercise (Bergstrom *et al.*, 1967; Ivy, 1998) and it appears that a positive correlation exists between the amount of carbohydrate ingested and the rate of muscle glycogen resynthesis (Jentjens and Jeukendrup, 2003). This relationship persists up until a carbohydrate intake of approximately $1.2 \text{ g}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$, at which point the rate of muscle glycogen synthesis plateaus slightly in excess of $40 \text{ mmol}\cdot\text{kg dry mass}^{-1}\cdot\text{h}^{-1}$ for recovery periods of 2-6 h in duration (van Loon *et al.*, 2000a).

In comparison to the extensive literature regarding the metabolic influences of carbohydrate ingestion, there is relatively little published data with reference to the ingestion of CHO-PRO mixtures. Despite the augmented insulin response when $\geq 0.3 \text{ g}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$ of protein is ingested alongside carbohydrate during recovery, it has become increasingly evident that the 'maximal' rates of muscle glycogen resynthesis following ingestion of $1.2 \text{ g CHO}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$ cannot be exceeded through ingesting this additional protein (Jentjens *et al.*, 2001; Van Hall *et al.*, 2000b). Conversely, the addition of protein to smaller amounts of carbohydrate (i.e. $\leq 0.8 \text{ g CHO}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$) has been reported to increase the sub-maximal rates of muscle glycogen resynthesis elicited by the carbohydrate alone (Ivy *et al.*, 2002; van Loon *et al.*, 2000a; Zawadzki *et al.*, 1992).

The above findings regarding CHO-PRO ingestion and muscle glycogen resynthesis may help explain why the recovery of exercise capacity has also been reported to be improved following ingestion of such supplements (Saunders *et al.*, 2004; Williams *et al.*, 2003). Indeed, the rates of muscle glycogen resynthesis

reported by van Loon *et al.* (2000a) in response to either moderate carbohydrate, carbohydrate with protein or high carbohydrate solutions follow an identical pattern across treatments as does the exercise capacity data reported in the previous chapter of this thesis (section 6.3.1, p. 158). However, the mixture of protein hydrolysate and amino acids that was used by these other authors proved to be remarkably effective in promoting muscle glycogen resynthesis, accelerating the synthetic rate above that recorded in the moderate carbohydrate trial by approximately $18.8 \text{ mmol}\cdot\text{kg dry mass}^{-1}\cdot\text{h}^{-1}$ (van Loon *et al.*, 2000a). Given that certain amino acids are likely to stimulate muscle glycogen resynthesis more effectively than others (van Loon *et al.*, 2000b), it remains to be established whether the specific CHO-PRO mixture used in the previous study of this thesis provided its ergogenic benefit through facilitating muscle glycogen resynthesis or through some other mechanism.

In addition, while current understanding is relatively advanced regarding the influence of CHO-PRO ingestion on muscle glycogen resynthesis during recovery, less is known about the influence of these supplements on substrate metabolism during exercise subsequent to recovery. Those studies attempting to maximise the rate of muscle glycogen resynthesis during recovery often cite evidence to support the contention that an elevated pre-exercise muscle glycogen content will extend the capacity to perform moderate to high intensity exercise (Ahlborg *et al.*, 1967; Bergstrom *et al.*, 1967). However, it is possible that an initial bout of prolonged exercise may influence the metabolic response to subsequent performance such that muscle glycogen availability no longer dictates exercise capacity. A recent study assessed endurance capacity following a 4 h recovery from prolonged exercise and reported a moderate positive correlation ($r = 0.53$, $P \leq 0.05$) between pre-exercise liver glycogen content and exercise time to exhaustion (Casey *et al.*, 2000), a finding which may be associated to the earlier observation that an improved availability of blood glucose has the capacity to postpone fatigue (Coyle *et al.*, 1986). Taken together, these data raise the interesting possibility that the interaction of the initial exercise bout and CHO-PRO ingestion may modify the relative contributions of liver and muscle glycogen towards overall metabolism during subsequent exercise.

To this end, the present study was designed to examine the relative influences of CHO and CHO-PRO ingestion on the rate of muscle glycogen resynthesis during a 4 h recovery from prolonged treadmill running were assessed in the present study. Subsequent to recovery, a further 60 min of treadmill running was performed to evaluate whether the rate of muscle glycogen utilisation for this period was different between experimental treatments.

7.2 Methods

7.2.1 Participants

Six male volunteers were recruited to take part in this study. These participants were healthy individuals who had all previously been involved with similar experiments and whose habitual training involved a substantial cardiovascular/endurance component. Physiological characteristics describing this cohort are presented in Table 7.1.

Table 7.1: Participant details

Characteristic	Mean \pm SD
Age (yr)	22 \pm 1.3
Height (m)	1.76 \pm 0.1
Mass (kg)	73.8 \pm 6.7
$\dot{V}O_2$ max (ml·kg ⁻¹ ·min ⁻¹)	61 \pm 6

7.2.2 Experimental Design

In a randomised and counterbalanced design, 2 main trials were performed with a 2 week interval separating them. Over the 48 h leading up to the first of these trials, participants were asked to complete a dietary record which they were subsequently requested to adhere to over the same period prior to trial 2 (2772 \pm 1136 kcal·day⁻¹, 58 \pm 11% CHO, 22 \pm 9% fat, 19 \pm 5% protein: mean \pm SD). Main trials involved a 90 min run at 70% $\dot{V}O_2$ max (R₁) followed by a 4 h recovery, during which participants rested in the laboratory while consuming either CHO alone or a CHO-PRO mixture in a double blind manner. Muscle biopsies were obtained at the beginning and end of recovery to assess the rate of muscle glycogen resynthesis over the intervening period. Furthermore, a third muscle biopsy was taken following a second treadmill run (R₂: 60 min at 70% $\dot{V}O_2$ max) to evaluate the rate of muscle glycogen utilisation during exercise subsequent to recovery.

7.2.3 Experimental protocol

The experimental procedures applied during this study are illustrated in Figure 7.1. Participants arrived in the laboratory between 8-9 am following a 10 h overnight fast and were asked to complete a pre-trial consent form (Appendix Aii). After providing a urine sample, each participant's post-void nude body mass was recorded (Avery Ltd., UK) before a cannula was inserted into an antecubital vein and a 10 ml resting venous blood sample obtained from 5 of the 6 participants. The cannula was kept patent throughout each trial by frequent flushing with isotonic saline. Prior to exercise, the Douglas bag technique was used to collect a 5 min resting expired gas sample (Williams and Nute, 1983). Participants were required to stand for 15 min prior to the collection of all resting gas and blood samples. A 5 min run at 60% $\dot{V}O_2$ max was used as a standardised warm-up prior to running at a speed equivalent to 70% $\dot{V}O_2$ max for 90 min (R_1). One minute expired gas samples, heart rates (Polar 8810, Finland) and RPE (Borg, 1973) followed by 10 ml venous blood samples were taken at 30 min intervals throughout R_1 . Water intake was permitted *ad libitum* during trial 1 and matched in trial 2 (0.79 ± 0.27 l; mean \pm SD). Nude body mass was recorded immediately following R_1 to assess hydration status through percentage change in mass. Participants were then asked to rest in a supine position while a 3-5 mm skin incision was made to the anterior portion of the thigh and a muscle biopsy obtained from the vastus lateralis using the percutaneous needle biopsy technique (Bergstrom, 1962). A more comprehensive account of muscle biopsy sampling procedures is included in the General Methods section 3.5.

The first volume of prescribed solution was ingested as soon as the first muscle biopsy had been removed from the leg. The remaining 7 volumes were provided at 30 min intervals during the 4 h recovery such that the last solution was ingested 30 min prior to the second muscle biopsy, participants were permitted 15 min to consume each volume. Expired gas samples and venous blood samples, along with ratings of gut fullness and thirst (6-20 scale; Appendices Bii and Biii), were taken during the recovery period every hour prior to feedings. In addition, each participant's urine was collected throughout the 4 h recovery period and was stored in a vessel containing 5 ml of 10% thymol-*l*-isopropanol as a preservative. Approximately 3 h 45 min following the initial muscle biopsy, a further 2 separate

skin incisions were made to the same leg and the second biopsy was sampled precisely 4 h following the first. Nude body mass was then recorded and, after the standard warm-up, participants were asked to continue running on the treadmill for a further 60 min at 70% $\dot{V}O_2$ max (R_2). As in R_1 , water intake was *ad libitum* during trial 1 and matched in trial 2 (0.39 ± 0.19 l; mean \pm SD). Physiological measurements were obtained at 15 minute intervals during this second run. Nude body mass was recorded immediately after R_2 and the remaining incision site was used to obtain a final muscle biopsy. The 3 muscle biopsies taken in any given trial were sampled from the same leg, with the use of dominant/non-dominant legs being counterbalanced between trials. Ambient temperature and humidity were recorded at 30 min intervals throughout all testing periods using a hygrometer (Zeal, UK) and were not different between trials. Average values recorded were 20.5 ± 0.5 °C and $55.6 \pm 1.9\%$ (mean \pm SD). Full details of all sampling and analysis are included in the General Methods section 3.5.

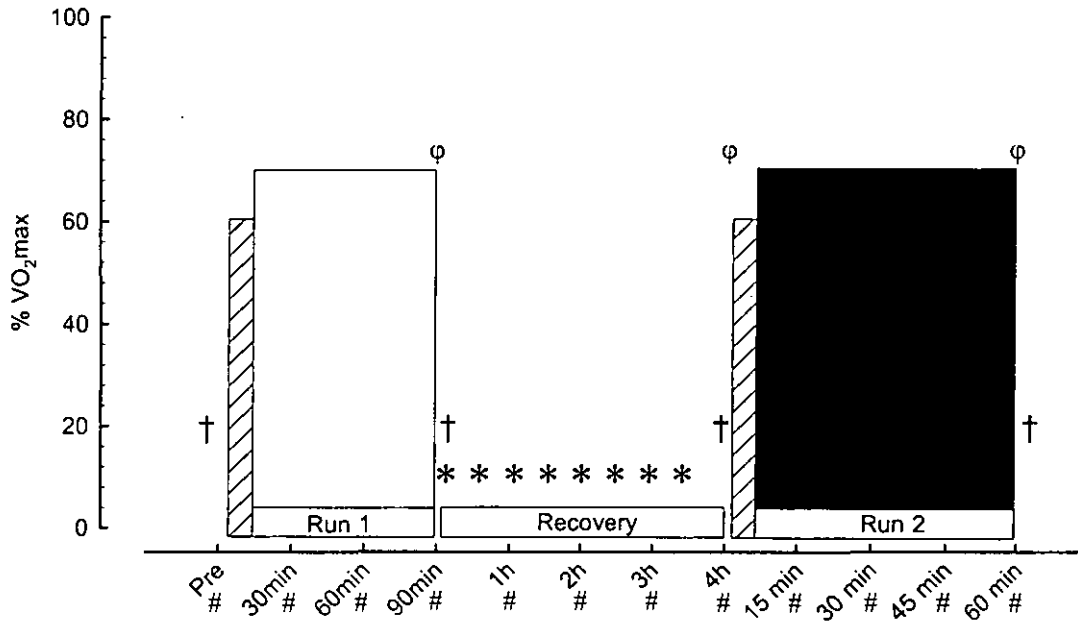


Figure 7.1: A schematic representation of the experimental protocol. Key: * = solution provision, # = expired gas sample/blood sample & RPE, † = body mass, φ = muscle biopsy, hatched area = warm-up (5 min at 60% $\dot{V}O_2$ max), open area = R₁ (90 min at 70% $\dot{V}O_2$ max) and closed area = R₂ (60 min at 70% $\dot{V}O_2$ max).

7.2.4 Solution Composition

The CHO and CHO-PRO solutions used in this study were identical to those described in Chapter 6 (section 6.2.4). These solutions were provided in equal volumes between trials ($590 \pm 53 \text{ ml}\cdot\text{h}^{-1}$; mean \pm SD) relative to each participant's body mass such that the amount of carbohydrate (sucrose) ingested was $0.8 \text{ g}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$, which equates to a total of $236 \pm 21 \text{ g}$ CHO during recovery (mean \pm SD). The CHO-PRO mixture included 3.3% ($0.3 \text{ g PRO}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$) whey protein isolate (i.e. $\approx 3:1$ carbohydrate-protein ratio), thus providing $89 \pm 8 \text{ g}$ PRO during recovery (mean \pm SD) and increasing the metabolisable energy content by 37.5%. The estimated amount of energy that the CHO and CHO-PRO solutions made available for metabolism was therefore $3.2 \text{ kcal}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$ and $4.3 \text{ kcal}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$, respectively. As in the previous study, the CHO and CHO-PRO solutions were matched for flavour (orange and passion fruit), consistency and odour. The specific amino acid profile of the CHO-PRO solution is included in Chapter 6 (Figure 6.2, p. 156).

7.2.5 Statistical Analyses

The data of Tsintzas *et al.* (2003) were utilised to estimate that a sample size of 6 has a 63% power to detect differences in the rate of muscle glycogen resynthesis of 43.8 mmol glucosyl units·kg dry mass⁻¹·h⁻¹, assuming a SD of differences of approximately 45.6, using a paired t-test with a 1 sided significance level. Both the incremental area under curve data that was calculated to assess the insulinaemic response to each solution during recovery (Wolever, 2004) and the lipid oxidation data for R₂ were found to be non-normally distributed and a Wilcoxon test was therefore applied to compare the median values between trials for these data sets, along with any other ordinal/discrete variables (i.e. RPE, gut fullness and thirst) requiring non-parametric statistical analysis. Paired t-tests were used to analyse any other results involving a single comparison of 2 means (i.e. glycaemic responses and rates of muscle glycogen resynthesis/utilisation) while a 2 way general linear model for repeated measures (Drink × Time) was used to identify overall differences between experimental conditions. The Greenhouse-Geisser correction was utilised for epsilon <0.75, while the Huynh-Feldt correction was adopted for less severe asphericity. Where significant F values were found, the Holm-Bonferroni step-wise correction was applied to determine the location of variance where necessary (Atkinson, 2002; Ludbrook, 1998). Unless otherwise stated, all data are expressed in text as mean ± SEM and the error bars included on figures are confidence intervals (CI) that have been corrected to remove between subject variance (Masson and Loftus, 2003). Specific details of, and justification for, these statistical methods are included in the General Methods section 3.8.

7.3 Results

7.3.1 Mixed Muscle Metabolites

Total muscle glycogen concentrations were not different between the CHO and CHO-PRO treatments at any time point, although an effect of time was established ($F = 83.3$, $P < 0.001$). *Post hoc* statistical analysis confirmed that both treatments significantly elevated muscle glycogen storage during recovery before a considerable quantity of muscle glycogen was metabolised during R_2 (Figure 7.2). This pattern is supported by the data included in Figures 7.3 and 7.4 which illustrate the rates of muscle glycogen resynthesis and subsequent degradation, respectively. All other mixed muscle metabolite data is provided in Table 7.2, which presents the 2 sub-glycogen pools separately along with concentrations of adenosine triphosphate (ATP), phosphocreatine (PCr), creatine (Cr), glucose-6-phosphate (G-6-P) and muscle lactate. Resynthesis rates for both proglycogen and macroglycogen exhibited large inter-individual variations such that the trend for greater proglycogen accumulation during recovery with CHO-PRO ingestion could not be detected statistically. Conversely, an effect of treatment was identified in terms of macroglycogen (main effect: $F = 6.7$, $P = 0.05$), signifying higher concentrations throughout the CHO-PRO trials. However, it is likely that this effect is primarily an artefact of the disparity in macroglycogen concentrations immediately following R_1 rather than a true effect of treatment.

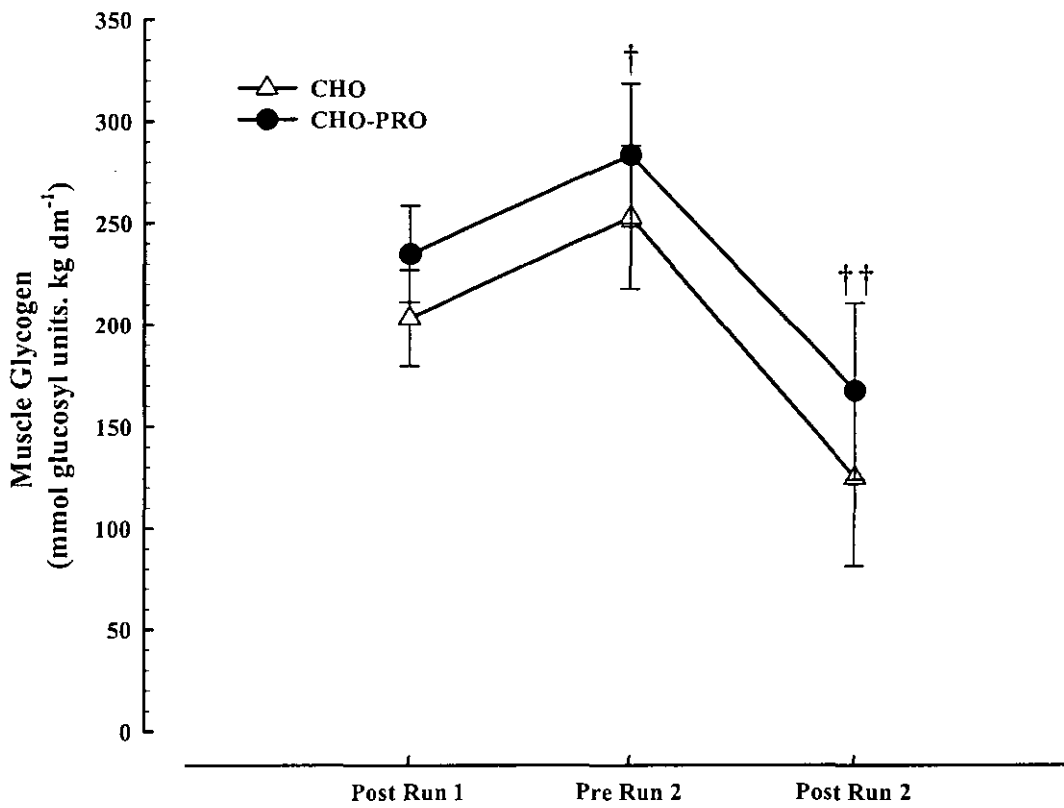


Figure 7.2: Muscle glycogen concentrations following R₁, recovery and R₂. Participants (n = 6) received either CHO or CHO-PRO supplements during recovery. Values are means \pm CI. † denotes values different from Post R₁ ($P < 0.001$); †† denotes values different from Pre R₂ ($P < 0.001$).

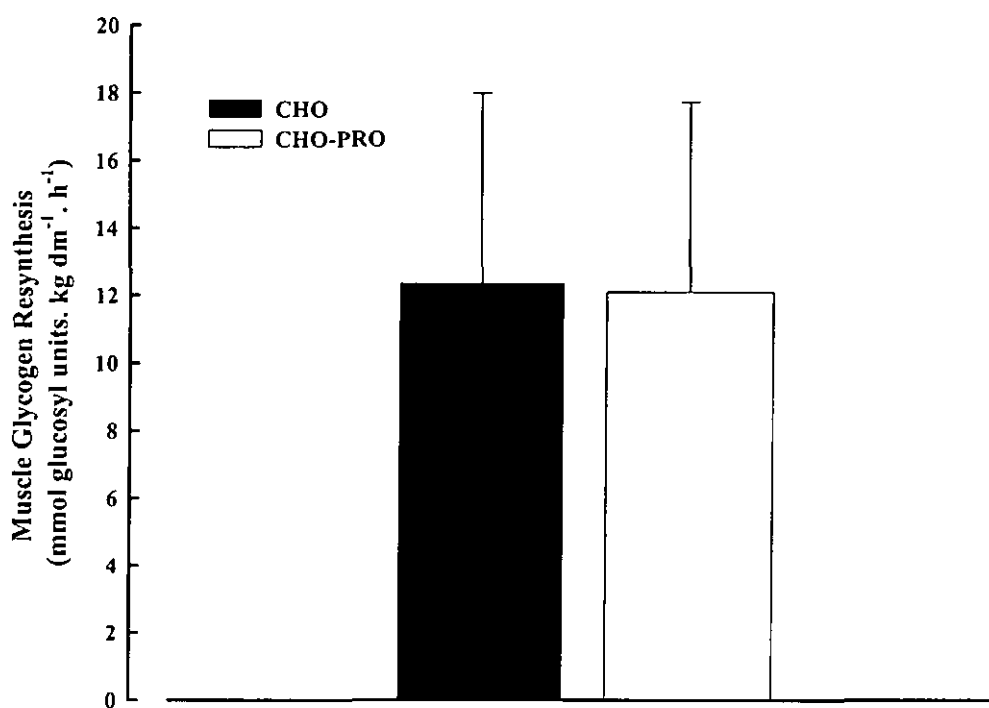


Figure 7.3: Rates of muscle glycogen resynthesis during 4 h recovery (n = 6). Values are means \pm CI.

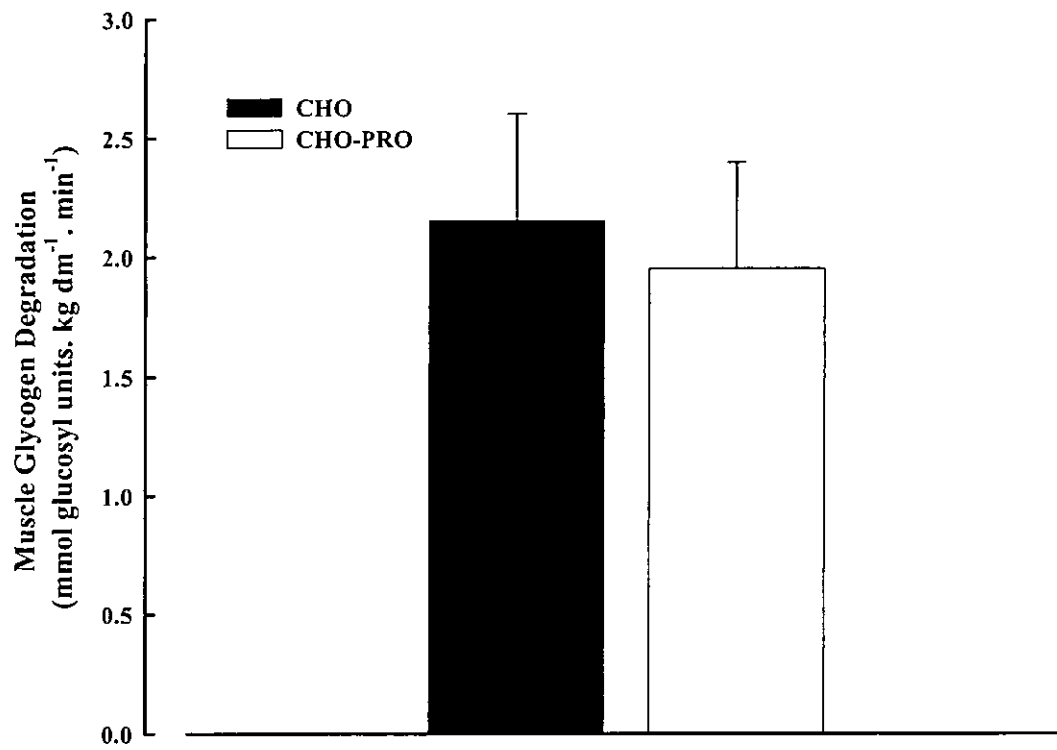


Figure 7.4: Rates of muscle glycogen degradation during R₂ (n = 6). Values are means \pm CI.

Table 7.2: Mixed muscle metabolites following R₁, recovery and R₂. Participants (n = 6) received either CHO or CHO-PRO supplements during recovery. Values are means ± SEM and are given in mmol glucosyl units·kg dry mass⁻¹ for glycogen and mmol units·kg dry mass⁻¹ for all other metabolites. Values are different between trials for macroglycogen (*P* = 0.05) with no detectable free glucose present in these samples.

	CHO			CHO-PRO		
	Post R ₁	Pre R ₂	Post R ₂	Post R ₁	Pre R ₂	Post R ₂
Proglycogen	189 ± 15	216 ± 12	115 ± 26	196 ± 15	234 ± 15	154 ± 19
Macroglycogen	13.9 ± 6.7	36.1 ± 10.1	9.1 ± 5.0	39.0 ± 9.5	48.7 ± 9.9	13.2 ± 4.8
ATP	27.3 ± 1.1	27.6 ± 1.2	25.5 ± 1.5	25.8 ± 2.7	26.0 ± 1.5	29.0 ± 2.2
PCr	79.8 ± 3.9	72.2 ± 2.5	73.5 ± 6.2	74.4 ± 4.9	65.8 ± 4.8	75.6 ± 5.2
Cr	38.2 ± 4.7	45.8 ± 4.3	44.5 ± 7.4	43.5 ± 6.6	52.2 ± 5.2	42.4 ± 8.0
G-6-P	1.3 ± 0.2	1.5 ± 0.2	1.3 ± 0.2	1.5 ± 0.3	1.8 ± 0.5	1.3 ± 0.4
Lactate	6.1 ± 2.0	9.5 ± 1.8	5.3 ± 1.0	9.7 ± 3.6	11.5 ± 3.0	7.5 ± 1.4

7.3.2 Serum Insulin

The additional protein included in the CHO-PRO solution resulted in higher overall insulin concentrations than when the solution with no additional protein was ingested (main effect: $F = 13.1$, $P = 0.02$). This effect appeared to arise from the elevated insulin concentrations during both recovery and R_2 , although no specific effects at any particular time-point could be detected. As such, the incremental area under the curve for the recovery alone was calculated to assess the specific insulinaemic response over this period. Further to the established main effect, median insulin responses during recovery were different between trials ($P = 0.04$): CHO = 42.8 (22.2 - 47.3) $\text{nmol}\cdot 240 \text{ min}^{-1}\cdot \text{l}^{-1}$ and CHO-PRO = 48.5 (42.9 - 64.1) $\text{nmol}\cdot 240 \text{ min}^{-1}\cdot \text{l}^{-1}$.

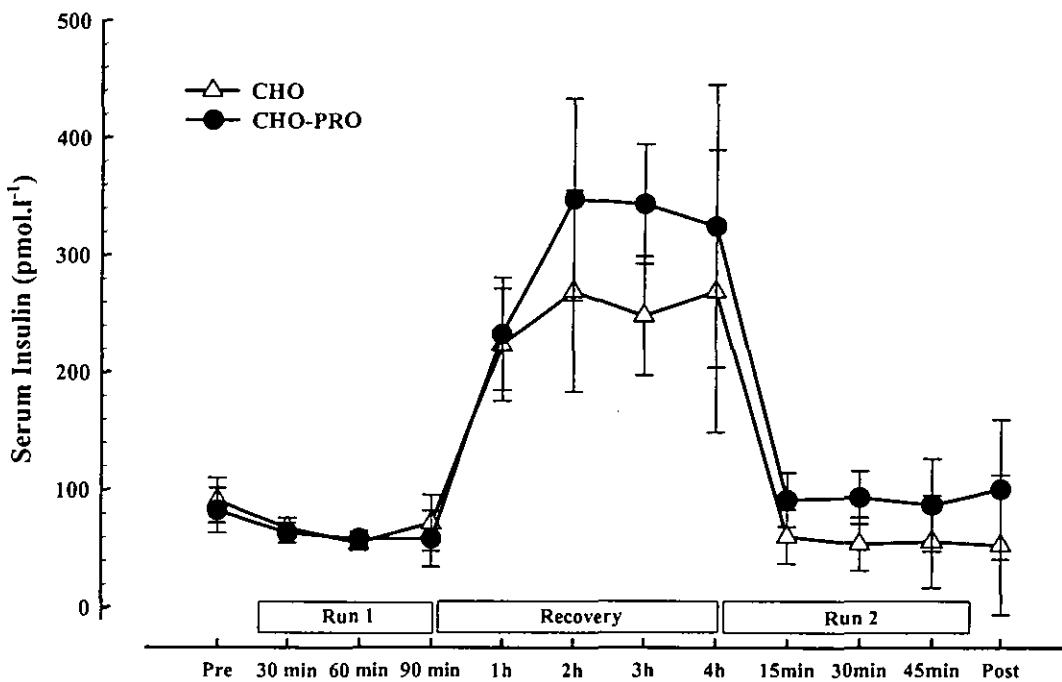


Figure 7.5: Serum insulin concentrations during R_1 , recovery and R_2 . Participants ($n = 5$) received either CHO or CHO-PRO supplements during recovery. Values are means \pm CI and are different between trials ($P = 0.02$). To convert from SI units into $\mu\text{IU}\cdot\text{ml}^{-1}$ divide by 6.95.

7.3.3 Plasma Glucose

Ingestion of the CHO-PRO solution did not substantially increase plasma glucose concentrations at any point during recovery. In contrast, ingestion of the CHO solution resulted in an initial glucose peak approximately 1 h following the commencement of feeding ($P = 0.08$ versus CHO-PRO trial), before concentrations reduced gradually over the remainder of recovery (Figure 7.6). Despite this varied pattern of glucose turnover between trials ($F = 6.5$, $P = 0.01$), the incremental area under curve for the recovery period was similar with each solution (Figure 7.7). With the onset of exercise subsequent to recovery, plasma glucose concentrations decreased transiently in both trials. However, this decrement was most pronounced in the CHO trial and the disparity between treatments was maintained throughout R₂, with significant differences noted at the 30 min sampling point ($P = 0.01$; Figure 7.6).

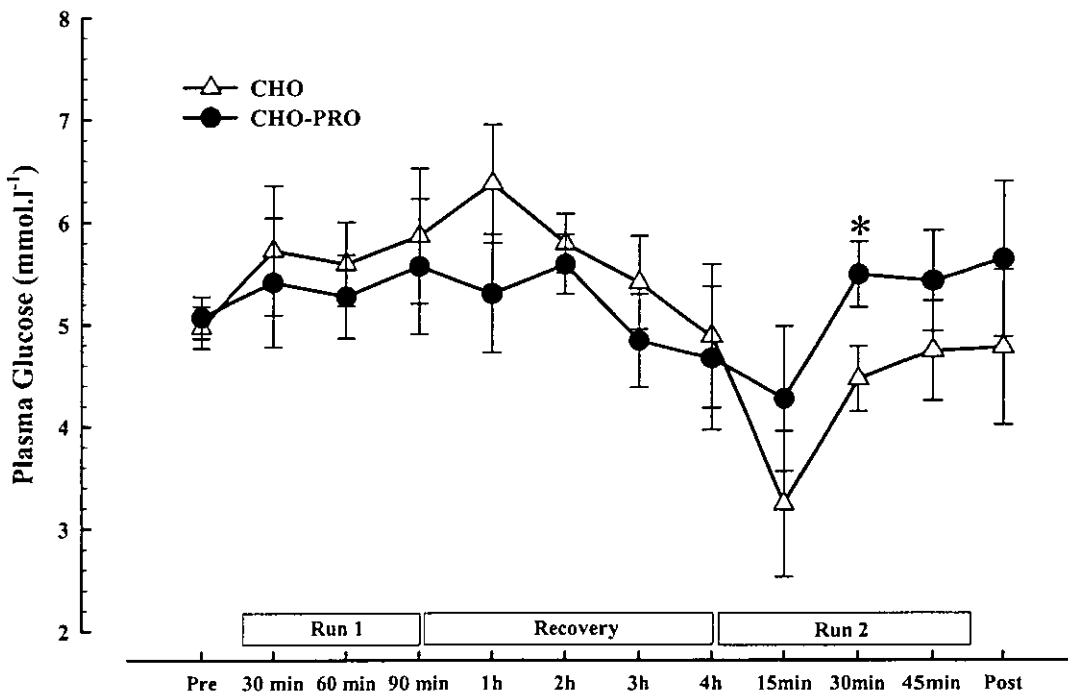


Figure 7.6: Plasma glucose concentrations during R₁, recovery and R₂. Participants ($n = 5$) received either CHO or CHO-PRO supplements during recovery. Values are means \pm CI and * denotes values different between trials ($P = 0.01$).

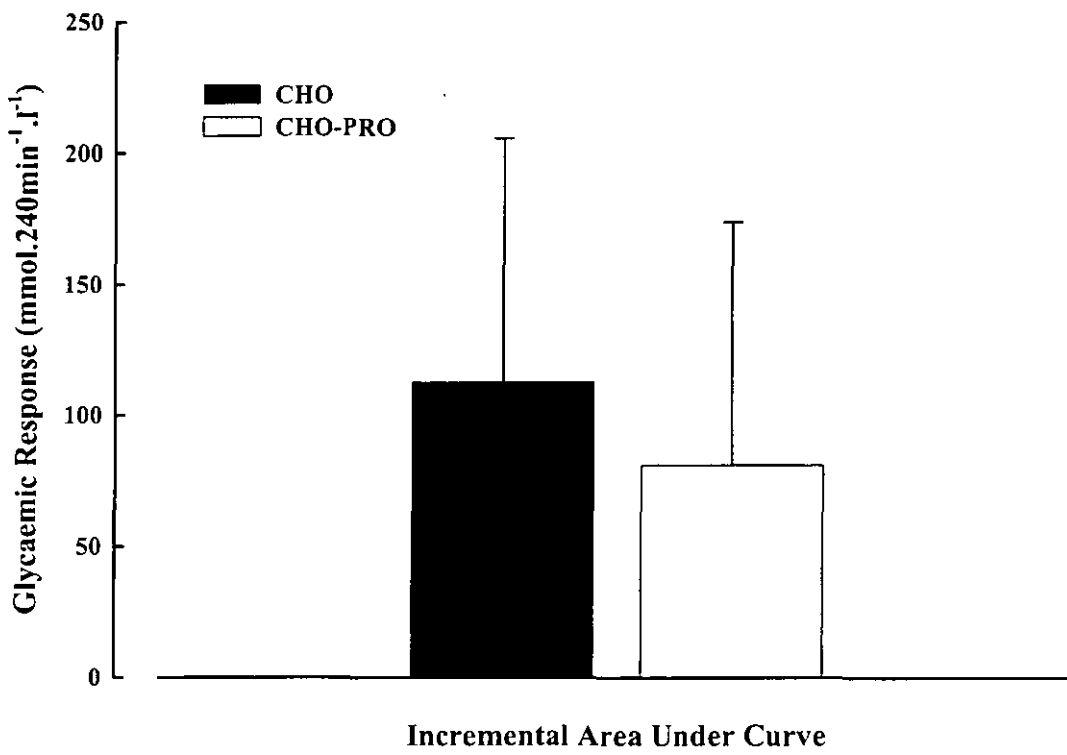


Figure 7.7: Glycaemic responses during 4 h recovery (n = 5). Values are means ± CI.

7.3.4 Plasma Urea

Plasma urea concentrations did not vary substantially from basal levels at any time point during the CHO trial. Conversely, during the CHO-PRO trial, urea began to accumulate in the plasma at the start of recovery ($F = 32.8$, $P < 0.001$). This initial rate of increase in response to CHO-PRO ingestion was maintained until the end of the trial, with differences between trials recorded throughout R_2 ($P \leq 0.02$; Figure 7.8).

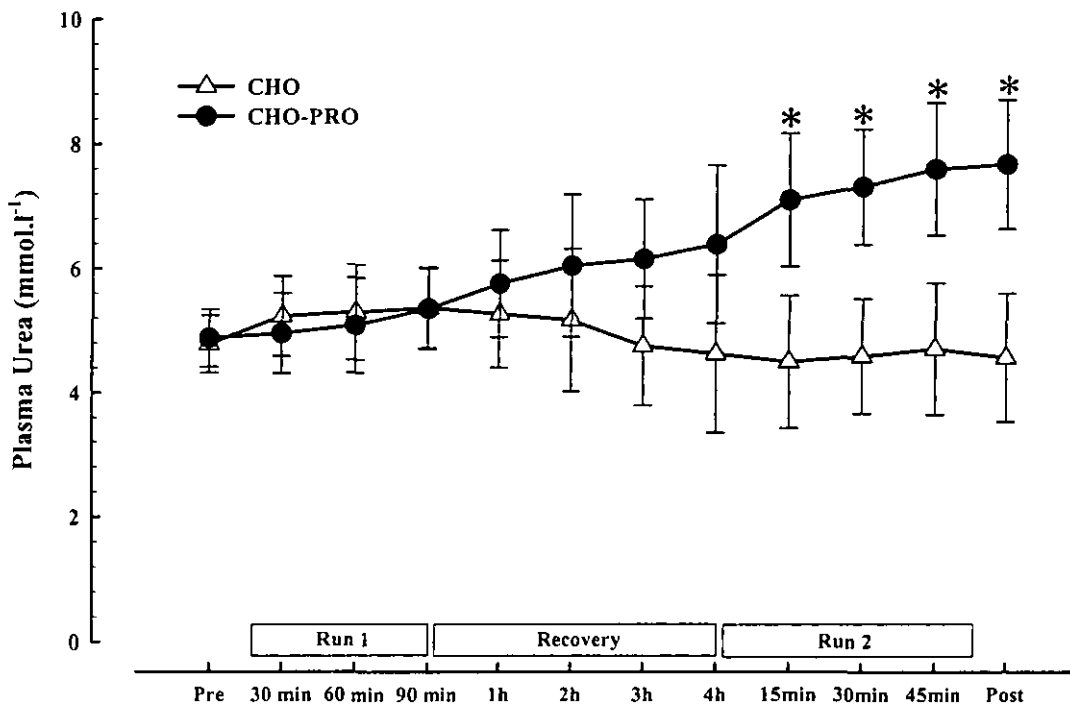


Figure 7.8: Plasma urea concentrations during R_1 , recovery and R_2 . Participants ($n = 5$) received either CHO or CHO-PRO supplements during recovery. Values are means \pm CI and * denotes values different between trials ($P \leq 0.02$).

7.3.5 Plasma Free Fatty Acids and Glycerol

Concentrations of free fatty acids and glycerol were elevated during R_1 before rapidly decreasing with the onset of feeding. During R_2 , free fatty acid concentrations were not deemed to be statistically different between treatments (Figure 7.9). In contrast, glycerol concentrations were notably lower during R_2 following CHO-PRO ingestion ($F = 5.8$, $P = 0.02$) and a specific difference between trials was identified at the 45 min sampling point ($P = 0.04$; Figure 7.10).

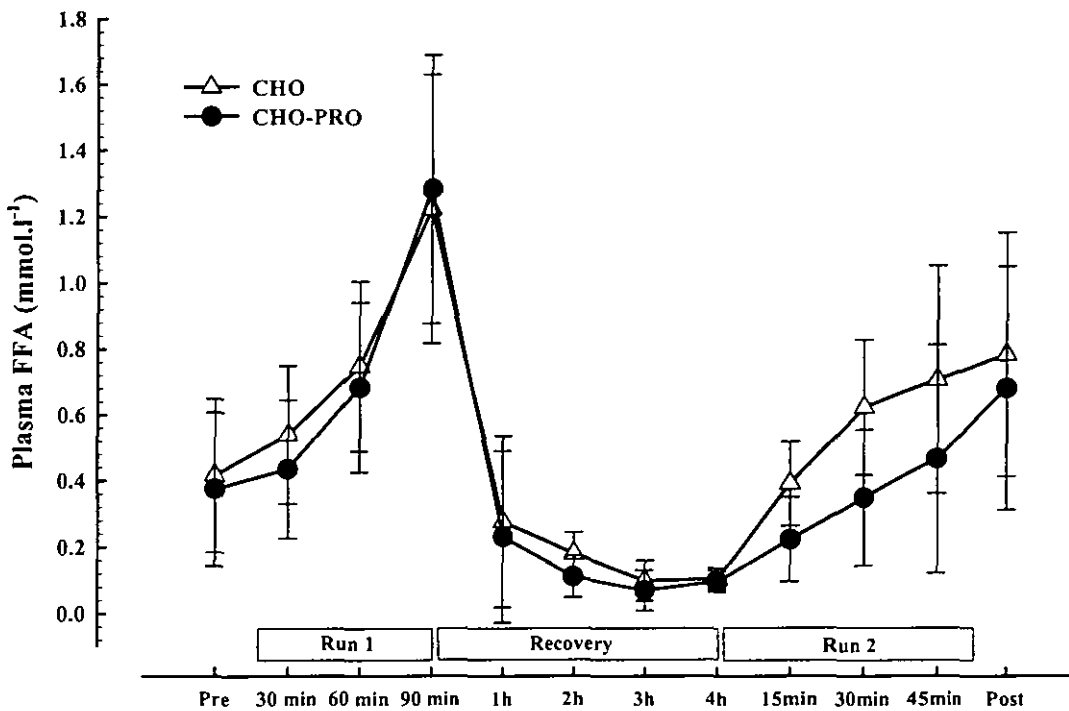


Figure 7.9: Plasma free fatty acid concentrations during R_1 , recovery and R_2 . Participants ($n = 5$) received either CHO or CHO-PRO supplements during recovery. Values are means \pm CI.

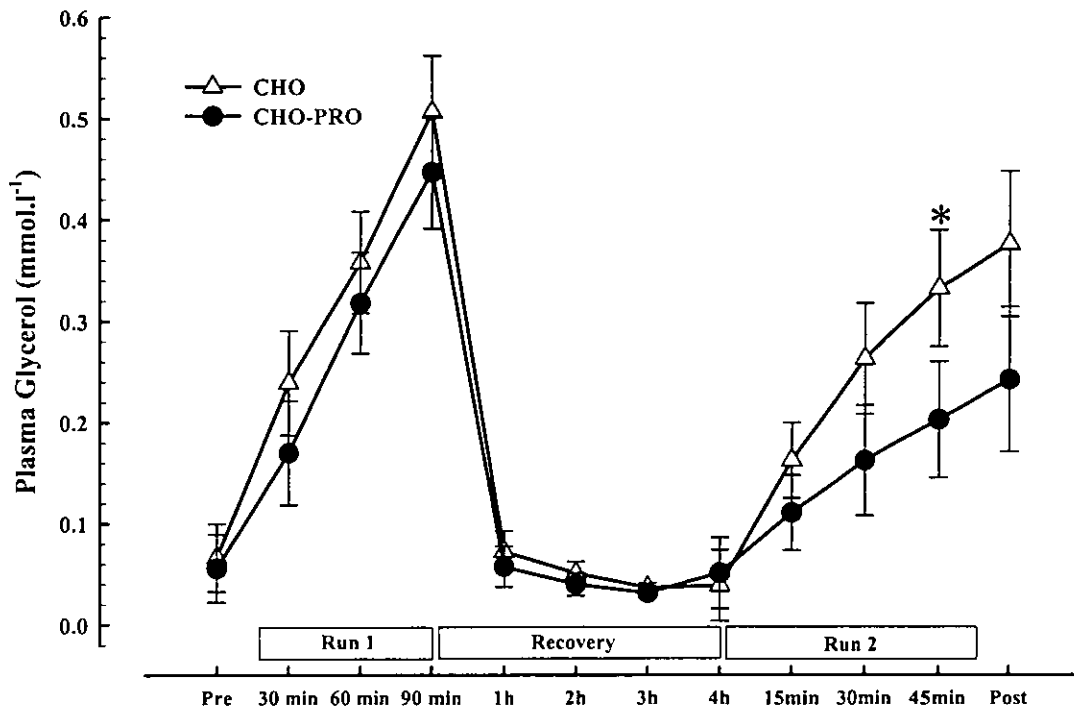


Figure 7.10: Plasma glycerol concentrations during R₁, recovery and R₂. Participants (n = 5) received either CHO or CHO-PRO supplements during recovery. Values are means ± CI and * denotes values different between trials ($P = 0.04$).

7.3.6 Blood Lactate

Blood lactate concentrations increased under both experimental treatments during R₁ and then decreased during recovery (Figure 7.11). There was a tendency in the latter stages of recovery for lower lactate concentrations with CHO-PRO ingestion than with CHO ingestion but this effect did not reach statistical significance. The subsequent blood lactate response to R₂ was also not different between trials.

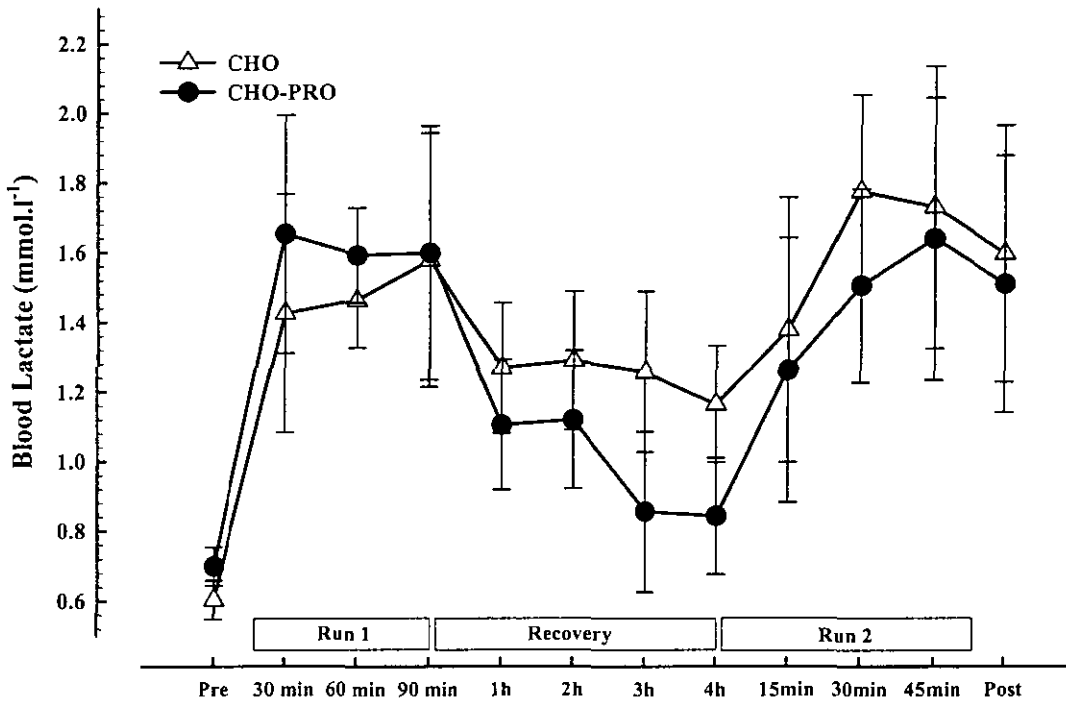


Figure 7.11: Blood lactate concentrations during R₁, recovery and R₂. Participants ($n = 5$) received either CHO or CHO-PRO supplements during recovery. Values are means \pm CI.

7.3.7 Substrate Metabolism

Protein oxidation rates were estimated from urine/plasma urea concentrations during recovery and revealed a greater rate of protein oxidation with ingestion of the CHO-PRO solution than with ingestion of the CHO solution ($P = 0.01$; Figure 7.12). Subsequent corrected estimations of RER were not different between trials and are presented in Table 7.3 along with the estimated contributions of lipid and carbohydrate towards overall metabolism. During R_2 , ingestion of the CHO-PRO solution resulted in higher rates of carbohydrate oxidation ($P \leq 0.03$) and lower rates of lipid oxidation ($P = 0.01$) than ingestion of the CHO solution. Figure 7.13 clearly illustrates that the differences between trials in terms of total carbohydrate oxidation were entirely attributable to variations in the contribution of carbohydrate sources other than muscle glycogen. Specifically, the oxidation rate of blood glucose and lactate during R_2 was elevated from $1.1 \pm 0.2 \text{ g}\cdot\text{min}^{-1}$ in the CHO trial to $1.6 \pm 0.1 \text{ g}\cdot\text{min}^{-1}$ in the CHO-PRO trial ($P = 0.02$).

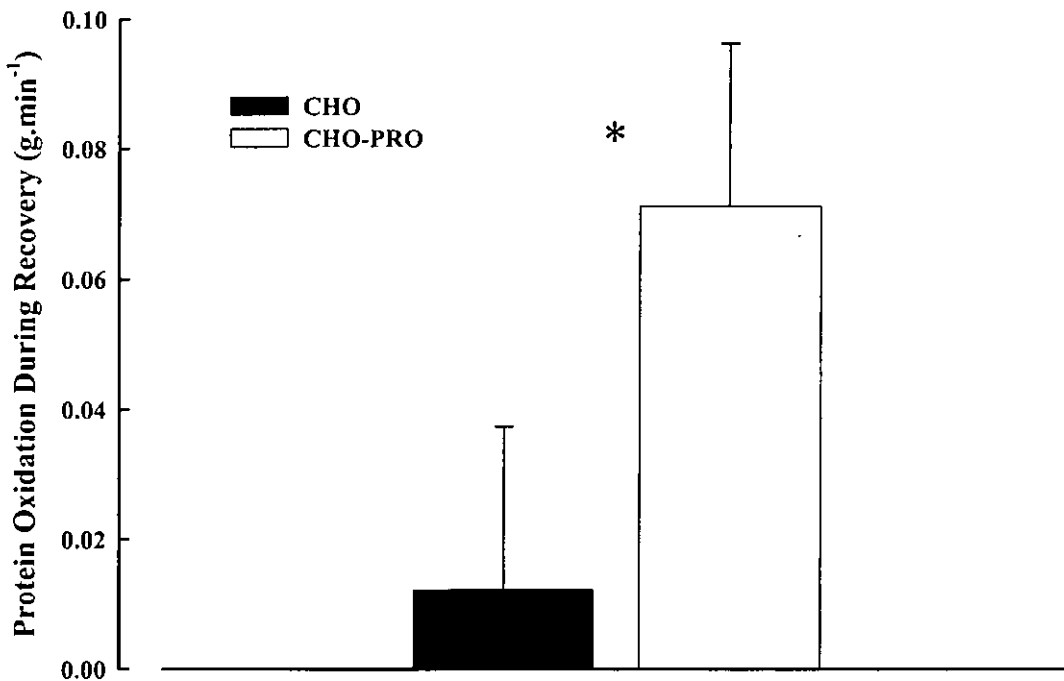


Figure 7.12: Rates of protein oxidation during 4 h recovery ($n = 5$). Values are means \pm CI and * denotes values different between trials ($P = 0.01$).

Table 7.3: Substrate metabolism and respiratory exchange ratios during R₁, recovery and R₂. Recovery data is corrected for estimated protein oxidation rates. Participants (n = 6) received either CHO or CHO-PRO supplements during recovery. (N.B: at Last min R₂ n = 5).

Values are means ± SEM and * denotes values different between trials ($P \leq 0.03$).

	Pre	Run 1				Recovery				Run 2		
		30 min	60 min	90 min	1 hr	2 hr	3 hr	4 hr	15 min	30 min	45 min	Last min
Carbohydrate oxidation (g.min⁻¹)										*		*
CHO	0.2±0.1	2.5±0.2	2.7±0.1	2.8±0.2	0.3±0.1	0.3±0.03	0.4±0.04	0.5±0.1	3.4±0.2	3.0±0.2	3.0±0.3	2.7±0.2
CHO-PRO	0.3±0.1	3.1±0.2	3.1±0.1	2.9±0.1	0.3±0.02	0.3±0.03	0.3±0.1	0.4±0.1	3.6±0.1	3.7±0.2	3.4±0.2	3.5±0.2
Lipid oxidation (g.min⁻¹)										*		*
CHO	0.1±0.03	0.6±0.1	0.5±0.03	0.5±0.1	0.1±0.03	0.1±0.01	0.1±0.01	0.02±0.01	0.1±0.1	0.4±0.1	0.4±0.1	0.5±0.1
CHO-PRO	0.1±0.02	0.4±0.1	0.3±0.1	0.5±0.1	0.1±0.01	0.1±0.01	0.1±0.01	0.04±0.03	0.2±0.1	0.3±0.03	0.3±0.03	0.2±0.1
RER					Non-protein respiratory exchange ratios							
CHO	0.85±0.05	0.90±0.01	0.91±0.01	0.92±0.01	0.87±0.03	0.91±0.02	0.92±0.01	0.97±0.02	1.02±0.04	0.93±0.01	0.93±0.02	0.91±0.01
CHO-PRO	0.89±0.04	0.94±0.01	0.94±0.01	0.92±0.01	0.86±0.01	0.87±0.02	0.88±0.02	0.96±0.04	0.98±0.01	0.98±0.01	0.95±0.01	1.00±0.04

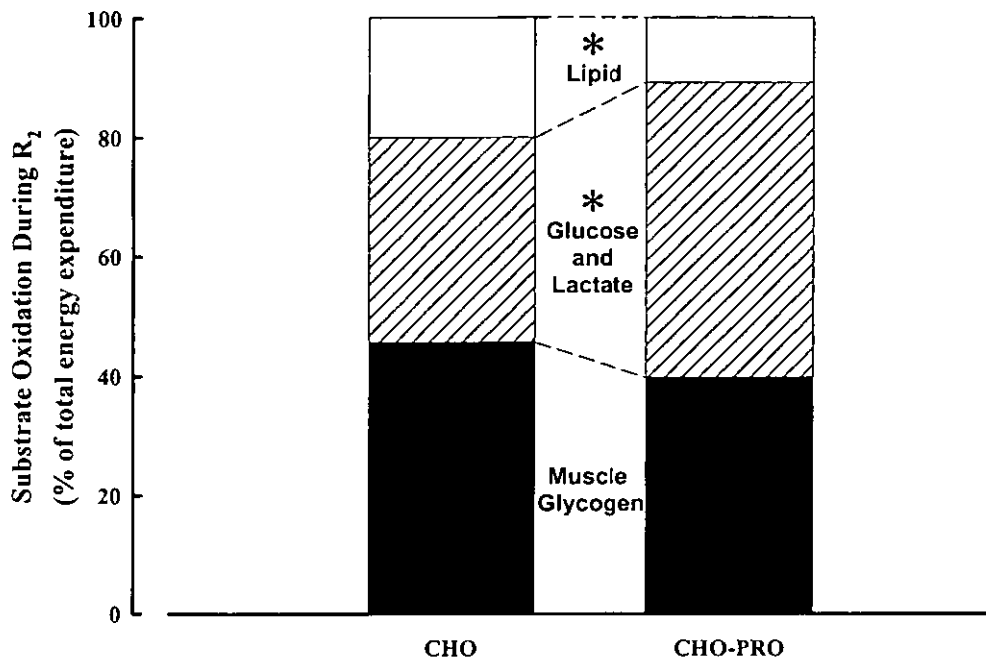


Figure 7.13: Rates of muscle glycogen, glucose/lactate and lipid oxidation during R₂ (n = 6). Values are means and * denotes values different between trials ($P = 0.03$).

7.3.8 Serum Cortisol

In both trials, serum cortisol concentrations were maintained at basal levels for the majority of R_1 but increased sharply towards the end of this exercise session (Figure 7.14). Thereafter, concentrations declined gradually over the ensuing 3-4 h of recovery before increasing steadily during the second bout of exercise (R_2). This pattern of response was standard between trials.

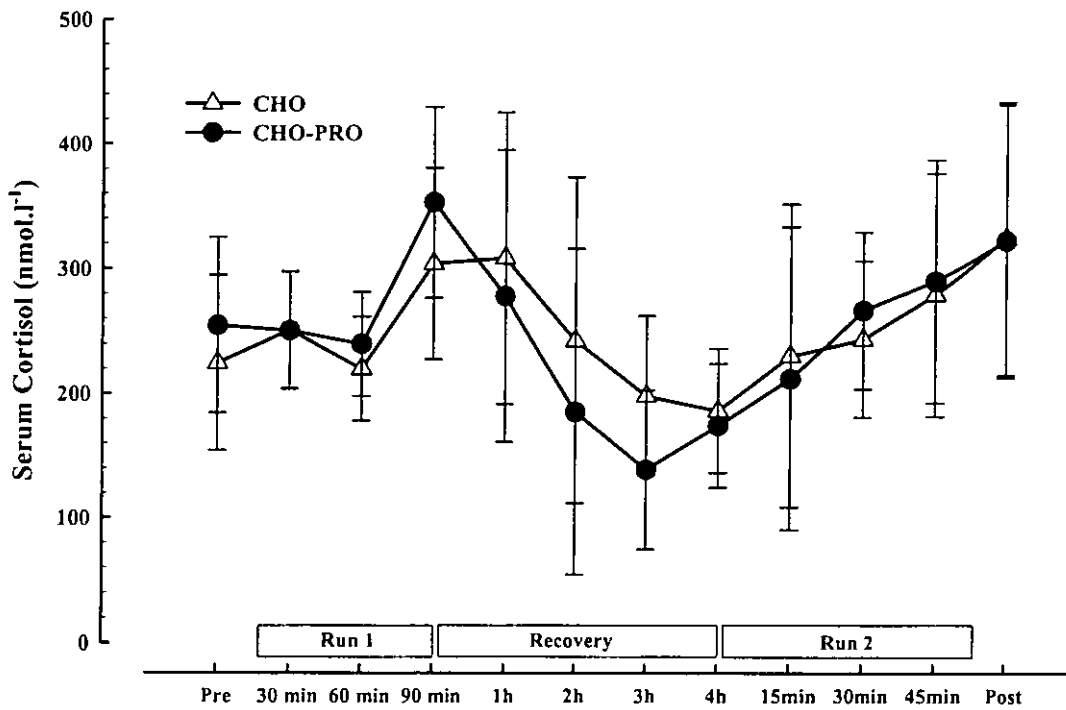


Figure 7.14: Serum cortisol concentrations during R_1 , recovery and R_2 . Participants ($n = 5$) received either CHO or CHO-PRO supplements during recovery. Values are means \pm CI.

7.3.9 Plasma Myoglobin

Plasma myoglobin concentrations did not decrease substantially during recovery following the increases recorded during R_1 (Figure 7.15). Subsequently, the progressive myoglobin release induced by R_2 appeared to supplement the already elevated concentrations of this haemprotein, culminating in peak values of 27.5 ± 8.8 $\text{nmol}\cdot\text{l}^{-1}$ (CHO) and 27.3 ± 9.1 $\text{nmol}\cdot\text{l}^{-1}$ (CHO-PRO) in the immediate post-exercise period.

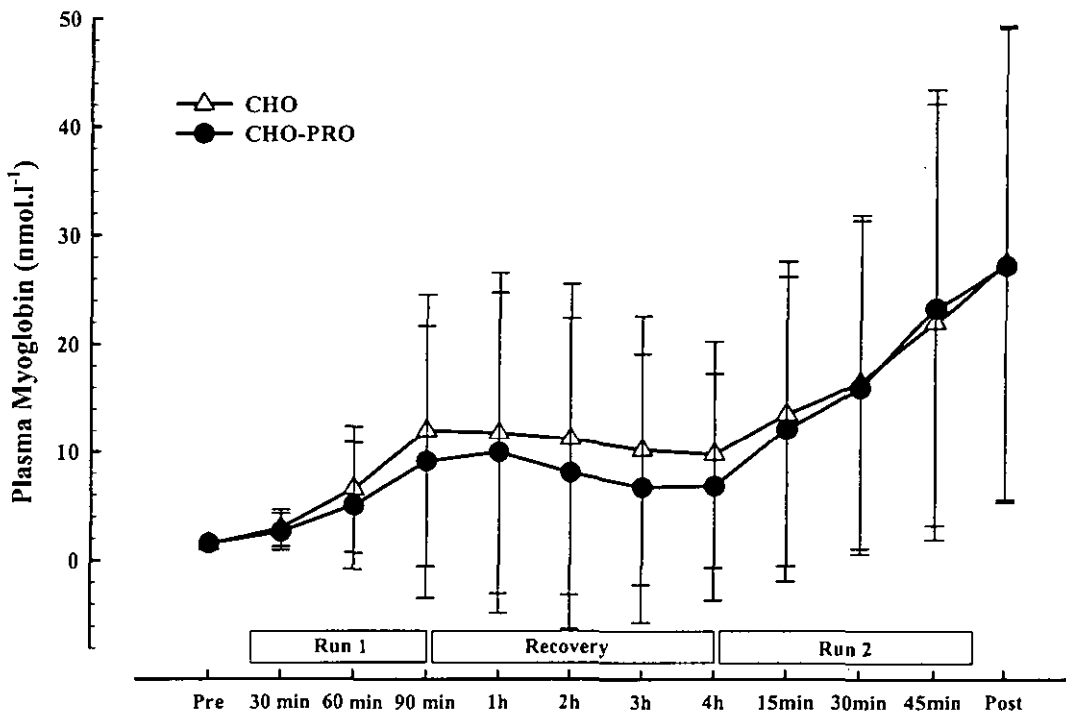


Figure 7.15: Plasma myoglobin concentrations during R_1 , recovery and R_2 . Participants ($n = 5$) received either CHO or CHO-PRO supplements during recovery. Values are means \pm CI.

7.3.10 Indices of Hydration Status

The performance of R₁ resulted in mean body mass (BM) losses of $-2.0 \pm 0.4\%$ BM in the CHO trial and $-1.9 \pm 0.3\%$ BM in the CHO-PRO trial, with the urine samples taken prior to this first exercise session indicating an adequate degree of pre-trial hydration (CHO = 612.8 ± 401.1 mosmol·kg⁻¹; CHO-PRO = 583.1 ± 336.5 mosmol·kg⁻¹; mean \pm SD). Following rehydration during recovery, similar decrements in body mass of $-1.5 \pm 0.1\%$ BM (CHO) and $-1.3 \pm 0.2\%$ BM (CHO-PRO) were recorded for R₂. Plasma volume was reduced with each exercise session but increased with fluid consumption during the intervening recovery (time effect: $F = 9.0$, $P = 0.001$). This response was uniform across the CHO and CHO-PRO treatments and the combined values of both trials were found to differ significantly from baseline at 30 min, 60 min and 90 min of R₁ ($P \leq 0.04$) and at 4 h of recovery ($P = 0.05$; Figure 7.16). Total urine volumes passed during the 4 h recovery period were 1204 ± 227 ml in the CHO trial and 953 ± 52 ml in the CHO-PRO trial.

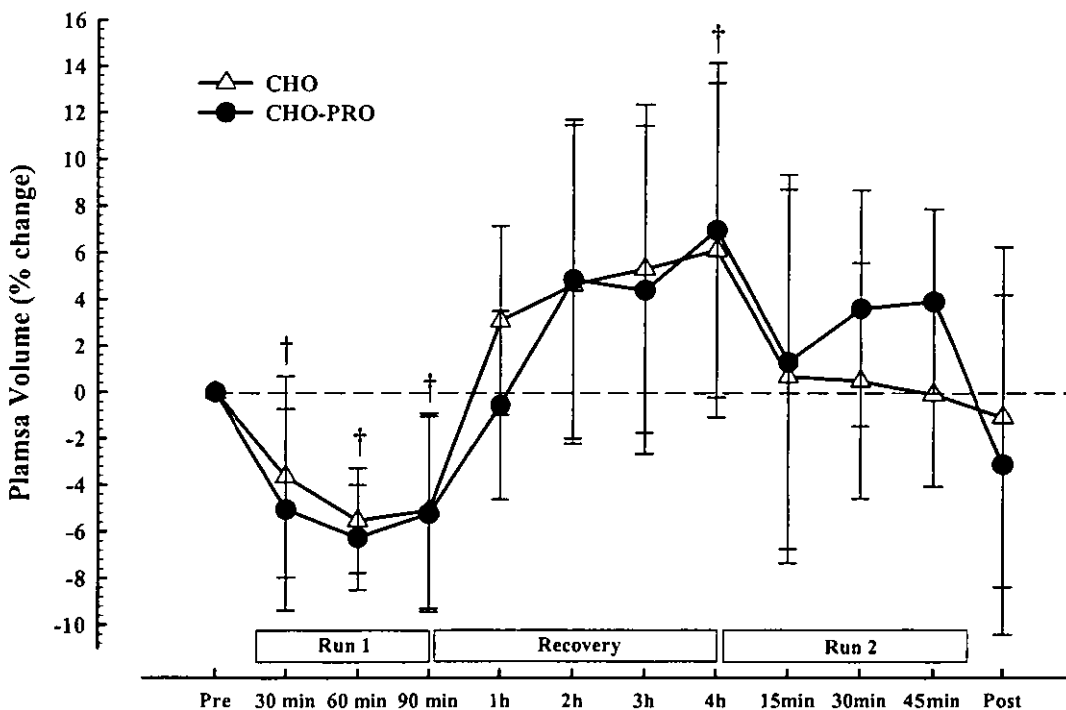


Figure 7.16: Changes in plasma volume during R₁, recovery and R₂. Participants ($n = 5$) received either CHO or CHO-PRO supplements during recovery. Values are means \pm CI and † denotes values different from baseline ($P \leq 0.05$).

7.3.11 Subjective Data

No statistical differences between trials could be detected in terms of the perceived exertion reported by participants during each exercise bout. Nonetheless, in the final minute of R₂, participants tended to indicate a greater degree of perceived exertion (scale 6-20) when CHO had been ingested during recovery (16.7 ± 1.1) compared to when CHO-PRO had been ingested (15.3 ± 0.9). While the 2 experimental solutions did not influence participants' reported sensations of thirst any differently, ingestion of the CHO-PRO solution produced higher overall ratings of gut fullness than ingestion of CHO alone ($P = 0.03$; Table 7.4).

Table 7.4: Subjective ratings of gut fullness during recovery and R₂. Participants ($n = 6$) received either CHO or CHO-PRO supplements during recovery.

(N.B: at Last min R₂ $n = 5$). Values are means \pm SEM and are different between trials ($P = 0.03$).

	Recovery				Run 2			
	1 h	2 h	3 h	4 h	15 min	30 min	45 min	Last min
Gut Fullness (scale 6-20)								
CHO	9 ± 1	10 ± 1	11 ± 1	11 ± 1	12 ± 1	11 ± 1	11 ± 1	10 ± 1
CHO-PRO	11 ± 1	12 ± 1	13 ± 1	14 ± 1	12 ± 1	12 ± 1	11 ± 1	10 ± 1

7.3.12 Control Data

The treadmill speeds selected for R₁ were found to elicit relative exercise intensities of $73.7 \pm 5.5\%$ $\dot{V}O_2$ max in the CHO trials and $74.3 \pm 3.5\%$ $\dot{V}O_2$ max in the CHO-PRO trials (mean \pm SD). Similarly, relative exercise intensities of $72.7 \pm 4.5\%$ $\dot{V}O_2$ max and $74.7 \pm 3.2\%$ $\dot{V}O_2$ max were recorded during R₂ in the CHO and CHO-PRO trials, respectively (mean \pm SD). The heart rate responses to these exercise bouts were very similar, exhibiting a gradual rise with increasing exercise duration. Independent of trial, heart rates increased from 169 ± 9 beats \cdot min⁻¹ to 178 ± 8 beats \cdot min⁻¹ during R₁ and from 170 ± 9 beats \cdot min⁻¹ to 180 ± 11 beats \cdot min⁻¹ during R₂ (mean \pm SD).

7.4 Discussion

Ingesting a carbohydrate solution with an additional $0.3 \text{ g}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$ of whey protein isolate during a 4 h recovery from prolonged exercise resulted in a similar rate of muscle glycogen resynthesis as when a matched carbohydrate solution with no additional protein was ingested. In addition, the inclusion of protein in the solutions had no effect on muscle glycogen degradation rates during exercise subsequent to recovery. However, the ingestion of the CHO-PRO solution did result in improved maintenance of euglycaemia and an increased rate of carbohydrate oxidation during the second exercise session than did carbohydrate ingestion alone.

The observation that muscle glycogen resynthesis was similar between treatments is not consistent with previous findings; all other research on this topic has reported an increase in the rate of muscle glycogen resynthesis when protein has been added to recovery solutions that provide $\leq 0.8 \text{ g CHO}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$ (Ivy *et al.*, 2002; Van Hall *et al.*, 2000a; van Loon *et al.*, 2000a; Zawadzki *et al.*, 1992). The reason for this discrepancy is not immediately apparent but may be related to the exercise mode that was selected for the present protocol. Studies using prolonged cycling to induce muscle glycogen depletion prior to recovery have established that adding protein to larger quantities of carbohydrate (i.e. $1.2 \text{ g CHO}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$) does not enhance muscle glycogen storage during recovery, possibly because such rates of carbohydrate intake maximise the glucose transport capacity of previously exercised muscle even without added protein (Jentjens *et al.*, 2001; Van Hall *et al.*, 2000b). Evidence supports the possibility that the treadmill running in comparison with cycling may reduce maximal glucose transport capacity following exercise as a consequence of the increased eccentric component of muscular contraction (Asp *et al.*, 1997b; King *et al.*, 1993; Kirwan *et al.*, 1992). If this is assumed to be true, then the maximal rate of muscle glycogen resynthesis might also be expected to be limited at a lower than usual rate (Costill *et al.*, 1990; Doyle *et al.*, 1993; O'Reilly *et al.*, 1987) and it would follow that a smaller than usual quantity of carbohydrate would be sufficient to maximise this process. Although speculative, this explanation would account for the virtually identical rates of muscle glycogen resynthesis between trials in the present study despite differences in serum insulin concentrations during recovery.

Alternatively, it may be that the treatment differences in serum insulin concentration in the present study were simply not of sufficient magnitude to produce a concomitant effect on the rate of muscle glycogen resynthesis. While even modest increases in circulating insulin can increase glucose uptake, it has been proposed that a minimum insulin concentration of $\approx 340 \text{ pmol}\cdot\text{l}^{-1}$ may be necessary to shift the primary fate of intracellular glucose from net oxidation to net storage (Young *et al.*, 1988). It was certainly the case that the peak insulin concentrations which occurred after 1 h of recovery in the present study only exceeded this proposed figure in the CHO-PRO trial (CHO = $269 \pm 26 \text{ pmol}\cdot\text{l}^{-1}$ and CHO-PRO = $347 \pm 37 \text{ pmol}\cdot\text{l}^{-1}$) but by no means were these increments comparable with those reported in studies where the rate of glycogen resynthesis has been affected. For instance, the ingestion of $0.8 \text{ g}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$ in the study of van Loon *et al.* (2000a) resulted in similar peak insulin concentrations to those observed in the present study, but when these authors added $0.4 \text{ g}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$ of amino acids there was an 88% increase in insulinaemic response over 5 h and peak insulin concentrations were in excess of $600 \text{ pmol}\cdot\text{l}^{-1}$ (van Loon *et al.*, 2000a). It is not clear why the protein provided by these authors proved to be such an effective insulin secretagogue in comparison with solution currently under investigation but it may be related either to minor variations in the amount of protein provided (i.e. 0.3 versus $0.4 \text{ g}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$) or to subtle differences in the precise amino acid composition of the protein that was ingested. Nonetheless, the inconsistent insulin concentrations between the study of van Loon *et al.* (2000a) and those of the present study are likely to be at least partially responsible for the varied rates of muscle glycogen resynthesis following CHO-PRO ingestion in each respective study.

Although the insulin concentrations observed in the CHO-PRO trial of the present study may not have been sufficiently elevated to increase muscle glycogen storage overall, a trend ($P = 0.15$) was apparent for a greater rate of muscle proglycogen storage in the CHO-PRO trial ($9.7 \pm 2.4 \text{ mmol glucosyl units}\cdot\text{kg dry mass}^{-1}\cdot\text{h}^{-1}$) than in the CHO trial ($6.8 \pm 1.7 \text{ mmol glucosyl units}\cdot\text{kg dry mass}^{-1}\cdot\text{h}^{-1}$). Of the 2 forms of muscle glycogen, proglycogen is most responsive to insulin and would therefore be expected to account for the majority of muscle glycogen synthesis in response to acute nutritional intervention over 4 h (Adamo *et al.*, 1998; Wee *et al.*, 2005). On the other hand, macroglycogen is known to be responsible for muscle

glycogen supercompensation following prior depletion, which is interesting given that the response of macroglycogen resynthesis to each treatment was opposite to that of proglycogen in the present study. It is possible that synthesis of macroglycogen may have been preferentially stimulated in the CHO trial due to the lower absolute concentrations following R_1 in this trial than in the CHO-PRO trial, although this difference between treatments is negligible when considered as a fraction of overall muscle glycogen availability (Table 7.2). Nonetheless, it is interesting to note that macroglycogen only represented 6.8 and 16.6% of total muscle glycogen availability following R_1 in the CHO and CHO-PRO trials, respectively. These relatively low proportions may support the contention that macroglycogen was heavily used during R_1 since this acid soluble fraction has previously been shown to account for up to 25% of total muscle glycogen content at rest (Jansson, 1981). This would therefore be consistent with the data of Adamo *et al.* (1998) who have reported macroglycogen concentrations in the region of 11-13% following exhaustive exercise. In summary, the distinct responses of macroglycogen and proglycogen to CHO-PRO supplementation are an interesting phenomenon and further research is required to determine the specific physiological roles of these separate pools of muscle glycogen.

It should be noted that any gluconeogenic efflux arising from the deamination of amino acids during recovery will have overestimated the rate of protein oxidation presented in Figure 7.12. However, the NPRER that was calculated using this estimated protein oxidation rate remains accurate since the overall respiratory exchange ratio both for direct oxidation of protein and oxidation of glucose that is derived from protein is 0.8 (Frayn, 1983). The limitation to this method occurs if newly synthesised glucose is stored rather than oxidised since this process has a respiratory quotient of 0.4 and therefore artificially reduces the NPRER (Jequier *et al.*, 1987). It is therefore of interest that the NPRER displayed a slight tendency to be lower throughout recovery in the CHO-PRO trial than in the CHO trial, raising the possibility that the amino acids ingested in the CHO-PRO trial may have been deaminated and stored in the form of liver glycogen. While the lack of any significant differences in NPRER only allows such a conclusion to be drawn very tentatively ($F = 1.5$, $P = 0.15$), many of the other variables measured during the present study are consistent with this suggestion. Firstly, it is unlikely that the reduced NPRER during recovery in the CHO-PRO trial was a consequence of increased lipid metabolism

since no trial differences in either FFA or glycerol concentrations were apparent at this time and the elevated insulin concentrations following CHO-PRO ingestion would be expected to inhibit lipolysis (Castan *et al.*, 1999). In addition, the observation that plasma urea began to rise immediately upon ingestion of the CHO-PRO solution provides evidence that the exogenously ingested amino acids were being deaminated and, given the relative hyperinsulinaemia during recovery, it is likely that a large portion of the newly generated glucose would be retained by the liver (Pencek *et al.*, 2004). Unfortunately, measurement of liver glycogen concentrations along with gluconeogenic hormones such as glucagon was not possible in the present study and the above hypothesis remains to be examined directly. Nonetheless, the majority of available literature attests that insulin can be expected to play a more central role than glucagon in the regulation of glucose metabolism and the current results can therefore be taken in provisional support for the suggestion that the protein fraction of the CHO-PRO mixture might have contributed carbon for the synthesis of liver glycogen during recovery.

The above speculation regarding increased liver glycogen resynthesis during recovery in the CHO-PRO trial may also explain the differences between trials in terms of total carbohydrate oxidation during R₂. Figure 7.13 neatly illustrates that blood glucose and lactate oxidation must have occurred at a greater rate following CHO-PRO ingestion since muscle glycogen utilisation during R₂ was very similar between treatments and lipid oxidation was reduced. The hypoglycaemia which occurred in the early stages of R₂ would be expected to stimulate endogenous glucose production (Coker *et al.*, 2002) and it is therefore conceivable that hepatic glucose output would be higher in the CHO-PRO treatment due to an increased availability of liver glycogen. While it is understood that the slightly elevated insulin concentrations during R₂ in the CHO-PRO trial would usually oppose hepatic glucose output (Camacho *et al.*, 2004), it is thought that hypoglycaemia overrides this effect through reducing the action of insulin on the liver (Koyama *et al.*, 2002). The progressive increase in free fatty acids during R₂ of the CHO-PRO trial lends further support for this contention since increments in free fatty acids are known to stimulate gluconeogenesis even under conditions of hyperinsulinaemia (Lam *et al.*, 2003). Therefore, the differences between trials in plasma glucose concentration during R₂

may well reflect an improved counterregulatory response to hypoglycaemia following CHO-PRO ingestion.

The finding that ingestion of the CHO-PRO solution produced more stable blood glucose concentrations than ingestion of the CHO solution but without compromising the rate of muscle glycogen resynthesis presents the interesting possibility that including protein in a carbohydrate solution might simultaneously provide the combined benefits of high and low glycaemic index (GI) carbohydrates. To be precise, the increased insulin response associated with ingesting high GI carbohydrate during recovery results in a greater rate of muscle glycogen storage than does ingestion of similar amounts of low GI carbohydrate (Burke *et al.*, 1993; Kiens *et al.*, 1990). However, the highly insulinotropic nature of high GI carbohydrates can also cause metabolic disturbances such as hypoglycaemia during subsequent exercise (Costill *et al.*, 1977; Foster *et al.*, 1979), a potential disadvantage which is less likely when low GI carbohydrates are ingested (Febbraio *et al.*, 2000; Sparks *et al.*, 1998; Wee *et al.*, 1999; Wee *et al.*, 2005). It is therefore clear from the present results that a CHO-PRO mixture can provide the latter benefit of low GI carbohydrates in terms of reduced metabolic disturbance while at the same time imparting the benefit of high GI carbohydrates through maintaining relatively high rates of muscle glycogen resynthesis during recovery. These combined effects of CHO-PRO ingestion on glucose homeostasis can clearly facilitate athletic performance but have also been applied as an effective nutritional intervention in clinical populations. Specifically, it has recently been demonstrated that the impaired insulinaemic response to carbohydrate ingestion in patients with type 2 diabetes can be successfully treated through the coingestion of amino acids along with that carbohydrate, thus functioning to increase glucose disposal and reduce postprandial hyperglycaemia (Frid *et al.*, 2005; Manders *et al.*, 2005; van Loon *et al.*, 2003a).

The reason that the peak in glucose concentrations during recovery tended to be lower in the CHO-PRO trial than in the CHO trial is probably related to the rate of glucose appearance from the GI tract under each condition. Intact protein is known to empty from the gut slightly slower than carbohydrate (Thomas, 1957) and has been found to delay gastric emptying when added to a glucose solution (Berry *et al.*, 2002). While no direct evidence to verify this contention is available, participants' subjective

ratings of gut fullness were significantly higher when ingesting CHO-PRO than when ingesting CHO which may infer differences in gastric volume during recovery. Importantly, if it is assumed that intestinal glucose absorption was delayed with the CHO-PRO treatment, then it is possible that this factor might also have contributed to the trial differences in glucose concentration during R₂. Specifically, glucose appearance from the GI tract may have been maintained until R₂ in the CHO-PRO trial, thus supplementing plasma glucose concentrations more effectively than in the CHO trial.

In summary, contrary to the wealth of available literature, the addition of protein to a carbohydrate recovery solution providing 0.8 g CHO·kg⁻¹·h⁻¹ did not further accelerate the rate of muscle glycogen resynthesis during a 4 h recovery from prolonged exercise. Furthermore, the rate of muscle glycogen degradation during exercise subsequent to recovery was also not different between the CHO and CHO-PRO treatments despite a higher contribution of overall carbohydrate oxidation towards metabolism following CHO-PRO ingestion. It is concluded that the added protein increased the oxidation rate of other carbohydrate sources such as glucose and lactate during R₂, possibly as a result of an elevated appearance of glucose from the liver and/or gastrointestinal tract during exercise.

CHAPTER 8

General Discussion

This series of studies has revealed that exercise capacity can be restored more completely within a 4 h recovery from prolonged exercise if the energy content of a carbohydrate solution providing $0.8 \text{ g CHO}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$ is increased through the addition of $0.3 \text{ g}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$ of either mixed amino acids or further carbohydrate. The addition of amino acids was consistently found to augment the insulinaemic response to varying levels of carbohydrate intake, although no difference in the rate of muscle glycogen resynthesis during recovery was apparent between these treatments. Furthermore, the presence of these amino acids in the recovery solutions did not alter the rate of muscle glycogen degradation during exercise subsequent to recovery, instead resulting in an increased oxidation of alternative sources of carbohydrate. Other notable findings were that ingestion of CHO-PRO rather than CHO during recovery resulted in an immediate increase in plasma urea concentrations and also increased the concentrations of human growth hormone following the exercise capacity test. It is also noteworthy that differences in exercise capacity following recovery were only evident when the intensity of that exercise test did not exceed $\approx 70\% \dot{V}O_{2\text{max}}$.

The most striking of the above findings is that ingestion of a CHO-PRO solution during a short-term recovery can promote a more rapid restoration of exercise capacity than ingestion of a solution containing the carbohydrate fraction in isolation. More importantly, it appears that this ergogenic benefit of CHO-PRO ingestion may be independent of an effect on muscle glycogen resynthesis during recovery. This contention stems from the combined results of Studies 3 and 4 in which the addition of protein to a carbohydrate solution improved exercise capacity but without accelerating the rate of muscle glycogen resynthesis, respectively.

However, the authority with which the above conclusion can be drawn depends on the degree to which Studies 3 and 4 can be assumed to reflect one another. In support of this assumption, the descriptive characteristics of participants recruited for Study 4 were almost identical to those of the participants who ran to volitional exhaustion during Study 3 and the experimental designs of these 2 studies were also identical, other than the open ended nature of the exercise test in Study 3. The overall

consequence of this standardisation was that virtually all of the physiological variables that were common to both studies displayed a very similar pattern between treatments. In light of this, it is reasonable to assume that the muscle glycogen concentrations measured in Study 4 would have followed a similar pattern during Study 3. This line of reasoning equally leads to the suggestion that the participants in Study 4 would have been able to exercise for longer during R₂ had this exercise session required them to run to the point of exhaustion rather than being a fixed duration exercise task. This possibility therefore presents a number of interesting points of discussion regarding the potential causes of fatigue during R₂ in Study 3 along with the mechanism through which the ingestion of CHO-PRO may have operated to delay these processes.

The CHO-PRO mixture did not elicit a greater rate of muscle glycogen resynthesis during recovery than that achieved through ingestion of a matched amount of carbohydrate alone. As such, the absolute amount of muscle glycogen that was available at the onset of R₂ was not different between the CHO and CHO-PRO treatments and the differences in exercise capacity cannot therefore be attributed to differences in pre-exercise muscle glycogen status. Neither can the increased exercise capacity in the CHO-PRO trial of Study 3 be attributed to muscle glycogen sparing during R₂, at least during the first 60 min of exercise, since Study 4 established that rates of muscle glycogen degradation were almost identical over this period between the CHO and CHO-PRO treatments. Once these possibilities have been eliminated, what remains is the prospect that some influence of the CHO-PRO solution must have operated to prolong physical performance in the late stages of exercise, i.e. after the 60 min sampling point applied in Study 4. Figure 8.1 presents 2 possible mechanisms (*a* and *b*) through which muscle glycogen concentrations may have dissociated between treatments such that performance could be prolonged in the CHO-PRO trial.

The broken lines on Figure 8.1 represent the predicted changes in muscle glycogen concentration following the 60 min sampling point in each trial, although it should be acknowledged that the suggested rates of muscle glycogen degradation between 60 min and the point of fatigue are probably overestimates since the rate of glycogen utilisation would be expected to decrease with increasing exercise duration (Coyle *et al.*, 1986; Hargreaves *et al.*, 1995; Romijn *et al.*, 1993). Nonetheless, given

the similarity of glycogen concentrations between trials at 60 min, this overestimation is likely to be of a similar magnitude between the 2 solutions and therefore would not confound interpretation of this figure. What is clearly demonstrated in Figure 8.1 is that muscle glycogen concentrations must have dissociated between treatments in the late stages of exercise, either through a reduced rate of muscle glycogen utilisation in the CHO-PRO trial (option *a*) or through lower absolute muscle glycogen concentrations at the point of fatigue in the CHO-PRO trial (option *b*). To address the former of these possibilities, fatigue may potentially have been postponed by an increased availability of liver glycogen in the latter stages of R₂. According to this explanation, when critically low muscle glycogen concentrations occurred after approximately 84 min of the exercise capacity test, further performance would be entirely dependent on the availability of other carbohydrate sources. It is therefore conceivable that ingestion of CHO-PRO rather than CHO during recovery may have resulted in an enhanced rate of liver glycogen resynthesis, thus facilitating hepatic glucose output once muscle glycogen was depleted during R₂. Evidence from Ahlborg and Felig (1982) certainly supports that, once muscle glycogen has been depleted, hepatic glucose output can only be sustained by an adequate supply of liver glycogen (Ahlborg and Felig, 1982). Furthermore, the study of Coyle *et al.* (1986) has previously demonstrated that exercise can be performed for prolonged periods with minimal reliance on muscle glycogen provided that an adequate supply of blood glucose is available to maintain carbohydrate oxidation (Coyle *et al.*, 1986). It is therefore notable that blood glucose concentrations and calculated rates of carbohydrate oxidation were greater during R₂ in the CHO-PRO trials than in the CHO trials both in Study 3 and Study 4. In fact, the finding that muscle glycogen degradation rates were almost identical between the CHO and CHO-PRO trials infers that the observed increase in overall carbohydrate oxidation must have arisen from an increased oxidation of other carbohydrate sources (i.e. blood glucose/lactate).

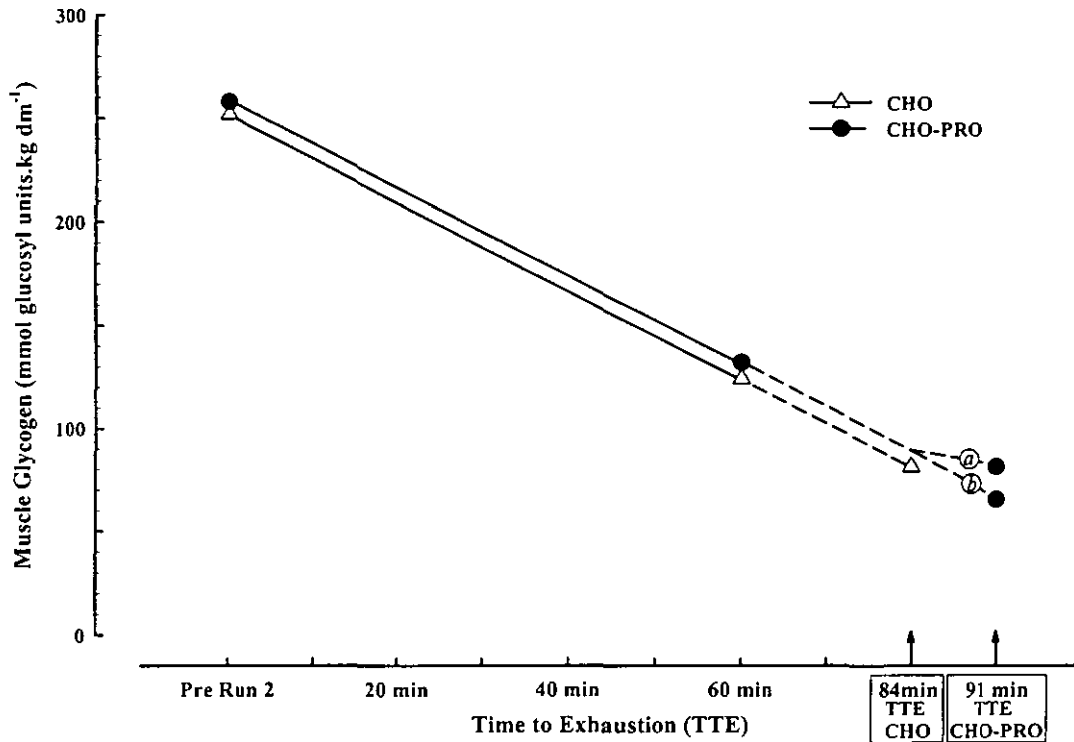


Figure 8.1: Possible dissociation of muscle glycogen concentrations between treatments to account for the prolonged performance in the CHO-PRO trial. The solid lines represent the rates of muscle glycogen degradation measured in Study 4 while the broken lines represent the possible changes in muscle glycogen degradation after the 60 min sampling point based on the run times recorded in Study 3. Option *a* = muscle glycogen sparing through oxidation of other carbohydrate sources late in exercise; option *b* = lower critical level of muscle glycogen depletion prior to the onset of fatigue.

Despite the potential similarity between the current findings and those of Coyle *et al.* (1986), in that fatigue may have been postponed with no further reliance on muscle glycogen (option *a*), it was proposed by these authors that fatigue in such circumstances would coincide with the development of hypoglycaemia (Coyle *et al.*, 1986). However, there was no evidence of hypoglycaemia at the point of fatigue in any of the trials during Study 3, which would appear to conform to the more recent suggestion of Claassen *et al.* (2005) that the beneficial effect of improved glucose availability late in exercise is unrelated either to maintenance of carbohydrate oxidation or avoidance of hypoglycaemia. The alternative proposal from these authors is that fatigue may be a consequence of combined muscle *and* liver glycogen depletion resulting in an increased cerebral sensitivity to relatively small fluctuations in glucose concentration (Claassen *et al.*, 2005), an explanation that would certainly be more compatible with the present results and also with data from other authors in relation to intermittent exercise (Foskett *et al.*, 2004). Furthermore, this notion of cerebral sensitivity to overall carbohydrate status would also fit nicely with recent findings indicating that simply the taste of carbohydrate can impart an ergogenic benefit (Carter *et al.*, 2004). Taken in combination, all the above studies support that fatigue is initiated as a safety mechanism in response to compromised overall carbohydrate status rather than as a consequence of muscle glycogen depletion *per se*. In this sense the possibility illustrated as option *b* gains credibility since the CNS might permit muscle glycogen to reach slightly lower concentrations before terminating exercise in circumstances where sufficient liver glycogen is available to safely continue physical activity without compromising glucose homeostasis. This interaction between net carbohydrate status, the CNS and the onset of fatigue is presented graphically in Figure 8.2.

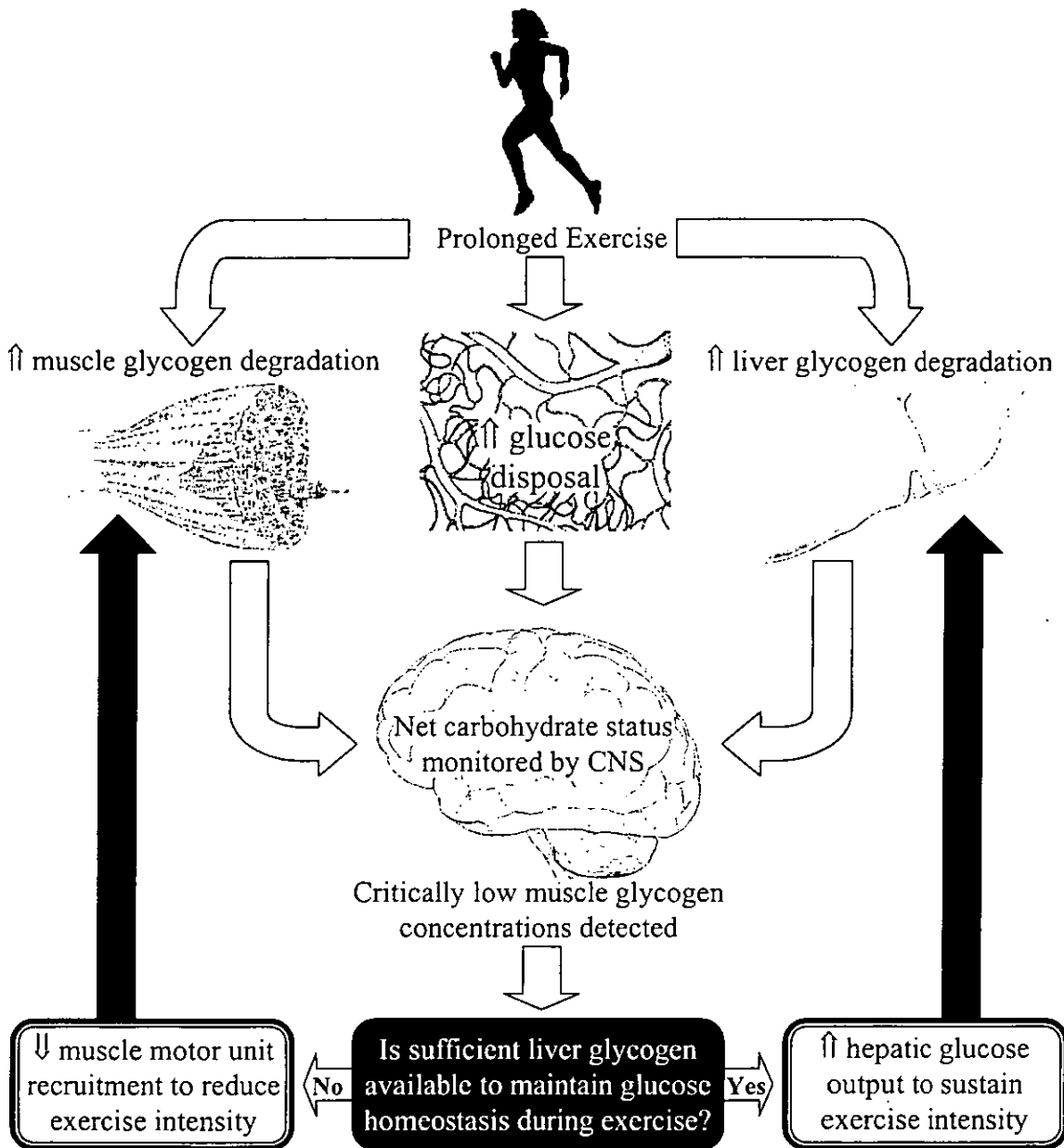


Figure 8.2: Proposed safety mechanism resulting from signalling between net carbohydrate status, the CNS and the onset of fatigue.

Support for the process illustrated in Figure 8.2 comes from Casey *et al.* (2000) who have described how exercise capacity may be more strongly associated with overall carbohydrate status than with muscle glycogen status alone (Casey *et al.*, 2000). Intuitively, the monitoring of liver glycogen availability by the CNS can be expected to play a primary role in the development of fatigue since maintenance of glucose homeostasis would take priority over maintaining exercise intensity in almost all circumstances. The implication of this model is that effective nutritional supplementation during a short-term recovery will need to replace both muscle and liver glycogen to prevent this safety mechanism being initiated prematurely during subsequent exercise. In relation to the present results, however, it cannot be fully established whether exercise capacity was enhanced as a direct result of increased liver glycogen storage during recovery when ingesting the solutions with added protein. As discussed in both Chapters 6 and 7, indirect evidence for such an effect might be taken from the observation of elevated urea concentrations coupled with the decreased RER during recovery in the CHO-PRO trials, which may reflect the storage of amino acids as liver glycogen. Nonetheless, while this explanation would certainly account for the improved maintenance of blood glucose during R₂, it is clear that further investigation will be necessary to directly assess the impact of CHO-PRO ingestion on the rate of liver glycogen resynthesis during recovery.

Just as the safety mechanism proposed in Figure 8.2 implies that muscle glycogen cannot be assumed to be the sole determinant of exercise capacity, it would be equally simplistic to suggest that compromised liver glycogen availability will be the primary cause of fatigue in every situation. Instead, a more adaptable system would involve the CNS monitoring various components of net carbohydrate status independently, with the relative impact of each of these signals mediated by a number of factors. An interesting possibility is that certain influences from a prior bout of exercise might carry over into the second exercise session such that the CNS will be more sensitive to changes in carbohydrate availability. It is apparent from this example that fatigue will be the overall product of a complex interaction between peripheral signalling of challenged carbohydrate status and the sensitivity of the CNS to these signals. Therefore, the differences in exercise capacity reported in Study 3 may not only reflect differences in carbohydrate storage during recovery but also alterations in central drive during R₂. Indeed, the central fatigue hypothesis provides

a clear mechanism through which the inclusion of amino acids in a recovery solution might offset sensations of fatigue when carbohydrate status is compromised (Davis, 1995; Davis and Bailey, 1997). According to this hypothesis, the increase in plasma FFA which occurs in response to carbohydrate depletion would displace tryptophan from its binding site on albumin (Yamamoto *et al.*, 1997), thus favouring the transport of this particular amino acid across the blood-brain barrier and facilitating the synthesis of certain neurotransmitters (e.g. 5-HT) that are associated with sensations of fatigue (Bailey *et al.*, 1993; Pardiidge, 1983). There are therefore 2 possible mechanisms through which the CHO-PRO mixture used in Study 3 may have influenced the above process to postpone fatigue. Firstly, it is likely that circulating amino acid concentrations would be higher during R₂ in the CHO-PRO trials than in the CHO trials which would competitively inhibit the transport of tryptophan across the blood-brain barrier. Evidence in support of this contention can be taken from studies which have demonstrated that ingestion of amino acids alone can both reduce perceived exertion and improve performance (Blomstrand *et al.*, 1997; Blomstrand *et al.*, 1991), which may also explain why participants tended to report lower RPE scores following CHO-PRO rather than either CHO or CHO-CHO ingestion during Study 3. The second mechanism through which the additional protein may have operated to attenuate sensations of fatigue may relate back to the findings cited earlier regarding carbohydrate availability. Specifically, both Studies 3 and 4 demonstrated that the increased glucose concentrations during exercise following ingestion of CHO-PRO rather than CHO were also associated with slightly decreased circulating concentrations of FFA. According to the central fatigue hypothesis, a greater proportion of tryptophan would therefore remain bound to albumin in the CHO-PRO trials and this might reduce the synthesis of 5-HT and consequently delay the onset of fatigue (Blomstrand *et al.*, 2005). In addition, given that an inhibitory influence of brain glucose on 5-HT synthesis has been demonstrated (Bequet *et al.*, 2002), it is also reasonable to suggest that peripheral carbohydrate status might be detected by the CNS at least partially through a direct assessment of blood glucose availability.

However, it is also emerging that IL-6 release from skeletal muscle during exercise might provide an additional mechanism through which the CNS can detect critically low levels of carbohydrate availability (Nieman *et al.*, 2005; Pedersen and Febbraio, 2005). Particularly since the release of this cytokine has been associated

with the activity of AMPK in muscle fibres that have been depleted of muscle glycogen (MacDonald *et al.*, 2003). It has been postulated that such increases in IL-6 might contribute to the development of central fatigue as described above (Gleeson, 2000) and, with relevance to Figure 8.2, it is also known that IL-6 serves a secondary purpose in stimulating hepatic glucose production (Febbraio *et al.*, 2004). This latter finding is of utmost importance regarding the regulation of glucose metabolism during exercise because low muscle glycogen concentrations *per se* do not increase the rate of blood glucose oxidation without increases in blood glucose concentration (Arkinstall *et al.*, 2004; Weltan *et al.*, 1998a, 1998b). Therefore, IL-6 may represent the signalling mechanism through which an impending intra-muscular energy crisis is detected by both the brain and the liver in order to increase hepatic glucose output and thus sustain energy production through increasing the oxidation of blood glucose. Nonetheless, in light of the similarity in muscle glycogen concentrations between trials in Study 4, IL-6 release from muscle might also be expected to be similar between treatments. What would differ between trials, however, would be the ability of the liver to respond to this signal with an increase in glucose production, prompting the CNS to reduce motor unit recruitment and thus restrict further energy expenditure after different exercise durations following CHO or CHO-PRO ingestion.

Further to the above discussion regarding the possible mechanisms through which the inclusion of protein may have prolonged exercise capacity in Study 3, this investigation also unearthed the interesting finding that recovery of exercise capacity was equally enhanced through increasing the rate of carbohydrate ingestion from 0.8 g CHO·kg⁻¹·h⁻¹ to 1.1 g CHO·kg⁻¹·h⁻¹. Given the proposed positive correlation between exercise capacity and pre-exercise muscle glycogen availability (Bergstrom *et al.*, 1967), this finding is not entirely unexpected since a similar increase in carbohydrate intake has been previously shown to accelerate the rate of muscle glycogen resynthesis during a short-term recovery (van Loon *et al.*, 2000a). However, efforts from other authors to translate these differences in muscle glycogen resynthesis into an effect on restoration of treadmill running capacity have not been successful (Fallowfield and Williams, 1997; Wong and Williams, 2000). The finding of a similar recovery of endurance capacity following ingestion of either 0.2 g CHO·kg⁻¹·h⁻¹ or 0.5 g CHO·kg⁻¹·h⁻¹ by Wong and Williams (2000) is of particular interest because a subsequent investigation from Tsintzas *et al.* (2003) has indicated

that an increase in carbohydrate intake of this magnitude would be highly likely to increase the rate of muscle glycogen resynthesis during recovery without increasing the rate of muscle glycogen degradation during subsequent exercise (Tsintzas *et al.*, 2003). Therefore, it remains difficult to explain why effects on exercise capacity have not been identified in those studies in which varying amounts of carbohydrate have been ingested during recovery.

One potential explanation is that the exercise capacity tests employed by previous investigators may not have been sufficiently reliable to detect small but worthwhile differences in run time to exhaustion between treatments (Hopkins *et al.*, 1999; Hopkins *et al.*, 2001). Indeed, the reliability of tests involving exercise to the point of fatigue at a fixed sub-maximal intensity have been questioned (Krebs and Powers, 1989; McLellan *et al.*, 1995) and have lead other authors to promote the use of self-paced time-trials as a more reliable alternative (Russell *et al.*, 2004; Schabert *et al.*, 1998a; Schabert *et al.*, 1998b). The concern underlying this suggestion is that any given exercise test will only be capable of detecting intervention effects that are larger than the coefficient of variation (CV) for that test (Hopkins *et al.*, 1999). Therefore, while such exercise capacity tests can easily detect the large intervention effect which occurs when carbohydrate is compared with a placebo (Fallowfield *et al.*, 1995), it is conceivable that the relatively small enhancement of exercise capacity which occurs in response to various rates of carbohydrate ingestion may not be detected (Fallowfield and Williams, 1997; Wong and Williams, 2000). The upper panel of Figure 8.3 (A) illustrates that, even in the face of a large degree of error variance, differences in recovery of exercise capacity can be detected between a placebo solution and a range of carbohydrate solutions, but with no distinction between the varying levels of carbohydrate intake. However, it would be inappropriate to conclude that exercise capacity tests *per se* are inherently unreliable since the exercise capacity data gathered during Study 3 was obviously of sufficient sensitivity to detect not only the effect of increasing carbohydrate intake by just $0.3 \text{ g}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$ but also to detect the even smaller influence on exercise capacity of adding a matched quantity of amino acids. The capability of this increase in test-retest reliability to reveal small but worthwhile intervention effects is shown in the lower panel of Figure 8.3 (B). Overall, it seems logical to suggest that the reliability of any exercise test will be directly proportional to the degree of control which is exerted

over extraneous variables, which therefore dictates that the degree of control required for a given study will be inversely proportional to the magnitude of the smallest effect that is deemed to be worthwhile.

The precise reason for the apparently improved reliability of the exercise capacity test in Study 3 in comparison with the previous studies of Fallowfield and Williams (1997) and Wong and Williams (2000) is not immediately obvious but may be related to the characteristics of the participants who were recruited for these respective investigations. Specifically, in both the studies cited above, the mean run time to exhaustion at 70% $\dot{V}O_2$ max following a 4 h recovery was approximately 60 min regardless of the amount of carbohydrate consumed during recovery (Fallowfield and Williams, 1997; Wong and Williams, 2000). In contrast, the participants recruited for Study 3 were all able to run for at least 60 min during R₂, with many running far longer and producing mean run times to exhaustion in excess of 80 min for all 3 trials. It is possible, therefore, that the participants used in these earlier investigations may have terminated their exercise capacity tests before a true metabolic endpoint had been established. This is likely to be true for at least some of the participants recruited by Wong and Williams (2000) as evidenced by the large range of run times reported in this study (27.9-105.6 min).

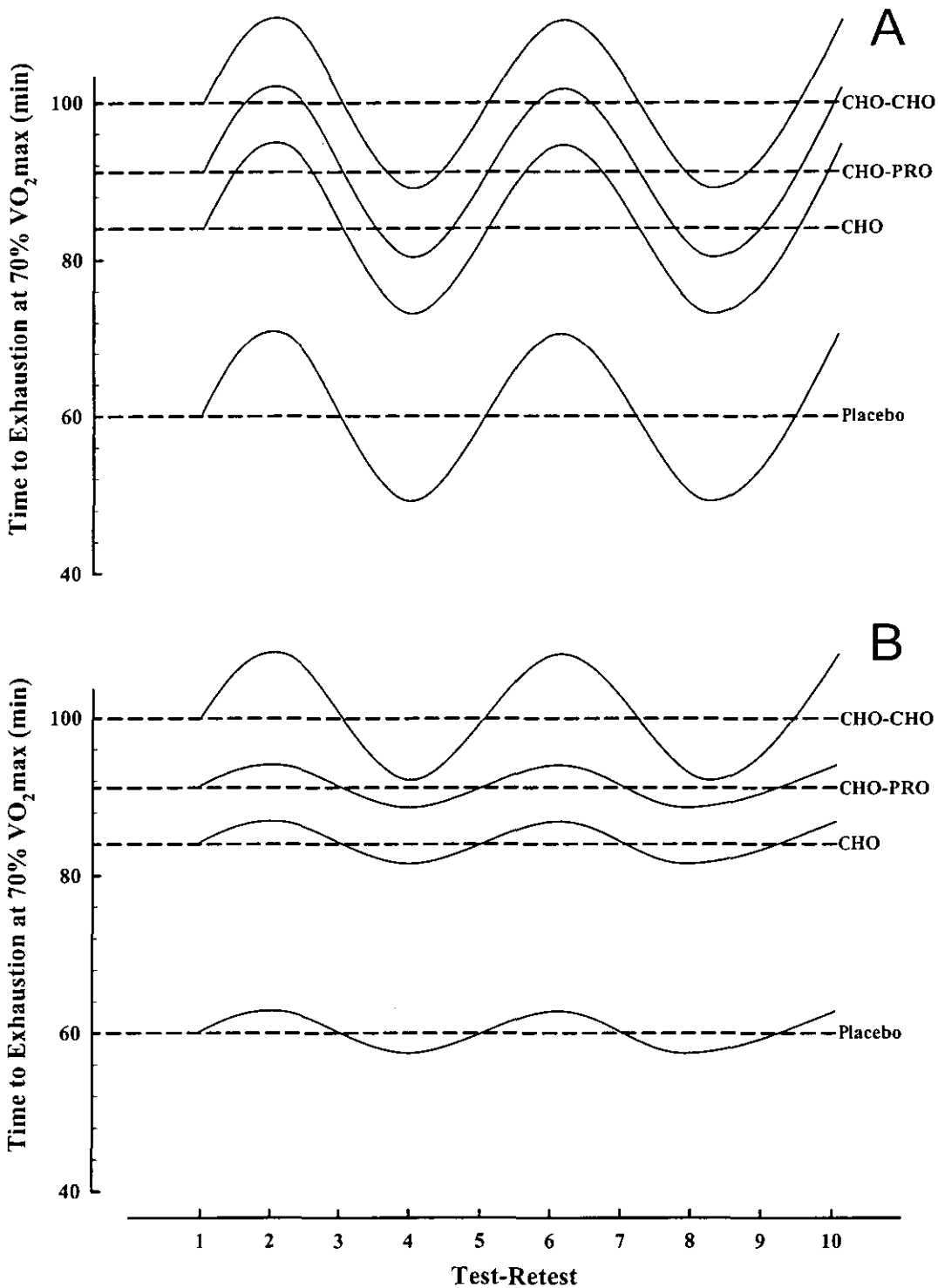


Figure 8.3: Reliability of exercise capacity tests under varying degrees of control. Broken lines denote the ‘true’ exercise time to exhaustion for the target population following ingestion of each supplement and the solid lines denote the test-retest error variance for a given individual. The upper panel (A) is representative of those studies which have identified differences in recovery of exercise capacity between carbohydrate and placebo solutions (Fallowfield *et al.*, 1995) but have not established differences between varying rates of carbohydrate intake (Fallowfield and Williams, 1997; Wong and Williams, 2000). The error variance for this upper figure is based a CV ranging between 17-27% that has been reported for similar tests (Schabert *et al.*, 1998b). The lower panel (B) represents the degree of control employed in Investigation 3, which was capable of detecting differences between the CHO solution and the CHO-PRO and CHO-CHO solutions.

It is also noteworthy that no significant differences in time to fatigue were detectable between the CHO-PRO and CHO-CHO treatments in Study 3, this is despite a relatively large absolute difference between the means (i.e. 91.2 versus 99.9 min, respectively). Given that this effect was only marginally short of attaining statistical significance ($P = 0.07$), it might be suggested that additional participants should have been recruited to reveal a statistical difference. However, it should be borne in mind that 5 of the 8 participants originally recruited for Study 3 experienced severe gastrointestinal distress during the exercise capacity test following ingestion of the CHO-CHO solution, so much so that 2 of these participants were forced to withdraw from the study. Therefore, it is reasonable to suggest that the reliability of the exercise capacity test in the CHO-CHO trial may have been compromised in that sensations of gastrointestinal discomfort might have precluded the establishment of a valid metabolic endpoint (see error variance Figure 8.3B). In this sense, to recruit a few additional participants in order to attain statistical significance would assume that these new recruits would reproduce the effect observed in those participants who reported no gastrointestinal distress, when in fact it is equally as likely that any further data collected could simply increase the degree of random error. Nonetheless, the tendency for longer run times following ingestion of CHO-CHO rather than CHO-PRO indicates that it might be erroneous to conclude that these 2 solutions are equally effective in restoring exercise capacity. However, it would require an unrealistically large sample size to detect this difference given the confounding influence of the concentrated carbohydrate solution on the reliability of the exercise capacity test. Overall, even if the conclusion of Study 3 (i.e. CHO-PRO = CHO-CHO) does represent a type II error, it is not costly in practical terms since the negative side-effects associated with the CHO-CHO solution would favour recommendation of the CHO-PRO solution regardless of any potential ergogenic differences between the 2 treatments.

A final issue relating to the reliability of exercise capacity tests is the possibility that those conducted at 85% $\dot{V}O_2$ max during Studies 1 and 2 may not have been sufficiently reliable to detect differences between the CHO and CHO-PRO solutions. Interestingly, the studies which challenge the reliability of exercise capacity testing by Krebs *et al.* (1989) and McLellan *et al.* (1995) acquired their CV (20.3% and 17.1%, respectively) from exercise tests conducted at 80% $\dot{V}O_2$ max. It is

therefore possible that significant differences between treatments were apparent in Study 3 but not in Studies 1 and 2 entirely as a consequence of differences in the reliability of the capacity tests at various exercise intensities. However, this suggestion cannot be made with any authority because other variables relating to the composition of the CHO-PRO mixture were also refined prior to Study 3. Therefore it remains a possibility that the ergogenic benefit of including protein in a carbohydrate recovery solution may be dependant on the absolute amount of protein that is ingested during recovery.

The insulin concentrations recorded during recovery throughout this thesis certainly appear to suggest that the wheat protein hydrolysate used in Study 2 may have been a slightly less effective insulin secretagogue than the whey protein isolate that was used in the latter 2 studies. Evidence from van Loon *et al.* (2000b) has previously demonstrated that the precise amino acid composition of ingested protein can have a profound impact on the resultant insulinaemic response. Specifically, it was suggested by these authors that the insulinaemic response to any given amino acid mixture will be strongly dependent on the quantities of leucine, phenylalanine and tyrosine which are included (van Loon *et al.*, 2000b). Furthermore, despite the finding that glutamine may facilitate leucine evoked insulin secretion by acting as a fuel for pancreatic islet cells (Borel *et al.*, 1998; Malaisse-Lagae *et al.*, 1982), the work from van Loon *et al.* (2000b) concludes that enough glutamine may be present in humans *in vivo* to serve this function (van Loon *et al.*, 2000b). Taken together, these findings may partially explain why the whey protein isolate was slightly more effective than the wheat protein hydrolysate in stimulating insulin secretion since the glutamine content of the former was lower, thus allowing for a greater content of the more insulintropic essential amino acids. However, the augmented insulin response in Studies 3 and 4 cannot be exclusively attributed to the composition of protein that was ingested because the amount of protein included in the CHO-PRO mixtures was also increased following Study 2. Based on the available evidence, the increase in protein ingestion rate from $\leq 0.2 \text{ g PRO}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$ in Studies 1 and 2 to $0.3 \text{ g PRO}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$ in Studies 3 and 4 would be predicted to increase insulin release. This is because the synergistic influence of combined carbohydrate and protein ingestion on insulinaemic response has only been reported in those studies which have provided $\geq 0.3 \text{ g PRO}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$ (Jentjens *et al.*, 2001; Van Hall *et al.*, 2000a; Van Hall *et al.*, 2000b; van Loon *et*

al., 2000a; van Loon *et al.*, 2000c; Zawadzki *et al.*, 1992), while CHO-PRO solutions containing less than this amount of protein have not produced such large increases in insulin concentration (Carrithers *et al.*, 2000; Ivy *et al.*, 2002; Ivy *et al.*, 2003; Tamopolsky *et al.*, 1997).

Importantly, even though the whey protein isolate was only marginally more effective than the wheat protein hydrolysate in augmenting the insulin response during recovery, the increase above that of the CHO solution was sufficient that the fate of intracellular glucose would be expected to shift from oxidative to non-oxidative disposal (Young *et al.*, 1988). It is therefore surprising that muscle glycogen resynthesis was not different between treatments, although elevations in circulating insulin may have also contributed to non-oxidative disposal of glucose within the liver. First pass extraction of glucose by the liver is thought to be a highly efficient process irrespective of insulin concentration but an increased availability of insulin could possibly have increased liver glycogen storage through facilitating hepatic glucose retention (Wasserman and Cherrington, 1991). In this way, the CHO-PRO solution may have enhanced liver glycogen resynthesis during recovery both through providing amino acids as gluconeogenic substrate and then ensuring that any newly formed glucose was stored rather than mobilised.

The above possibility raises the question as to whether the CHO-CHO solution in Study 3 may also have increased the rate of liver glycogen resynthesis above that of the CHO solution since the addition of further carbohydrate resulted in a similar augmentation of insulin release as did the inclusion of protein. It would therefore be interesting to have muscle glycogen data for the CHO-CHO solution to determine whether this solution improved exercise capacity through the same mechanism as the CHO-PRO solution or via some other mechanism. The majority of scientific literature seems to suggest that the rate of muscle glycogen resynthesis would be greater following ingestion of $1.1 \text{ g CHO}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$ than following ingestion of $0.8 \text{ g CHO}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$ (see Figure 2.5, p. 59), so it might be concluded that the CHO-PRO and CHO-CHO solutions enhanced recovery through mutually exclusive mechanisms. However, it should be noted that the data supporting this contention relates to recovery periods following prolonged cycling, which may not be entirely applicable to the present results. It is particularly interesting that carbohydrate ingestion

following prolonged treadmill running has been reported by Tsintzas *et al.* (2003) to result in rates of muscle glycogen resynthesis that are relatively low in comparison with reports from other authors involving carbohydrate ingestion following prolonged cycling (Maehlum *et al.*, 1978; Tarnopolsky *et al.*, 1997; Tsintzas *et al.*, 2003). It is therefore possible that an initial bout of treadmill running may limit the rate of muscle glycogen resynthesis during recovery below that which is possible following prolonged cycling, potentially as a consequence of damage to contractile tissue as a result of the increased eccentric component of muscular contraction during treadmill running (Costill *et al.*, 1990; Doyle *et al.*, 1993; O'Reilly *et al.*, 1987; Zehnder *et al.*, 2004). In concordance with this line of thought is the observation of substantial increases in plasma myoglobin concentrations following R₁ in every study of this thesis, thus providing clear evidence of disruption to the plasma membrane. If this were the case then it becomes more understandable why the glucose that could not be stored in damaged muscle tissue might find an alternative site of storage within the liver.

In addition to the degree of muscle damage that may have been induced by R₁, it is also likely that the rates of muscle glycogen resynthesis during recovery may have been restricted in the present series of studies by the fact that the exercise session performed prior to recovery was of fixed duration rather than to the point of volitional exhaustion. It has been well established that one of the most potent effectors of muscle glycogen synthesis is a low availability of muscle glycogen itself, which would explain why some of the most rapid rates of muscle glycogen resynthesis have been reported in those studies which have reduced muscle glycogen below 100 mmol glucosyl units·kg dry mass⁻¹ prior to recovery (Blom, 1989; Casey *et al.*, 1995; Van Hall *et al.*, 2000b). Notably, the initial exercise session used in all the studies cited above required participants to exercise to the point of fatigue, which leaves open the possibility that CHO-PRO solutions may be of greatest value following more prolonged periods of exercise when an effective recovery is most dependent on exogenous sources of carbohydrate.

A final point of discussion which arises from this series of investigations is the magnitude of growth hormone response to each respective exercise session (i.e. R₁ versus R₂). In both the studies in which this variable was measured, growth hormone

concentrations were not vastly different between the first and second exercise sessions in each trial (Chapters 5 and 6). It might be expected that the growth hormone response to the second exercise bout should be attenuated due to an accumulation of hypothalamic somatostatin during R_1 (Lanzi and Tannenbaum, 1992b), which would explain the attenuated growth hormone release which has been observed when repeated bouts of aerobic exercise have been performed with only 1 h of recovery (Sartorio *et al.*, 2005). However, this auto-feedback mechanism is only thought to persist for approximately 4 h, thus explaining the relative lack of any attenuated growth hormone release during R_2 in the current investigations (Lanzi and Tannenbaum, 1992a). Converse to the above hypothesis, other authors have offered support for the contention that repeated bouts of aerobic exercise may in fact progressively desensitise the growth hormone response to this auto-negative feedback loop, thus augmenting the degree of growth hormone secretion with each ensuing exercise session (Kanaley *et al.*, 1997; Ronsen *et al.*, 2001). The precise reasons for these apparently inconsistent findings remain to be fully elucidated but may be at least partially related to individual differences in the participants recruited for each investigation (Mejri *et al.*, 2005; Wideman *et al.*, 2000b). Alternatively, the relative attenuation or augmentation of the growth hormone response to repeated exercise may be mediated by exercise intensity (Pritzlaff *et al.*, 1999), a suggestion which would certainly account for the marked attenuation of growth hormone response during repeated bouts of maximal intensity exercise (Stokes *et al.*, 2005).

To summarise the main results of this thesis in their entirety, it is firstly important to note that differences between recovery solutions were only recorded in terms of exercise capacity when the test used to assess this variable was conducted at running speeds equivalent to 70% $\dot{V}O_2$ max. It is therefore suggested that exercise capacity tests at this intensity may be a more appropriate surrogate measure of carbohydrate status than exercise tests set at higher relative exercise intensities, such as were employed during Studies 1 and 2. However, future research will be necessary to fully confirm this suggestion since the specific composition of the CHO-PRO mixtures used in the current series of studies did vary slightly between the first and last 2 experiments. It is therefore possible that the ergogenic benefit which was revealed in Study 3 might be the combined product both of a more effective recovery solution and a more appropriate exercise test. Nonetheless, even if the mixture of

amino acids used in Studies 3 and 4 was superior to that used in the earlier studies, the wheat protein hydrolysate and the whey protein isolate were both capable of increasing the insulinaemic response during recovery above that elicited by a solution containing carbohydrate alone. Therefore, the results gathered in this thesis consistently support earlier evidence demonstrating a synergistic influence of combined carbohydrate and protein ingestion on insulin secretion (Rabinowitz *et al.*, 1966).

From a practical perspective, the most prominent finding which arises from this sequence of experiments is that recovery of prolonged running capacity can be enhanced during a short-term recovery either through adding $0.3 \text{ g}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$ of mixed amino acids to a moderately concentrated carbohydrate solution or through increasing the carbohydrate content of that solution by an amount equal to that of the additional protein. While other authors have previously demonstrated a similar ergogenic benefit of mixed carbohydrate and protein ingestion when assessed in comparison with a lower carbohydrate solution (Williams *et al.*, 2003), the present data represents the first evidence of this effect when the CHO and CHO-PRO solutions are matched for carbohydrate content. In addition, the further comparison between the CHO and CHO-CHO solutions in Study 3 also confirms the previously uncorroborated hypothesis that a dose-response relationship might exist between carbohydrate ingestion rate and the restoration of exercise capacity following prolonged exercise. However, with regard to the comparison between the CHO and CHO-PRO solutions, a subsequent more invasive examination revealed that the rate of muscle glycogen resynthesis was not accelerated when amino acids were included in the carbohydrate solution that was ingested during recovery. It can therefore be concluded that the addition of protein to a carbohydrate solution can improve recovery of exercise capacity but that this effect is unrelated to changes in muscle glycogen availability.

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APPENDICES

Appendix A Health Questionnaires

- (i) Pre-study
- (ii) Pre-trial

Appendix B Subjective Rating Scales

- (i) Rating of perceived exertion (RPE)
- (ii) Gut fullness scale
- (iii) Thirst scale

Appendix C Dietary Record Booklet

Appendix D Expired air analysis

- (i) Calculation of $\dot{V}O_2$ and $\dot{V}CO_2$ via the Haldane transformation
- (ii) Calculation of lipid and carbohydrate oxidation via indirect calorimetry
- (iii) Correction of respiratory exchange ratio for estimated protein oxidation

Appendix E Muscle Biopsy Analysis

Appendix F Calculation of IAUC

Appendix G Calculation of Confidence Intervals

HEALTH SCREEN FOR STUDY VOLUNTEERS

Name or Number

It is important that volunteers participating in research studies are currently in good health and have had no significant medical problems in the past. This is to ensure (i) their own continuing well-being and (ii) to avoid the possibility of individual health issues confounding study outcomes.

Please complete this brief questionnaire to confirm fitness to participate:

1. **At present**, do you have any health problem for which you are:

(a) on medication, prescribed or otherwise.....	Yes <input type="checkbox"/>	No <input type="checkbox"/>
(b) attending your general practitioner.....	Yes <input type="checkbox"/>	No <input type="checkbox"/>
(c) on a hospital waiting list.....	Yes <input type="checkbox"/>	No <input type="checkbox"/>

2. **In the past two years**, have you had any illness which require you to:

(a) consult your GP	Yes <input type="checkbox"/>	No <input type="checkbox"/>
(b) attend a hospital outpatient department.....	Yes <input type="checkbox"/>	No <input type="checkbox"/>
(c) be admitted to hospital	Yes <input type="checkbox"/>	No <input type="checkbox"/>

3. **Have you ever** had any of the following:

(a) Convulsions/epilepsy.....	Yes <input type="checkbox"/>	No <input type="checkbox"/>
(b) Asthma.....	Yes <input type="checkbox"/>	No <input type="checkbox"/>
(c) Eczema	Yes <input type="checkbox"/>	No <input type="checkbox"/>
(d) Diabetes	Yes <input type="checkbox"/>	No <input type="checkbox"/>
(e) A blood disorder	Yes <input type="checkbox"/>	No <input type="checkbox"/>
(f) Head injury	Yes <input type="checkbox"/>	No <input type="checkbox"/>
(g) Digestive problems.....	Yes <input type="checkbox"/>	No <input type="checkbox"/>
(h) Heart problems	Yes <input type="checkbox"/>	No <input type="checkbox"/>
(i) Problems with bones or joints	Yes <input type="checkbox"/>	No <input type="checkbox"/>
(j) Disturbance of balance/coordination.....	Yes <input type="checkbox"/>	No <input type="checkbox"/>
(k) Numbness in hands or feet	Yes <input type="checkbox"/>	No <input type="checkbox"/>
(l) Disturbance of vision.....	Yes <input type="checkbox"/>	No <input type="checkbox"/>
(m) Ear / hearing problems.....	Yes <input type="checkbox"/>	No <input type="checkbox"/>
(n) Thyroid problems	Yes <input type="checkbox"/>	No <input type="checkbox"/>
(o) Kidney or liver problems.....	Yes <input type="checkbox"/>	No <input type="checkbox"/>
(p) Allergy to nuts	Yes <input type="checkbox"/>	No <input type="checkbox"/>

4. **Has any**, otherwise healthy, member of your family under the age of 35 died suddenly during or soon after exercise? Yes No

If YES to any question, please describe briefly if you wish (eg to confirm problem was/is short-lived, insignificant or well controlled.)

.....

.....

Thank you for your co-operation!

Health Questionnaire

Please complete the following brief questions to confirm your fitness to participate:

At present do you have any health problems for which you are:

1) On medication, prescribed or otherwise YES NO .

2) Attending your general practitioner YES NO .

Have you any symptoms of ill health, such as those associated with a cold or other
common infection? YES NO .

If you have answered yes to any of the above questions please give more details
below:

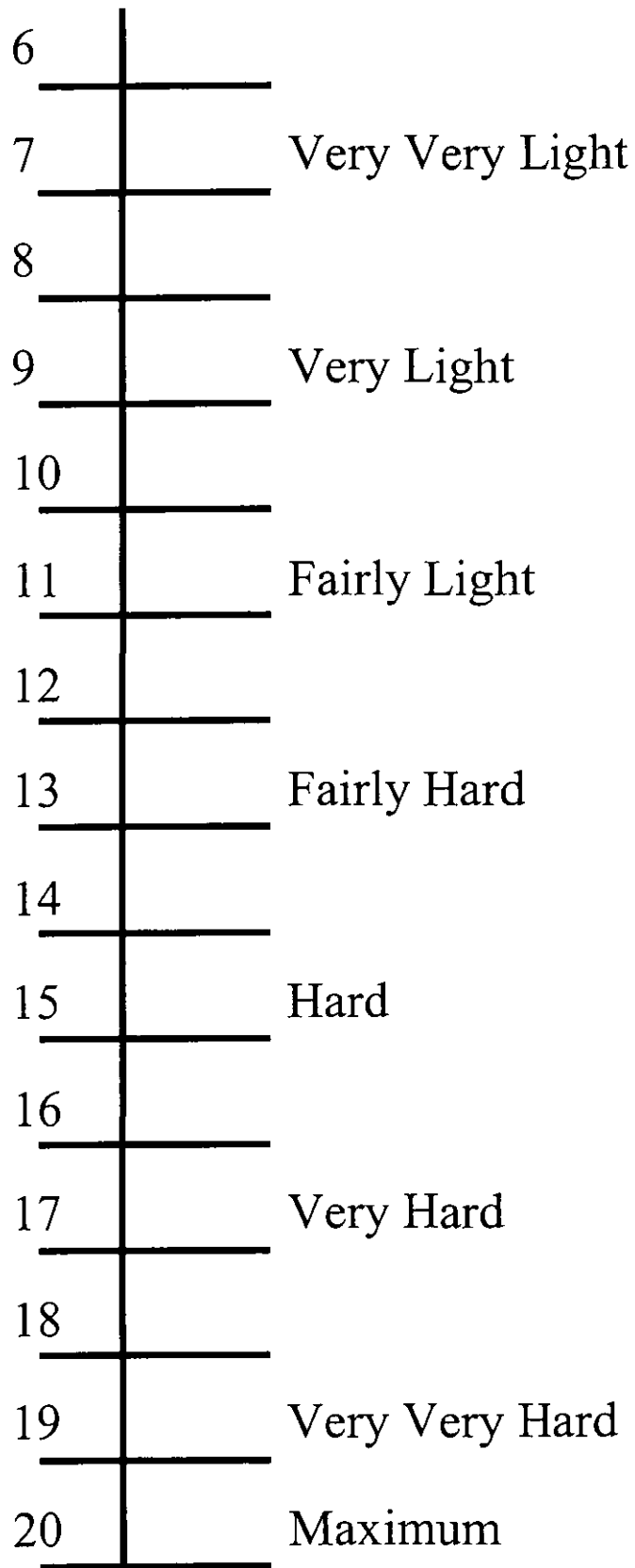
Do you want to take part in today's experiments? YES NO .

Signature: _____

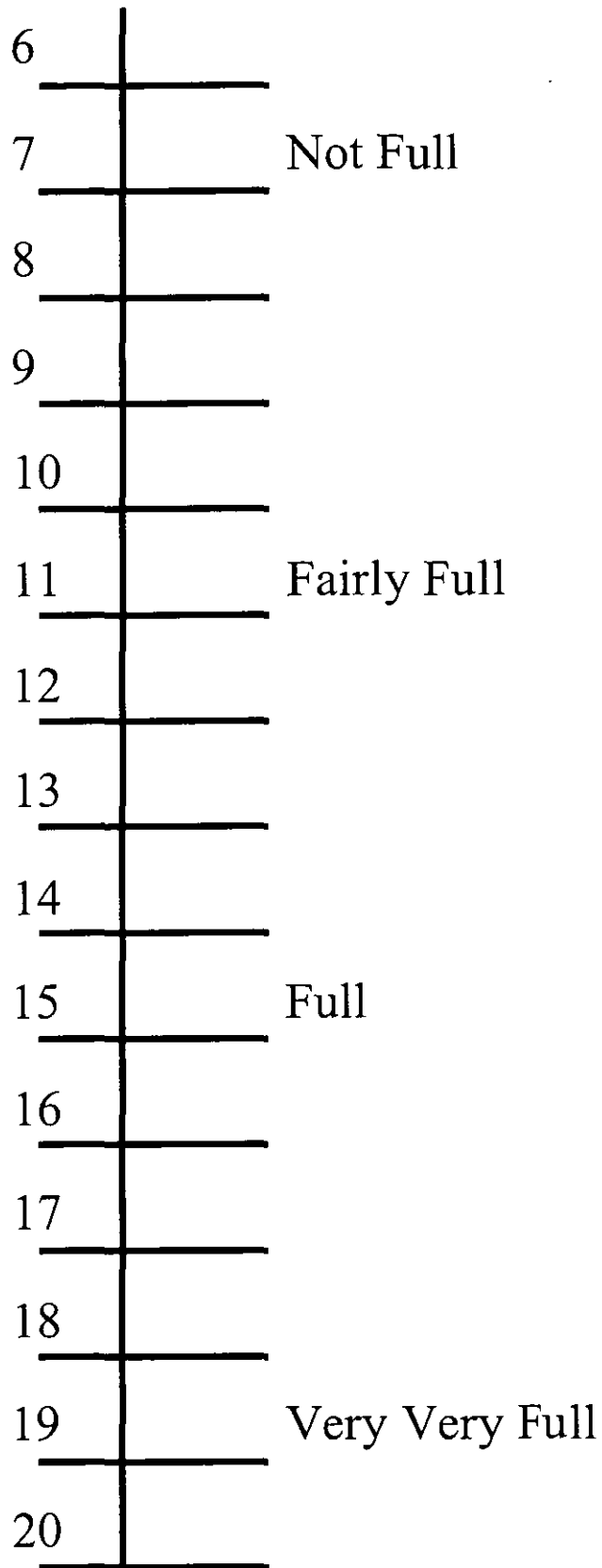
Date:



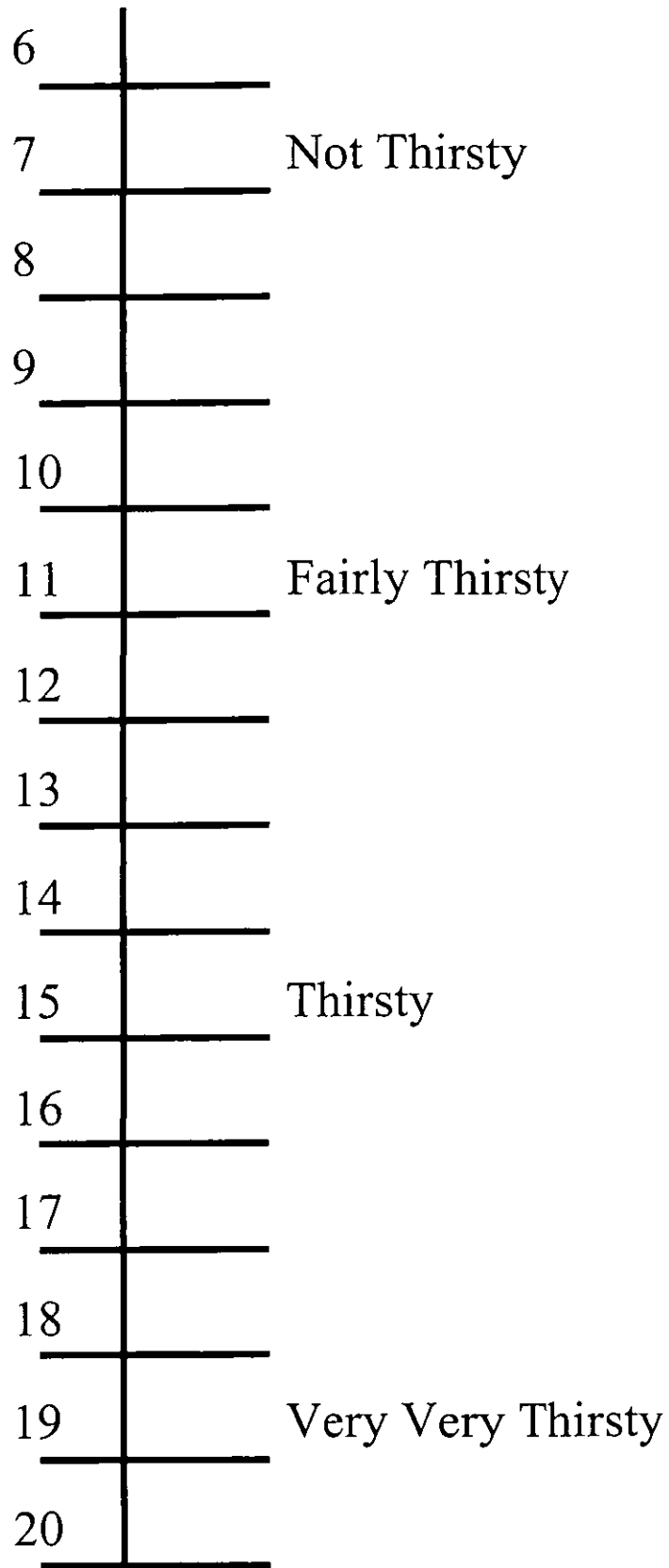
Rating of Perceived Exertion



Gut Fullness Scale



Thirst Scale



CONFIDENTIAL

NAME AND ADDRESS

.....
.....
.....
.....
.....

AGE.....YRS

DATE OF START OF DIET/...../.....

FOOD RECORD DIARY

Please record everything you eat and drink during the 2 days prior to trial 1. Instructions are given inside.

Information about your diet will be treated in confidence and results will be returned to you as soon as possible.

The most important contribution you can make as a participant in this study is to accurately record your dietary intake and replicate it precisely between trials.

INSTRUCTION FOR USING THE FOOD DIARY

Everything that you eat and drink over the 2 days prior to main trials should be weighed and the weight and type of food or drink recorded.

For solid foods, the food should be placed on the scale on a plate or container. The plate or container must be weighted empty first and the scales can then be zeroed. Each item of food can then be added to the plate and weighted individually, returning the scales to zero between each item.

eg. Plate 150g zero scale.
Roast Beef 100g zero scale.
Potato 150g zero scale.
Gravy 30g zero scale.

For drinks, a cup or glass must first be weighed and then the scale can be returned to zero and the drink added. Please remember to record separately the weight of tea, milk and sugar put into a drink.

Do not forget to weight and record second helpings and between meal snacks.

Any leftovers (eg. apple cores) should also be weighted and recorded in the leftovers column.

Eating Out – Most people eat foods away from home each day, please do not forget to record these. Take your diary and scales with you where ever it is possible. If this is too inconvenient just record the type of food eaten with an estimated weight – but please say when a weight has been estimated.

Most snack foods will have the weight of the food on the packet so they do not need weighing if you eat the whole packet yourself.

Names and descriptions of foods should be as detailed as possible, including the brand name and any other information available.

Start a new page in your diary for each day, and record each item on a separate line. Record the time of day in the first column of each line.

eg. 10.30 am Mcvities Biscuits (2) 50g

The space provided at the foot of each page for general comments is for you to give any further information about your diet and your training/activity for that day.

eg. Steady run, morning 1 hour.
Missed lunch due to stomach pains.

Please try to be as accurate as possible and try to eat your usual diet. Try and think ahead so that you do not eat any foods prior to your first trial which you will not have access to prior to subsequent trials.

Expired Air Analysis

Calculation of $\dot{V}O_2$ and $\dot{V}CO_2$ via the Haldane transformation

The Douglas bag technique was used to measure the relative fractions of expired O_2 and CO_2 (i.e. $F_{EO_2}\%$ and $F_{ECO_2}\%$) and the total volume expired per minute was corrected to the standard temperature and pressure for a dry gas (\dot{V}_E). The total volume inspired per minute (\dot{V}_I) can then be calculated as shown below:

$$\text{Concentration} = \text{Mass} / \text{Volume}$$

therefore;

$$F_{IN_2}\% = \text{Mass } N_2 \text{ Inspired} / \dot{V}_I$$

and;

$$F_{EN_2}\% = \text{Mass } N_2 \text{ Expired} / \dot{V}_E$$

Wilmore and Costill (1973) support that nitrogen can be assumed to be metabolically inert, which allows above equations can be combined and rearranged to give:

$$\dot{V}_I = (F_{EN_2}\% / F_{IN_2}\%) \times \dot{V}_E$$

Given that the atmospheric concentrations of O_2 and CO_2 are known (20.93% and 0.03%, respectively) and that the expired fractions of O_2 and CO_2 have been measured, $F_{IN_2}\%$ and $F_{EN_2}\%$ can therefore be determined as follows:

$$F_{IN_2}\% = 100 - (20.93 + 0.03)$$

and;

$$F_{EN_2}\% = 100 - (F_{EO_2}\% + F_{ECO_2}\%)$$

Once \dot{V}_I has been determined using $F_{IN_2}\%$ and $F_{EN_2}\%$, $\dot{V}O_2$ and $\dot{V}CO_2$ can be calculated using the following equations:

Oxygen Uptake ($\dot{V}O_2$)

$$F_{IO_2}\% = (20.93 / 100) \times \dot{V}_I$$

$$F_{EO_2}\% = (F_{EO_2}\% / 100) \times \dot{V}_E$$

$$\dot{V}O_2 = F_{IO_2}\% - F_{EO_2}\%$$

Carbon Dioxide Production ($\dot{V}CO_2$)

$$F_{ICO_2}\% = (0.003 / 100) \times \dot{V}_I$$

$$F_{ECO_2}\% = (F_{ECO_2}\% / 100) \times \dot{V}_E$$

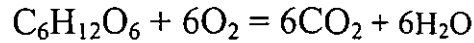
$$\dot{V}CO_2 = F_{ECO_2}\% - F_{ICO_2}\%$$

These values can then be used to determine the respiratory exchange ratio (RER) using the equation:

$$RER = \dot{V}CO_2 / \dot{V}O_2$$

Calculation of lipid and carbohydrate oxidation via indirect calorimetry

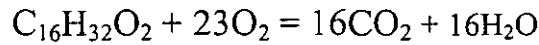
Oxidation of 1 gram of glycogen requires 0.828 litres of O₂ and produces 0.828 litres of CO₂.



RER = 1

(N.B: only 0.746 litres of O₂ and CO₂ are used and produced for the oxidation of glucose but all equations in this thesis assume the above value for glycogen)

Oxidation of 1 gram of lipid (e.g. palmitic acid) requires 1.989 litres of O₂ and produces 1.419 litres of CO₂.



RER = 0.7

Therefore the below simultaneous equation can be applied to determine the oxidation rate of lipid (Lox) and carbohydrate (Cox) in grams per minute:

$$\dot{V}\text{O}_2 = (0.828 \times \text{Cox}) + (1.989 \times \text{Lox})$$

$$\dot{V}\text{CO}_2 = (0.828 \times \text{Cox}) + (1.419 \times \text{Lox})$$

Once rearranged these formulae can be expressed as:

$$\text{Lox} = (\dot{V}\text{O}_2 - \dot{V}\text{CO}_2) / (1.989 - 1.419)$$

$$\text{Cox} = (\dot{V}\text{O}_2 - (1.989 \times \text{Lox})) / 0.828$$

(N.B: If the RER for any given calculation is found to be greater than 1 then Lox can be assumed to be zero and Cox will be calculated simply as $\dot{V}\text{O}_2 / 0.828$).

Correction of RER for estimated protein oxidation

The RER values recorded during recovery in Chapter 7 were corrected using urinary urea excretion to estimate the rate of protein oxidation according to the methods described by Jequier *et al.* (1987). Immediately upon completion of R_1 in each trial, participants were required to empty their bladders before all subsequent urine that was passed during the 4 h recovery was collected in a vessel containing 5 ml of 10% thymol-isopropanol as a preservative. The total volume of urine passed during recovery was recorded and a mixed 5 ml sample was analysed for urea content ($\text{mmol}\cdot\text{l}^{-1}$). The following conversions were then applied:

1. Divide urea concentration $\text{mmol}\cdot\text{l}^{-1}$ by 35700 to yield $\text{g}\cdot\text{ml}^{-1}$
(Urea nitrogen conversion factor $\text{mmol}\cdot\text{l}^{-1}$ to $\text{mg}\cdot\text{dl}^{-1} = 0.357$)
2. Multiply by 0.47 to yield nitrogen $\text{g}\cdot\text{ml}^{-1}$
(Nitrogen only comprises 47% of urea- only an approximation since urea only represents $\approx 90\%$ of urine nitrogen)
3. Multiply by total urine volume to yield total nitrogen loss during recovery (g)
4. Divide by recovery time in minutes to yield nitrogen loss $\text{g}\cdot\text{min}^{-1}$
5. Multiply by 6.25 to yield protein oxidation rate
(Nitrogen only comprises $\approx 16\%$ of protein)

This estimate of protein oxidation rate can now be corrected for changes in the bodily urea pool during recovery by incorporating the data gathered through plasma urea analysis.

6. Subtract post- from pre-recovery plasma urea concentration
(Will give a negative number if urea concentrations increased during recovery)
7. Multiply the difference by 60 to convert $\text{mmol}\cdot\text{l}^{-1}$ into $\text{g}\cdot\text{l}^{-1}$
(mw urea = 60)
8. Multiply by 0.47 to yield nitrogen $\text{g}\cdot\text{l}^{-1}$
(Nitrogen only comprises 47% of urea)
9. Multiply by 57% of body mass to yield whole body nitrogen in g
(Total body water $\approx 57\%$ of body mass)
10. Divide by $1000 \times$ recovery time in minutes to yield plasma change in nitrogen $\text{g}\cdot\text{min}^{-1}$

The rate of protein oxidation established in step 5 can now be corrected for changes in the urea pool through the addition of the result of step 10.

Oxidation of 1 gram of protein requires 0.966 litres of O₂ and produces 0.782 litres of CO₂, resulting in an RER of 0.8 for protein oxidation. Given that only ≈16% of protein is nitrogen, this equates to **6.04** and **4.89** litres of O₂ and CO₂ per gram of nitrogen (**N**), respectively. This information can therefore be added to the equations presented earlier to adjust the calculated rates of lipid and carbohydrate oxidation such that the $\dot{V}O_2$ and $\dot{V}CO_2$ for protein (i.e. $P\dot{V}O_2$ and $P\dot{V}CO_2$) are disregarded:

$$\dot{V}O_2 = (0.828 \times Cox) + (1.989 \times Lox) + (6.04 \times N)$$

$$\dot{V}CO_2 = (0.828 \times Cox) + (1.419 \times Lox) + (4.89 \times N)$$

Again, once solved these equations yeild:

$$Lox = (1.754 \times \dot{V}O_2) - (1.754 \times \dot{V}CO_2) - (2.017 \times N)$$

$$Cox = (4.212 \times \dot{V}CO_2) - (3.005 \times \dot{V}O_2) - (2.449 \times N)$$

Basically, $P\dot{V}O_2$ and $P\dot{V}CO_2$ are subtracted from $\dot{V}O_2$ and $\dot{V}CO_2$ to yield non-protein $\dot{V}O_2$ and $\dot{V}CO_2$ which can then be used to establish corrected substrate oxidation rates and non-protein respiratory exchange ratios (NPRER) in the manner described in Appendices Di and Dii.

N.B: If amino acids are used as gluconeogenic substrate during the urine collection period then the rate of protein oxidation will be overestimated. However, the NPRER that is calculated using this overestimate remains accurate providing that the newly synthesised glucose is oxidised (RER = 0.8) rather than stored (RER = 0.4).

Muscle Biopsy Analysis Procedures

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Required Chemicals:

Dithiothreitol	(DTT)
Nicotinamide adenine dinucleotide phosphate	(NADP)
Adenosine diphosphate	(ADP)
D-glucose	(Glu)
Glucose-6-phosphate	(G-6-P)
Adenosine triphosphate	(ATP)
Phosphocreatine	(PCr)
Phosphoenolpyruvate	(PEP)
Nicotinamide adenine dinucleotide (Reduced)	(NADH)
Creatine	(Cr)
Nicotinamide adenine dinucleotide	(NAD)
Lactic Acid	(LA)

Required Enzymes:

Glucose-6-phosphate dehydrogenase	(G-6-PDH)
Hexokinase	(HK)
Creatinephosphokinase	(CPK)
Lactate dehydrogenase	(LDH)
Pyruvate kinase	(PK)

Assay Reagents & Buffers

Metabolite Extraction Reagents

- 0.5 M perchloric acid (HClO_4) + 1 Mm ethylenediaminetetraacetic acid (EDTA)
10.062 ml of 70% HClO_4
+ 74.44 mg EDTA in 200 ml dw
- 2.2 M potassium hydrogen carbonate (KHCO_3)
22.026 g of KHCO_3 in 100ml dw

Muscle Hydrolysis Reagents

- 1 M hydrochloric acid (HCl)
9.823 ml of 10.18 HCl in 100 ml dw
- 6 M sodium hydroxide (NaOH)
24 g NaOH in 100 ml dw

Assay Buffers

- **G1** (glycogen) pH = 8.2 (KOH)
7 g triethanolamine (TEA)
+0.8 g potassium hydroxide (KOH)
+2.4 g magnesium acetate ($\text{Mg}(\text{AC})_2 \cdot 4\text{H}_2\text{O}$)
+0.14 g $\text{EDTA} \cdot \text{Na}_2 \cdot 2\text{H}_2\text{O}$ in 100 ml dw
- **D1** (G-6-P, ATP, PCr) pH = 7.5-7.6 (KOH)
18.6 g TEA
+2.2 g $\text{Mg}(\text{AC})_2 \cdot 4\text{H}_2\text{O}$
+0.4 g disodium ethylenediaminetetraacetic acid ($\text{EDTA} \cdot \text{Na}_2 \cdot 2\text{H}_2\text{O}$) in 100 ml dw
- **D2** (lactate) pH = 9.5
6.5 ml of hydrazine hydrate ($\text{N}_2\text{H}_4 \cdot \text{H}_2\text{O}$)
+7.5 g of glycine (Gly)
+0.2 g of $\text{EDTA} \cdot \text{Na}_2 \cdot 2\text{H}_2\text{O}$ in 100 ml dw
- **D3** (creatine)
15 g potassium chloride (KCl) in 100 ml dw
- **D4** (creatine)- pH = 9.0-9.1 (KOH or NaOH)
2.4 g glycine
+0.4 g $\text{Mg}(\text{AC})_2 \cdot 4\text{H}_2\text{O}$ in 100 ml dw
- **D5** (G-6-P, ATP, PCr, creatine)
0.5 g KHCO_3
+50 mg bovine serum albumin (BSA) in 100ml dw

Fragmentation & Freeze-Drying of Muscle Sample

Required equipment:

- Kidney dish
- Scalpels
- Tweezers
- Liquid nitrogen
- Freeze-drier
- Petroleum ether (1 ml.sample⁻¹)
- Pipette (≈5 µl)

The standard muscle metabolite assay (i.e. glycogen, creatine, lactate, ATP, PCr & G-6-P) can be conducted on ≈2 mg of powdered muscle and approximately 8 mg of frozen muscle will yield this amount. Therefore, 16-20 mg (extra 2 mg for additional assays) should be removed from the initial muscle biopsy using scalpels and tweezers to fragment the sample. This procedure should be performed in a kidney dish with the sample submerged in a small volume of liquid nitrogen. Both fragments can then be stored in separate eppendorfs in liquid nitrogen.

The powder fragments are then freeze-dried over 24 h at -50°C and between -10¹ and -10² mmHg. Ensure that all eppendorfs have 4-5 holes in the top to ensure that all moisture can escape and include a small volume of liquid nitrogen to keep samples cool while the freeze-drier reaches the desired temperature & pressure.

Remove samples from freeze-drier and check each individual sample to ensure that they have freeze-dried effectively (they should be white & brittle rather than red and sticky). Remove pierced lids and add approximately 1 ml of petroleum ether; this will remove any fat to facilitate powdering but also reduces the quantity of blood metabolites in each sample since blood cells will stay in suspension longer than muscle cells (corrections for total creatine account for any further blood metabolites which remain in the sample). Attach new lids with no holes and vortex thoroughly. Remove as much ether as possible using a pipette and then leave the eppendorfs open in the fume cupboard for ≈30 min to allow the last few µl to evaporate. These samples must now be stored at -80°C in a bag containing silica gel.

Powdering of Muscle Samples

Required equipment:

- Pestle and mortar
- Scalpel
- Tweezers
- Scales
- 70% methylated spirit

Prepare the bench with the pestle and mortar on a cloth to ensure a clear work area (ideally, spotlights and a magnifier will be used). All powdering tools should be cleaned in 70% methylated spirit and dry in the air. Remove cleaned, freeze-dried samples from -80°C and allow to reach room temperature (approximately 20 min) before emptying each one into the mortar. Initially the scalpel should be used to remove any obvious connective tissue (there will be very little in human samples) and to scrape off any dried on blood (will have retained its red colour- again, non-essential since later creatinine corrections will account for any blood metabolites). Slice the sample into pieces using the scalpel and then use 2 sets of tweezers to grind up these slices into a rough powder. Collect this rough powder into the centre of the mortar and grid up with the pestle (any remaining connective tissue will be revealed at this stage as it will not reduce to powder). Repeat the process using scalpel, tweezers then pestle until a very fine powder results (usually takes 5-10 min).

Label 2 (P+A) eppendorfs and then de-static both, along with any spatulas to be used in transferring powder. Calibrate scales and then zero using the tube labelled P. Transfer >2 mg of powder into the eppendorf into the tube and record the weight (see muscle extraction sheet). Re-zero scales using tube labelled A and record the weight of the remaining powder. The first tube (P) will be used for metabolites and requires in excess of 2 mg while the second tube (A) requires slightly more powder for additional analyses (e.g. phosphorylase). Most biopsies will provide sufficient powder for both tubes but if <2 mg is available for any additional analyses then use all of it for metabolites.

Following powdering, samples are again stored at -80°C in a bag containing silica gel. Powdering tools should now be cleaned in 70% methylated spirit and left to dry in the air before powdering the next sample. It will take approximately 15-20 min to complete each sample.

Metabolite Extraction from Powdered Muscle Samples

Required equipment:

- Ice tray
- Eppendorf centrifuge
- 0.5 M perchloric acid (HClO₄) + 1 Mm ethylenediaminetetraacetic acid (EDTA)
- 2.2 M potassium hydrogen carbonate (KHCO₃)
- Pipettes ($\approx 5 \mu\text{l}$ & $\approx 200 \mu\text{l}$)
- Litmus paper

Label 2 new eppendorfs (X & E) for each sample to be extracted. Only attempt to extract ≈ 6 samples at a time to allow frequent vortexing later. These 6 samples should be removed from -80°C and allowed to reach room temperature (≈ 20 min) in a bag containing silica gel. An ice tray can be prepared and samples will be stored on ice for the remainder of this procedure. Centrifuges should also be pre-cooled while samples defrost.

Spin all tubes at 14,000 rpm for 1 min and tap down gently to dislodge pellet without dispersing loose powder. Using the powder masses recorded on muscle extraction sheet, add $100 \mu\text{l}$ of HClO₄/EDTA mixture per mg of powdered muscle (the volume added can also be recorded on this sheet). Start a timer immediately and begin gently vortexing all samples for 5 seconds each, keeping them on ice in between mixes. It is important to ensure that pellets remain submerged in HClO₄/EDTA throughout this stage. After 10 min of regular vortexing, centrifuge the tubes at 14,000 rpm for 3 min at $0-4^{\circ}\text{C}$ (if centrifuge is not yet cooled then the vortexing stage can be extended).

Transfer samples back to ice and use a pipette to remove as much extract as possible (usually $\approx 15 \mu\text{l}$ less than volume added). This extract should be stored in the eppendorf labelled X and the volume recorded on muscle extraction sheet.

-note: it is best to leave a small quantity of extract around the pellet rather than risk touching the pellet since the extract only holds $\approx 15\%$ of glycogen (i.e. macroglycogen) while the pellet contains $\approx 85\%$ (i.e. proglycogen). Therefore it is best if any contamination occurs due to extract being left on the pellet. Often the last 2-5 μl to be removed should be discarded.

Both fractions are then stored on ice.

As quickly as possible, KHCO₃ must be added to stop the reaction initiated by HClO₄ (thus preventing any further reductions in metabolites). Using the volumes

recorded in the previous step, 25% of the volume already in the tube should be added (i.e. 285 μl of extract will require 71.25 μl of KHCO_3). Add these figures together and include on muscle extraction sheet for later reference. The addition of KHCO_3 will liberate CO_2 and a white salt will form. Each tube should be vortexed quickly upon the introduction of KHCO_3 and then be left on ice with lids loosely fitted for approximately 5 min (litmus paper can be used to check that the reaction is complete).

Once the reaction is over, screw on lids and tubes can be spun for 3 min at 14,000 rpm. The clear supernatant can now be transferred to the final eppendorf (E) using the previous recording on muscle extraction sheet as a guide for how much will be available (usually ≈ 30 μl less than total volume). The final volume in tube E can now be recorded on muscle extraction sheet simply as a guideline for how many assays will be possible (>150 μl will typically be required for the standard array of metabolites). Tube X containing the salt pellet can be discarded and the products should be our original tube (P) containing the pellet for proglycogen analysis and the final tube (E) containing our extract for all other metabolites.

Acid Hydrolysis of Muscle Samples for Glycogen Analysis

Required equipment:

- Ice tray
- Dry bath (up to 100°C)
- 1 M hydrochloric acid (HCl)
- 6 M sodium hydroxide (NaOH)
- Pipette (≈200 µl)
- Eppendorf centrifuge

Pre-heat dry bath to 100°C and defrost all pellets and extracted samples quickly in hot water (i.e. tubes P & E) before storing on ice. Label a new tube (H) for each extract (E) and transfer 20 µl of each extract into its corresponding tube (H). 100 µl of HCl can now be added to every 20 µl of extract (H) and 100 µl of HCl per mg of muscle powder can be added to each pellet (P); refer to muscle extraction sheet to determine the mass of each pellet (i.e. 2.71 mg of muscle powder will require 271 µl of HCl).

Ensure that the lids of all eppendorfs are tightly screwed before mixing both samples (P & H) gently (avoid leaving any sample in the lid) and then incubate in the dry bath for 2 h at 100°C. Following incubation, all tubes should be centrifuged for 1 min at 14,000 rpm before being left at room temperature for 5-10 min to cool. The acid hydrolysed extract (H) is now neutralised with 15 µl of NaOH (do not add any NaOH to the pellet).

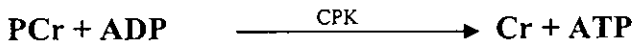
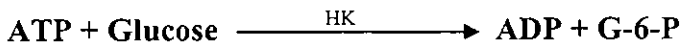
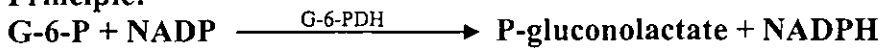
In addition to the hydrolysis procedures, 20 µl of the hydrolysed pellet (P) can be transferred to a new eppendorf labelled (D) and 180 µl of dw added to give a 10:1 dilution. Similarly, 40 µl of the master extract (E) can be transferred into another newly labelled eppendorf (S) and 200 µl of dw added to give a 6:1 dilution.

The end result is 5 labelled eppendorfs for each muscle biopsy originally taken:

E=	Undiluted (Master) Extract	- <u>Lactate</u>
P=	Hydrolysed Pellet	-n/a
H=	Hydrolysed Extract	- <u>Macroglycogen</u>
D=	10 x diluted hydrolysed pellet	- <u>Proglycogen</u>
S=	6 x diluted hydrolysed extract	- <u>Creatine, PCr, ATP & G-6-P</u>

Measurement of G-6-P, ATP & PCr

Principle:



Method:

- Weigh and dilute all components of reagent mixture, enzymes and standards according to G-6-P/ATP/PCr Assay Sheet. All should be thoroughly vortexed and stored on ice.
- Once reagent mixture, enzymes and standards are prepared, samples (S) can be defrosted quickly in hot water, vortexed and spun for 3 min at 14,000 rpm.
- Before pipetting, the spectrophotometer (plate reader) should be switched on to allow the internal temperature to reach $>20^{\circ}\text{C}$. All samples should be read at 340 nm.
- Pipette 20 μl of Sigma RNase free H_2O for blanks into wells A1-A5.
- Pipette 20 μl of 6:1 diluted samples into wells B1 onwards.
- Pipette 200 μl of reagent mixture into all wells.
- Add 7 μl of each standard into wells A6-A8 (total 21 μl in each well).
- Mix and read A1.
- Add 3 μl of G-6-PDH, mix well and incubate in the plate reader ($>20^{\circ}\text{C}$).
- Mix again and read A2 at 5 minute intervals beginning at 10 minutes to establish plateau (reaction should be complete by 20 min).
- Add 5 μl of HK, mix well and incubate in the plate reader ($>20^{\circ}\text{C}$).
- Mix again and read A3 at 5 minute intervals beginning at 15 minutes to establish plateau (reaction should be complete by 20 min).
- Add 3 μl of CPK, mix well and incubate in the plate reader ($>20^{\circ}\text{C}$).
- Mix again and read A4 at 5 minute intervals beginning at 30 minutes to establish plateau (reaction should be complete by 40 min).

G-6-P/ATP/PCr Assay Sheet

Cat. no#	<u>Reaction mixture</u>	Volume per well (μl)	x total wells +10% (Tot.) <small>added</small>	Required concentration (RC)	Volume requires (>mg)	Weighed amount (WA)	Volume to add (μl)
-	D1 buffer	22.2	[]	-	-	-	-
(Boeh. 19777)	DTT	4.4	[]	7.8 mg.ml ⁻¹	>		
(Boeh. 128058)	NADP	8.9	[]	20.9 mg.ml ⁻¹	>		
(Boeh. 236675)	ADP	0.9	[]	5.1 mg.ml ⁻¹	>		
(Fisons 05003)	Glu	8.9	[]	22.5 mg.ml ⁻¹	>		
(Sigma)	RNAse free H ₂ O	154.7	[]	-	-	-	-
	Σ	200		-	$\frac{\text{Tot. x RC}}{1000}$	-	$\frac{\text{WA x 1000}}{\text{RC}}$
	<u>Enzymes</u>						
(Sigma G5885)	G-6-PDH	3		-	-	-	-
(Sigma H1131)	HK	5	* ^a	-	-	-	-
(Sigma S3755)	CPK	3	* ^b	15 mg.ml <u>D5 buffer</u> ⁻¹	>		
	<u>Standards</u>	mw	10 mM	Weighed amount	Volume to add (μl)	Dilution	
(Boeh. 121027)	G-6-P	304.2	3.042 mg.ml ⁻¹			100 μl 10 mM + 900 μl H ₂ O = 1 mM	
(Boeh. 127523)	ATP	605.2	6.052 mg.ml ⁻¹				
(Boeh. 127574)	PCr	327.2	3.272 mg.ml ⁻¹				

*^a Hexokinase is diluted 1 part enzyme to 1 part dw (so transfer to eppendorf 50% of required enzyme).

*^b Creatinephosphokinase is diluted 2 parts enzyme to 1 part dw (so weigh for 67% of required enzyme).

G-6-P/ATP/PCr Calculations

G-6-P (mmol. kg dm⁻¹):

$$\frac{\text{Vol. post-enzyme} \times (A2-BLKA2) - \text{Vol. pre-enzyme} \times (A1-BLKA1) \times \text{Extraction factor} \times \text{Dilution factor}}{\text{Extinction coefficient} \times \text{Sample Vol.}}$$

$$\text{Excel Formula} = (((223 \times (A2-BLKA2)) - (220 \times (A1-BLKA1))) \times (125 \times 6)) / (6.22 \times 20)$$

ATP (mmol. kg dm⁻¹):

$$\frac{\text{Vol. post-enzyme} \times (A3-BLKA3) - \text{Vol. pre-enzyme} \times (A2-BLKA2) \times \text{Extraction factor} \times \text{Dilution factor}}{\text{Extinction coefficient} \times \text{Sample Vol.}}$$

$$\text{Excel Formula} = (((228 \times (A3-BLKA3)) - (223 \times (A2-BLKA2))) \times (125 \times 6)) / (6.22 \times 20)$$

PCr (mmol. kg dm⁻¹):

$$\frac{\text{Vol. post-enzyme} \times (A4-BLKA4) - \text{Vol. pre-enzyme} \times (A3-BLKA3) \times \text{Extraction factor} \times \text{Dilution factor}}{\text{Extinction coefficient} \times \text{Sample Vol.}}$$

$$\text{Excel Formula} = (((231 \times (A4-BLKA4)) - (228 \times (A3-BLKA3))) \times (125 \times 6)) / (6.22 \times 20)$$

Where:

Extraction factor = 125 (i.e. 100 µl.mg⁻¹ of HClO₄/EDTA mixture + 25% KHCO₃ for extraction).

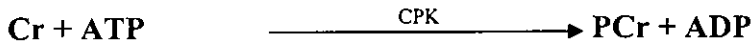
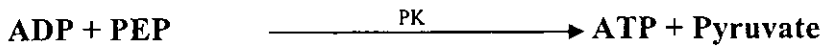
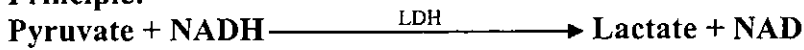
Dilution factor = 6 (i.e. tube S = 200 µl H₂O + 40 µl master extract).

Extinction coefficient = 6.22 (i.e. 6.22 cm². µmol is the absorbency of NADH when read at 340 nm- note: 3.44 cm². µmol at 366 nm).

Note: Standard calculations include no extraction or dilution factors and the standard volume will be 7 µl.

Measurement of Creatine

Principle:



Method:

- Weigh and dilute all components of reagent mixture, enzyme and standard according to Creatine Assay Sheet. All should be thoroughly vortexed and stored on ice.
- Once reagent mixture, enzyme and standard are prepared, samples (S) can be defrosted quickly in hot water, vortexed and spun for 3 min at 14,000 rpm.
- Before pipetting, the spectrophotometer (plate reader) should be switched on to allow the internal temperature to reach $>20^{\circ}\text{C}$. All samples should be read at 340 nm.
- Pipette 15 μl of Sigma RNase free H_2O for blanks into wells A1-A5.
- Pipette 15 μl of standard into wells A6-A8.
- Pipette 15 μl of 6:1 diluted samples into wells B1 onwards.
- Pipette 225 μl of reagent mixture into all wells (viscous, pipette onto side of well).
- Mix and read A1.
- Add 5 μl of CPK, mix well and incubate in the plate reader ($>20^{\circ}\text{C}$).
- Mix again and read A2 at 5 minute intervals beginning at 20 minutes to establish plateau (reaction should be complete by 30 min).

Note: The reaction in this assay converts NADH into NAD. Therefore our absorbencies will reflect the disappearance of NADH (i.e. decrease until plateau).

Creatine Assay Sheet

Cat. no#	<u>Reaction mixture</u>	Volume per well (μl)	x total wells +10% (Tot.) <small>added</small>	Required concentration (RC)	Volume requires (>mg)	Weighed amount (WA)	Volume to add (μl)
-	<u>D4 buffer</u>	75	[]	-	-	-	-
-	<u>D3 buffer</u>	3.75	[]	-	-	-	-
(Sigma A2383)	ATP	15	[]	15.4 mg.ml ⁻¹	>		
(Sigma P7252)	PEP	11.25	[]	11.6 mg.ml ⁻¹	>		
(Boch. 107735)	NADH	3.75	[]	9 mg.ml ⁻¹	>		
(Boch. 107085)	LDH	0.375	[]	-	-	-	-
(Boch. 128155)	PK	0.375	[]	-	-	-	-
(Sigma)	RNase free H ₂ O	115.5	[]	-	-	-	-
	Σ	225		-	$\frac{\text{Tot. x RC}}{1000}$	-	$\frac{\text{WA x 1000}}{\text{RC}}$
	<u>Separate Enzyme</u>						
(Sigma S3755)	CPK	5		15 mg.ml <u>D5 buffer</u> ⁻¹	>		
	<u>Standard</u>	<u>mw</u>	<u>30 mM</u>	<u>Weighed amount</u>	<u>Volume to add (μl)</u>	<u>Dilution</u>	
(Sigma C0780)	Creatine Anhydrous	131.1	3.933 mg.ml ⁻¹			50 μl 30 mM + 950 μl H ₂ O = 1.5 mM	

Creatine Calculations

Creatine (mmol. kg dm⁻¹):

$$\frac{\text{Vol. pre-enzyme} \times (A1 - BLKA1) - \text{Vol. post-enzyme} \times (A2 - BLKA2) \times \text{Extraction factor} \times \text{Dilution factor}}{\text{Extinction coefficient} \times \text{Sample Vol.}}$$

$$\text{Excel Formula} = (((240 \times (A1 - BLKA1)) - (245 \times (A2 - BLKA2))) \times (125 \times 6)) / (6.22 \times 15)$$

Where:

Extraction factor = 125 (i.e. 100 μl .mg⁻¹ of HClO₄/EDTA mixture + 25% KHCO₃ for extraction).

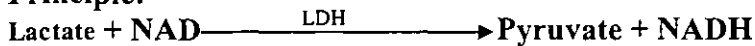
Dilution factor = 6 (i.e. tube S = 200 μl H₂O + 40 μl master extract).

Extinction coefficient = 6.22 (i.e. 6.22 cm². μmol is the absorbency of NADH when read at 340 nm- note: 3.44 cm². μmol at 366 nm).

Note: Standard calculations include no extraction or dilution factors and the standard volume will also be 15 μl .

Measurement of Lactate

Principle:



Method:

- Weigh and dilute all components of reagent mixture, enzyme and standard according to Lactate Assay Sheet. All should be thoroughly vortexed and stored on ice.
- Once reagent mixture, enzyme and standard are prepared, samples (E) can be defrosted quickly in hot water, vortexed and spun for 3 min at 14,000 rpm.
- Before pipetting, the spectrophotometer (plate reader) should be switched on to allow the internal temperature to reach $>20^{\circ}\text{C}$. All samples should be read at 340 nm.
- Pipette 10 μl of Sigma RNase free H_2O for blanks into wells A1-A5.
- Pipette 10 μl of standard into wells A6-A8.
- Pipette 10 μl of samples into wells B1 onwards.
- Pipette 200 μl of reagent mixture into all wells.
- Mix and read A1.
- Add 3 μl of LDH (must be from pig heart), mix well and incubate in the plate reader ($>20^{\circ}\text{C}$).
- Mix again and read A2 at 5 minute intervals beginning at 15 minutes to establish plateau (reaction should be complete by 30 min but can be checked up to 1h).

Lactate Assay Sheet

Cat. no#	<u>Reaction mixture</u>	Volume per well (μl)	x total wells +10% (Tot.) added	Required concentration (RC)	Volume requires (>mg)	Weighed amount (WA)	Volume to add (μl)
-	D2 buffer	75	<input type="checkbox"/>	-	-	-	-
(Boeh. 127965)	NAD	25	<input type="checkbox"/>	16.6 mg.ml ⁻¹	>		
(Sigma)	RNAse free H ₂ O	100	<input type="checkbox"/>	-	-	-	-
	Σ	200		-	$\frac{\text{Tot.} \times \text{RC}}{1000}$	-	$\frac{\text{WA} \times 1000}{\text{RC}}$
	Enzyme						
(Boeh. 107069)	LDH	3		-	-	-	-
	Standards	mw	20 mM	Weighed amount	Volume to add (μl)	Dilution	
(Sigma L6402)	Lactic acid (98%)	90.08	1.8376 mg.ml ⁻¹			50 μl 20 mM + 950 μl H ₂ O = 1 mM	

Lactate Calculations

Lactate (mmol. kg dm⁻¹):

$$\frac{\text{Vol. post-enzyme} \times (A2 - BLKA2) - \text{Vol. pre-enzyme} \times (A1 - BLKA1) \times \text{Extraction factor}}{\text{Extinction coefficient} \times \text{Sample Vol.}}$$

$$\text{Excel Formula} = (((213 \times (A2 - BLKA2)) - (210 \times (A1 - BLKA1))) \times 125) / (6.22 \times 10)$$

Where:

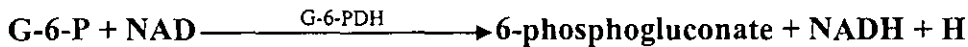
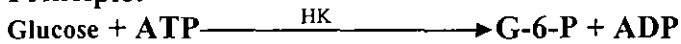
Extraction factor = 125 (i.e. 100 $\mu\text{l} \cdot \text{mg}^{-1}$ of $\text{HClO}_4/\text{EDTA}$ mixture + 25% KHCO_3 for extraction).

Extinction coefficient = 6.22 (i.e. 6.22 $\text{cm}^2 \cdot \mu\text{mol}^{-1}$ is the absorbency of NADH when read at 340 nm- note: 3.44 $\text{cm}^2 \cdot \mu\text{mol}^{-1}$ at 366 nm).

Note: Standard calculations include no extraction factor and the standard volume will also be 10 μl .

Measurement of Prolycogen & Macroglycogen

Principle:



Method:

- Weigh and dilute all components of reagent mixture, enzyme and standard according to Glycogen Assay Sheet. All should be thoroughly vortexed and stored on ice.
 - Once reagent mixture, enzyme and standard are prepared, samples (D & H) can be defrosted quickly in hot water, vortexed and spun for 3 min at 14,000 rpm.
 - Before pipetting, the spectrophotometer (plate reader) should be switched on to allow the internal temperature to reach $>20^{\circ}\text{C}$. All samples should be read at 340 nm.
 - Pipette 20 μl of Sigma RNase free H_2O for blanks into wells A1-A5.
 - Pipette 20 μl of standard into wells A6-A8.
- Note: A set of blanks and standards is required on each new plate.
- Pipette 20 μl of samples into wells B1 onwards.
 - Pipette 200 μl of reagent mixture into all wells.
 - Mix and read A1.
 - Add 2 μl of G-6-PDH/HK mixture, mix well and incubate in the plate reader ($>20^{\circ}\text{C}$).
 - Mix again and read A2 at 5 minute intervals beginning at 10 minutes to establish plateau (reaction should be complete by 20 min).

Note: During high intensity exercise (i.e. $>85\% \dot{V}\text{O}_2 \text{ max}$), it may be necessary to conduct an assay for free glucose on the muscle extract (E) since some free glucose may contribute to the recorded concentration of soluble glucose in this fraction (i.e. macroglycogen).

Glycogen Assay Sheet

Cat. no#	<u>Reaction mixture</u>	Volume per well (μ l)	x total wells +10% (Tot.) <small>added</small>	Required concentration (RC)	Volume requires (>mg)	Weighed amount (WA)	Volume to add (μ l)
-	<u>G1 buffer</u>	64	[]	-	-	-	-
(Sigma A2383)	ATP	4	[]	27.72 mg.ml^{-1}	>		
(Boeh. 197777)	DTT	4	[]	9.36 mg.ml^{-1}	>		
(Boeh. 127965)	NAD	8	[]	19.92 mg.ml^{-1}	>		
(Sigma)	RNAse free H ₂ O	120	[]	-	-	-	-
	Σ	200		-	$\frac{\text{Tot. x RC}}{1000}$	-	$\frac{\text{WA x 1000}}{\text{RC}}$
	<u>Enzymes</u>						
(Sigma G5885)	G-6-PDH	1	*	-	-	-	-
(Sigma H1131)	HK	1	*	-	-	-	-
	<u>Standards</u>	mw	15 mM	Weighed amount	Volume to add (μl)	Dilution	
(Fisons 05003)	Glu	180.16	2.7 mg.ml^{-1}			$100 \mu\text{l } 15 \text{ mM} + 900 \mu\text{l H}_2\text{O} = 1.5 \text{ mM}$	

* The enzyme mixture is 1 part G-6-PDH to 1 part hexokinase (i.e. 2 μ l of mixture added to each well).

Glycogen Calculations

Proglycogen (mmol. kg dm⁻¹):

$$\frac{[(A2 \times \text{Vol. post-enzyme}) - (A1 \times \text{Vol. pre-enzyme})] - [(BLKA2 \times \text{Vol. post-enzyme}) - (BLKA1 \times \text{Vol. pre-enzyme})] \times \text{Hydrolysis factor (D)} \times \text{Dilution factor}}{\text{Extinction coefficient} \times \text{Sample Vol.}}$$

$$\text{Excel Formula} = (((A2*222)-(A1*220))-((BLKA2*222)-(BLKA1*220)))*100*10/(6.22*20)$$

Macroglycogen (mmol. kg dm⁻¹):

$$\frac{[(A2 \times \text{Vol. post-enzyme}) - (A1 \times \text{Vol. pre-enzyme})] - [(BLKA2 \times \text{Vol. post-enzyme}) - (BLKA1 \times \text{Vol. pre-enzyme})] \times \text{Hydrolysis factor (H)} \times \text{Extraction factor}}{\text{Extinction coefficient} \times \text{Sample Vol.}}$$

$$\text{Excel Formula} = (((A2*222)-(A1*220))-((BLKA2*222)-(BLKA1*220)))*6.75*125/(6.22*20)$$

Where:

Hydrolysis factor (D) = 100 (i.e. 100 µl mg⁻¹ of HCl for pellet hydrolysis).

Hydrolysis factor (H) = 6.75 (i.e. 100 µl HCl + 15 µl NaOH + 20 µl for extract hydrolysis).

Dilution factor = 10 (i.e. tube D = 180 µl H₂O + 200 µl hydrolysed pellet).

Extraction factor = 125 (i.e. 100 µl.mg⁻¹ of HClO₄/EDTA mixture + 25% KHCO₃ for extraction).

Extinction coefficient = 6.22 (i.e. 6.22 cm². µmol is the absorbency of NADH when read at 340 nm- note: 3.44 cm². µmol at 366 nm).

Note: Standard calculations include no hydrolysis, extraction or dilution factors and the standard volume will also be 20 µl.

Correction of Muscle Metabolites for Total Creatine

G-6-P, ATP, PCr, creatine and glycogen concentrations must be corrected for the total concentration of creatine (i.e. PCr + Cr) recorded in each sample (n.b. PCr & Cr are therefore self-correcting). Based on the assumption that there will be little variability in total creatine concentrations for a given individual over a short period of time, a single value can be established for each participant. This value can either be taken as the highest value recorded for that participant or as the average total creatine recorded over all sampling-points ($\approx 124 \text{ mmol.kg dm}^{-1}$ *). The equation below can then be used to correct muscle metabolite results for the influence of any contaminating blood metabolites:

$$\text{Corrected Result} = \frac{\text{Uncorrected Result} \times \text{TCr}}{\text{tCr}}$$

where:

TCr = Total creatine for that participant.

tCr = Total creatine at the corresponding sampling-point.

Note: Muscle lactate concentrations are not corrected for total creatine.

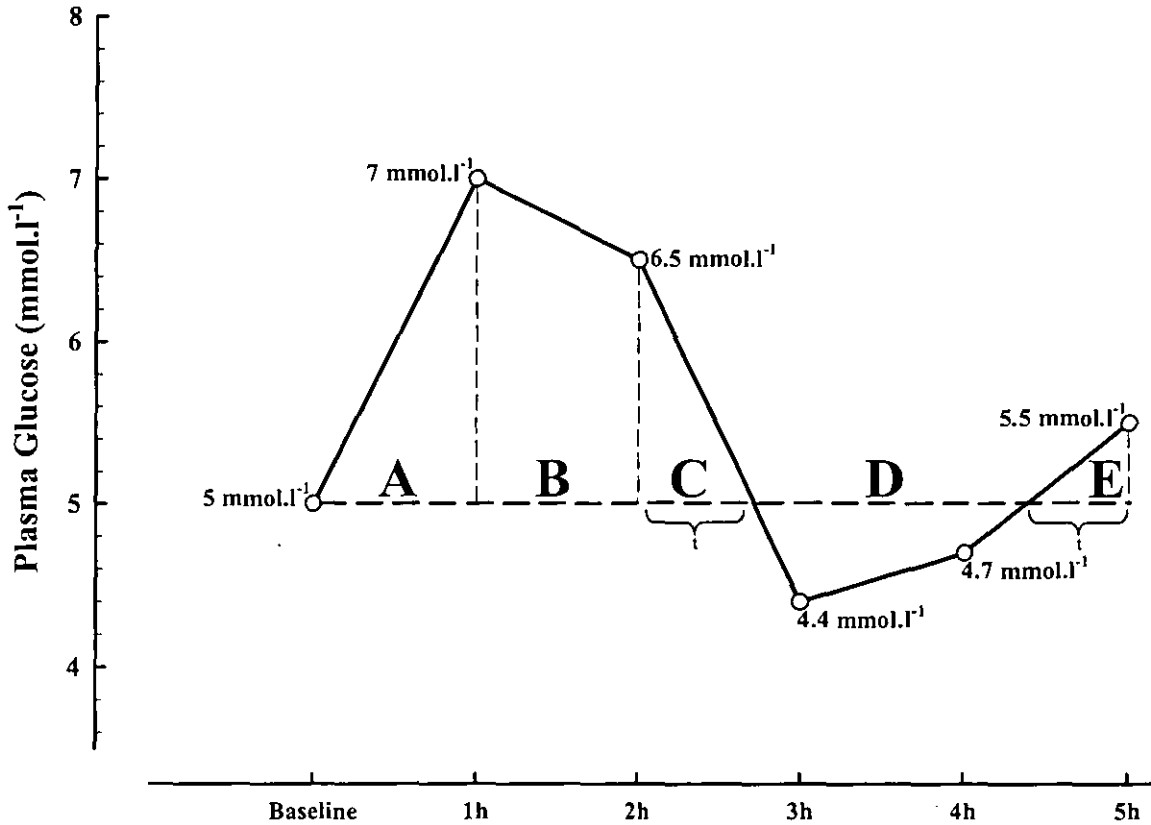
Typical Resting Concentrations of Muscle Metabolites

Metabolite	Mean (mmol. kg dm ⁻¹)	±SD
AMP	0.1	0.05
ADP	3.2	0.5
ATP	24	2.6
G-6-P	1.7	0.7
PCr	75.5	7.6
Cr	49	6.7
PCr + Cr	124.4*	11.2
Glucose	1.7	0.7
Glycogen	349	69.0
Pyruvate	0.4	0.21
Lactate	5.1	2.5

Mean metabolite contents of the quadriceps femoris at rest using 4 different methods of analysis.

Calculation of Incremental Area Under Curve

The incremental area under curve (IAUC) was calculated throughout this thesis in accordance with the method recommended by Wolever (2004), as described below:



The total IAUC for the above example will be the sum of periods A+B+C+D+E

$$\begin{aligned}
 \text{Triangle A} &= (\text{start conc.} + \text{end conc.}) \times \frac{1}{2} \text{ time (min)} - \text{baseline area} \\
 &= (5 + 7) \times 30 - (5 \times 60) \\
 &= 60 \text{ mmol} \cdot 60 \text{ min} \cdot \text{l}^{-1}
 \end{aligned}$$

$$\begin{aligned}
 \text{Trapezoid B} &= (7 + 6.5) \times 30 - (5 \times 60) \\
 &= 105 \text{ mmol} \cdot 60 \text{ min} \cdot \text{l}^{-1}
 \end{aligned}$$

$$\begin{aligned}
 \text{Triangle C} &= (\text{start conc.} - \text{baseline conc.}) \times t / 2 \\
 &= (6.5 - 5) \times 42.9 / 2 \\
 &= \mathbf{32.2 \text{ mmol} \cdot 60 \text{ min} \cdot \text{l}^{-1}}
 \end{aligned}$$

$$\begin{aligned}
 \text{where } t &= (\text{start conc.} - \text{baseline conc.}) / (\text{start conc.} + \text{end conc.}) \times \text{time (min)} \\
 &= (6.5 - 5) / (6.5 + 4.4) \times 60 \\
 &= 42.9 \text{ min}
 \end{aligned}$$

Period D the concentrations both at the start and the end of this period are below baseline so IAUC = $\mathbf{0 \text{ mmol} \cdot 60 \text{ min} \cdot \text{l}^{-1}}$

$$\begin{aligned}
 \text{Triangle E} &= (\text{end conc.} - \text{baseline conc.}) \times t / 2 \\
 &= (5.5 - 5) \times 37.5 / 2 \\
 &= \mathbf{9.4 \text{ mmol} \cdot 60 \text{ min} \cdot \text{l}^{-1}}
 \end{aligned}$$

$$\begin{aligned}
 \text{where } t &= (\text{end conc.} - \text{baseline conc.}) / (\text{end conc.} + \text{start conc.}) \times \text{time (min)} \\
 &= (5.5 - 5) / (5.5 + 4.7) \times 60 \\
 &= 37.5 \text{ min}
 \end{aligned}$$

Therefore the total IAUC for the 5 h period =

$$\begin{aligned}
 &60 \text{ mmol} \cdot 60 \text{ min} \cdot \text{l}^{-1} \\
 &+ 105 \text{ mmol} \cdot 60 \text{ min} \cdot \text{l}^{-1} \\
 &+ 32.2 \text{ mmol} \cdot 60 \text{ min} \cdot \text{l}^{-1} \\
 &+ 0 \text{ mmol} \cdot 60 \text{ min} \cdot \text{l}^{-1} \\
 &+ 9.4 \text{ mmol} \cdot 60 \text{ min} \cdot \text{l}^{-1} \\
 \hline
 &\mathbf{206.6 \text{ mmol} \cdot 300 \text{ min} \cdot \text{l}^{-1}}
 \end{aligned}$$

Calculation of Confidence Intervals

The error bars displayed on all figures in this thesis are confidence intervals (CI) that have been corrected to remove between subject variance according to the methods described by Loftus and Masson (1994). These CI can be calculated using commercially available statistical software (SPSS 11.0, USA) as shown below:

- Begin with data set to be compared, if you are comparing multiple points over time then take the data from a given time point in each trial, e.g.

Trial 1	Trial 2	Trial 3
10	13	13
6	8	8
11	14	14
22	23	25
16	18	20
15	17	17
1	1	4
12	15	17
9	12	12
8	9	12

- Paste these data into SPSS in columns as shown above and perform a general linear model (GLM):

Tests of Within-Subjects Effects

Measure: MEASURE_1

Source		Type III Sum of Squares	df	Mean Square	F	Sig.
DRINK	Sphericity Assumed	52.267	2	26.133	42.506	.000
	Greenhouse-Geisser	52.267	1.690	30.928	42.506	.000
	Huynh-Feldt	52.267	2.000	26.133	42.506	.000
	Lower-bound	52.267	1.000	52.267	42.506	.000
Error(DRINK)	Sphericity Assumed	11.067	18	.615		
	Greenhouse-Geisser	11.067	15.209	.728		
	Huynh-Feldt	11.067	18.000	.615		
	Lower-bound	11.067	9.000	1.230		

- Note the Mean Square for error and the corresponding degrees of freedom (df): 0.615 & 18, respectively.
- Use a t Distribution table to determine a t value using the df.
For the above example $df = 18$ so $t = 2.101$.

- A normalised confidence interval can now be created using the formula:

$$CI = M_j \pm \sqrt{\frac{MS_{SXC}}{n}} \text{ [criterion } t(df_{SXC})]$$

where: MS_{SXC} = mean squared error
 n = number of subjects in each trial
 criterion t = t distribution value

So for the present example this formula will be:

$$= \sqrt{0.615/10} \times 2.101$$

$$= 0.52$$

- Therefore the overall CI that will be plotted about each mean will be:

	Trial 1	Trial 2	Trial 3
Mean \pm CI	11 \pm 0.52	13 \pm 0.52	14 \pm 0.52

- Interpretation of these CI is not entirely objective and is not intended to correspond with traditional null hypothesis testing techniques. In general, however, plotted means whose confidence intervals overlap by no more than half the distance of 1 side of an interval are likely to be deemed statistically different via t -test.
- Confidence intervals calculated for within-subjects designs in this way do not infer that there is a 95% probability that the mean of the general population lies within that interval (as do conventional CI). Instead, what is illustrated by these CI is the probability that the pattern of recorded means is reflective of the general population (i.e. statistical significance). The reason that this relationship does not always correspond with standard statistical methods is that these CI also provide additional information regarding the statistical power of each comparison.

Additional Issue: Violations of Sphericity

- When 3 or more treatments are to be compared, the F ratio calculated using a general linear model will only be accurate when sphericity can be assumed. The degree of sphericity can be assessed through referring to the Greenhouse-Geisser epsilon. If this value is <0.75 then, similar to hypothesis testing, the Greenhouse-Geisser corrected MS_{SXC} and df should be used. If the Greenhouse-Geisser epsilon is >0.75 then the Huynh-Feldt corrected MS_{SXC} and df are used, as was the case in the example on the previous page.
- However, the table below presents the severe violation of sphericity that was observed in relation to the exercise capacity data recorded in Study 3 (Chapter 6):

	Greenhouse-Geisser	Huynh-Feldt
Epsilon	.557	.604

- The Greenhouse-Geisser epsilon of 0.557 is therefore applied to the GLM output below to increase the MS_{SXC} and reduce the df:

Tests of Within-Subjects Effects

Measure: MEASURE_1

Source		Type III Sum of Squares	df	Mean Square	F	Sig.
FACTOR1	Sphericity Assumed	793.750	2	396.875	6.082	.019
	Greenhouse-Geisser	793.750	1.115	711.928	6.082	.050
	Huynh-Feldt	793.750	1.207	657.580	6.082	.045
	Lower-bound	793.750	1.000	793.750	6.082	.057
Error(FACTOR1)	Sphericity Assumed	652.583	10	65.258		
	Greenhouse-Geisser	652.583	5.575	117.063		
	Huynh-Feldt	652.583	6.035	108.126		
	Lower-bound	652.583	5.000	130.517		

- Therefore the confidence intervals to be plotted around each mean can now be calculated using this corrected omnibus error term as previously:

$$CI = \sqrt{117.1/6} \times 2.447$$

$$= 10.8$$

- So for the run times in study 3 this would equal:

	CHO	CHO-PRO	CHO-CHO
Mean \pm CI	83.7 \pm 10.8	91.2 \pm 10.8	99.9 \pm 10.8

- This correction of the omnibus error term maintains an accurate illustration of the pattern across all treatments and is therefore more useful on a line graph where the comparisons of interest can vary over time.
- However, when constructing a bar graph, the specific differences between each pair of means are more important and it would therefore be inappropriate to use a corrected omnibus error term when sphericity has been violated severely (i.e. Greenhouse-Geisser epsilon < 0.75).
- In such situations it will be more appropriate to compute a separate GLM for each comparison of interest and then use their respective MS_{sxc} and df to construct distinct pair of CI for each contrast. For example, in Study 3 it was of interest to illustrate the specific comparisons between the CHO-PRO trial and each other trial separately. Therefore the CI were constructed and plotted as follows:

CHO-PRO versus CHO

$$MS_{sxc} = 12.6$$

$$df = 5$$

$$t = 2.571$$

$$n = 6$$

$$CI = \sqrt{12.6/6} \times 2.571$$

$$= 3.7$$

$$CHO = 83.7 \pm 3.7$$

$$CHO-PRO = 91.2 \pm 3.7$$

CHO-PRO versus CHO-CHO

$$MS_{sxc} = 70.2$$

$$df = 5$$

$$t = 2.571$$

$$n = 6$$

$$CI = \sqrt{70.2/6} \times 2.571$$

$$= 8.8$$

$$CHO-CHO = 99.9 \pm 8.8$$

$$CHO-PRO = 91.2 \pm 8.8$$

